

1 **Characterization of the Golgi c10orf76-PI4KB complex, and its** 2 **necessity for Golgi PI4P levels and enterovirus replication**

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4 McPhail, J.A.¹, Lyoo, H.R.^{2*}, Pemberton, J.G.^{3*}, Hoffmann, R.M.¹, van Elst W.²,
5 Strating J.R.P.M.², Jenkins, M.L.¹, Stariha, J.T.B.¹, van Kuppeveld, F.J.M.^{2#}, Balla, T.^{3#}, and
6 Burke, J.E.^{1#}

7 * - These authors contributed equally

8 # - Corresponding authors

9

10 **Affiliations**

11 1 Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC,
12 Canada

13 2 Department of Infectious Diseases & Immunology, Virology Division, Faculty of
14 Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

15 3 Section on Molecular Signal Transduction, Eunice Kennedy Shriver National Institute
16 of Child Health and Human Development, National Institutes of Health, Bethesda, MD,
17 USA

18

19 **Corresponding authors:**

20 Burke, John E. (jeburke@uvic.ca), Tel: 1-250-721-8732

21 Balla, Tamas (ballat@mail.nih.gov), Tel: 301-435-5637

22 van Kuppeveld, Frank J.M. (F.J.M.vanKuppeveld@uu.nl), Tel: 31-30-253-4173

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24 **Running title**

25 Role of c10orf76-PI4KB at the Golgi

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33 **Keywords**

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35 phosphoinositide, phosphatidylinositol 4-phosphate, PI4P, PKA, enterovirus, coxsackievirus
36 A10, viral replication, hydrogen deuterium exchange mass spectrometry, HDX-MS, Golgi

37

38 **Highlights**

- 39 • c10orf76 forms a direct complex with PI4KB, with the interface formed by a disorder-
40 to-order transition in the kinase linker of PI4KB
- 41 • The c10orf76 binding site of PI4KB can be phosphorylated by PKA, with
42 phosphorylation leading to decreased affinity for c10orf76
- 43 • Complex-disrupting mutants of PI4KB and c10orf76 reveal that PI4KB recruits
44 c10orf76 to the Golgi/TGN
- 45 • Depletion of c10orf76 leads to decreases in both active Arf1 and Golgi PI4P levels
- 46 • Enteroviruses that rely on c10orf76 for replication depend on formation of the
47 c10orf76-PI4KB complex

48

49 **Summary**

50 The lipid kinase PI4KB, which generates phosphatidylinositol 4-phosphate (PI4P), is a key
51 enzyme in regulating membrane transport and is also hijacked by multiple picornaviruses to
52 mediate viral replication. PI4KB can interact with multiple protein binding partners, which are
53 differentially manipulated by picornaviruses to facilitate replication. The protein c10orf76 is a
54 PI4KB-associated protein that increases PI4P levels at the Golgi, and is essential for the viral
55 replication of specific enteroviruses. We used hydrogen deuterium exchange mass
56 spectrometry to characterize the c10orf76-PI4KB complex and reveal that binding is mediated
57 by the kinase linker of PI4KB, with formation of the heterodimeric complex modulated by
58 PKA-dependent phosphorylation. Complex-disrupting mutations demonstrate that PI4KB is
59 required for membrane recruitment of c10orf76 to the Golgi, and that an intact c10orf76-PI4KB
60 complex is required for the replication of c10orf76-dependent enteroviruses. Intriguingly,
61 c10orf76 was also required for proper Arf1 activation at the Golgi, providing a putative
62 mechanism for the c10orf76-dependent increase in PI4P levels at the Golgi.

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64

65 Introduction

66 Phosphoinositides are essential regulatory lipids that play important roles in myriad
67 cellular functions. The phosphoinositide species phosphatidylinositol-4-phosphate (PI4P) is
68 widely distributed and involved in the coordinated regulation of membrane trafficking, cell
69 division, and lipid transport [1,2]. Multiple human pathogens manipulate PI4P levels to
70 mediate their intracellular replication, including *Legionella* [3] and multiple picornaviruses
71 [4,5]. PI4P in humans is generated through the action of four distinct phosphatidylinositol-4-
72 kinases: PI4KII α (PI4K2A), PI4KII β (PI4K2B), PI4KIII α (PI4KA) and PI4KIII β (PI4KB) [6-
73 8]. PI4KB is localized at the Golgi and trans-Golgi-network (TGN), with PI4P pools in the
74 Golgi apparatus generated by both PI4K2A and PI4KB [9]. While the localization and activity
75 of PI4K2A is regulated through its palmitoylation, local membrane composition, and
76 cholesterol levels [10], the activity of PI4KB is regulated by multiple protein-protein
77 interactions [11-14]. These regulatory protein-protein interactions are in turn manipulated by
78 many pathogenic RNA viruses that have evolved the ability to hijack PI4KB and generate
79 PI4P-enriched replication organelles, which are essential for viral replication [15]. For most
80 picornaviruses, manipulation of PI4P levels is driven by the action of the viral 3A protein and
81 its interactions with PI4KB-binding proteins [13,16-18].

82 PI4KB plays both important catalytic and non-catalytic functions, with its regulation
83 controlled by interactions with multiple protein binding partners, including acyl CoA Binding
84 Domain 3 (ACBD3), Rab11, 14-3-3, and c10orf76 (chromosome 10, open-reading frame 76,
85 also referred to as Armadillo-like helical domain-containing protein 3 (ARMH3)). PI4KB is a
86 multi-domain lipid kinase containing a disordered N-terminus, a helical domain, and a bi-lobal
87 kinase domain [14,19]. Biophysical and biochemical studies have defined the domains of
88 PI4KB that mediate complex formation with a number of binding partners. The helical domain
89 of PI4KB forms a non-canonical interaction with the small GTPase Rab11a that mediates
90 localization of a pool of Rab11 to the Golgi and TGN [14,20]. PI4KB is primarily localized to
91 the Golgi through the interaction of its N-terminus with ACBD3 [12,13]. PI4KB is activated
92 downstream of ADP-ribosylation factor 1 (Arf1) [21], however, no evidence for a direct Arf1-
93 PI4KB interface has been found, suggesting that this may be an indirect effect. PI4KB contains
94 phosphorylation sites in disordered linkers between domains, including Ser294 in the helical-
95 kinase linker of PI4KB, which is phosphorylated by protein kinase D (PKD). Phosphorylation
96 of Ser294 drives binding of 14-3-3, which stabilizes PI4KB, prevents degradation, and
97 increases Golgi PI4P levels [22-24]. Ser496 in the N-lobe linker of PI4KB is phosphorylated

98 by protein kinase A (PKA) [25], and drives PI4KB localization from the Golgi to nuclear
99 speckles [26]. c10orf76 was identified as a putative PI4KB interacting partner in
100 immunoprecipitation experiments [17,27], with knockout of c10orf76 leading to decreased
101 Golgi PI4P levels [28]. The function of this protein is unknown, however, it contains a domain
102 of unknown function (DUF1741) that is well conserved in many eukaryotes.

103 Enterovirus proteins do not interact directly with PI4KB – they instead recruit PI4KB-
104 regulatory proteins. A key component of manipulating PI4KB to generate PI4P-enriched
105 replication organelles is the interaction of viral 3A proteins with host PI4KB-binding proteins.
106 The 3A proteins from enteroviruses (i.e. Poliovirus, Rhinovirus, Coxsackievirus, Rhinovirus
107 and Enterovirus 71) and Aichivirus recruit PI4KB to replication organelles through an
108 interaction with ACBD3 [11,13,16-18,29-32]. The viral 3A protein from Aichivirus forms a
109 direct interaction with the GOLD domain of ACBD3, leading to redistribution of PI4KB to
110 replication organelles [11,31]. Enteroviruses also manipulate other lipid signaling pathways,
111 with viral 3A proteins able to recruit the protein Golgi-specific brefeldin A-resistance guanine
112 nucleotide exchange factor 1 (GBF1) that activates Arf1 [4,33-35], and subvert Rab11-positive
113 recycling endosomes to replication organelles [36]. A new component of the PI4KB hijacking
114 process, c10orf76, was identified as a key host factor in the replication of coxsackievirus A10
115 (CVA10) replication, but not coxsackievirus B1 (CVB1) [28].

116 We hypothesized that a direct c10orf76-PI4KB interaction may be critical for the
117 regulation of Golgi PI4P levels and play a role in enterovirus replication. To elucidate the role
118 of c10orf76 in PI4KB-mediated signaling, we utilized a synergy of hydrogen deuterium
119 exchange mass spectrometry (HDX-MS) and biochemical assays to characterize the novel
120 c10orf76-PI4KB complex *in vitro*. This allowed us to engineer complex-disrupting mutations
121 that were subsequently used to define the role of the c10orf76-PI4KB complex in Golgi PI4P-
122 signaling and viral replication *in vivo*. We find that PI4KB and c10orf76 form a high affinity
123 complex mediated by a disorder-to-order transition of the kinase linker of PI4KB, with
124 complex affinity modulated by PKA phosphorylation of the c10orf76 binding site on PI4KB.
125 Knockout of c10orf76 lead to decreased PI4P levels, and disruption of Arf1 activation in cells.
126 Complex-disrupting mutations revealed that c10orf76 is recruited to the Golgi by PI4KB, and
127 that viral replication of enteroviruses that require c10orf76 is mediated by the c10orf76-PI4KB
128 complex.

129

130 **Results**

131 **c10orf76 forms a direct, high affinity complex with PI4KB**

132 c10orf76 was previously identified as a putative PI4KB-binding partner through
133 immunoprecipitation experiments [17,27], however, it was not clear if this was through a direct
134 interaction. To identify a potential direct interaction between PI4KB and c10orf76 *in vitro*, we
135 purified recombinant full-length proteins using a baculovirus and *Spodoptera frugiperda* (*Sf9*)
136 expression system. Experiments on PI4KB used the slightly smaller isoform 2 variant (1-801,
137 uniprot: Q9UBF8-2), compared to PI4KB isoform 1 (1-816 uniprot: Q9UBF8-1), similar to
138 previous structural studies [14]. His-pulldown assays using NiNTA-agarose beads and purified
139 recombinant proteins showed a direct interaction between PI4KB and His-tagged c10orf76
140 (**Fig. 1A**). Pulldown experiments carried out with the PI4KB-binding partners Rab11 and
141 ACBD3 revealed that c10orf76 could form PI4KB-containing ternary complexes with both
142 (**Fig. S1**), indicating a unique c10orf76 binding interface on PI4KB compared to Rab11 and
143 ACBD3. To examine the stoichiometry of the c10orf76-PI4KB complex, we subjected apo
144 c10orf76 and c10orf76 with PI4KB to size exclusion chromatography. Apo c10orf76 (79 kDa)
145 eluted from the size exclusion column at a volume consistent with a monomer, while the
146 c10orf76-PI4KB complex eluted at a volume consistent with a 1:1 complex (**Fig. 1B**). Since
147 cellular knockout of c10orf76 has been shown to reduce PI4P levels *in vivo* [28], we
148 investigated the effect of c10orf76 on PI4KB lipid kinase activity with biochemical membrane
149 reconstitution assays using phosphatidylinositol (PI) vesicles. Intriguingly, c10orf76 was a
150 potent inhibitor of PI4KB, with inhibition being dose-dependent and possessing an IC₅₀ of ~90
151 nM (**Fig. 1C**). This inhibitory effect was observed on both pure phosphatidylinositol (PI)
152 vesicles, and vesicles that mimic the composition of the Golgi (20% PI, 10% PS, 45% PE, 25%
153 PC) (**Fig. 1D**). This paradoxical PI4KB-inhibitory result *in vitro* conflicts with observed Golgi
154 PI4P decreases in c10orf76 deficient cells [28]. This suggests that biochemical assays may not
155 fully recapitulate the environment of the Golgi. To further define the role of this complex we
156 focused on defining the molecular basis of this interface, allowing for generation of binding-
157 deficient mutants for downstream cellular and viral experiments.

158

159 **HDX-MS reveals that PI4KB and c10orf76 form an extended interface involving a**
160 **disorder-to-order transition of the PI4KB N-lobe linker**

161 To identify the putative interface between PI4KB and c10orf76, we employed
162 hydrogen-deuterium exchange mass spectrometry (HDX-MS) to map regions protected in both
163 proteins upon complex formation. HDX-MS is an analytical technique that measures the
164 exchange rate of amide hydrogens in proteins. Because one of the main determinants for amide

165 exchange is the presence of secondary structure, their exchange rate is an excellent readout of
166 protein dynamics. HDX-MS is thus a potent tool to determine protein-protein, protein-ligand,
167 and protein-membrane interactions [37-39]. H/D exchange was carried out for three different
168 conditions: apo PI4KB, apo c10orf76, and a 1:1 complex of PI4KB with c10orf76. Deuterium
169 incorporation experiments were carried out at four different timepoints (3, 30 and 300 seconds
170 at 23°C and 3 seconds at 1°C). Deuterium incorporation is determined by quenching the
171 exchange reaction in a solution that dramatically decreases the exchange rate, followed by rapid
172 digestion, peptide separation, and mass analysis. A total of 185 peptides covering 96.9% of the
173 PI4KB sequence, and 108 peptides covering 73.9% of the c10orf76 sequence were generated
174 (**Fig. 1E-H-source data 1, Fig. S2**). Significant differences in deuterium exchange between
175 conditions were defined as changes in exchange at any timepoint that met the three following
176 criteria: greater than 7% change in deuterium incorporation, a greater than 0.5 Da difference in
177 peptide mass, and a p-value of less than 0.05 (unpaired student's t-test).

178 Multiple regions of PI4KB were protected from amide exchange in the presence of
179 c10orf76, revealing an extended binding interface (**Fig. 1E,F,H; Fig. S2**). The most prominent
180 difference in exchange was at the C-terminus of the disordered N-lobe linker (residues 486-
181 498), where the presence of c10orf76 led to a significant ordering of this region. This region
182 had no protection from amide exchange in the apo state, revealing it to be disordered, with a
183 very strong stabilization (>80% decrease in exchange) in the presence of c10orf76, indicating
184 a disorder-to-order transition upon c10orf76 binding (**Fig. 1H**). This N-lobe kinase linker is
185 dispensable for lipid kinase activity, as it can be removed with a minimal effect on PI4KB
186 catalytic activity [19]. In addition to this change there were multiple smaller decreases in
187 exchange in the helical domain (131-138, 149-157, 159-164, and 183-204) and kinase domain
188 (676-688, 725-734, and 738-765). The helical domain of PI4KB mediates binding to Rab11.
189 However, the PI4KB-Rab11 complex was still able to form in the presence of c10orf76 (**Fig.**
190 **S1**). The decreases in exchange with c10orf76 observed in the kinase domain were located in
191 the activation loop (676-688) and the C-lobe (738-765), which may mediate the inhibition
192 observed *in vitro*. The protected surface on PI4KB extensively spans the membrane face of the
193 kinase, which may prevent PI4KB from directly interfacing with the membrane and accessing
194 PI in the presence of c10orf76, at least in the absence of other binding partners *in vitro* (**Fig.**
195 **1F**).

196 The presence of PI4KB also caused multiple differences in H/D exchange in c10orf76,
197 with increased exchange at the N-terminus (56-62) as well as decreased exchange N-terminal

198 of, and within, the domain of unknown function (DUF1741; 403-408, 534-547, 632-641) (**Fig.**
199 **1G,H; Fig. S2**). There are no clear structural determinants of c10orf76, with limited homology
200 to any previously solved structure, however, it is predicted to consist of a primarily helical fold
201 arranged into armadillo repeats. The uncharacterized DUF1741 domain of c10orf76 is present
202 throughout many eukaryotes, however, even though the DUF1741 domain is strongly
203 conserved in evolution, c10orf76 is the only protein that contains this domain in humans.

204 The largest observed change in deuterium incorporation in either protein was in the
205 PI4KB N-lobe linker (486-496). Interestingly, this region contains a consensus PKA motif
206 (RRxS) that corresponds to Ser496 (Ser511 in PI4KB isoform 1), which is phosphorylated *in*
207 *vivo* and conserved back to the teleost fishes (**Fig. 2A**) [26]. Systems level analysis of PKA
208 signaling networks also show that phosphorylation of this site is decreased >90% in PKA
209 knockout cells, indicating that it is likely a direct PKA target [25]. To better understand the
210 regulation of the c10orf76-PI4KB complex, we sought to characterize the effects of Ser496
211 phosphorylation.

212

213 **PI4KB is directly phosphorylated at Ser496 by PKA to modulate the affinity of the** 214 **c10orf76-PI4KB complex**

215 There are three well-validated phosphorylation sites on PI4KB: Ser294, Ser413, Ser496
216 [40]. To test the role of phosphorylation of PI4KB at Ser496, we generated stoichiometrically
217 phosphorylated PI4KB at only Ser496 using an *in vitro* phosphorylation approach that relied
218 on the production of the purified mouse PKA catalytic subunit in *E. coli*. To minimize
219 complications from any background phosphorylation that occurs in *Sf9* cells, we used PI4KB
220 expressed in *E. coli* to ensure the starting protein substrate was non-phosphorylated. Analysis
221 of the *Sf9*-produced PI4KB revealed significant phosphorylation of Ser294, Ser413, Ser430
222 and Ser496, while *Sf9*-produced c10orf76 had evidence of phosphorylation of Ser14, and an
223 additional Ser/Thr phosphorylation in the 325-351 region, although the specific residue is
224 ambiguous from the MS data. No phosphorylation was identified from *E. coli* produced
225 proteins, as expected (**Fig. S3**). Dose response assays for the phosphorylation of PI4KB Ser496
226 using *E. coli*-produced protein were then carried out with increasing concentrations of purified
227 PKA, and the resulting product was analyzed by mass spectrometry for the site-specific
228 incorporation of the phosphate moiety (**Fig. 2B**). Ser496 in PI4KB was phosphorylated
229 efficiently by PKA, with >99% phosphorylation at Ser496 occurring with a 1:500 ratio of PKA
230 to PI4KB and no detectable phosphorylation at the other major PI4KB phosphorylation sites
231 (**Fig. S3**). Lipid kinase assays were then carried out using different concentrations of c10orf76

232 for both phosphorylated and non-phosphorylated PI4KB. The phosphorylated form had a 3-
233 fold increase in the IC₅₀ value, suggesting that Ser496 phosphorylation decreases c10orf76
234 binding affinity, with no shift in the IC₅₀ value for the S496A PI4KB mutant (**Fig. 2C**). Kinase
235 assays carried out on both Ser496 phosphorylated PI4KB and non-phosphorylated PI4KB
236 showed that there is no direct effect of the phosphorylation events on basal lipid kinase activity
237 (**Fig. 2D**). PKA-mediated phosphorylation-dependent changes in the affinity of protein-protein
238 complexes have been previously described [41,42]. We utilized HDX-MS to test if the altered
239 inhibition profile we saw was due to decreased affinity between c10orf76 and PI4KB. These
240 experiments were carried out at a single time point of D₂O exposure (5 seconds at 20°C) with
241 differing levels of c10orf76 present. Plotting the difference in deuterium incorporation versus
242 c10orf76 concentration gives a characteristic binding isotherm for both phosphorylated and
243 non-phosphorylated PI4KB; displaying a ~3-fold decreased affinity for the phosphorylated
244 form of PI4KB (85 nM vs 30 nM, **Fig. S3**) Phosphomimic variants of Ser496 in PI4KB mutants
245 did not alter the affinity for c10orf76, so they could not be utilized to study this effect *in vivo*
246 (data not shown). To better characterize the role of the c10orf76-PI4KB complex *in vivo*, we
247 sought to generate c10orf76-PI4KB complex-disrupting mutations.

248

249 **Rationally engineered PI4KB and c10orf76 mutants that disrupt complex formation**

250 The c10orf76 binding site within the N-lobe kinase linker of PI4KB identified by HDX-
251 MS is highly conserved in vertebrates, with much of the region also conserved in *D.*
252 *melanogaster*, but not in *C. elegans* (**Fig. 2A**). We used a combination of both the sequence
253 conservation and HDX-MS results to design a complex-disrupting mutant. The RL residues at
254 494-495 were mutated to EA (RL494EA), effectively causing both a charge reversal and
255 decrease in hydrophobicity. The RL494EA mutant disrupted binding to His-tagged c10orf76
256 bait in a His pulldown assay (**Fig. 2E**) and prevented inhibition by c10orf76 in kinase assays
257 (**Fig. 2F**). This mutant had exactly the same basal kinase activity as the WT PI4KB on both PI
258 vesicles and Golgi-mimetic vesicles (**Fig. 2G**), strongly suggesting that the mutant kinase is
259 properly folded. In an attempt to design rational mutations of c10orf76 that also disrupted
260 binding to PI4KB, multiple mutations were tested in regions 403-408, 534-547 and 632-641
261 that were identified using HDX-MS. Combining the HDX-MS data and sequence homology,
262 we designed a triple alanine mutant at the end of a putative helix (QYANAFL) that was well
263 conserved in vertebrates (**Fig. 2J**), close to the HDX-MS protection (FLH residues 409-411 to
264 AAA, referred to as FLH mutant afterwards). The FLH mutant expressed well, significantly

265 reduced binding to PI4KB in a His-pulldown assay (**Fig. 2E**), and also showed a marked
266 reduction in its ability to inhibit PI4KB activity (**Fig. 2I**). To confirm the c10orf76 FLH mutant
267 does not affect global protein structure, we compared deuterium incorporation of the c10orf76
268 wild-type and FLH mutant and observed no changes in deuterium incorporation seen outside
269 of the predicted helix containing the FLH residues (**Fig. S4**). The engineering of complex-
270 disrupting mutants that do not alter catalytic activity or protein folding provided an excellent
271 tool to test the importance of the c10orf76-PI4KB complex in cells.

272

273 **PI4KB recruits c10orf76 to the Golgi**

274 To define the role of the c10orf76-PI4KB interface in cellular localization we utilized
275 fluorescently-tagged variants of the wild-type and complex-disrupting mutants of both PI4KB
276 and c10orf76. Fluorescence microscopy of HEK293 cells expressing GFP-tagged wild-type
277 PI4KB revealed that it primarily localizes to the Golgi (**Fig. 3A**). GFP-PI4KB RL494EA,
278 which is deficient in c10orf76 binding, also localized mainly to the Golgi, which suggests that
279 c10orf76 plays a minimal role in the Golgi recruitment of PI4KB (**Fig. 3A**). The wild-type
280 GFP-c10orf76 also localizes to the Golgi. However, the PI4KB binding-deficient FLH mutant
281 is redistributed to the cytosol; revealing an important role for PI4KB in the proper cellular
282 localization of c10orf76 (**Fig. 3B**). To further analyze the role of PI4KB in the recruitment of
283 c10orf76, we utilized a chemically-inducible protein heterodimerization system that relies on
284 the selective interaction of the FKBP12 (FK506 binding protein 12) and FRB (a 9 kDa fragment
285 of mTOR that binds rapamycin) modules upon treatment with rapamycin [12,43]. Specifically,
286 we fused the FRB domain to residues 34–63 of a CFP-tagged mitochondrial localization signal
287 from mitochondrial A-kinase anchor protein 1 (AKAP1), and fused mRFP-FKBP12 onto the
288 wild-type or mutant variants of human PI4KB (**Fig. 3C**). These constructs allowed us to
289 examine the localization of the wild-type or mutant GFP-c10orf76 following the acute
290 sequestration of PI4KB to the outer mitochondrial membrane, where other Golgi-associating
291 proteins are not be present. Treatment with rapamycin (100 nM) caused the rapid recruitment
292 of mRFP-FKBP12-PI4KB to the mitochondria, which also caused the rapid co-recruitment of
293 c10orf76 (**Fig. 3D; Video 1**); suggesting that PI4KB is the only component necessary for
294 membrane recruitment of c10orf76. Experiments using mRFP-FKBP12 PI4KB RL494EA
295 showed that although the mutant kinase is relocated to the mitochondria, GFP-c10orf76 does
296 not co-localize (**Fig. 3E; Fig. 3F, Video 2**). Taken together, these live-cell studies corroborated
297 the protein interaction studies completed *in vitro* and also demonstrate that the newly defined

298 c10orf76-PI4KB interface is required for proper localization of c10orf76 to the Golgi.
299 Compellingly, these findings reveal a potential novel function of PI4KB in the recruitment of
300 c10orf76.

301

302 **c10orf76 regulates Arf1 activation and maintains Golgi PI4P levels**

303 The paradoxical finding that the loss of c10orf76 leads to increased PI4P levels in cells,
304 yet decreased catalytic activity of PI4KB *in vitro*, suggested that there was an unknown lipid
305 or protein constituent in cells that is not present in our *in vitro* experiments. To determine the
306 role of c10orf76 in cells we examined the distribution of different Golgi-localized signaling
307 components in c10orf76-deficient (knockout) HAP1 cells. In agreement with previous studies
308 [28], we found that there were decreased PI4P levels at the Golgi in c10orf76 knockout cells,
309 as indicated by decreased Golgi staining by an anti-PI4P antibody (**Fig. 4A**). Intriguingly, there
310 was an apparent increase in Golgi localized PI4KB in the c10orf76 knockout cells (**Fig. 4A**),
311 similar to what occurs upon treatment with a PI4KB inhibitor [44], clearly indicating that
312 decreased PI4P production was not due to loss of PI4KB recruitment in the absence of
313 c10orf76. We tested the localization of different Golgi markers to verify that decreased PI4P
314 was not due to disruption of Golgi morphology. Markers for the cis Golgi (GM130), cis/medial
315 Golgi (Giantin), and the trans-Golgi network (TGN46) all showed similar localization in both
316 WT and c10orf76 knockout cells (**Fig. 4B**). The distribution of the ER-Golgi intermediate
317 compartment marker ERGIC53 was also similar, suggesting that Golgi morphology was
318 maintained in the c10orf76 knockout HAP1 cells (**Fig. 4B**).

319 We next tested the localization of the Arf1-GEF GBF1, as active GTP-bound Arf1 is a
320 putative activator of PI4KB [21]. In c10orf76 knockout cells there was a redistribution of
321 GBF1, with GBF1 being more diffuse, with less localized at the Golgi (**Fig. 4C**). The
322 generation of active GTP-bound Arf1 by Arf-GEFs leads to recruitment of multiple effector
323 proteins, with one of most well characterised being the coatamer proteins, which form COPI
324 coated vesicles that mediate Golgi to ER trafficking. Antibody staining with the CM1 antibody,
325 which only recognizes the native form of coatamer, showed similar Golgi distribution for both
326 WT and c10orf76 knockout cells. However, antibodies recognizing COP- β and - α/γ subunits,
327 which associate with GTP-bound Arf1, not only showed staining in the Golgi, but also diffuse
328 staining in the cytosol in c10orf76 knockout cells which was not observed in wild type cells
329 (**Fig. 4C**). Together, these results suggest that c10orf76 plays a key role in Arf1 activation,
330 likely providing a mechanism for increased PI4P levels driven by c10orf76.

331

332 **Replication of c10orf76-dependent enteroviruses requires intact c10orf76-PI4KB**
333 **interaction**

334 All enteroviruses depend on PI4KB kinase activity for replication. Despite the physical
335 and functional connection between PI4KB and c10orf76, enteroviruses showed different
336 dependencies on c10orf76 [28]. Specifically, while Coxsackievirus A10 (CVA10) replication
337 was impaired in c10orf76 knockout cells, the replication of CVB1 was not. Furthermore,
338 c10orf76 was identified as a pro-viral factor for replication of poliovirus (PV1) [45]. We set
339 out to investigate the importance of the c10orf76-PI4KB interaction for replication of CVA10
340 and PV1. We first made a side-by-side comparison of virus replication in HAP1 wildtype and
341 c10orf76 knockout cells in a single cycle of replication. The replication of CVA10 was
342 significantly impaired in c10orf76 deficient cells, with partial inhibition of PV1 replication,
343 and no impairment for replication of CVB3 (**Fig. 5A**). Due to the notoriously difficult nature
344 of transfecting HAP1 cells, we determined the importance of the c10orf76-PI4KB interaction
345 for virus replication in HeLa PI4KB knockout cells transfected with different PI4KB
346 expression plasmids as previously described [29]. Expression of wild type PI4KB efficiently
347 restored the replication of all viruses (**Fig. 5B**). Expression of the PI4KB RL494EA mutant
348 that is deficient in binding c10orf76 fully rescued replication in CVB3, only partially rescued
349 PV1 replication, and failed to rescue CVA10 replication. These observations suggest that the
350 c10orf76-PI4KB interaction is necessary for CVA10, and to a lesser extent, PV1 replication
351 and thereby implies that functions of c10orf76 are selectively hijacked by specific viruses.

352

353 **Discussion**

354 Defining the full complement of cellular roles for PI4KB is an important objective in
355 characterizing the integrated control of secretion and membrane trafficking at the Golgi, and
356 also provides a framework for understanding how PI4P can be manipulated by viruses. We
357 have identified the c10orf76-PI4KB interaction as an important Golgi signaling complex and
358 a critical factor in the replication of specific enteroviruses. Multiple mechanisms have been
359 previously described for how PI4KB participates in Golgi signaling and membrane trafficking,
360 including detailed insights into protein binding partners, post-translational modifications, and
361 regulated recruitment to specific membrane compartments. PI4KB was originally identified in
362 yeast (yeast protein PIK1) as an essential gene [46], with its activity playing a key role in
363 secretion from the Golgi [47]. The mammalian isoform was identified soon afterwards through

364 its sensitivity to wortmannin [48-50]. The first identified Golgi activator of PI4KB was the
365 GTPase Arf1 [21]. However, no direct interaction has been established, which indicates a
366 potential indirect mechanism of activation. Phosphorylation of PI4KB by PKD at Ser294
367 mediates binding to 14-3-3 proteins, with this leading to an increase in PI4KB activity [22,23],
368 that has been suggested to correspond with an increase in PI4KB stability [24]. The most well
369 validated protein binding partner that regulates Golgi recruitment of PI4KB is ACBD3
370 (previously referred to as GCP60) [13]. ACBD3 forms a direct, high-affinity interface with
371 PI4KB that is mediated by a disorder-to-order transition in the N-terminus of PI4KB upon
372 binding to the Q domain of ACBD3 [11,12]. The recruitment of PI4KB to the Golgi by ACBD3
373 is controlled through the direct interaction of the GOLD domain of ACBD3 with the Golgi
374 resident transmembrane protein Giantin [51]. In addition to regulatory protein interactions,
375 PI4KB is predicted to contain an ALPS motif at the C-terminus that mediates lipid binding to
376 unsaturated membranes [9]. PI4KB plays key non-catalytic roles through its interaction with
377 the GTPase Rab11, with PI4KB required for localizing a pool of Rab11 to the Golgi and TGN
378 [52]. This interaction is mediated through a non-canonical, nucleotide-independent binding
379 interface with the helical domain of PI4KB [14]. However, there are still many unexplained
380 aspects of PI4KB recruitment and regulation, highlighted by the increased recruitment of
381 PI4KB to the Golgi following treatment with PI4KB inhibitors that is concomitant with a
382 decrease in Golgi PI4P levels [44].

383 The protein c10orf76 was originally identified as a putative PI4KB interacting partner
384 through co-immunoprecipitation experiments using tagged PI4KB [17,27]. Tests of genetic
385 essentiality identified c10orf76 as a central molecular hub at the Golgi, with it being
386 synthetically lethal in combination with the loss of several different Golgi-signaling proteins,
387 and also showing a genetic link to PI4KB [28]. That study also found that c10orf76 is essential
388 in the KBM7 CML cell line, but not in HAP1 cells, with this relationship also being true for
389 PI4KB. Additional evidence on the essentiality of this protein is highlighted by the
390 homozygous mutant of ARMH3, the mouse homolog of c10orf76, which is lethal at the pre-
391 weaning stage [53]. c10orf76 is highly conserved in vertebrates and we find a strong correlation
392 between the conservation of the kinase linker region of PI4KB and the PI4KB-binding site in
393 c10orf76, suggesting that a key role of c10orf76 is linked to its ability to form a complex with
394 PI4KB. PI4KB recruitment to the Golgi is not mediated by c10orf76, but instead it appears that
395 PI4KB is responsible for the Golgi-recruitment of c10orf76. *In vitro*, c10orf76 led to decreased
396 lipid kinase activity of PI4KB. However, knockout of c10orf76 in cells led to reduced PI4P
397 levels. This discrepancy could be due to the lack of other interacting partners *in vitro*, such as

398 Arf1/GBF1. c10orf76 knockout led to an increased cytosolic fraction of Arf1 effectors and the
399 Arf GEF GBF1. Our work reveals c10orf76 as a novel player in Arf1 regulation, with c10orf76
400 required for maintaining active Arf1 and corresponding Golgi PI4P levels.

401 Enteroviruses hijack numerous lipid signaling processes within infected cells to
402 mediate their replication through the generation of replication organelles, with recruitment of
403 PI4KB [4] and GBF1 [33] playing key roles in this process. Recruitment of these cellular host
404 factors in enteroviruses is primarily mediated through the action of membrane-bound viral 3A
405 proteins, which form either direct or indirect interactions that are important for facilitating
406 replication organelle formation. One of the most well-conserved 3A binding partners in
407 enteroviruses is the Golgi resident protein ACBD3, which interacts with the central part of 3A
408 and recruits as well as activates PI4KB [11,13,18,29,31]. The N-terminal part of the 3A
409 proteins from several enteroviruses (*e.g.*, poliovirus and coxsackie virus B3) directly binds and
410 recruits GBF1, but this interaction is less conserved, severely reduced, or even absent in the
411 3A proteins of rhinoviruses due to subtle amino acid differences in their N-terminus [18,33].
412 We find that c10orf76 is required for replication of coxsackie virus A10 and, to a lesser extent,
413 poliovirus and that c10orf76-dependent viruses rely on the c10orf76-PI4KB interface.
414 Poliovirus is the causative agent of poliomyelitis, and coxsackie virus A10 is an important
415 cause of outbreaks of hand-foot-and-mouth disease, but which is also associated with severe,
416 and sometime fatal, clinical symptoms such as aseptic meningitis. Remarkably, replication of
417 coxsackie virus B1 [28] and coxsackie virus B3 (this study) is independent of c10orf76. Why
418 the c10orf76-PI4KB interface is necessary for replication of some enteroviruses, but not others,
419 is unknown. The differential dependence on c10orf76 could possibly be explained by distinct
420 affinity of 3A proteins from different viruses towards GBF1. Alternatively, each virus may
421 require specific threshold PI4P level for efficient formation of its replication complexes or
422 replication organelles. More research on the dependence of viral replication on either GBF1 or
423 c10orf76-mediated alteration of PI4P levels is required to better understand how enteroviruses
424 hijack these complex membrane trafficking processes.

425 Direct inhibition of PI4KB is likely not a useful antiviral strategy due to unexpected
426 deleterious side effects of PI4KB inhibition in animal models [54]. The targeting of other
427 cellular host factors used to manipulate PI4KB signaling or feedback is a potential avenue for
428 development of novel antiviral therapeutics. Identification of a direct high-affinity c10orf76-
429 PI4KB complex that regulates the cellular localization of c10orf76 represents key insight into
430 the multifaceted regulation of PI4KB signaling. The important role of the c10orf76-PI4KB
431 complex in the replication of select enteroviruses represents a novel molecular platform which

432 is targeted by viruses that hijack lipid signaling. The involvement of c10orf76 in Arf1
433 dynamics, as well as the dependence on PI4KB for Golgi localization of c10orf76, reveals a
434 potential role of the c10orf76-PI4KB complex in Arf1 activation and subsequent PI4P
435 production.

436

437 **Materials and Methods**

438 **Protein expression and purification**

439 *c10orf76 and PI4KB*

440 The human *C10orf76* gene (Uniprot Q5T2E6) was synthesized by GeneArt
441 (ThermoFisher). c10orf76 and PI4KB (Uniprot Q9UBF8-2) were each expressed with an N-
442 terminal 6xHis-tag followed by a TEV protease site. The c10orf76 and PI4KB proteins purified
443 for HDX-MS were expressed in *Spodoptera frugiperda* (*Sf9*) cells by infecting 1-4 L of cells
444 at a density of 1.5×10^6 cells/mL with baculovirus encoding the kinase. After 60-72 hours
445 infection at 27°C, *Sf9* cells were harvested and washed in phosphate-buffered saline (PBS).
446 The c10orf76 and PI4KB proteins utilized for assays, mutational analysis and studying PKA
447 phosphorylation were expressed in Rosetta (DE3) *E. coli* (c10orf76) or BL21 C-41(DE3) *E.*
448 *coli* (PI4KB) induced overnight at 16 °C with 0.1 mM IPTG at an OD₆₀₀ of 0.6. Cell pellets
449 containing c10orf76 or PI4KB were sonicated in NiNTA Buffer (20 mM Tris-HCl pH 8.0, 100
450 mM NaCl, 20 mM imidazole, 5% (v/v) glycerol, 2 mM β-mercaptoethanol) containing protease
451 inhibitors (Millipore Protease Inhibitor Cocktail Set III, Animal-Free) for 5 minutes on ice.
452 Triton X-100 (0.1% v/v) was added to the cell lysate and the lysed cell solution was centrifuged
453 for 45 minutes at 20,000 x g at 2°C. Supernatant was filtered through a 5 μm filter and loaded
454 onto a 5 mL HisTrap™ FF crude (GE) column in NiNTA buffer. The column was washed with
455 1.0 M NaCl and 20 mM imidazole in NiNTA buffer and protein was eluted with 200-250 mM
456 imidazole in NiNTA buffer. Eluted c10orf76 or PI4KB was pooled and concentrated onto a 5
457 mL HiTrap™ Q column (GE) equilibrated with Q buffer (20 mM Tris-HCl pH 8.0, 100 mM
458 NaCl, 5% glycerol v/v, 2 mM β-mercaptoethanol) and eluted with an increasing concentration
459 of NaCl. Protein was pooled and concentrated using an Amicon 30K concentrator and
460 incubated overnight on ice with the addition of TEV protease. Size exclusion chromatography
461 (SEC) was performed using a Superdex™ 200 10/300 GL increase (GE) column equilibrated
462 in SEC buffer (20 mM HEPES pH 7.5, 150 mM NaCl and 0.5 mM TCEP). Fractions containing
463 the protein of interest were pooled, concentrated, spun down to remove potential aggregate and
464 flash frozen in liquid nitrogen for storage at -80 °C. c10orf76-PI4KB complex SEC trace was

465 generated by mixing c10orf76 and PI4KB in a 1:1 ratio after individual anion exchange runs
466 and then injecting onto the Superdex™ 200 10/300 GL increase (GE) column. Elution volumes
467 of protein standards were obtained from the GE Instruction 29027271 AH Size exclusion
468 chromatography columns document. See *Protein Kinase A (PKA) treatment of PI4KB* for
469 details on producing the phosphorylated variant of PI4KB.

470

471 *ACBD3, Rab11a and PKA*

472 ACBD3 and Rab11a were expressed with N-terminal GST tags, with Protein kinase A
473 (*M. musculus* PKA catalytic subunit alpha; Addgene 14921) expressed with an N-terminal His
474 tag. ACBD3, Rab11a, and PKA were expressed in BL21 C-41(DE3) *E. coli* cells, with ACBD3
475 and Rab11 expression carried out overnight at 16 °C with 0.1 mM IPTG, and PKA expression
476 was carried out for 4 hours at 28 °C with 1 mM IPTG. ACBD3, Rab11, and PKA were purified
477 as previously published [11,14,55]. In brief, cell pellets containing expressed ACBD3 or
478 Rab11a were sonicated in Q Buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 5% (v/v) glycerol,
479 2 mM β -mercaptoethanol) containing protease inhibitors (Millipore Protease Inhibitor Cocktail
480 Set III, Animal-Free) for 5 minutes on ice. Triton X-100 (0.1% v/v) was added to the cell lysate
481 and the lysed cell solution was centrifuged for 45 minutes at 20,000 x g at 2°C. Supernatant
482 was filtered through a 5 μ m filter and incubated with 1-4mL of Glutathione Sepharose™ 4B
483 beads (GE) for 1-2 hours at 4°C. Beads were then washed with Q buffer, and GST-tagged
484 proteins were eluted with 20 mM glutathione in Q buffer. Protein was further purified using
485 anion exchange and size-exclusion chromatography as described above and final protein was
486 spun down to remove potential aggregate and flash frozen in liquid nitrogen for storage at -80
487 °C. Nickel purification of PKA proceeded as described for PI4KB, and nickel elute was
488 concentrated, spun down to remove potential aggregate and flash frozen in liquid nitrogen for
489 storage at -80 °C.

490

491 **Nickel and GST Pulldown Assays**

492 For His pulldowns, NiNTA agarose beads (Qiagen) (20 μ L) were washed three times
493 by centrifugation and resuspension in NiNTA buffer. His-tagged bait protein was then added
494 to a concentration of 1-3 μ M and incubated with the beads on ice for 10 minutes in a total
495 volume of 50 μ L. Beads were washed three times with 150 μ L NiNTA buffer at 4 °C. Non-
496 His-tagged prey protein was then added to a final concentration of 1-2 μ M in a total volume of
497 50 μ L, at which point 10 μ L was taken for SDS-PAGE analysis. The mixture was incubated

498 on ice for an additional 30 minutes and then washed four times with 120 μ L NiNTA buffer at
499 4 °C at which time an aliquot was taken as the output for SDS-PAGE analysis.

500 For GST pulldowns, Glutathione Sepharose™ 4B beads (GE healthcare) were washed
501 three times by centrifugation and resuspension in Q buffer. GST-tagged bait protein (or control
502 GST) was then added to a concentration of 3-6 μ M in 50 μ L and incubated with the beads on
503 ice for 10 minutes in a total volume of 50 μ L. Beads were washed three times with 150 μ L Q
504 buffer at 4 °C. Non-GST-tagged prey protein were then added to a final concentration of 2-4
505 μ M in a total volume of 50 μ L, at which point the input was taken for SDS-PAGE analysis.
506 The mixture was incubated on ice for an additional 30 minutes and then washed four times
507 with 120 μ L Q buffer at 4 °C, at which time an aliquot was taken as the output for SDS-PAGE
508 analysis.

509

510 **Vesicle Preparation and Lipid Kinase Assays**

511 Lipid kinase assays were carried out using the Transcreener® ADP² FI Assay
512 (BellBrook Labs) following the published protocol as previously described [11]. In brief,
513 substrate stocks were made up containing 1.0 mg/mL PI vesicles or 4.0 mg/mL Golgi-mimetic
514 vesicles (10% PS, 20% PI, 25% PE, 45% PC) and were extruded through a 100 nm Nanosizer
515 Extruder (T&T Scientific) and then combined with in a buffer containing 20 mM Hepes pH
516 7.5, 100 mM KCl and 0.5 mM EDTA (200 μ M ATP with 1.0 mg/mL PI vesicles, 20 μ M ATP
517 with 1.0 mg/mL Golgi-mimetic vesicles). Kinase reactions were started by adding 2 μ L of this
518 substrate stock in a 384-well black low volume plates (Corning 4514). Proteins were thawed
519 on ice and spun down to remove precipitate. Proteins were diluted individually to 4X the
520 desired concentration in Kinase Buffer (40 mM Hepes pH 7.5, 200 mM NaCl, 20 mM MgCl₂,
521 0.8% Triton-X, and 0.2 mM TCEP) on ice. Proteins were then mixed together or with additional
522 Kinase buffer resulting in 2X desired concentrations of each protein. To start the reaction, 2
523 μ L of 2X protein stock was added to 2 μ L of 2X substrate stock in plates. After mixing, the 4
524 μ L reactions consisted of 30 mM HEPES pH 7.5 (RT), 100 mM NaCl, 50 mM KCl, 10mM
525 MgCl₂, 0.25 mM EDTA, 0.4% (v/v) Triton-X, 0.1 mM TCEP, 10 μ M ATP and 0.5 mg/mL
526 vesicles. PI4KB was run at a final concentration of 15 nM, 20 nM or 40 nM and c10orf76 was
527 run in 4-fold curves from 1 μ M – 3.9 nM or 5-fold curves from 2 μ M – 1.6 nM. Reactions
528 proceeded at 23°C for 20-30 minutes. Reactions were stopped using 4 μ L of the transcreener
529 stop buffer (1X Stop & Detect Buffer B, 8 nM ADP Alexa594 Tracer, 97 μ g/ml ADP2
530 Antibody-IRDye® QC-1). Fluorescence intensity was measured using a Spectramax M5 plate

531 reader with $\lambda_{\text{exc}} = 590$ nm and $\lambda_{\text{em}} = 620$ nm (20nm bandwidth). Data was plotted using
532 Graphpad Prism software, with IC_{50} values determined by nonlinear regression (curve fit). No
533 detectable nonspecific ATPase activity was detected in reactions containing 250 nM wild-type
534 PI4KB without vesicle substrate.

535

536 **Mapping the c10orf76-PI4KB binding interface using HDX-MS**

537 HDX reactions were conducted in 50 μ L reactions with a final concentration of 400
538 nM of protein per sample (c10orf76-PI4KB, 400 nM each). Reactions were initiated by the
539 addition of 45 μ L of D₂O Buffer Solution (10 mM HEPES pH 7.5, 50 mM NaCl, 97% D₂O)
540 to 5 μ L of protein solution, to give a final concentration of 87% D₂O. Exchange was carried
541 out for four timepoints, (3s at 1°C and 3s, 30s, and 300s at 23 °C). Exchange was terminated
542 by the addition of acidic quench buffer giving a final concentration 0.6 M guanidine-HCl and
543 0.8% formic acid. All experiments were carried out in triplicate. Samples were immediately
544 frozen in liquid nitrogen and stored at -80°C until mass analysis.

545

546 **Comparison of FLH409AAA and WT c10orf76 secondary structure**

547 HDX-MS reactions were performed with 40 μ L final volume with a protein
548 concentration of 0.25 μ M in each sample. Reactions were started by the addition of 39 μ L D₂O
549 buffer (100mM NaCl, 35 mM Hepes, 91.7% D₂O) to 1 μ L of protein (Final: 89.4% D₂O).
550 Reactions were quenched by the addition of 30 μ L of acidic quench buffer (3% formic acid, 2M
551 Guanidine) resulting in final 1.28% Formic acid and 0.85M guanidine-HCl. Proteins were
552 allowed to undergo exchange reactions for either 3s or 300s at 23°C prior to addition of quench
553 buffer and flash freezing in liquid N₂. All samples were set and run in triplicate. Samples were
554 stored at -80°C until injection onto the UPLC for MS analysis.

555

556 **HDX-MS data analysis**

557 Protein samples were rapidly thawed and injected onto a UPLC system kept in a cold
558 box at 2°C. The protein was run over two immobilized pepsin columns (Applied Biosystems;
559 porosyme, 2-3131-00) stored at 10°C and 2°C at 200 μ L/min for 3 min and the peptides were
560 collected onto a VanGuard precolumn trap (Waters). The trap was subsequently eluted in line
561 with an Acquity 1.7 μ m particle, 100 \times 1 mm² C18 UPLC column (Waters), using a gradient
562 of 5-36% B (buffer A 0.1% formic acid, buffer B 100% acetonitrile) over 16 minutes. MS
563 experiments were performed on an Impact QTOF (Bruker) and peptide identification was done

564 by running tandem MS (MS/MS) experiments run in data-dependent acquisition mode. The
565 resulting MS/MS datasets were analyzed using PEAKS7 (PEAKS) and a false discovery rate
566 was set at 1% using a database of purified proteins and known contaminants. HD-Examiner
567 Software (Sierra Analytics) was used to automatically calculate the level of deuterium
568 incorporation into each peptide. All peptides were manually inspected for correct charge state
569 and presence of overlapping peptides. Deuteration levels were calculated using the centroid of
570 the experimental isotope clusters. Attempts at generating fully deuterated protein samples to
571 allow for the control of peptide back exchange levels during digestion and separation were
572 made for all proteins. Protein was incubated with 3M guanidine for 30 minutes prior to the
573 addition of D₂O, where they were further incubated for an hour on ice. The reactions were then
574 quenched as before. Generation of a fully deuterated sample was successful for PI4K using this
575 method, however generation of fully deuterated c10orf76 failed. Results for c10orf76 are
576 therefore presented as relative levels of deuterium incorporation and the only control for back
577 exchange was the level of deuterium present in the buffer (87%). The average error of all time
578 points and conditions for each HDX project was less than 0.2 Da. Therefore, changes in any
579 peptide at any time point greater than both 7% and 0.5 Da between conditions with an unpaired
580 t-test value of p<0.05 was considered significant. The full details of H/D exchange for all
581 peptides are shown in Source data, with statistics described in **Supplemental Table 1**.

582

583 **Protein Kinase A (PKA) Treatment of PI4KB**

584 PKA (mouse catalytic subunit) was serially diluted and different concentrations were
585 incubated with PI4KB in 20 µL reactions on ice for 1 hour (20 µg PI4KB, 20 mM MgCl₂, 200
586 µM ATP and either 840 ng, 168 ng, 34 ng, 7 ng or 0 ng PKA). Reactions were terminated by
587 the addition of acidic quench buffer giving a final concentration 0.6 M guanidine-HCl and
588 0.8% formic acid and then flash frozen in liquid N₂ prior to MS phosphorylation analysis.

589 To generate *E. coli* expressed, PKA phosphorylated PI4KB for use in kinase assays and
590 HDX-MS, phosphorylation of Ser496 was carried out using 1.0 mg PI4KB, 20 mM MgCl₂,
591 200 µM ATP and 4.2 µg PKA in NiNTA buffer, with the reaction allowed to proceed for 1
592 hour on ice. The reaction was quenched with 20 mM EDTA, and immediately loaded onto a
593 GE 1 mL HisTap FF crude to remove His-tagged PKA. Phosphorylated PI4KB was
594 concentrated followed by size exclusion chromatography as described for PI4KB above. In
595 tandem, a non-phosphorylated PI4KB control was purified in the same manner except MgCl₂,
596 ATP, and PKA were not added. Protein was flash frozen in liquid N₂ for storage at -80 °C.

597

598 **HDX-MS dose response of c10orf76 of phosphorylated PI4KB**

599 Phosphorylated and non-phosphorylated PI4KB were generated and purified as described
600 above. HDX reactions were conducted in 130 μ l reaction volumes with a final concentration
601 of 20nM PI4KB (phosphorylated or non-phosphorylated) per sample, with 0 nM, 5 nM, 10 nM,
602 20 nM, 40 nM, 80 nM, 160 nM and 320 nM c10orf76. Exchange was carried out for 5 seconds,
603 in triplicate for each concentration of c10orf76. Hydrogen deuterium exchange was initiated
604 by the addition of 80 μ l of D₂O buffer solution (10 mM HEPES (pH 7.5), 50 mM NaCl, 97%
605 D₂O) to the protein solution, to give a final concentration of 60% D₂O. Exchange was
606 terminated by the addition of 20 μ l ice cold acidic quench buffer at a final concentration 0.6 M
607 guanidine-HCl and 0.9% formic acid. Samples were immediately frozen in liquid nitrogen at
608 – 80 °C. Data analyzed as described above in *HDX-MS data analysis*.

609

610 **Phosphorylation Analysis**

611 LC-MS/MS analysis of phosphorylated variants of PI4KB was carried out as described
612 in the HDX-MS data analysis section. MS/MS datasets were analyzed using PEAKS7 to
613 identify phosphorylated peptides in PI4KB and c10orf76. A false discovery rate was set at
614 0.1% using a database of purified proteins and known contaminants. To measure PI4KB
615 phosphorylation levels using Bruker Data analysis, the phosphorylated and non-
616 phosphorylated peptides of interest were extracted, and the total area of each peptide was
617 manually integrated to determine the amount of phosphorylated vs non-phosphorylated species
618 under given experimental conditions. No phosphorylation was detected in *E. coli* derived
619 PI4KB. For *Sf9* derived PI4KB Ser294 phosphorylation, the peptides KRTAS*NPKVENEDE
620 (290-303) and KRTAS*NPKVENEDEPVRLADERE (290-312) were averaged, for Ser413
621 phosphorylation DTTSVPARIPENRIRSTRS*VENLPECGITHE (395-425) was used, for
622 Ser430 phosphorylation GITHEQRAGS*F (430-441) was used, and for Ser496
623 phosphorylation IAAGDIRRRLS*EQLAHTPTA (486-505) and IAAGDIRRRLS*EQ-
624 LAHTPTAF (486-506) were averaged. No phosphorylation was detected in *E. coli* derived
625 c10orf76. For *Sf9* derived c10orf76 Ser14 phosphorylation, LRKSS*ASKKPLKE (10-22) was
626 used, and for the 325-351 phosphorylation (exact location of phosphorylation ambiguous)
627 VTPVSPAPTTPTPLGTTTPSSD (326-348), VTPVSPAPTTPTPLGTTTPSSDVISS
628 (325-351) and VTPVSPAPTTPTPLGTTTPSS (325-347) were averaged.

629

630 **Alignments**

631 Protein sequences from the Uniprot database were aligned using Clustal Omega [56]
632 and figures were generated using ESPript [57]. Uniprot PI4KB entries used: *H. sapiens*
633 (Q9UBF8-2), *M. musculus* (Q8BKC8), *D. rerio* (Q49GP3), *D. melanogaster* (Q9BKJ2), *C.*
634 *elegans* (Q20077). Uniprot c10orf76 entries used: *H. sapiens* (Q5T2E6), *M. musculus*
635 (Q6PD19), *D. rerio* (Q6PGW3), *D. melanogaster* (Q7KSU3).

636

637 **DNA Constructs and Antibodies**

638 The following antibodies were used to examine protein localization in WT and
639 c10orf76 knockout HAP1 cells. Mouse monoclonal antibodies included anti-GBF1 (BD
640 Biosciences), anti-CM1 (a gift from Felix Wieland, Heidelberg University, Germany), anti-
641 GM130 (BD Biosciences), anti-Giantin (Enzo Life Science), anti-ERGIC53 (Enzo Life
642 Science), anti- β COP (Sigma), anti-PI4P (Echelon). Rabbit polyclonal antibodies included anti-
643 PI4KB (Millipore), anti-COPI α/γ (a gift from Felix Wieland), anti-TGN46 (Novus
644 Biologicals). Conjugated goat anti-rabbit and goat anti-mouse Alexa Fluor 488, 596, or 647
645 (Molecular Probes) were used as secondary antibodies.

646 GFP-PI4KB, GFP-PI4KB RL494EA, GFP-c10orf76, and GFP-c10orf76 FLH409AAA
647 were cloned using Gibson assembly [58] into the pEGFP-C1 vector (Clontech). mRFP-
648 FKBP12-PI4KB and mRFP-FKBP12-PI4KB RL494EA were generated by amplifying the
649 mRFP-FKBP12 insert from mRFP-FKBP12-5ptpase domain [59] and replacing the N-terminal
650 GFP in either GFP-PI4KB or GFP-PI4KB RL494EA using a single digest with NdeI. AKAP-
651 FRB-CFP, which is used to selectively recruit FKBP12-tagged proteins to the outer
652 mitochondrial membrane, has been described previously [60].

653

654 **Cell Culture, Transfection, and Live-Cell Confocal Microscopy of Rapamycin** 655 **Recruitment**

656 HEK293-AT1 cells, which stably express the AT1a rat Angiotensin II receptor [61],
657 were cultured in Dulbecco's Modified Eagle Medium (DMEM-high glucose) containing 10%
658 (vol/vol) FBS and supplemented with a 1% solution of penicillin/streptomycin. This cell line
659 is regularly tested for *Mycoplasma* contamination using a commercially-available detection kit
660 (InvivoGen) and, after thawing, the cells are treated with plasmocin prophylactic (InvivoGen)
661 at 500 μ g/ml for the initial three passages (6-9 days) as well as supplemented with 5 μ g/ml of
662 plasmocin prophylactic for all subsequent passages.

663 For confocal microscopy, HEK293-AT1 cells (3×10^5 cells/well) were plated on 29 mm
664 circular glass-bottom culture dishes (#1.5; Cellvis) pre-coated with 0.01% poly-L-lysine

665 solution (Sigma). The cells were allowed to attach overnight prior to transfection of plasmid
666 DNAs (0.1-0.2 $\mu\text{g}/\text{well}$) using Lipofectamine 2000 (Invitrogen) and Opti-MEM (Invitrogen)
667 according to the manufacturer's instructions. Please note that studies using the rapamycin-
668 inducible protein hetero-dimerization system used a 1:2:1 ratio of plasmid DNA for
669 transfection of the FKBP12-tagged PI4KB enzyme, AKAP-FRB-CFP recruiter, and GFP-
670 c10orf76 variant (total DNA: 0.4 $\mu\text{g}/\text{well}$). After 18-20 hr of transfection, cells were incubated
671 in 1 mL of modified Krebs-Ringer solution (containing 120 mM NaCl, 4.7 mM KCl, 2 mM
672 CaCl_2 , 0.7 mM MgSO_4 , 10 mM glucose, 10 mM HEPES, and adjusted to pH 7.4) and images
673 were acquired at room temperature using a Zeiss LSM 710 laser-scanning confocal microscope
674 (Carl Zeiss Microscopy). Rapamycin treatment of cells was carried out at a final concentration
675 of 100 nM. Image acquisition was performed using the ZEN software system (Carl Zeiss
676 Microscopy), while the image preparation was done using the open-source FIJI platform [62].
677

678 **Cell Culture, Transfection, and Live-Cell Confocal Microscopy of HAP1 WT and** 679 **c10orf76 Knockout Cells**

680 *Cells and viruses*

681 HAP1 WT cells and HAP1 c10orf76 knockout cells were obtained from Horizon Discovery.
682 HeLa R19 cells were obtained from G. Belov (University of Maryland and Virginia-Maryland
683 Regional College of Veterinary Medicine, US). HeLa PI4KB knockout cells were described
684 previously [29]. HAP1 cells were cultured in IMDM (Thermo Fisher Scientific) supplemented
685 with 10% fetal calf serum (FCS) and penicillin–streptomycin. HeLa cells were cultured in
686 DMEM (Lonza) supplemented with 10% FCS and penicillin–streptomycin. All cells were
687 grown at 37°C in 5% CO_2 . The following enteroviruses were used: CVA10 (strain Kowalik,
688 obtained from the National Institute for Public Health and Environment; RIVM, The
689 Netherlands), CVB3 (strain Nancy, obtained by transfection of the infectious clone p53CB3/T7
690 as described previously [63], PV1 (strain Sabin, ATCC). Virus titers were determined by end-
691 point titration analysis and expressed as 50% tissue culture infectious dose (TCID_{50}).
692

693 *Replication rescue assay*

694 HeLa cells were transfected with plasmids carrying WT or mutant PI4KB (RL494EA),
695 Golgi-targeting EGFP (pEGFP-GalT) or kinase-dead PI4KB (PI4KB-KD) as a negative
696 control. At 24 h post-transfection, the cells were infected with CVA10, CVB3, and PV1. At 8
697 h p.i., the infected cells were frozen, and virus titers were determined by end-point titration
698 analysis and expressed as 50% tissue culture infectious dose (TCID_{50}).

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Immunofluorescence microscopy of WT and c10orf76 knockout HAP1 cells

HAP1 cells were grown on ibiTreat slides μ -slide 18-wells (Ibidi) one day prior to infection. Cells were fixed by submersion in a 4% paraformaldehyde solution for 15 minutes. Nuclei were stained with DAPI. Confocal imaging was performed with a Leica SpeII confocal microscope.

Author contributions

JAM, RMH, MLJ, and JTBS expressed and purified proteins. JAM and JEB designed complex-disrupting mutations. JAM carried out pulldowns and kinase assays. JAM, MLJ, RMH and JEB carried out HDX-MS and analysis. HRL and WvE performed viral infection assays, and HRL characterized c10orf76 knockout cells. JGP and TB performed cellular c10orf76 recruitment experiments. JAM, JRPMS, TB, FJMK, and JEB designed the research. JAM and JEB wrote the manuscript with input from all authors.

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Conflict of interest

The authors declare that they have no conflict of interest

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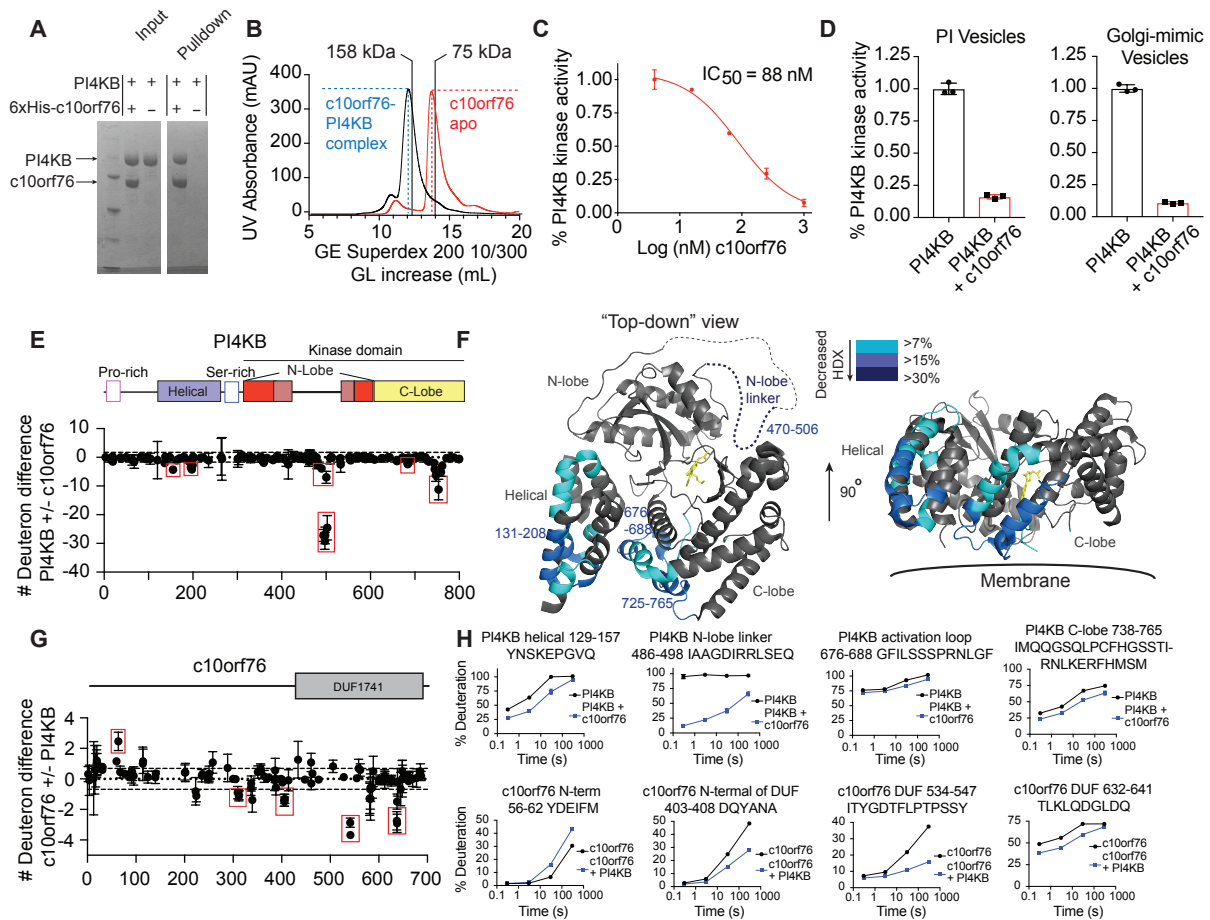
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929 **Figure Legends**



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932 **Figure 1. c10orf76 directly binds to PI4KB through an extended interface focused at the**
 933 **N-lobe kinase linker of PI4KB**

934 **(A)** Recombinant c10orf76 directly binds to PI4KB *in vitro*. His-pulldown assays of
 935 baculovirus/*Sf9* produced 6xHis-c10orf76 (3 μ M) were carried out with untagged PI4KB (2.5
 936 μ M).

937 **(B)** PI4KB and c10orf76 form a stable complex. The complex of c10orf76-PI4KB eluted from
 938 a S200 superdex 10/300 GL increase gel filtration column (GE) at a volume consistent with a
 939 heterodimer (169 kDa), while c10orf76 alone eluted at a volume consistent with a monomer
 940 (79 kDa). Lines with MW values indicate elution of MW standards (158 kDa aldolase, 75 kDa
 941 conalbumin).

942 **(C)** PI4KB is potently inhibited by c10orf76 in a dose-dependent manner *in vitro*. Kinase
 943 assays of PI4KB (20 nM) in the presence of varying concentrations of c10orf76 (1.6 nM-1 μ M)
 944 were carried out on pure PI lipid vesicles (0.5 mg/L) in the presence of 100 μ M ATP. The data
 945 was normalized to the kinase activity of PI4KB alone. IC₅₀ values were determined by one

946 binding site, nonlinear regression (curve fit) using Graphpad. Error bars represent standard
947 deviation (n=3).

948 **(D)** PI4KB is potently inhibited by c10orf76 on pure PI vesicles and vesicles mimicking Golgi
949 composition. Kinase assays of PI4KB and c10orf76 were carried out on lipid substrate
950 composed of pure PI vesicles (0.5 mg/mL) with 100 μ M ATP, and Golgi mimic vesicles (0.5
951 mg/ml, 10% PS, 20% PI, 25% PE, 45% PC) with 10 μ M ATP. PI4KB was present at 20 and
952 300 nM in the PI and Golgi substrate assays respectively, with c10orf76 present at 500 nM in
953 both experiments. The data is normalized to the kinase activity of PI4KB alone. Error bars
954 represent standard deviation (n=3).

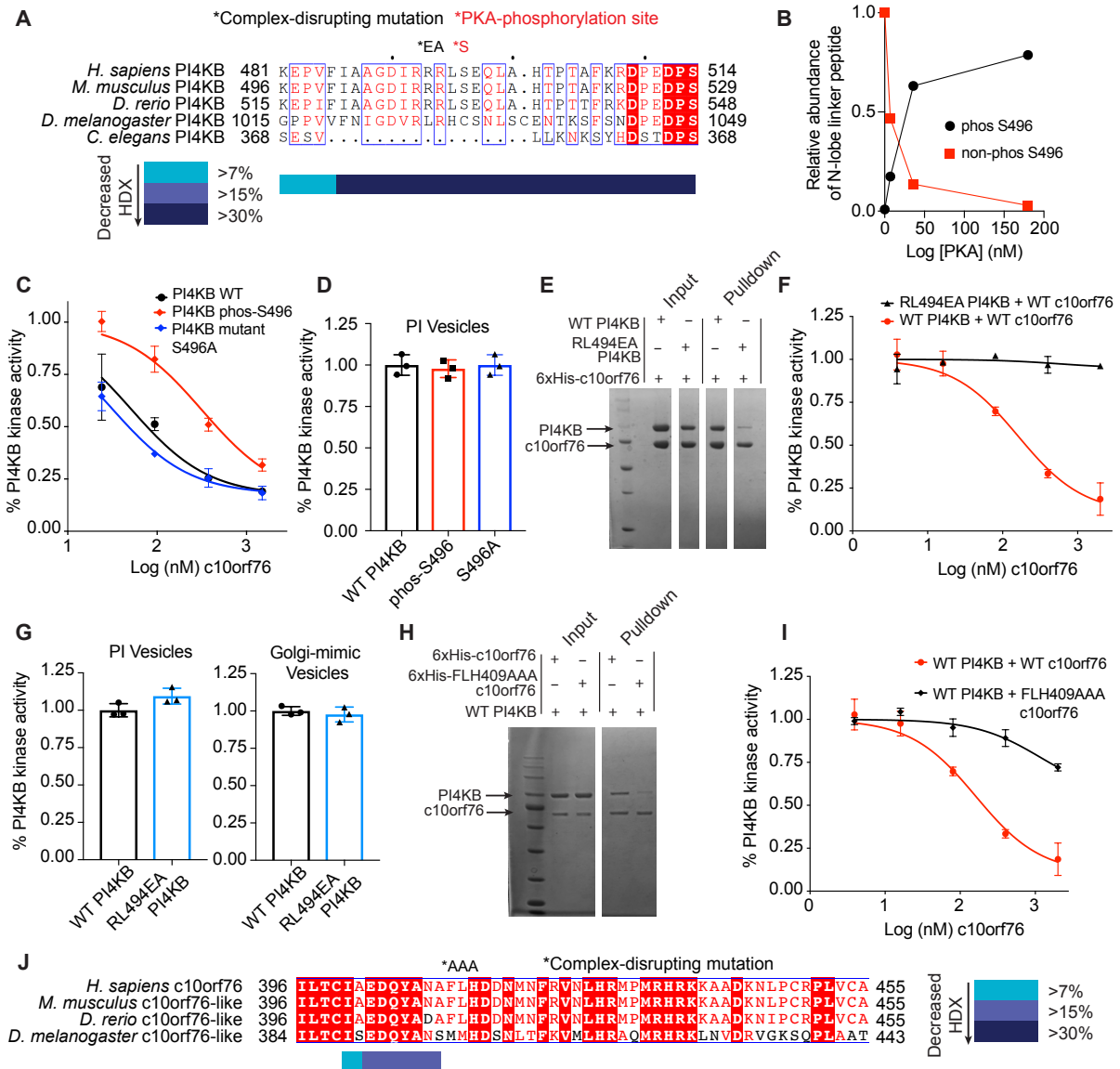
955 **(E)** Changes in deuterium incorporation PI4KB in the presence of c10orf76 showed a profound
956 ordering of the kinase domain N-lobe linker and smaller changes in the helical domain and C-
957 lobe of the kinase domain. The sum of the difference mapped as the difference in number of
958 deuterons incorporated for PI4KB (400 nM) in the presence and absence of c10orf76 (400 nM)
959 over all time points (3s at 1 $^{\circ}$ C; 3s, 30s, and 300s at 23 $^{\circ}$ C). Each dot represents a peptide
960 graphed on the x-axis according to the central residue. The red boxes highlight key regions that
961 showed significant changes (>7% decrease in exchange, >0.5 Da difference, and unpaired two-
962 tailed student t-test $p < 0.05$). For all panels error bars represent standard deviation (n=3).

963 **(F)** c10orf76 binding induces differences in HDX throughout multiple domains of PI4KB.
964 Regions of >7% difference in deuterium exchange in the presence of c10orf76 are colored onto
965 the structure of PI4KB according to the legend (PDB: 4D0L). The N-lobe linker of the kinase
966 domain is disordered in the structure and is represented by a dotted line.

967 **(G)** Changes in the deuterium incorporation of c10orf76 in the presence of PI4KB. H/D
968 exchange reactions displayed as the sum of the difference in HDX in the number of deuterons
969 for c10orf76 (400 nM) in the presence of PI4KB (400 nM) at all time points (3s at 1 $^{\circ}$ C; 3s,
970 30s, and 300s at 23 $^{\circ}$ C) analyzed. Red boxes highlight regions that showed significant changes
971 (>7% decrease in exchange, >0.5 Da difference, and unpaired two-tailed student t-test $p < 0.05$).

972 **(H)** The PI4KB N-lobe linker undergoes a disorder-to-order transition upon binding c10orf76.
973 Selected peptides (including the sequence, domain information, and numbering) of both PI4KB
974 and c10orf76 displayed as the % deuteration incorporation over time.

975



976

977 **Figure 2. The PI4KB c10orf76 interface is conserved and can be post-translationally**

978 **modified by PKA, with rationally designed mutations disrupting complex formation**

979 (A) The N-lobe kinase linker region of PI4KB is strongly conserved back to *D. rerio*. The N-
 980 lobe linker region of PI4KB sequences of the organisms indicated were analysed using Clustal
 981 Omega/ ESript 3. The consensus PKA motif (RRXS) that is conserved back to *D. rerio* is
 982 indicated on the sequence, as well as the RL494EA point mutation.

983 (B) The N-lobe kinase linker of PI4KB can be efficiently phosphorylated by PKA.
 984 Recombinant PKA at different concentrations (0, 7, 34, 168, or 840 ng) was incubated with
 985 recombinant (*E. coli*) wild-type PI4KB (20 µg) for 1 hour with 200 µM ATP and the amount
 986 of phosphorylation was followed using mass spectrometry. Relative abundance of Ser496
 987 phosphorylated PI4KB was calculated using the relative intensity (total area) of the
 988 phosphorylated vs non-phosphorylated peptide (486-506).

989 **(C)** PI4KB phosphorylation by PKA alters the affinity for c10orf76. The kinase activity of
990 different variants of PI4KB (15 nM) was measured in the presence of varying amounts of
991 c10orf76 (1.6 nM-2 μ M) with 100% PI lipid substrate (0.5 mg/L) and 100 μ M ATP. The data
992 was normalized to the kinase activity of PI4KB alone Error bars represent standard deviation
993 (n=3).

994 **(D)** PI4KB has the same kinase activity when Ser496 is phosphorylated or mutated to alanine.
995 Kinase assay of PI4KB non-phosphorylated, phos-Ser496 or S496A (15 nM) on pure PI lipid
996 vesicles (0.5 mg/L) with 100 μ M ATP. The data was normalized to the kinase activity of WT
997 PI4KB. Error bars represent standard deviation (n=3).

998 **(E)** Engineered RL494EA PI4KB mutant shows decreased binding to c10orf76. His-pulldown
999 assays of 6xHis-c10orf76 (3 μ M) with wild-type or RL494EA PI4KB (1-2 μ M).

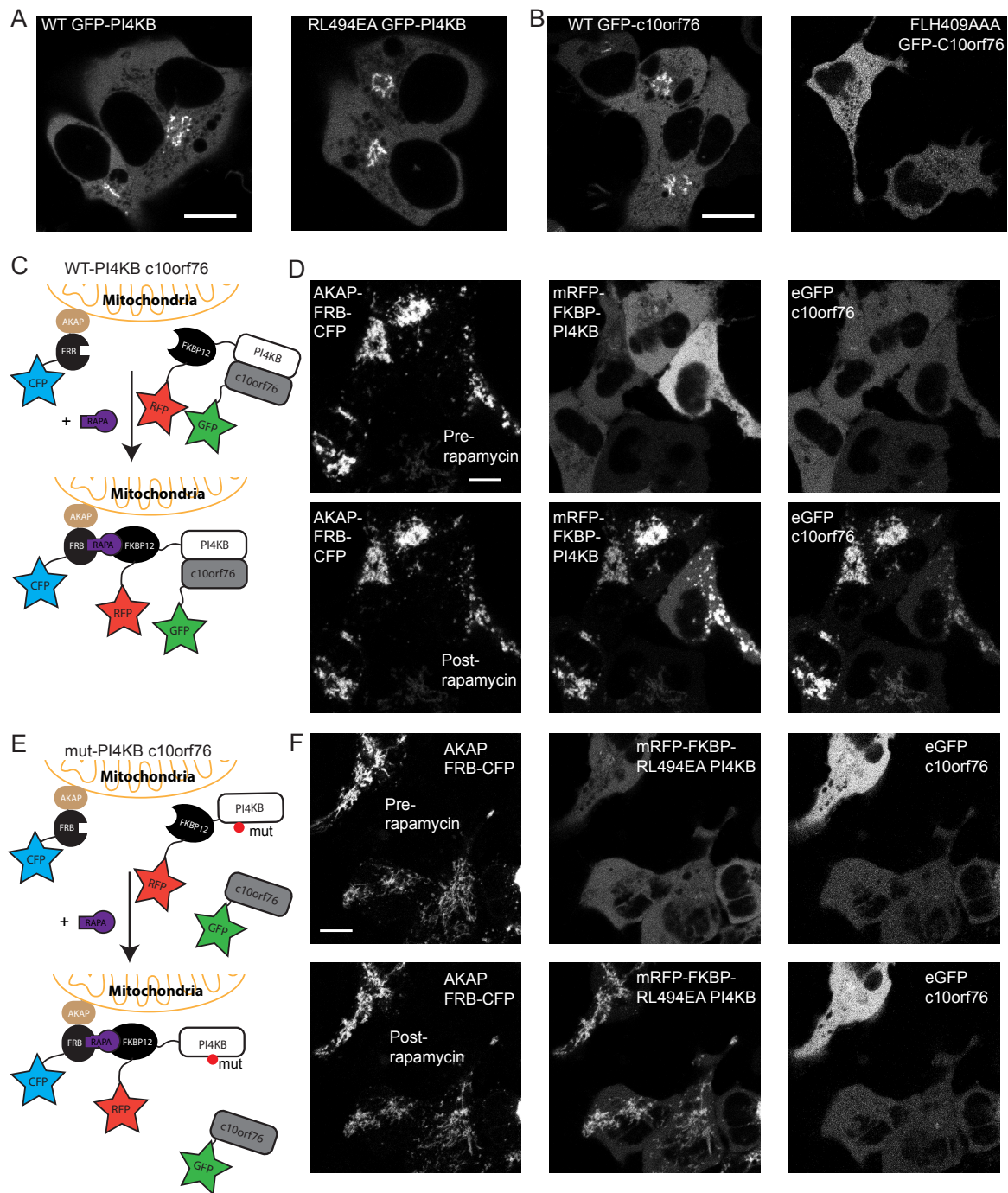
1000 **(F)** RL494EA PI4KB activity is not inhibited by c10orf76. Kinase assays of either wild type
1001 or mutant RL494EA PI4KB (40 nM) were carried out with varying concentrations of c10orf76
1002 (3.9 nM-2 μ M) with 100% PI lipid vesicles (0.5 mg/L) and 100 μ M ATP. The data was
1003 normalized to the kinase activity of PI4KB alone. Error bars represent standard deviation (n=3).

1004 **(G)** Wild-type PI4KB and RL494EA PI4KB mutant have the same lipid kinase activity. Kinase
1005 assays of either wild-type and mutant PI4KB (10 nM) were carried out with 100% PI lipid
1006 vesicles (0.5 mg/L), 100 μ M ATP, and PI4KB (300 nM) on Golgi-mimic vesicles (0.5 mg/mL)
1007 with 10 μ M ATP. The data was normalized to the kinase activity of WT PI4KB. Error bars
1008 represent standard deviation (n=3).

1009 **(H)** FLH409AAA-c10orf76 mutant shows decreased affinity for PI4KB. His-pulldown assays
1010 of 6xHis-c10orf76 (1 μ M) with wild-type PI4KB (1 μ M). Samples washed a total of 4 times.

1011 **(I)** Kinase assay shows FLH409AAA c10orf76 inhibition of PI4KB is greatly reduced. Kinase
1012 assay of PI4KB (40 nM) and a concentration curve of c10orf76 (3.9 nM-2 μ M) on pure PI lipid
1013 vesicles (0.5 mg/L) with 100 μ M ATP. The data was normalized to the kinase activity of PI4KB
1014 alone. Error bars represent standard deviation (n=3).

1015 **(J)** The PI4KB-binding region of c10orf76 is strongly conserved back to *D. rerio*. Clustal
1016 Omega/ ESript 3 alignment of the FLH409 region of c10orf76 that binds PI4KB.
1017
1018



1019

1020 **Figure 3. PI4KB recruits c10orf76 to the Golgi *in vivo***

1021 (A) Transfections of HEK293 cells revealed that both wild-type GFP-PI4KB and RL494EA
1022 GFP-PI4KB localize to the Golgi.

1023 (B) WT c10orf76 also localized to the Golgi, however, the PI4KB binding deficient mutant of
1024 c10orf76 (FLH409AAA) predominantly localized to the cytosol.

1025 (C) Cartoon schematic of rapamycin-inducible mitochondria recruitment. The AKAP1-FRB-
1026 CFP construct is localized to the outer mitochondrial membrane, while the RFP-FKBP12-
1027 PI4KB and GFP-c10orf76 are localized in the Golgi as well as within the cytoplasm where

1028 they can form a complex. Upon addition of rapamycin, the RFP-FKBP12-PI4KB construct is
1029 translocated to the mitochondria.

1030 **(D)** Mitochondria recruitment experiment with wild-type PI4KB and c10orf76. Left: AKAP1-
1031 FRB-CFP is localized to the mitochondria before (top) and 5 minutes after rapamycin (100
1032 nM) treatment (bottom). Middle: RFP-FKBP12-PI4KB is located in the cytosol before
1033 rapamycin (top) and translocates to the mitochondria after rapamycin induction (bottom).
1034 Right: GFP-c10orf76 is located in the cytosol before rapamycin (top) and translocates to the
1035 mitochondria after rapamycin induction (bottom).

1036 **(E)** Schematic of the rapamycin-inducible mitochondria recruitment experiment with mutant
1037 PI4KB and WT c10orf76.

1038 **(F)** Mitochondria recruitment experiment with mutant PI4KB and WT c10orf76. Left: AKAP1-
1039 FRB-CFP is localized to the mitochondria before (top) and 5 minutes after (bottom) rapamycin
1040 treatment. Middle: RFP-FKBP12-PI4KB(RL494EA) is located in the cytosol before
1041 rapamycin (top) and translocates to the mitochondria after rapamycin induction (bottom).
1042 Right: GFP-c10orf76 is located in the cytosol before (top) and after (bottom) rapamycin
1043 induction. Bars represent 10 μ m.

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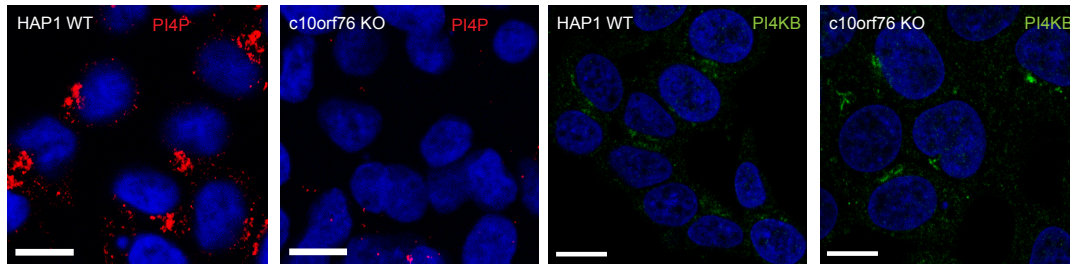
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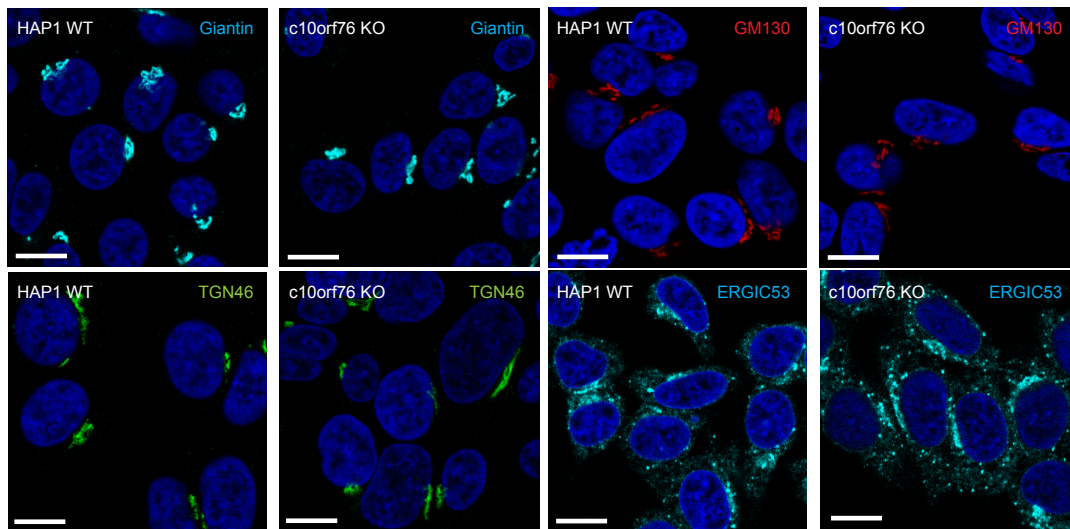
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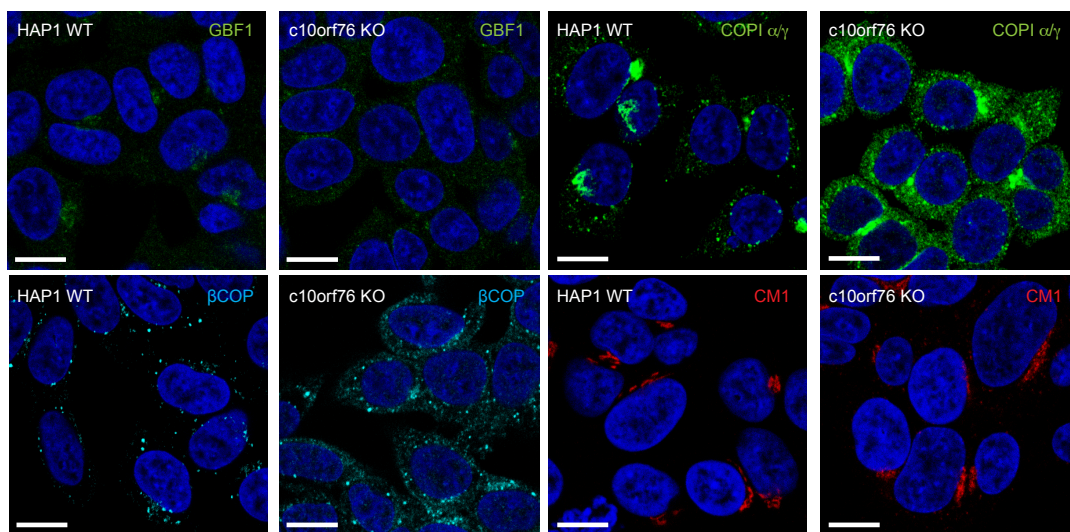
A Localisation of PI4P and PI4KB in WT and c10orf76 KO HAP1 cells



B Localisation of Golgi/TGN/ER markers in WT and c10orf76 KO HAP1 cells



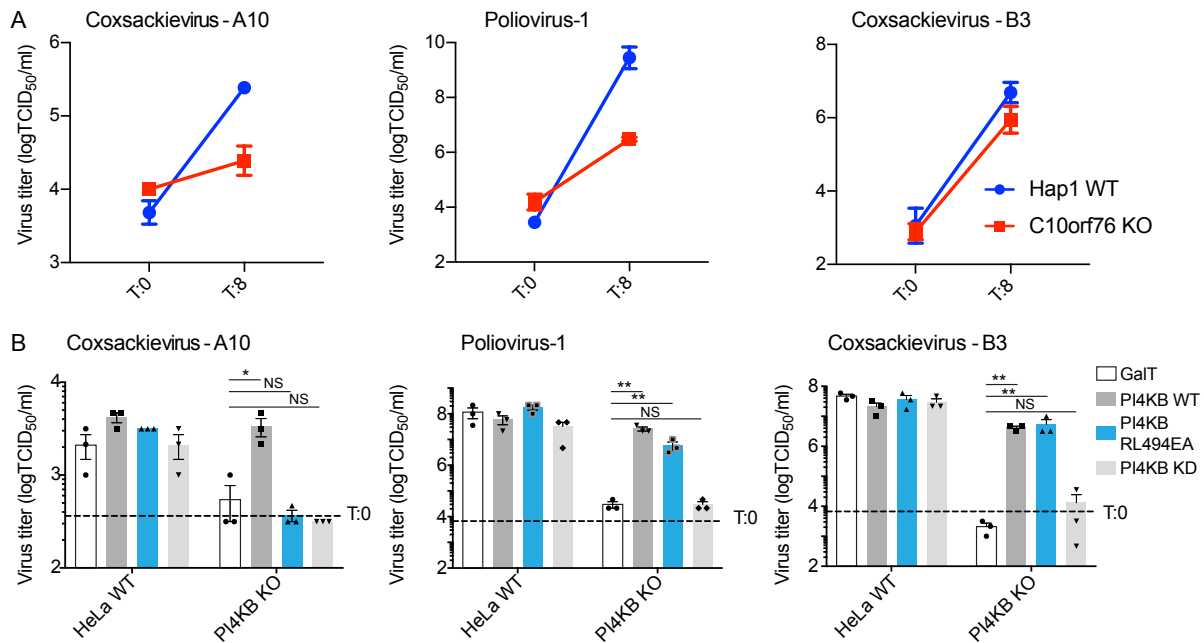
C Localisation of Arf1 GEFs and effectors in WT and c10orf76 KO HAP1 cells



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1057 **Figure 4. Knockout of c10orf76 in HAP1 cells leads to decreased PI4P levels and**
1058 **disruption of GBF1 / active Arf1 localization despite minimal effects on Golgi**
1059 **morphology.**

1060 HAP1 cells were fixed and stained with antibodies examining PI4P and PI4KB (A), Golgi
1061 morphology markers (B), and markers of Arf1 activation (C). The coatomer proteins COPI α/γ
1062 and β COP act as a readout for GTP-bound Arf1, while the native coatomer was detected with
1063 the CM1 antibody. Nuclei were stained with DAPI (blue). Bars represent 10 μ m.



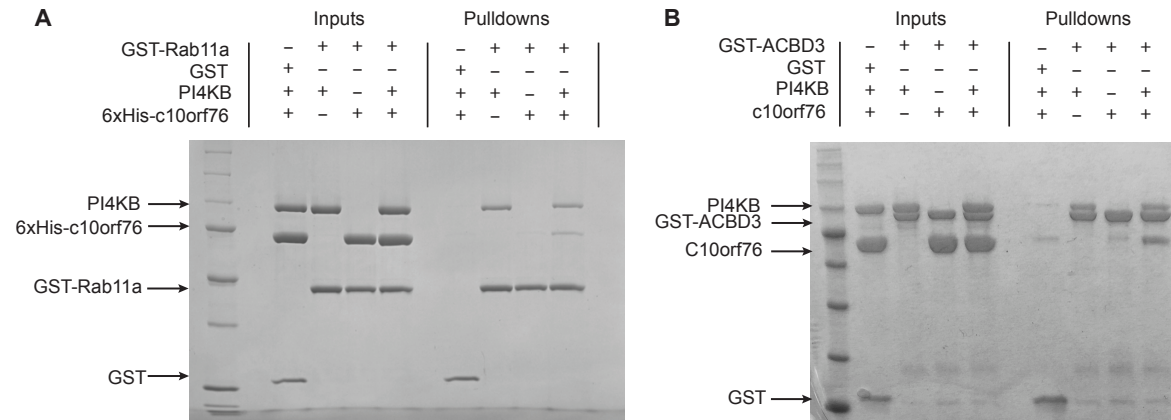
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1065 **Figure 5. The c10orf76-PI4KB complex is essential for Cocksackievirus A10 replication**

1066 (A) Viral infection assays determining viral titers by end-point titration at 0 hours and 8 hours
 1067 in HAP1 wild-type or c10orf76 knockout cells. Left: Cocksackievirus A10 infection. Middle:
 1068 Poliovirus-1 infection. Right: Cocksackievirus B3 infection.

1069 (B) Viral infection assays determining virus titers by end point titration at 8 hours in HeLa
 1070 wild-type and PI4KB knockout cells upon transfection of wild-type PI4KB, the complex-
 1071 disrupting RL494EA PI4KB mutant or the kinase dead D674A PI4KB mutant. Left:
 1072 Cocksackievirus A10 infection. Middle: Poliovirus-1 infection. Right: Cocksackievirus B3
 1073 infection. Values were statistically evaluated compared to the GalT control using a one-way
 1074 ANOVA. **, P<0.01; *, P<0.05; N.S., P>0.05. For all panels error bars represent standard
 1075 error (n=3).

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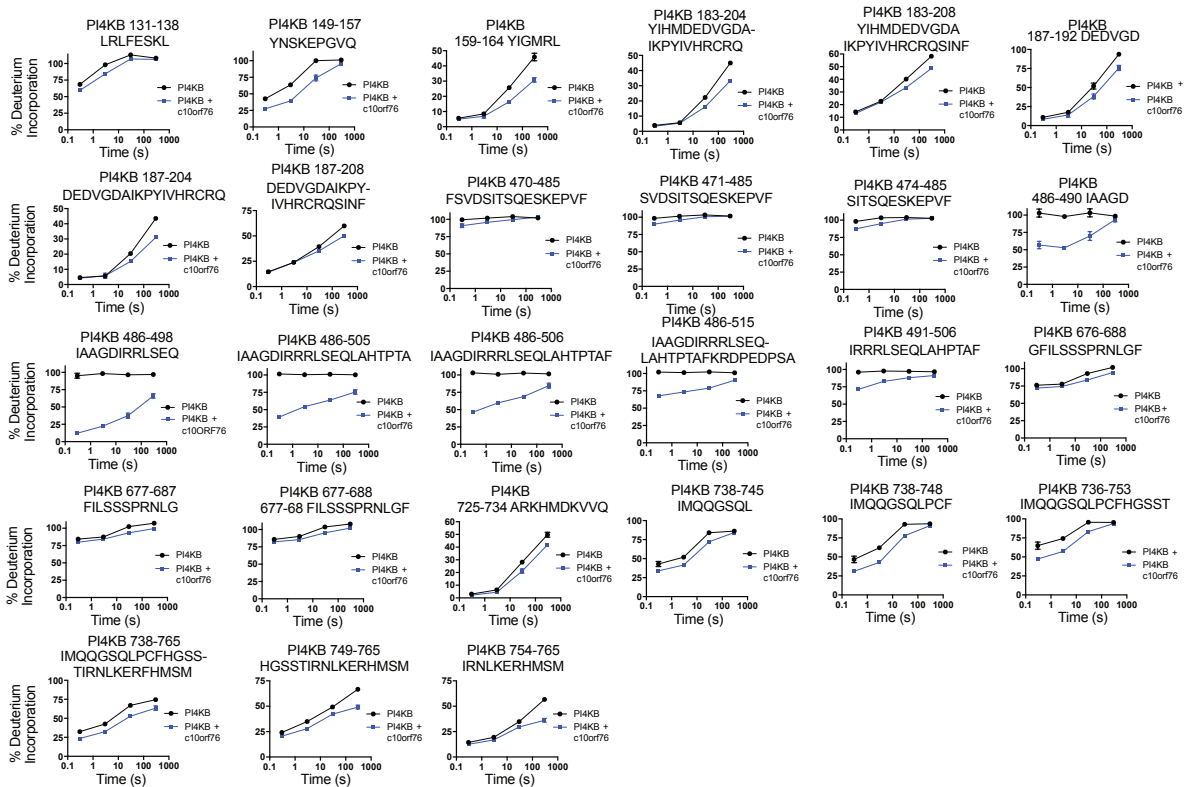


Supplemental Figure 1. PI4KB forms ternary complexes with c10orf76, Rab11a and ACBD3

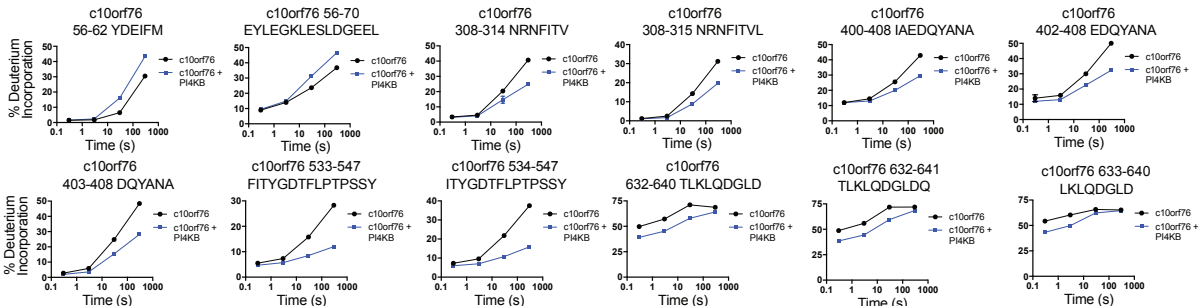
(A) PI4KB can form ternary complexes with Rab11a and c10orf76 *in vitro*. GST-pulldown assays were carried out using GST-Rab11a(Q70L) (6 μ M) or GST alone (3 μ M) as the bait, using 6xHis-c10orf76 (4 μ M), PI4KB (2 μ M) as the prey.

(B) PI4KB can form ternary complexes with ACBD3 and c10orf76 *in vitro*. GST-pulldown assays were carried out using GST-ACBD3 (4 μ M) or GST alone (4 μ M) as the bait, and 6xHis-c10orf76 (3 μ M) and PI4KB (2 μ M) as the prey. Samples were washed a total of 4 times in all experiments.

A PI4KB peptides showing HDX differences in presence of c10orf76 (significant difference >7 %D and 0.5 #D, unpaired student T-test <0.05)



B c10orf76 peptides showing HDX differences in presence of PI4KB (significant difference >7 %D and 0.5 #D, unpaired student T-test <0.05)



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14 **Supplemental Figure 2. PI4KB and c10orf76 form an extended interface with spanning**
15 **multiple regions.**

16 All peptides of both PI4KB (A) and c10orf76 (B) with a significant difference in H/D exchange
17 with >7% decrease in exchange, >0.5 Da difference, and unpaired two-tailed student t-test
18 $p < 0.05$ at any time point (3s at 1 °C; 3s, 30s, and 300s at 23 °C).

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25 **Supplemental Table 1. Full statistics on all hydrogen deuterium exchange experiments**
26 **according to the guidelines from the International Conference on HDX-MS.**

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Data Set	PI4KB	c10orf76	FLH409AAA c10orf76 mutant
HDX reaction details	%D ₂ O=87.4% pH _(read) = 7.5 Temp= 23°C	%D ₂ O=87.4% pH _(read) = 7.5 Temp= 23°C	%D ₂ O=90.5% pH _(read) = 7.5 Temp= 23°
HDX time course	3s at 1°C 3s, 30s, 300s at 23°C	3s at 1°C 3s, 30s, 300s at 23°C	3s, 300s at 23°C
HDX controls	N/A	N/A	N/A
Back-exchange	Corrected using a fully deuterated (FD) sample	Corrected based on %D ₂ O	Corrected based on %D ₂ O
Number of peptides	185	108	111
Sequence coverage	96.9%	73.9%	72.8%
Average peptide length/ redundancy	Length = 13.8 Redundancy = 3.2	Length = 12.1 Redundancy = 1.9	Length = 10.7 Redundancy = 1.7
Replicates	3	3	3
Repeatability	Average StDev = 1.2%	Average StDev = 0.6%	Average StDev = 1%
Significant differences in HDX	>7% and >0.5 Da and unpaired t- test <0.05	>7% and >0.5 Da and unpaired t-test <0.05	>7% and >0.5 Da and unpaired t-test <0.05

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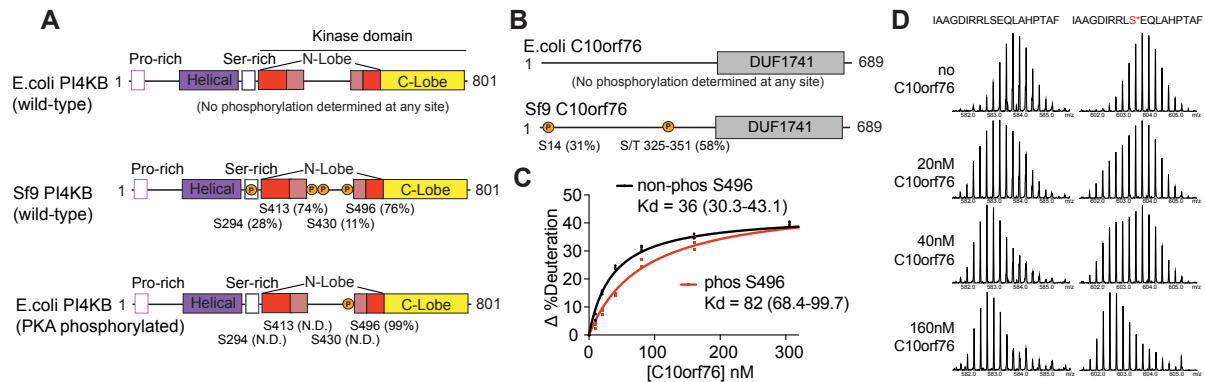
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39 **Supplemental Figure 3. PKA phosphorylation of PI4KB Ser496 reduces affinity for**
 40 **c10orf76.**

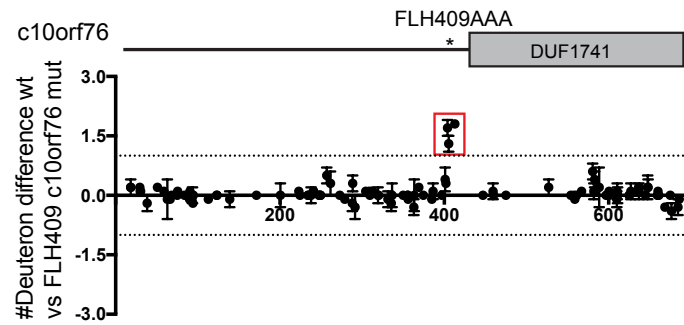
41 **(A)** PKA strongly phosphorylates Ser496 over Ser294 and Ser413 sites. Relative abundance of
 42 phosphorylated PI4KB at Ser294, Ser413 and Ser496 sites were calculated using the relative
 43 intensity (total area) of the phosphorylated vs non-phosphorylated peptides (peptides 290-303,
 44 290-312, 395-425, 430-441, 486-505 and 486-506) for PI4KB expressed in *Sf9*, expressed in
 45 *E. coli*, and expressed in *E. coli* and treated with PKA. N.D. indicates no phosphorylation was
 46 identified. N.D. indicates no phosphorylation was identified.

47 **(B)** c10orf76 contains two phosphorylation sites when produced in *Sf9*. Relative abundance of
 48 S14 was calculated using the relative intensity (total area) of the phosphorylated vs non-
 49 phosphorylated peptide (10-22). Relative abundance of the second phosphorylation site in the
 50 325-351 region was calculated using the relative intensity (total area) average over three
 51 phosphorylated vs non-phosphorylated peptides; the definitive Ser/Thr phosphorylation
 52 residue could not be determined.

53 **(C)** Phosphorylation of Ser496 reduces PI4KB affinity for c10orf76. Deuterium incorporation
 54 of the PI4KB kinase linker region peptide 488-508 (20 nM) at a single time point (5 seconds
 55 of D₂O exposure at 23°C) was monitored in the presence of increasing concentrations of
 56 c10orf76 (0-320 nM c10orf76). K_d values were generated using a one binding site, nonlinear
 57 regression (curve fit), and are shown with 95% confidence intervals. Error bars represent
 58 standard deviation (n=3).

59 **(D)** Raw deuterium incorporation data for PI4KB peptide 488-508 used to generate panel C.
 60 The deuterium incorporation for the phosphorylated and non-phosphorylated variants of
 61 PI4KB are shown in the presence of different concentrations of c10orf76.

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64 **Supplemental figure 4. The FLH409AAA c10orf76 mutant maintains similar overall**
65 **secondary structure to wild-type with a destabilization at the mutation site.** Differences in
66 the changes in the deuterium incorporation of wild type and FLH409AAA mutant c10orf76.
67 H/D exchange reactions of c10orf76 (400 nM) were carried out for 3s and 300s, and the average
68 difference in number of deuterons incorporated between wild-type and FLH409AAA c10orf76
69 (400 nM) was graphed. Error bars represent standard deviation (n=3).