



42 **Abstract**

43 Ciliary receptors and their certain downstream signaling components undergo intraflagellar transport  
44 (IFT) as BBSome cargoes to maintain their ciliary dynamics for sensing and transducing extracellular  
45 stimuli inside the cell. Cargo laden BBSomes shed from retrograde IFT at the proximal ciliary region  
46 above the transition zone (TZ) followed by diffusing through the TZ for ciliary retrieval, while how the  
47 BBSome barrier passage is controlled remains elusive. Here, we show that the BBSome is a major  
48 effector of the Arf-like 3 (ARL3) GTPase in *Chlamydomonas*. Under physiological condition, ARL3<sup>GDP</sup>  
49 binds the membrane for diffusing into and residing in cilia. Following a nucleotide conversion, ARL3<sup>GTP</sup>  
50 dissociates with the ciliary membrane and binds and recruits the IFT-detached and cargo (phospholipase  
51 D, PLD)-laden BBSome at the proximal ciliary region to diffuse through the TZ and out of cilia. ARL3  
52 deficiency impairs ciliary signaling, e.g. phototaxis of *Chlamydomonas* cells, by disrupting BBSome  
53 ciliary retrieval, providing a mechanistic understanding behind BBSome ciliary turnover required for  
54 ciliary signaling.

55

56 **Keywords:** *ARL3; BBSome; intraflagellar transport; transition zone; cilia; phototaxis*

## 57 Introduction

58 Cilia and flagella are interchangeable terms referring to the axonemal microtubule-based subcellular  
59 organelles projecting from the cell surface of most eukaryotic cells. They act as antennas for sensing and  
60 transducing the extracellular stimuli into the cell, thus essential for maintaining many physiological and  
61 developmental signaling pathways (Goetz and Anderson, 2010; Nachury and Mick, 2019; Singla and  
62 Reiter, 2006). Therefore, ciliary malfunction causes a group of related genetic disorders including Joubert  
63 syndrome, Meckel-Gruber syndrome, nephronophthisis, and Bardet-Biedl syndrome (BBS), collectively  
64 named ciliopathies (Hildebrandt et al., 2011). Underlying ciliopathies is the fact that many G protein-  
65 coupled receptors (GPCRs), ion channels, and enzymes like receptor tyrosine kinases, platelet-derived  
66 growth factor receptor alpha, and insulin-like growth factor-1 position to and traffic inside the ciliary  
67 membrane by motor protein-driven intraflagellar transport (IFT) trains along the axoneme (Liu et al.,  
68 2020; Nachury and Mick, 2019; Schneider et al., 2005; Yeh et al., 2013). During this process, the  
69 BBSome composed of multiple BBS proteins links these ciliary transmembrane signaling proteins to IFT  
70 trains composed of repeating units of IFT-A (6 subunits) and -B (16 subunits subdivided into IFT-B1 and  
71 -B2 entities) complexes by acting as an IFT cargo adaptor (Fan et al., 2010; Jin et al., 2010; Lechtreck et  
72 al., 2009; Loktev et al., 2008; Nachury et al., 2007; Nakayama and Katoh, 2020; Taschner and Lorentzen,  
73 2016; Wang et al., 2009). Compared with these ciliary receptors, channels, and enzymes, their  
74 downstream lipidated signaling cascade factors [e.g. heterotrimeric G protein transducin ( $G\alpha,\beta,\gamma$ ), inositol  
75 polyphosphate-5-phosphatase (INPP5E), nephronophthisis 3 (NPHP3)] do not count on the IFT/BBS  
76 system for shuttling into cilia to bind the ciliary membrane. They instead use the ADP-ribosylation factor  
77 (Arf)-like 3 (ARL3) pathway to achieve this goal.

78 As a member of the Arf subfamily of the Ras superfamily of small GTPases, ARL3 is conserved  
79 among the ciliated species but absent from the non-ciliated organisms, localizes throughout the cell, and  
80 is enriched in cilia (Avidor-Reiss et al., 2004; Efimenko et al., 2005; Pazour et al., 2005). Vertebrate and  
81 human ARL3 has three types of effectors including phosphodiesterase 6 delta subunit (PDE6D),  
82 uncoordinated-119A/B (UNC119A/B), and binder of Arl2 (BART)/binder of Arl2-like 1 (BARTL1),  
83 acting in general as carrier/solubilizing proteins to bind and shuttle the cytoplasmic lipidated cargoes of  
84 different groups into cilia (ElMaghloob et al., 2021; Linari et al., 1999; Lokaj et al., 2015; Wright et al.,  
85 2011). It was known that PDE6D binds and transports the C-terminal prenylated (farnesylated or  
86 geranylgeranylated) cargoes [e.g. the catalytic  $\alpha$  and  $\beta$  subunits of PDE6, INPP5E, transducin  $\gamma$  subunit  
87 ( $T\gamma$ ), and rhodopsin kinase (GRK1)] into cilia (Li and Baehr, 1998; Thomas et al., 2014; Zhang et al.,  
88 2007; Zhang et al., 2004). UNC119A/B instead binds and transports the N-terminal myristoylated cargoes  
89 [e.g. NPHP3, cystin, and transducin  $\alpha$  subunits (GNAT-1 and GNAT-2)] into cilia (Wright et al., 2011;  
90 Zhang et al., 2011a). Compared with PDE6D and UNC119A/B, BART/BARTL1 has no cargoes

91 determined thus far, while BART was recently identified to act as a ARL3-specific co-guanine nucleotide  
92 exchange factor (GEF) to contribute to convert GDP-bound ARL3 (ARL3<sup>GDP</sup>) to GTP-bound ARL3  
93 (ARL3<sup>GTP</sup>) (ElMaghloob et al., 2021). During cargo ciliary targeting, the carrier protein first binds the  
94 lipidated cargo in the cytoplasm and the carrier-cargo complex then shuttles towards cilia by an unknown  
95 mechanism. Upon arriving at the transition zone (TZ) region, the activated ARL3 (ARL3<sup>GTP</sup>) binds its  
96 carrier protein effector at a site allosterically different from the one for lipidated cargo binding, and this  
97 induces conformational changes of the carrier protein, leading to the release of the cargo for binding to  
98 the ciliary membrane (Fansa et al., 2016; Ismail et al., 2012; Watzlich et al., 2013). After this, ARL3<sup>GTP</sup>  
99 (the activated form) is bound to and stimulated by retinitis pigmentosa 2 (RP2), the ARL3-specific  
100 GTPase activating protein (GAP), to hydrolyze for releasing the carrier protein from ARL3 (Velte et al.,  
101 2008). ARL3<sup>GDP</sup> (the inactivated form) is then reactivated by the ARL3-specific GEF, ARL13b, and  
102 ARL3<sup>GTP</sup> recycles back to bind the carrier-cargo complex for cargo releasing in cilia (Gotthardt et al.,  
103 2015; Zhang et al., 2016).

104 Besides its role in releasing a variety of lipidated signaling factors for ciliary membrane binding,  
105 ARL3 was observed to be essential for mouse rhodopsin and worm and human polycystin-1 and -2  
106 (PKD1 and PKD2) to target to cilia (Schrick et al., 2006; Su et al., 2014; Zhang et al., 2013). Given that  
107 these ciliary transmembrane signaling proteins cycle through cilia by IFT via binding the BBSome, ARL3  
108 could mediate their ciliary dynamics through the BBSome (Abd-El-Barr et al., 2007; Liu et al., 2020;  
109 Nachury, 2018; Nishimura et al., 2004; Su et al., 2014). Our previous study has shown that Rab-like 5  
110 (RABL5) GTPase IFT22 coordinates with ARL6/BBS3 for recruiting the BBSome, as a BBS3 effector, to  
111 the basal body in a GTP-dependent manner in *Chlamydomonas reinhardtii* (Sun et al., 2021; Xue et al.,  
112 2020). BBS3 itself also diffuses into cilia and binds the ciliary membrane via its N-terminal amphipathic  
113 helix in a GTP-dependent manner (Gillingham and Munro, 2007; Liu et al., 2021). At the ciliary tip,  
114 BBS3 binds and recruits the BBSome to the ciliary membrane in a GTP-dependent manner, making it  
115 spatially available to couple with the membrane-anchored signaling proteins, e.g. phospholipase D (PLD),  
116 for ciliary exit by IFT (Liu et al., 2021; Sun et al., 2021). Prior to this event, IFT/BBS remodels to allow  
117 for the BBSome to undergo a disassembly/reassembly cycle (Sun et al., 2021). During this cycle, the  
118 heterodimer IFT25/27 composed of IFT25 and RABL4/IFT27 is indispensable for the disassembled  
119 BBSome subunits to reassemble at the ciliary tip (Dong et al., 2017; Sun et al., 2021). Interestingly,  
120 mammalian GPCRs undergo retrograde IFT from the ciliary tip to a proximal ciliary region right above  
121 the TZ (Nachury, 2018; Ye et al., 2018). At this region, the GPCR-loaded BBSome sheds from IFT and  
122 diffuses through the TZ for ciliary retrieval in a RABL2-dependent manner (Dateyama et al., 2019; Duan  
123 et al., 2021; Nachury, 2018; Ye et al., 2018). However, the molecular mechanism underlying how active

124 transport of the BBSome across the TZ diffusion barrier is controlled remains elusive thus far (Nozaki et  
125 al., 2019; Ye et al., 2018).

126 In this study, we identified the BBSome acts as a major ARL3 effector at the ciliary base right above  
127 the TZ but not in the cell body of *C. reinhardtii*. ARL3 mimics other Arf-like GTPases for diffusing into  
128 cilia and reversibly binding the ciliary membrane via its N-terminal amphipathic helix and the G2 residue  
129 but in a GDP-dependent manner (Liu et al., 2021). Once in cilia, ARL3 undergoes GTPase cycling and  
130 the activated ARL3<sup>GTP</sup> binds the retrograde IFT-detached and PLD-laden BBSome at the proximal ciliary  
131 region right above the TZ and recruits it to diffuse through the TZ for ciliary retrieval. Since disruption of  
132 BBSome ciliary dynamics generates cell defective in phototaxis, our finding thus fills a gap in our  
133 understanding of how ARL3 mediates phototaxis through controlling BBSome ciliary turnover in *C.*  
134 *reinhardtii* (Sun et al., 2021). Our data also shed lights on the molecular mechanism of how ARL3  
135 deficiency could cause BBS disorder in humans.

## 136 **Results**

### 137 **ARL3 diffuses into cilia**

138 *Chlamydomonas* ARL3 shares significant homology with its orthologues in ciliated species and is more  
139 closely related to homologues of worms, *leishmania*, and *Trypanosoma* than mammals and humans  
140 phylogenetically (Fig. S1 A and B). ARL3 was shown to be a negative regulator of ciliation in *leishmania*  
141 and mouse (Cuvillier et al., 2000; Efimenko et al., 2005; Hanke-Gogokhia et al., 2016). In worms,  
142 depletion of ARL3 causes IFT-B and KIF17 motor to dissociate through histone deacetylase 6  
143 (HDAC6)-dependent pathway and then disrupts IFT (Li et al., 2010; Zhang et al., 2013). To clarify  
144 whether ARL3 affects IFT and ciliation in *C. reinhardtii*, we examined the ARL3 CLiP mutant  
145 (LMJ.RY0420.182282) that we named *arl3*. The *arl3* cell contains a 2,217-bp paromomycin gene  
146 insertion in the fourth exon of the ARL3 gene (Fig. S2 A-C). With the newly developed ARL3 antibody  
147 available, this insertion was verified to prevent ARL3 from being synthesized, as shown by  
148 immunoblotting, demonstrating that *arl3* is a ARL3-null mutant (Fig. S3 A). *arl3* cells grown cilia of  
149 normal length, excluding ARL3 from mediating ciliation (Fig. S3 B). Supportive of this conclusion, *arl3*  
150 cells retained IFT-A subunits IFT43 and IFT139, IFT-B1 subunits IFT22 and IFT70, and IFT-B2 subunits  
151 IFT38 and IFT57 at wild-type (WT) levels both in whole cell and ciliary samples in the steady state (Fig.  
152 1 A). To examine if ARL3 affects IFT ciliary dynamics, we generated transgenic strains  
153 *arl3::IFT43::HA::YFP*, *arl3::IFT22::HA::YFP*, and *arl3::IFT38::YFP*, which expresses IFT43, IFT22, or  
154 IFT38 fused at their C-terminus to hemagglutinin (HA) and/or yellow fluorescent protein (YFP)  
155 (IFT43::HA::YFP, IFT22::HA::YFP, and IFT38::YFP) in *arl3* cells. When expressed at the same level as  
156 when three HA::YFP/YFP-tagged proteins of different IFT subcomplexes were expressed alone in CC-  
157 125 control cells (resulting strains CC-125::IFT43::HA::YFP, CC-125::IFT22::HA::YFP, and CC-  
158 125::IFT38::YFP) (Fig. S3 C) (Xue et al., 2020), they entered cilia (Fig. 1 B) and underwent typical  
159 bidirectional IFT of *C. reinhardtii* as reflected by total internal reflection fluorescence (TIRF) assays,  
160 excluding ARL3 from mediating IFT ciliary dynamics (Fig. 1 C and D) (Xue et al., 2020). After knowing  
161 this, we asked whether and how *Chlamydomonas* ARL3 enters cilia. To answer this question, we  
162 expressed ARL3::HA::YFP in *arl3* cells at WT ARL3 level of CC-5325 control cells (resulting strain  
163 *arl3::ARL3::HA::YFP*) (Fig. S3 D). The *arl3::ARL3::HA::YFP* cells retained ARL3::HA::YFP in cilia at  
164 WT ARL3 level (Fig. 1 E) and ARL3::HA::YFP diffused into cilia to reside along the whole length of  
165 cilia as reflected by TIRF assay (Fig. 1 F and Movie S1).

166

### 167 **ARL3<sup>GDP</sup> requires its N-terminal 15 amino acids and the G2 residue for membrane association and** 168 **diffusion into cilia**

169 Arf family GTPases associate with the inner membrane via its N-terminal amphipathic helix (Amor et al.,  
170 1994; Liu et al., 2010; Zhang et al., 2011b) and their N-terminal 15 residues are found essential for this  
171 association (Jin et al., 2010; Liu et al., 2021; Mourão et al., 2014). They were further reported to anchor to  
172 the membrane through myristylation on their glycine residue at the second amino acid position and  
173 disruption of myristylation by introducing a G2A mutation fully prevents them from associating with the  
174 membrane (Fig. S1 A) (Sahin et al., 2008; Vaughan and Moss, 1997). In addition, studies have identified  
175 the GTP-bound configuration as a prerequisite for Arf GTPases, i.e., ARL6/BBS3, to bind the membrane  
176 (Liu et al., 2021; Mourão et al., 2014). To dissect whether and how N-terminal residues, the G2 residue,  
177 and the nucleotide state confer ARL3 to bind the membrane, we applied bacteria to express ARL3 and a  
178 total of eight ARL3 variants, which contain the N-terminal 15 residue deletion ( $\Delta$ N15), the G2A  
179 mutation, or neither combined with the mutation Q70L, T30N, or none of them (Fig. 2 A). Q70L and  
180 T30N were introduced in ARL3 or its variants as they are constitutive-active (Q70L) and dominant-  
181 negative (T30N) mutations that can lock ARL3 in the GTP- and GDP-bound state, respectively (Veltel et  
182 al., 2008). When incubated with the synthetic liposomes, ARL3 associated with liposomes only in the  
183 presence of GDP, which locks ARL3 in a GDP-bound state (Fig. 2 B). Consistent with this observation,  
184 ARL3<sup>T30N</sup> rather than ARL3<sup>Q70L</sup> bound liposomes, revealing that ARL3, unlike ARL6/BBS3 that binds  
185 the membrane in a GTP-dependent manner, instead relies on GDP for membrane association (Fig. 2 B)  
186 (Liu et al., 2021; Veltel et al., 2008). In contrast, ARL3 $\Delta$ N15 and ARL3<sup>G2A</sup> both were deprived of  
187 binding liposomes and they remained unbound to liposomes even when the T30N mutation was  
188 introduced, demonstrating that the N-terminal amphipathic helix and the G2 residue both are required for  
189 ARL3 to bind the membrane (Fig. 2 B). To discern if the N-terminal 15 residues, the G2 residue, and  
190 GDP are essential for ARL3 to enter cilia, we expressed the above-mentioned ARL3 variants fused at  
191 their C-terminus to HA::YFP in *arl3* cells to generate eight strains including *arl3*::ARL3<sup>Q70L</sup>::HA::YFP,  
192 *arl3*::ARL3<sup>T30N</sup>::HA::YFP, *arl3*::ARL3 $\Delta$ N15::HA::YFP, *arl3*::ARL3 $\Delta$ N15<sup>Q70L</sup>::HA::YFP,  
193 *arl3*::ARL3 $\Delta$ N15<sup>T30N</sup>::HA::YFP, *arl3*::ARL3<sup>G2A</sup>::HA::YFP, *arl3*::ARL3<sup>G2AQ70L</sup>::HA::YFP, and  
194 *arl3*::ARL3<sup>G2AT30N</sup>::HA::YFP (Fig. 2 C). When expressed at WT ARL3 levels of CC-5325 control cells,  
195 ARL3<sup>Q70L</sup>::HA::YFP and ARL3<sup>T30N</sup>::HA::YFP both, unexpectedly, resembled ARL3::HA::YFP to enter  
196 cilia (Fig. 2 D). In contrast, depletion of N-terminal 15 residues and the G2A mutation alone prevented  
197 ARL3::HA::YFP from entering cilia, while Q70L rather than T30N mutation enabled both  
198 ARL3 $\Delta$ N15::HA::YFP and ARL3<sup>G2A</sup>::HA::YFP to enter cilia (Fig. 2 D). Upon entering cilia,  
199 ARL3<sup>T30N</sup>::HA::YFP existed in the membrane fraction, while ARL3::HA::YFP, ARL3<sup>Q70L</sup>::HA::YFP,  
200 ARL3 $\Delta$ N15<sup>Q70L</sup>::HA::YFP, and ARL3<sup>G2AQ70L</sup>::HA::YFP all resided in the matrix fraction (Fig. 2 E).  
201 Unlike ARL6/BBS3 that binds and recruits the BBSome to the basal body, thus showing a BBSome-like  
202 basal body distribution pattern (Liu et al., 2021), ARL3::HA::YFP and its cilium-entering variants did not

203 reside at the basal body as visualized by immunostaining (Fig. S4 A). TIRF assays noticed that  
204 ARL3<sup>T30N</sup>::HA::YFP diffused into cilia and resembled ARL3::HA::YFP to reside along the whole length  
205 of cilia (Fig. 1 F, 2 F, and Movies S1 and S2). In contrast, ARL3<sup>Q70L</sup>::HA::YFP,  
206 ARL3 $\Delta$ N15<sup>Q70L</sup>::HA::YFP, and ARL3<sup>G2AQ70L</sup>::HA::YFP diffused into cilia, while they mostly resided at a  
207 proximal ciliary region likely above the basal bodies (Fig. 2 F and Movies S3-S5). We failed to visualize  
208 their co-localization with the CEP290-labeled TZ region by immunostaining probably owing to their low  
209 ciliary abundance (Fig. 2 G), while we combined all these data together to conclude that ARL3 enters  
210 cilia via two different pathways. ARL3<sup>GDP</sup> binds the membrane for diffusing into cilia, while ARL3<sup>GTP</sup>  
211 diffuses into cilia independent of membrane association. Considering that ARL3::HA::YFP has a ciliary  
212 distribution pattern similar to its GDP-locked counterpart (Fig. 1 F and 2 F), ARL3, under physiological  
213 conditions, likely exists in a GDP-bound state and thus diffuses into cilia via the membrane association  
214 pathway in *C. reinhardtii*. Upon inside cilia, ARL3<sup>GDP</sup> was quickly converted to ARL3<sup>GTP</sup> by an unknown  
215 mechanism. That is why we observed ARL3::HA::YFP to reside along the whole length of cilia as shown  
216 by living TIRF assay, while it instead exists in the matrix but not membrane fraction of cilia in the steady  
217 state following ciliary fraction isolation (Fig. 2 E).

### 218 **ARL3<sup>GTP</sup> is required for the BBSome to move cross the TZ for ciliary retrieval**

219 Previous studies have identified ARL3 to be essential for maintaining ciliary dynamics of mouse  
220 rhodopsin and worm and human polycystin-1 and -2 (PKD1 and PKD2) (Schrack et al., 2006; Su et al.,  
221 2014; Zhang et al., 2013). These transmembrane signaling proteins cycle through cilia by IFT through  
222 binding the BBSome directly (Abd-El-Barr et al., 2007; Liu et al., 2020; Nachury, 2018; Nishimura et al.,  
223 2004; Su et al., 2014). Given that *Chlamydomonas* ARL3 does not affect IFT, we wondered whether  
224 ARL3 could mediate signaling protein dynamics in cilia via the BBSome pathway. To answer this  
225 question, we examined *arl3* cells and the rescuing strains *arl3*::ARL3::HA::YFP,  
226 *arl3*::ARL3<sup>Q70L</sup>::HA::YFP, and *arl3*::ARL3<sup>T30N</sup>::HA::YFP. As compared to CC-5325 control cells, four  
227 strains retained the BBSome subunits BBS1, BBS4, BBS5, BBS7, and BBS8 at WT levels (Fig. 3 A). Of  
228 note, these BBSome subunits accumulated in *arl3* cilia to levels ~7.5-fold higher than control cell cilia  
229 (Fig. 3 B). This observation was confirmed as rescuing ARL3 with ARL3::HA::YFP restored them back  
230 to normal, as shown in *arl3*::ARL3::HA::YFP cilia (Fig. 3 B). We further identified ARL3<sup>Q70L</sup>::HA::YFP  
231 rather than ARL3<sup>T30N</sup>::HA::YFP is able to restore these BBSome subunits back to normal in *arl3* cilia,  
232 revealing that ARL3<sup>GTP</sup> is required for maintaining BBSome ciliary dynamics (Fig. 3 B). Our previous  
233 study has shown that the BBSome disassembles at the ciliary tip followed by a reassembly process for  
234 loading onto retrograde IFT trains for transporting to the ciliary base (Sun et al., 2021). In the absence of  
235 ARL3, the BBSome remained as an intact entity in cilia, excluding ARL3 from mediating BBSome



236 remodeling at the ciliary tip (Fig. 3 C). To further discern how ARL3 mediates BBSome dynamics in cilia,  
237 we generated a ARL3- and BBS8-double null mutant that we named *arl3-bbs8* and expressed BBS8::YFP  
238 in *arl3-bbs8* cells (resulting strain *arl3-bbs8::BBS8::YFP*) at BBS8::YFP level of *bbs8::BBS8::YFP* cells,  
239 which expresses BBS8::YFP in BBS8-null *bbs8* cells (Fig. 3 D). When expressed at WT BBS8 level of  
240 CC-125 control cells, BBS8::YFP entered and retained in *bbs8::BBS8::YFP* cilia at the endogenous  
241 BBS8 level of control cells and so did for the BBSome subunits BBS1, BBS4, BBS5, and BBS7 (Fig. 3  
242 D). In contrast, ARL3 knockout did not affect cellular levels of BBS1, BBS4, BBS5, and BBS7 but  
243 caused them and BBS8::YFP to build up in *arl3-bbs8::BBS8::YFP* cilia (Fig. 3 D). Given that the  
244 BBSome (represented by BBS8::YFP) underwent typical bidirectional IFT of *Chlamydomonas* BBSome  
245 the same in cilia of both transgenic cells, ARL3 was then excluded from mediating BBSome  
246 transportation between the ciliary tip and base (Fig. 3 E and F and Movies S6-S7). Interestingly,  
247 BBS8::YFP was instead visualized to accumulate right above the CEP290-labeled TZ region of *arl3-*  
248 *bbs8::BBS8::YFP* cilia as compared to *bbs8::BBS8::YFP* cilia (Fig. 3 G). This buildup was defined to a  
249 proximal ciliary region obviously above the IFT46-labeled basal bodies (Fig. S4 B). Taking these data  
250 together, we conclude that ARL3 is dispensable for the BBSome to enter and traffic inside cilia, while the  
251 BBSome detaches from retrograde IFT at the proximal ciliary region right above the TZ, and it requires  
252 ARL3<sup>GTP</sup> for moving cross the TZ and out of cilia. This notion was verified as the endogenous BBS8 in  
253 *arl3* and *arl3::ARL3<sup>T30N</sup>::HA::YFP* cilia accumulated at the proximal ciliary region obviously above the  
254 IFT81-labeled basal bodies as compared to CC-5325, *arl3::ARL3::HA::YFP*, and  
255 *arl3::ARL3<sup>Q70L</sup>::HA::YFP* cilia (Fig. S4 C).

### 256 **The BBSome is a major ARL3 effector at the proximal ciliary region but not in the cell body**

257 Different from ARL3<sup>GDP</sup> that binds the ciliary membrane, ARL3<sup>GTP</sup> resembles the BBSome to reside in  
258 the ciliary matrix (Fig. 2 E). ARL3<sup>GDP</sup> resides along the whole length of cilia, while GTP loading largely  
259 restricts ARL3 to the proximal ciliary region, where the BBSome sheds from IFT and accumulates in the  
260 absence of ARL3 (Fig. 3 G and Fig. S4 B and C). As expected, neither ARL3::HA::YFP nor the two  
261 mutants can be visualized by immunostaining, preventing us from drawing a conclusion that they co-  
262 localize with the BBSome (represented by BBS8) at the proximal ciliary region right above the TZ (Fig. 4  
263 A), while the observation that ARL3<sup>GTP</sup> promotes outward movement of the BBSome across the TZ  
264 proposed that ARL3<sup>GTP</sup> may achieve this goal via interacting with the BBSome (Fig. 3). This notion was  
265 supported as partial ARL3::HA::YFP and ARL3<sup>Q70L</sup>::HA::YFP co-sedimented with the BBSome in  
266 *arl3::ARL3::HA::YFP* and *arl3::ARL3<sup>Q70L</sup>::HA::YFP* cilia in sucrose density gradients (Fig. 4 B). In  
267 contrast, ARL3<sup>T30N</sup>::HA::YFP remained to be separated from the BBSome in *arl3::ARL3<sup>T30N</sup>::HA::YFP*  
268 cilia, as shown by sucrose density gradient centrifugation assays (Fig. 4 B). Our previous studies have

269 shown that HMEKN buffer confers IFT-A, IFT-B1, and IFT-B2 subcomplexes to separate from one  
270 another, while the BBSome remains to be associated with IFT-B1 (Sun et al., 2021). In the presence of  
271 GTP $\gamma$ S that locks ARL3 in a GTP-bound state, ARL3::HA::YFP immunoprecipitated the BBSome  
272 subunits BBS1, BBS4, BBS5, BBS7, and BBS8 but not the IFT-B1 subunits IFT22 and IFT70 in  
273 *arl3::ARL3::HA::YFP* cilia (Fig. 4 C). In contrast, none of these proteins were recovered in the presence  
274 of GDP that locks ARL3 in a GDP-bound state, identifying IFT-B1-separated BBSomes indeed exist in  
275 cilia for ARL3<sup>GTP</sup> to interact with (Fig. 4 C). This notion was verified as ARL3<sup>Q70L</sup>::HA::YFP but not  
276 ARL3<sup>T30N</sup>::HA::YFP recovered the BBSome and none of them was able to recover IFT-B1, as shown in  
277 *arl3::ARL3<sup>Q70L</sup>::HA::YFP* and *arl3::ARL3<sup>T30N</sup>::HA::YFP* cilia (Fig. 4 C). Other than this, we further  
278 identified ARL3<sup>Q70L</sup>::HA::YFP fails to immunoprecipitate the BBSome subunits nor IFT proteins in the  
279 cell body extracts even in the presence of dithiothreitol (DTT) that separates the BBSome from IFT-B1  
280 B1 (Sun et al., 2021), revealing that ARL3<sup>GTP</sup> interaction with the IFT-detached BBSome only occurs in  
281 cilia but not in the cell body (Fig. 4 D and E). As identified by in vitro protein interaction assays, BBS1  
282 and BBS5 were shown to be the BBSome subunits most efficiently captured by ARL3<sup>Q70L</sup> but not  
283 ARL3<sup>T30N</sup> *in vitro*, revealing that ARL3, only when in a GTP-bound state, interacts with the IFT-  
284 separated BBSome directly (Fig. 4 F). We then conclude that the BBSome is the major ARL3 effector  
285 only when they both position at the proximal ciliary region.

### 286 **ARL3<sup>GTP</sup> recruits the BBSome for diffusing through the TZ for ciliary retrieval**

287 ARL3<sup>GTP</sup> promotes outward BBSome movement cross the TZ (Fig. 3). To have a full review on how  
288 ARL3 and the BBSome interplay for ciliary retrieval, we ought to dissect whether ARL3 ciliary dynamics  
289 is mediated by the BBSome. To solve this puzzle, we expressed ARL3::HA::YFP, ARL3<sup>Q70L</sup>::HA::YFP,  
290 and ARL3<sup>T30N</sup>::HA::YFP in ARL3- and BBS8-double null *arl3-bbs8* cells to generate three strains *arl3-*  
291 *bbs8::ARL3::HA::YFP*, *arl3-bbs8::ARL3<sup>Q70L</sup>::HA::YFP*, and *arl3-bbs8::ARL3<sup>T30N</sup>::HA::YFP*. By doing  
292 so, the BBSome was deprived of ciliary presence in these cells as BBS8 knockout disrupts BBSome  
293 assembly in the cell body, thus making the BBSome unavailable for entering cilia (Fig. 5 A). When  
294 expressed at WT ARL3 level of CC-125 control cells, ARL3::HA::YFP, ARL3<sup>Q70L</sup>::HA::YFP, and  
295 ARL3<sup>T30N</sup>::HA::YFP entered and retained at WT ARL3 level in cilia (Fig. 5 A). Like in ARL3-null *arl3*  
296 cells, ARL3<sup>T30N</sup>::HA::YFP resided in the membrane fraction, while ARL3::HA::YFP and  
297 ARL3<sup>Q70L</sup>::HA::YFP both existed in the matrix fraction in the absence of the BBSome (Fig. 5 B).  
298 Immunostaining, as expected, was unable to visualize these recombinant proteins to reside at the CEP290-  
299 labelled TZ region (Fig. S4 D). However, TIRF assays visualized that, like in *arl3* cells, all three  
300 fluorescent proteins entered cilia by diffusion in the *arl3-bbs8* double mutant cells (Fig. 5 C). Once inside  
301 cilia, ARL3::HA::YFP and ARL3<sup>T30N</sup>::HA::YFP distributed to the whole length of cilia, while

302 ARL3<sup>Q70L</sup>::HA::YFP mostly concentrated at the proximal ciliary region (Fig. 5 C). These data thus  
303 excluded the BBSome from mediating ARL3 ciliary dynamics.

304 Knockout of *Chlamydomonas* ARL3 does not disrupt IFT but causes BBSome accumulation at the  
305 proximal ciliary region right above the TZ, consistent with the observation that the human and murine  
306 BBSome sheds from retrograde IFT before diffusing through the TZ for ciliary retrieval (Fig. 5 D)  
307 (Nachury, 2018; Ye et al., 2018). To visualize whether ARL3<sup>GTP</sup> promotes BBSome diffusion through the  
308 TZ for ciliary retrieval in *C. reinhardtii*, we examined the IFT46::YFP-expressing *ift46::IFT46::YFP* and  
309 the BBS8::YFP-expressing *bbs8::BBS8::YFP* and *arl3-bbs8::BBS8::YFP* cells (Lv et al., 2017). Of note,  
310 TIRF assays identified retrograde IFT trains (represented by IFT46::YFP) transported from the ciliary tip  
311 all the way to the basal bodies, suggesting that they move cross the TZ for ciliary retrieval via IFT (Fig. 5  
312 E and Movie S8). The BBSome (represented by BBS8::YFP) performed normal IFT for trafficking from  
313 the ciliary tip to base (Fig. 5 E and Movie S9). When reaching the proximal ciliary region right above the  
314 TZ, the BBSome stopped performing IFT and shifted to diffuse through the TZ for ciliary retrieval (Fig. 5  
315 E and Movie S9). In the absence of ARL3, the BBSome (represented by BBS8::YFP) underwent normal  
316 bidirectional IFT, while its suspension for diffusing through the TZ but accumulating at the proximal  
317 ciliary region right above the TZ was easily observed, as shown in cilia of *arl3-bbs8::BBS8::YFP* cells  
318 (Fig. 5 E and Movie S10). In summary, the BBSome relies on ARL3<sup>GTP</sup> for diffusing through the TZ for  
319 ciliary retrieval but not *vice versa*.

320

### 321 **ARL3<sup>GTP</sup> recruits PLD-laden BBSomes to move cross the TZ for ciliary retrieval**

322 Our previous study and others have shown that the ciliary membrane anchored PLD couples with the  
323 BBSome at the ciliary tip followed by exiting cilia via IFT (Liu and Lechtreck, 2018; Liu et al., 2021). As  
324 compared to CC-125 control cells, *arl3* cells retained PLD at the WT level but accumulated it in cilia (Fig.  
325 6 A). Immunostaining identified PLD is not able to be visualized in CC-125 cilia but accumulates to  
326 become visible at the proximal ciliary above the IFT-81-labeled basal bodies in *arl3* cells (Fig. 6 B). The  
327 PLD abundance was restored to normal in cilia of *arl3::ARL3::HA::YFP* and *arl3::ARL3<sup>Q70L</sup>::HA::YFP*  
328 cells but not *arl3::ARL3<sup>T30N</sup>::HA::YFP* cells (Fig. 6 A). Accordingly, PLD became invisible in cilia of  
329 *arl3::ARL3::HA::YFP* and *arl3::ARL3<sup>Q70L</sup>::HA::YFP* cells as in cilia of CC-125 control cells but  
330 remained to be accumulated at the proximal ciliary region above the IFT-81-labeled basal bodies in  
331 *arl3::ARL3<sup>T30N</sup>::HA::YFP* cells (Fig. 6 B). Given that ARL3<sup>GTP</sup> directs the BBSome to behave the same  
332 way as PLD in these events (Fig. 3 A and B and Fig. S4 C), ARL3<sup>GTP</sup> is supposed to be desirable for  
333 promoting both PLD and the BBSome to move cross the diffusion barrier at the TZ for ciliary retrieval.  
334 Notably, ARL3::HA::YFP immunoprecipitated the BBSome and PLD but not IFT-A, IFT-B1, and IFT-  
335 B2 in the presence of GTPγS that locks ARL3 in a GTP-bound state (Fig. 6 C). In the presence of GDP

336 that locks ARL3 in a GDP-bound state, ARL3::HA::YFP recovered none of these proteins (Fig. 6 C).  
337 This observation was confirmed as ARL3<sup>Q70L</sup>::HA::YFP but not ARL3<sup>30N</sup>::HA::YFP immunoprecipitated  
338 the IFT-detached BBSome and PLD (Fig. 6 C). These data provided evidence to show that PLD remains  
339 to be a cargo of the IFT-detached BBSome during its diffusion through the TZ for ciliary retrieval (Fig. 5  
340 E). Therefore, ARL3<sup>GTP</sup> binds and recruits PLD-laden BBSomes to move cross the diffusion barrier at the  
341 TZ for ciliary retrieval.

342

### 343 **ARL3 mediates phototaxis through controlling BBSome ciliary retrieval**

344 Our previous study and others have shown that disruption of BBSome ciliary dynamics leads to the  
345 generation of *Chlamydomonas* cells defective in phototaxis (Liu and Lechtreck, 2018; Sun et al., 2021).  
346 The *arl3* cells were disabled for conducting phototaxis as they were derived from phototaxis-deficient  
347 CC-5325 cells (Fig. 7 A and B). To examine if ARL3 mediates phototaxis through controlling BBSome  
348 ciliary dynamics, we next applied microRNA (miRNA) vector to knock down the endogenous ARL3 to  
349 ~8.3% of WT level in CC-125 cells; we referred to this strain as ARL3<sup>miRNA</sup> (Fig. 7 C). Reflecting its  
350 cellular reduction, ARL3 was strongly reduced to ~8.0% of WT level in ARL3<sup>miRNA</sup> cilia (Fig. 7 C).  
351 Consistent with ARL3 knockout result, partial depletion of ARL3 did not appear to affect ciliary length  
352 (Fig. S5 A) nor altered cellular and ciliary abundance of IFT proteins (Fig. S5 B). As expected, BBS1,  
353 BBS4, BBS5, BBS7, and BBS8 were observed to accumulate in ARL3<sup>miRNA</sup> cilia, while ARL3  
354 knockdown did not alter their cellular contents (Fig. 7 C). After the BBSome (represented by BBS8) was  
355 determined to accumulate at a proximal region of ARL3<sup>miRNA</sup> cilia above the IFT81-labeled basal bodies,  
356 we concluded that ARL3 knockdown mimics ARL3 knockout for blocking BBSome to diffuse through  
357 the TZ for ciliary retrieval (Fig. 7 D). This notion was verified as rescue of ARL3 with ARL3::HA::YFP  
358 to WT level (resulting strain ARL3<sup>Res-WT</sup>) did not alter cellular contents of BBS1, BBS4, BBS5, BBS7,  
359 and BBS8 but restored them to WT levels in cilia (Fig. 7 C and D). We further observed that rescue of  
360 ARL3 with ARL3<sup>Q70L</sup>::HA::YFP but not ARL3<sup>T30N</sup>::HA::YFP (resulting strains ARL3<sup>Res-Q70L</sup> and  
361 ARL3<sup>Res-T30N</sup>) restored those BBSome subunits to WT levels in cilia, verifying that GTP-loading confers  
362 ARL3 to promote the BBSome to diffuse through the TZ for ciliary retrieval (Fig. 7 C and D). After  
363 obtaining these cells, we performed both population and single cell assays to determine their phototoxic  
364 responses and identified ARL3<sup>miRNA</sup> cells are non-phototactic, ARL3<sup>Res-WT</sup> and ARL3<sup>Res-Q70L</sup> cells, like  
365 CC-125 control cells, became normal in phototaxis, and ARL3<sup>Res-T30N</sup> cells remained to be non-  
366 phototactic (Fig. 7 E and F). Therefore, ARL3 controls *Chlamydomonas* phototaxis through maintaining  
367 BBSome ciliary dynamics by controlling BBSome diffusion through the TZ for ciliary retrieval in a GTP-  
368 dependent manner.

## 369 Discussion

370

371 As a Arf-like small GTPase, *Chlamydomonas* ARL3 relies on GDP, its N-terminal amphipathic helix,  
372 and the glycine myristylation site at the second amino acid position to associate with the cell membrane,  
373 which is a prerequisite for ARL3 to diffuse into cilia, and to reside along the whole length of cilia by  
374 anchoring to the ciliary membrane. Following a rapid activation process in cilia, ARL3<sup>GDP</sup> is converted to  
375 become ARL3<sup>GTP</sup> for releasing from the ciliary membrane. Following ciliary cycling, the BBSome sheds  
376 from retrograde IFT at the proximal ciliary region right above the TZ. By acting as a major ARL3 effector,  
377 the cargo (PLD)-laden BBSome was then bound to and recruited by ARL3<sup>GTP</sup> to diffuse through the TZ  
378 for ciliary retrieval (Fig. 7). Our data show that ARL3 maintains BBSome ciliary dynamics by moving it  
379 cross the diffusion barrier at the TZ and out of cilia, thus closing a gap in our understanding of how ARL3  
380 affects cell behavior, e.g., phototaxis, of *C. reinhardtii*.

381

### 382 How does ARL3 contribute to maintain BBSome dynamics in cilia?

383 The BBSome relies on IFT for maintaining its ciliary dynamics. During BBSome ciliary cycling, certain  
384 small GTPases contribute to maintain BBSome ciliary dynamics by mediating its coupling with IFT  
385 directly or indirectly. Our previous study identified RABL5/IFT22 binds ARL6/BBS3 to form an  
386 IFT22/BBS3 heterodimer in the cell body of *Chlamydomonas* cells and IFT22 binding is required for  
387 stabilizing BBS3 (Xue et al., 2020). As a major BBS3 effector, the BBSome is bound to and recruited by  
388 IFT22/BBS3 for targeting to the basal bodies (Jin et al., 2010; Xue et al., 2020). In such a way,  
389 IFT22/BBS3 controls BBSome amount available for entering cilia from the basal bodies, thus playing a  
390 critical role in maintaining BBSome ciliary dynamics (Xue et al., 2020). Upon reaching the ciliary tip via  
391 anterograde IFT, the BBSome disassembles first followed by reassembly, a process known as BBSome  
392 remodeling, before being able to load onto retrograde IFT trains for transporting to the ciliary base (Sun et  
393 al., 2021). During this process, RABL4/IFT27, by binding its stabilizing partner IFT25 to form an  
394 IFT25/27 heterodimer, cycles off IFT to promote BBSome reassembly (Liew et al., 2014; Sun et al., 2021;  
395 Wang et al., 2009). Therefore, IFT25/27 is critical for maintaining BBSome ciliary dynamics as it  
396 contributes to promote the BBSome to U turn at the ciliary tip. As for ARL3, it mimics ARL6/BBS3 to  
397 bind the membrane to diffuse into cilia and resides along the whole length of cilia by attaching to the  
398 ciliary membrane (Liu et al., 2021). Differing from ARL6/BBS3 that in a GTP-bound state binds the  
399 membrane, ARL3 relies on GDP for membrane binding (Liu et al., 2021). This is easy to understand as  
400 the cell may have developed an elaborated system to restrict ARL3 to bind its BBSome effector only at  
401 the proximal ciliary region right above the TZ where ARL3<sup>GTP</sup> is supposed to concentrate (Fig. 2 F and 5  
402 C). Upon reaching the proximal ciliary region right above the TZ via retrograde IFT, the BBSome drops

403 off retrograde IFT via a mechanism that remains unknown yet, consistent with the mammalian BBSome  
404 behavior in cilia (Duan et al., 2021; Nachury, 2018; Ye et al., 2018). GTP loading then enables ARL3 to  
405 bind and recruit the IFT-detached BBSome to move across the diffusion barrier at the TZ for ciliary  
406 retrieval. Therefore, ARL3 controls BBSome ciliary amount through mediating its diffusion through the  
407 TZ for ciliary removal, playing a critical role in maintaining BBSome ciliary dynamics.

408

#### 409 **The BBSome is a ARL3 effector only when they both position to the proximal ciliary region**

410 Cross ciliated species, PDE6D, UNC119A/B, and BART/BARTL1 are three types of ARL3 effectors  
411 known as carrier/solubilizing proteins for binding and shuttling cytoplasmic lipidated signaling protein  
412 cargoes into cilia (Linari et al., 1999; Lokaj et al., 2015; Wright et al., 2011). Once at the proximal ciliary  
413 region, ARL3 applies RP2 and ARL13b as its GAP and GEF, respectively, and is catalyzed to convert  
414 between being GTP- and GDP-bound (Gotthardt et al., 2015; Veltel et al., 2008; Zhang et al., 2016). This  
415 ARL3/effector/cargo cascade works efficiently for releasing the cytoplasmic lipidated cargoes to bind the  
416 ciliary membrane via their lipidated moieties. Reflecting its critical role in releasing signaling proteins  
417 downstream the transmembrane sensing receptors in cilia, ARL3 deficiency disrupts certain signal  
418 transduction pathway(s) to cause many diseases. i.e., inherited retinal degenerations (IRDs) (Fu et al.,  
419 2021; Ratnapriya et al., 2021). In this study, the BBSome was identified to be a major ARL3 effector in  
420 cilia, uncovering ARL3's role in mediating ciliary signaling but via the BBSome pathway. Remarkably,  
421 ARL3 applies the BBSome as its effector only when they both position to the proximal ciliary region  
422 right above the TZ but not in the cell body, suggesting that ARL3 is intrinsically prevented from  
423 interacting with the BBSome for conducting GTPase/effector function in the cell body. This could be  
424 achieved simply by retaining ARL3 at a GDP-bound configuration in the cell body. Although the  
425 underlying mechanism of how cells restrain ARL3 in a GDP-bound state in the cell body compartment  
426 remains unknown thus far, ARL3 is indeed observed to exist in a GDP-bound state in the cell body,  
427 disabling it for BBSome binding in the cell body. It has been known that IFT22/BBS3 binds the BBSome  
428 via a direct interaction between the BBSome and BBS3 in the cell body and recruits the BBSome as a  
429 BBS3 effector to the basal bodies (Xue et al., 2020). This observation explained well why ARL3 even in  
430 a GTP-bound configuration fails to bind the BBSome in the cell body (Fig. 4 D and E). The cell may  
431 direct BBSome trafficking in different cellular compartments through applying distinct GTPase pathways.

432

#### 433 **How is ARL3 activated to promote BBSome diffusion through the TZ?**

434 ARL3 in a GDP-bound state enters cilia but relies on GTP for binding and recruiting the BBSome to  
435 diffuse through the TZ for ciliary retrieval, suggesting that ARL3 must have to convert from being GDP-  
436 bound to being GTP-bound in cilia. *Chlamydomonas* ARL13b has been identified as a ARL3 GEF to

437 catalyze the conversion of ARL3<sup>GDP</sup> to ARL3<sup>GTP</sup> in cilia (Gotthardt et al., 2015). While ARL13b fits to  
438 the profile of the ARL3 GEF as it localizes to cilia and has in vitro GEF activity, functional correlation of  
439 ARL13b to ARL3 and structural studies for elucidating how ARL13b activates ARL3 at a molecular level  
440 were not recorded in the literature (ElMaghloob et al., 2021; Gotthardt et al., 2015). Most recently,  
441 ARL13b was found to be able to activate ARL3 but very weakly at physiological GTP:GDP levels and its  
442 stronger activation was achieved through applying BART as a so-called “co-GEF”, which stabilizes  
443 ARL3 to retain in a GTP-bound state (ElMaghloob et al., 2021). Unfortunately, knockout of human  
444 ARL13b alters BBSome ciliary dynamics but by reducing BBSome content in cilia, a result excluding  
445 ARL13b from promoting BBSome diffusion through the TZ for ciliary retrieval via ARL3 pathway  
446 (Fujisawa et al., 2021). Interestingly, although they both play critical roles in targeting INPP5E to the  
447 ciliary membrane in human cells, ARL3 and ARL13b instead participate in distinct steps of this event,  
448 again revealing their functional discrepancy in vivo (Fujisawa et al., 2021). These findings do not  
449 necessarily disapprove ARL13b for activating ARL3 as a possible functional ARL3 GEF in vivo, while  
450 ARL3/ARL13b cascade can be confidently excluded from promoting BBSome diffusion through the TZ  
451 for ciliary retrieval, at least in *C. reinhardtii*. This raised an interesting question, namely which factor  
452 other than ARL13b, if desirable, contributes to activate ARL3 specifically for promoting BBSome  
453 diffusion through the TZ. We currently had no answer for this question, while RABL2 deficiency causes  
454 BBSome to cease for moving out of cilia but to accumulate at the proximal region right above the TZ, the  
455 same BBSome intraciliary trafficking defect pattern as shown by ARL3 knock-out (Duan et al., 2021).  
456 This observation provides a clue, from the functional review, that RABL2 could likely be a ARL3 GEF in  
457 cilia, though his hypothesis remains to be confirmed.

458

#### 459 **How does ARL3 mediate phototaxis?**

460 *C. reinhardtii* cells apply the photoreceptors channelrhodopsin 1 (ChR1) and ChR2 to sense light for  
461 conducting phototaxis via ciliary beating (Berthold et al., 2008; Nagel et al., 2002; Nagel et al., 2003).  
462 Among these two sensory ion channels, ChR1 was identified as the major one, by residing in the eyespot,  
463 for sensing light to generate and transduce electrophysiological signal to cilia to direct their beating  
464 (Berthold et al., 2008). ChR1 was later shown to be able to target to cilia via an IFT-dependent manner,  
465 giving rise to a possibility that ChR1 might sense light for directing the cell to conduct phototaxis by  
466 residing in cilia but not the eyespot (Awasthi et al., 2016). We currently had no clue about if ChR1, like  
467 certain GPCRs (Ye et al., 2013; Ye et al., 2018), requires the BBSome for exporting out of cilia, while  
468 BBS mutants deprives the BBSome of being present in cilia, eventually disrupting phototaxis simply  
469 owing to biochemical defects of the ciliary membrane (Lechtreck et al., 2013; Lechtreck et al., 2009; Liu  
470 and Lechtreck, 2018). If the prediction that ChR1 ciliary positioning and its BBSome-dependent

471 dynamics maintenance in cilia are prerequisites for *Chlamydomonas* cells to conduct phototaxis is correct,  
472 these defects may arise from abnormal ciliary buildup of ChR1 due to its disrupted export out of cilia in  
473 the absence of the BBSome. Therefore, it is most likely that ARL3 mediates phototaxis via controlling the  
474 BBSome-dependent ChR1 dynamics maintenance in cilia of *Chlamydomonas* cells, which deserves to be  
475 carefully investigated in the future.



## 476 **Materials and methods**

477

### 478 **Antibodies, *Chlamydomonas* strains, and culture conditions**

479 Rabbit-raised polyclonal antibodies including  $\alpha$ -BBS1,  $\alpha$ -BBS4,  $\alpha$ -BBS5,  $\alpha$ -BBS7,  $\alpha$ -BBS8,  $\alpha$ -IFT22,  $\alpha$ -  
480 IFT38,  $\alpha$ -IFT43,  $\alpha$ -IFT46,  $\alpha$ -IFT57,  $\alpha$ -IFT70,  $\alpha$ -IFT139, and  $\alpha$ -PLD and mouse-raised  $\alpha$ -IFT81 have been  
481 described previously and were listed in Table EV1 (Dong et al., 2017; Liu et al., 2021; Sun et al., 2021).  
482 Rabbit-originated antibodies against ARL3 and CEP290 were produced by Beijing Protein Innovation,  
483 LLC (Beijing). The monoclonal antibodies against YFP (mAbs 7.1 and 13.1, Roche), HA (mAb 3F10  
484 clone, Roche),  $\alpha$ -tubulin (mAb B512, Sigma), and acetylated-tubulin (mAb 6-11B-1, Sigma-Aldrich)  
485 were commercially bought (Table S1). *C. reinhardtii* strains including CC-125, CC-5325, and *arl3* (the  
486 CLiP mutant LMJ.RY0420.182282) were purchased from the *Chlamydomonas* Genetic Center at the  
487 University of Minnesota, Twin Cities, MN (<http://www.chlamycollection.org>). BBS8-null mutant *bbs8*  
488 has been reported previously (Sun and Pan, 2019). All the strains used in this study are listed in Table S2.  
489 If not otherwise specialized, strains were grown in Tris acetic acid phosphate (TAP) or minimal 1 (M1)  
490 medium in a continuous light with constant aeration at room temperature. Depending on a specific strain,  
491 cells were cultured with or without the addition of 20  $\mu$ g/ml paromomycin (Sigma-Aldrich), 15  $\mu$ g/ml  
492 bleomycin (Invitrogen), or both antibiotics with 10  $\mu$ g/ml paromomycin and 5  $\mu$ g/ml bleomycin.

493

### 494 **Vectors and cell line construction**

495 ARL3 miRNA vector was created according to the method described previously (Hu et al., 2014). In brief,  
496 the miRNA sequences targeting the 3'-UTR region of *arl3* gene were designed using WMD3 software  
497 (<http://wmd3.weigelworld.org>) and were combined with the miRNA cre-MIR1157 (accession number  
498 MI0006219) to result in a 171-bp of ARL3 miRNA precursor sequence (Table S3). The ARL3 miRNA  
499 precursor sequence was synthesized by Genewiz (China) and ligated to the pHK263 plasmid (Hu et al.,  
500 2014), resulting in the ARL3 miRNA vector pMi-ARL3. Expression vectors were constructed on pBKS-  
501 gBBS3::HA::YFP-Ble that contained HA::YFP coding sequences followed immediately downstream by a  
502 sequence encoding the Rubisco 3'-UTR and the bleomycin cassette (*Ble*, zeocine resistant gene) (Dong et  
503 al., 2017). To generate ARL3::HA::YFP-expressing vector, a 3.4-kb ARL3 fragment consisting of the  
504 1.0-kb promoter sequence and its coding region was amplified from genomic DNA by using primer pair  
505 (gARL3-FOR1 and gARL3-REV1) as listed in Table S3 and inserted into the *Xba*I and *Eco*RI sites of  
506 pBKS-gBBS3::HA::YFP-Ble, resulting in pBKS-gARL3::HA::YFP-Ble. To generate  
507 ARL3 $\Delta$ N15::HA::YFP-expressing vector, two fragments were amplified by using primer pair (gARL3-  
508 FOR1 and gARL3 $\Delta$ N15-REV; gARL3 $\Delta$ N15-FOR and gARL3-REV1) as listed in Table S3 and inserted  
509 into the *Xba*I and *Eco*RI sites of pBKS-gBBS3::HA::YFP-Ble by three-way ligation, resulting in pBKS-

510 gARL3 $\Delta$ N15::HA::YFP-Ble. The desiring mutations including G2A, G2AT30N, G2AQ70L, T30N, and  
511 Q70L were introduced into pBKS-gARL3::HA::YFP-Ble and pBKS-gARL3 $\Delta$ N15::HA::YFP-Ble by site-  
512 directed mutagenesis using the primer pairs (ARL3<sup>G2A</sup>-FOR and ARL3<sup>G2A</sup>-REV; ARL3<sup>T30N</sup>-FOR and  
513 ARL3<sup>T30N</sup>-REV; ARL3<sup>Q70L</sup>-FOR and ARL3<sup>Q70L</sup>-REV, respectively) as listed in [Table S3](#). Afterward, the  
514 mutated DNAs were inserted into the *Xba*I and *Eco*RI sites of pBKS-gARL3::HA::YFP-Ble, resulting in  
515 pBKS-gARL3<sup>T30N</sup>::HA::YFP-Ble, pBKS-gARL3<sup>Q70L</sup>::HA::YFP-Ble, pBKS-gARL3<sup>G2A</sup>::HA::YFP-Ble,  
516 pBKS-gARL3<sup>G2AT30N</sup>::HA::YFP-Ble, pBKS-gARL3<sup>G2AQ70L</sup>::HA::YFP-Ble, pBKS-  
517 gARL3 $\Delta$ N15<sup>T30N</sup>::HA::YFP-Ble, and pBKS-gARL3 $\Delta$ N15<sup>Q70L</sup>::HA::YFP-Ble. To express  
518 IFT43::HA::YFP, a 2,800-bp DNA fragment composing of a 1,000-bp promoter and IFT43 coding  
519 sequence was amplified from genomic DNA by using the primer pair (gIFT43-FOR and gIFT43-REV) as  
520 listed in [Table S3](#), and inserted into *Not*I and *Eco*RI sites of pBluescript II KS(+) vector, resulting in  
521 pBKS-gIFT43. Afterwards, gIFT43 sequence was cut from pBKS-gIFT43 by *Not*I and *Eco*RI sites,  
522 HA::YFP-Ble sequence was cut from pBKS-gARL3::HA::YFP-Ble by *Eco*RI and *Kpn*I, and inserted  
523 those two fragments into the *Not*I and *Kpn*I sites of pBluescript II KS(+) vector by three-way ligation,  
524 resulting in pBKS-gIFT43::HA::YFP-Ble. To express IFT38::YFP, a 3,875-bp DNA fragment composing  
525 of a 1,000-bp promoter sequence and the IFT38 coding sequence was amplified from genomic DNA  
526 using primer pair (gIFT38-FOR and gIFT38-REV) as listed in [Table S3](#) and inserted into the *Bam*HI and  
527 *Eco*RI sites of pBluescript II KS(+) vector, resulting in pBKS-gIFT38. Next, IFT38 sequence was cut  
528 from pBKS-gIFT38, and inserted into the *Bam*HI and *Eco*RI sites of pBKS-gBBS5::YFP-Paro and  
529 pBKS-gBBS5::YFP-Ble, resulting in pBKS-gIFT38::YFP-Paro and pBKS-gIFT38::YFP-Ble,  
530 respectively. pBKS-gIFT22::HA::GFP-Paro and pBKS-gIFT22::HA::GFP-Ble have been described  
531 previously ([Xue et al., 2020](#)). To express IFT22::HA::YFP, the HA::GFP fragment was replaced with the  
532 HA::YFP fragment obtained from pBKS-gARL3::HA::YFP-Ble by *Eco*RI and *Xho*I digestion, resulting  
533 in pBKS-gIFT22::HA::YFP-Paro and pBKS-gIFT22::HA::YFP-Ble, respectively. To express BBS8::YFP,  
534 a 4,804-bp DNA fragment composing of 1,000-bp promoter and 3,804-bp BBS8 coding sequence was  
535 amplified from genomic DNA by using the primer pair (gBBS8-FOR and gBBS8-REV) as listed in [Table](#)  
536 [S3](#), and inserted into *Xba*I and *Eco*RI sites of pBluescript II KS(+) vector, resulting in pBKS-gBBS8.  
537 Then, BBS8 sequence cut from pBKS-gBBS8 by *Xba*I and *Eco*RI sites and YFP-Ble sequence cut from  
538 pBKS-gBBS5::YFP-Ble by *Eco*RI and *Kpn*I sites were inserted into the *Xba*I and *Kpn*I sites of  
539 pBluescript II KS(+) vector by three-way ligation, resulting in pBKS-gBBS8::YFP-Ble. After the  
540 verification by direct nucleotide sequencing, the new constructs were transformed into *C. reinhardtii*  
541 strain by electroporation as described previously and screening of the positive transformants was done  
542 according to the method described previously ([Xue et al., 2020](#)). The screening of ARL3 miRNA cells  
543 was initiated by checking the cellular level of the target proteins through immunoblotting of whole cell

544 extracts with ARL3 antibody. The miRNA strains showing a reduced level of the target proteins were  
545 selected for further phenotypic analysis.

546 For *arl3-bbs8* double mutant generation, 20 ml of each of *arl3*- and *bbs8*-null mutant cells were  
547 grown to a final concentration of  $2 \times 10^6$  cells/ml in M1 medium under continuous light at room  
548 temperature. The cells were collected by centrifugation at  $1000 \times g$  for 15 min and washed with M1-N  
549 (without nitrogen) medium. Afterwards, the cells were transferred to 20 ml of M1-N medium and aerated  
550 for 12 hrs under continuous light. After that, gametes of two opposite mating types were mixed in flask  
551 and incubated under light for 2 hrs. The cells sitting on the flask bottom were transferred to M1 plate  
552 containing 4% agar, air-dried, incubated overnight under continuous light, and then continued to be  
553 incubated for a week in the dark. The plates were then moved to incubate at  $-20^\circ\text{C}$  for two days followed  
554 by culturing at room temperature under continuous light for at least ten days. The *arl3*- and *bbs8*-double  
555 null mutant was screened by detecting the loss of ARL3 and BBS8 proteins through immunoblotting of  
556 whole cell extracts with both ARL3 and BBS8 antibodies.

557

#### 558 **Total RNA and genomic DNA manipulations**

559 Genomic DNA of *Chlamydomonas* cells was extracted and purified using a Wizard® Genomic DNA  
560 Purification Kit (Promega, Beijing) following the kit's protocol. To characterize the *arl3* cell (the CLiP  
561 mutant LMJ.RY0420.182282) at the genomic level, 20 ng of genomic DNA was applied as PCR template  
562 to amplify ARL3 genomic sequence. The PCR reactions were performed at  $95^\circ\text{C}$  for 5 min followed by  
563 30 cycles of  $95^\circ\text{C}$  for 20 sec,  $61^\circ\text{C}$  for 20 sec, and  $72^\circ\text{C}$  for 5 min with the primer pair gARL3-FOR1 and  
564 gARL3-REV1 as listed in Table S3. Total RNA of *Chlamydomonas* cells was extracted and purified  
565 according to our protocol reported previously (Dong et al., 2017). Five micrograms of RNA were reverse  
566 transcribed at  $42^\circ\text{C}$  for 1 h using M-MLV Reverse Transcriptase (Promega) and oligo(T)18 primers  
567 (Takara). ARL3 cDNA and cDNAs encoding the six BBS proteins BBS1, BBS2, BBS4, BBS5, BBS7,  
568 and BBS8 were amplified by PCR using primer pairs as listed in Table S3. The PCR reactions were  
569 performed at  $95^\circ\text{C}$  for 5 min followed by 30 cycles of  $95^\circ\text{C}$  for 20 sec,  $61^\circ\text{C}$  for 20 sec, and  $72^\circ\text{C}$  for 4  
570 min.

571

#### 572 **Ciliary length measurement**

573 *Chlamydomonas* cells growing to a concentration of  $\sim 10^7$  cells were collected and placed onto the surface  
574 of glass slides and covered by cover glass. The cells were observed under an IX83 inverted fluorescent  
575 microscopy at  $100\times$  amplification. Phase contrast images were then taken for ciliary length analysis.  
576 Ciliary length was measured by using ImageJ (version 1.42g, National Institutes of Health) according to

577 our protocol reported previously (Fan et al., 2010). The data was processed with GraphPad Prism 8.30  
578 (GraphPad Software). For each strain, a total of 20 cilia were measured.

579

### 580 **Isolation of cilia and cell bodies**

581 Isolation of cilia and cell bodies was performed according to our protocol reported previously (Fan et al.,  
582 2010). In brief, *Chlamydomonas* cells were grown in 10 liters of TAP medium to a final density of  $10^8$   
583 cells/ml, collected by centrifugation at  $1000 \times g$  for 15 min, and suspended in 150 ml of TAP (pH7.4).  
584 Cells were incubated for 2 h under strong light with bubbling before 0.5 M acetic acid was added to adjust  
585 the pH value to 4.5 for cell deciliation. Afterwards, 0.5 M KOH was added to adjust the pH value to 7.4.  
586 Cell body pellets and cilia in the supernatant were collected separately after centrifugation at  $600 \times g$  at  
587  $4^\circ\text{C}$  for 5 min. To avoid the possible cell body contamination, cilia were repeatedly washed with  
588 HMDEKN buffer (30 mM Hepes [pH 7.4], 5 mM  $\text{MgSO}_4$ , 1 mM DTT, 0.5 mM EGTA, 25 mM KCl, 125  
589 mM NaCl) by centrifugation at  $12,000 \times g$  for 10 min until the green color disappeared completely. All the  
590 experiments were done at  $4^\circ\text{C}$ .

591

### 592 **Preparation of ciliary fractions**

593 Ciliary fractions were prepared according to our protocol reported previously (Liu et al., 2021). In brief,  
594 cell body-depleted cilia were dissolved in HMDEKN buffer supplemented with protein inhibitors (PI) (1  
595 mM PMSF, 50  $\mu\text{g/ml}$  soy-bean trypsin inhibitor, 1  $\mu\text{g/ml}$  pepstatin A, 2  $\mu\text{g/ml}$  aprotinin, and 1  $\mu\text{g/ml}$   
596 leupeptin) and frozen in liquid nitrogen. After three cycles of frozen-and-thaw, the solution was  
597 centrifuged at  $12,000 \times g$  at  $4^\circ\text{C}$  for 15 min and the ciliary matrix fraction was collected as the  
598 supernatant. The pellets were then dissolved in HMEKDN (see above) buffer containing 0.5% nonidet P-  
599 40 (NP-40) and stayed on ice for 15 min. After centrifugation at  $12,000 \times g$  at  $4^\circ\text{C}$  for 10 min, the  
600 supernatant and pellet were collected as membrane and axonemal fractions, respectively.

601

### 602 **Sucrose density gradient centrifugation assay**

603 Ciliary samples were analyzed by sucrose density gradient centrifugation according to our protocol  
604 reported previously (Sun et al., 2021). Briefly, linear 12 ml of 10-25% sucrose density gradients were  
605 prepared in HMDEKN buffer supplemented with PI (see above) and 1% NP-40. Ciliary extracts were  
606 frozen and thawed for three cycles by using liquid nitrogen and centrifuged at  $12,000 \times g$  at  $4^\circ\text{C}$  for 10  
607 min. After the non-soluble debris was removed, 700  $\mu\text{l}$  of samples were loaded on the top of the gradients  
608 and separated at  $38,000 \times g$  for 14 hrs in a SW41Ti rotor (Beckman Coulter). After the gradients were  
609 fractioned into 24 0.5 ml aliquots, 20  $\mu\text{l}$  of each fraction was loaded into SDS-PAGE gel for  
610 electrophoresis and analyzed by immunoblotting as described below. The centrifugation was done at  $4^\circ\text{C}$ .

611 **Immunoblotting**

612 Whole cell, cell body, and ciliary samples were prepared for immunoblotting according to our protocol  
613 reported previously (Dong et al., 2017). If not otherwise specified, 20 µg of total protein from each  
614 sample was loaded in the 12% SDS-PAGE gel for electrophoresis. Primary and secondary antibodies  
615 were diluted for immunoblotting with a ratio as shown in Table S1. ImageJ software (version 1.42g,  
616 National Institutes of Health) was applied for quantifying the target proteins by measuring the  
617 immunoblot intensity according to our protocol reported previously (Xue et al., 2020). The immunoblot  
618 intensity was normalized to the intensity of a loading control protein.

619

620 **Immunoprecipitation**

621 Immunoprecipitation was performed according to our protocol reported previously (Liu et al., 2021). In  
622 brief, cell body and ciliary samples isolated from cells expressing HA::YFP, HA::YFP-tagged ARL3, or  
623 its variants were resuspended in HMDEKN or DTT-deprived HMEKN buffer supplemented with protein  
624 inhibitors (see above). The samples were lysed by adding NP-40 to a final concentration of 1% followed  
625 by centrifugation at 14,000 ×g, 4°C for 10 min. Afterward, the supernatants were collected for agitating  
626 with 5% BSA-pretreated camel anti-YFP antibody-conjugated agarose beads (V-nanoab Biotechnology)  
627 for 2 hrs at 4°C. After continuous washing with HMEKN buffer, HMEK buffer containing 50 mM NaCl,  
628 and HMEK without containing NaCl, the beads were collected by centrifugation at 2,500 ×g for 2 min,  
629 mixed with Laemmli SDS sample buffer, and boiled for 5 min before centrifugation at 2,500 ×g for 5 min.  
630 The immunoprecipitants in the supernatants were collected for immunoblotting analysis as described  
631 above.

632

633 **Protein-liposome binding assay**

634 Liposomes were prepared according to the protocol reported previously (Jin et al., 2010). The ARL3  
635 cDNA was inserted into the *EcoRI* and *XhoI* sites of pET-28a (Novagen) to result in pET-28a-cARL3.  
636 The desiring deletion of N-terminal 15 amino acid residues and the mutations G2A, Q70L, and T30N  
637 were introduced into ARL3 by regular PCR or site-directed mutagenesis using the primer pairs as listed in  
638 Table S3, resulting plasmids pET-28a-cARL3<sup>Q70L</sup>, pET-28a-cARL3<sup>T30N</sup>, pET-28a-cARL3ΔN15, pET-  
639 28a-cARL3ΔN15<sup>Q70L</sup>, pET-28a-cARL3ΔN15<sup>T30N</sup>, pET-28a-cARL3<sup>G2A</sup>, pET-28a-cARL3<sup>G2AQ70L</sup>, and pET-  
640 28a-cARL3<sup>G2AT30N</sup>, respectively. After these plasmids were transformed into the *Escherichia coli* strain  
641 BL21(DE3), the bacterially expressed 6×His tagged ARL3 and its variants were purified with Ni  
642 Sepharose™ 6 Fast Flow beads (GE Healthcare) and cleaved with thrombin (Solarbio) for 1 hr at 37°C to  
643 get rid of the 6×His tag according to our previous report (Xue et al., 2020). ARL3 association with  
644 liposome was conducted in binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% NP-40). In brief, 1

645  $\mu\text{g}$  of the purified ARL3 (in the presence of 100 mM of GTP $\gamma$ S or of GDP) or its variants protein, 20  $\mu\text{l}$   
646 of 1 mM PolyPIPosomes<sup>TM</sup> (Echelon Biosciences), and 1 ml of binding buffer were mixed, rotated for 10  
647 min at room temperature, and then centrifuged at 12,000  $\times$ g for 10 min. The liposome pellet was washed  
648 in 1 ml of binding buffer by centrifugation for three times. Afterward, equal portions of the resulting  
649 pellets were mixed with Laemmli SDS sample buffer and boiled for 5 min before resolving by SDS-  
650 PAGE and immunoblotting with  $\alpha$ -ARL3.

651

### 652 **Fixed imaging**

653 Immunofluorescence staining was performed according to our protocol reported previously (Wang et al.,  
654 2009). The primary antibodies against CEP290, BBS8, IFT46, IFT81, YFP, HA, and the secondary  
655 antibodies including Alexa-Fluor594-conjugated goat anti-rabbit, Alexa-Fluor488-conjugated goat anti-  
656 mouse, and Alexa-Fluor488-conjugated goat anti-rat (Molecular Probes, Eugene, OR) were listed in  
657 Table S1 with their suggested dilutions. Images were captured with an IX83 inverted fluorescent  
658 microscopy (Olympus) equipped with a back illuminated scientific CMOS camera (Prime 95B,  
659 Photometrics), a 100 $\times$ /1.40 NA oil objective lens (Olympus), and 488-nm and 561-nm lasers from  
660 Coherent OBIS Laser Module. All images were acquired and processed with CellSens Dimension  
661 (version 2.1, Olympus).

662

### 663 **Live-cell imaging**

664 Total internal reflection fluorescence (TIRF) microscopy was applied to visualize the motility of YFP-  
665 tagged IFT43, IFT22, IFT38, IFT46, BBS8, and ARL3 and its variants in cilia. YFP-tagged proteins was  
666 imaged at  $\sim$ 15 frames per second (fps) using an IX83 inverted fluorescent microscopy (Olympus)  
667 equipped with a through-the-objective TIRF system, a 100 $\times$ /1.49 NA TIRF oil immersion objective len  
668 (Olympus), a back illuminated scientific CMOS camera (Prime 95B, Photometrics), and 488-nm laser  
669 from Coherent OBIS Laser Module as detailed previously (Xue et al., 2020).

670

### 671 **Kymogram analysis**

672 Kymography was generated according to our protocol reported previously (Dong et al., 2017). In brief,  
673 YFP-tagged IFT, BBSome and ARL3 proteins were imaged with TIRF (15 Hz) for  $\sim$ 20 s. The videos  
674 obtained were processed with CellSens Dimension (version 2.1, Olympus) for generating kymographs. In  
675 kymographs, lines drawn along the long axis of the cilium (“the leg”) and the processive movement (“the  
676 hypotenuse”) represent the anterograde and retrograde IFT tracks. The angle of the lines was measured  
677 (“the included angle”). Comparison of the leg and the hypotenuse was applied for quantifying the  
678 frequency of an IFT- or BBS-containing train. Comparison of the hypotenuse and the included angle was

679 applied for measuring the velocity of IFT and BBSome trains. KymographClear was applied to  
680 deconvolve retrograde from anterograde trains for clearly showing BBSome diffusion through the  
681 transition zone (e.g., Fig. 5E) (Mangeol et al., 2016).

682

### 683 **Protein-protein interaction assay**

684 The cDNA encoding BBS9 was synthesized by Genewiz (China). The cDNAs encoding BBS1, BBS2,  
685 BBS4, BBS5, BBS7, BBS8, and BBS9 were inserted into the *Bam*HI and *Hind* III sites of pMal-C2x  
686 (Nova Lifetech) to generate pMal-C2x-cBBS1, pMal-C2x-cBBS2, pMal-C2x-cBBS4, pMal-C2x-cBBS5,  
687 pMal-C2x-cBBS7, pMal-C2x-cBBS8, and pMal-C2x-cBBS9. After these plasmids and the empty pMal-  
688 C2x plasmid were transformed into the *E. coli* strain BL21(DE3), the bacterially expressed N-terminal  
689 MBP-tagged BBS proteins were purified with Dextrin Sepharose™ High Performance with MBP-tagged  
690 protein purification resin (GE Healthcare). One hundred micrograms of MBP and the MBP-tagged BBS  
691 proteins were individually mixed with 100 µg of bacterially expressed ARL3<sup>Q70L</sup> or ARL3<sup>T30N</sup> (see above)  
692 to form a combination of 16 reactions. After incubated for 2 hrs at room temperature, the mixtures were  
693 purified with Dextrin Sepharose™ High Performance MBP-tagged protein purification resin (GE  
694 Healthcare). Ten micrograms of proteins from elutes was resolved on 12% SDS-PAGE gels and  
695 visualized with Coomassie blue staining. Immunoblotting assay was also performed to verify the  
696 interaction between BBS proteins and ARL3 variants with  $\alpha$ -ARL3.

697

### 698 **Population phototaxis assay**

699 Population phototaxis assays were performed on *Chlamydomonas* cells according to the protocol reported  
700 previously (Liu and Lechtreck, 2018). In brief, *Chlamydomonas* cells growing to a concentration of  $\sim 10^7$   
701 cells were harvested and 100 µl of the cell suspension were placed into the surface of Petri dishes of 3.5-  
702 cm diameter (706001; Wuxi NEST Biotech.) containing solid TAP medium. Afterwards, the cells were  
703 illuminated with a flashlight from one side for 4 min. Images were continuously taken once every two  
704 minutes with a standard digital camera (Nikon A70).

705

### 706 **Single-cell motion assay**

707 Single-cell motion assay was performed on *Chlamydomonas* cells according to the protocol reported  
708 previously (Liu and Lechtreck, 2018). Briefly, 20 µl of cell suspensions as obtained above were placed on  
709 superfrost™ plus microscope slides (12-550-15; Fisher Brand) and observed using an inverted  
710 microscope (IX83, Olympus) under non-phototactic red light illumination. The cells were then  
711 illuminated for 5s with phototactic active green light. Afterwards, five images were continuously taken  
712 once every 0.3s using a back illuminated scientific CMOS camera (Prime 95B, Photometrics). The five

713 sequential images each displayed in a different color were merged in ImageJ (version 1.42g, National  
714 Institutes of Health) to show the swimming tracks of single cells, allowing us to determine the angle and  
715 the direction of a cell's movements. Excel for Mac (version 16.52) were applied to analyze the data and  
716 generate polar histograms with 60° bins.

717

#### 718 **Statistical analysis**

719 Statistical analysis was done with GraphPad Prism 8.30 (GraphPad Software). For comparisons on  
720 velocities and frequencies of the YFP- and HA::YFP-labeled proteins and ciliary length measurement,  
721 one-sample unpaired student *t*-test was used on samples. The data were presented as mean ± S.D. n.s.  
722 represents non-significance.

723

#### 724 **Data availability**

725 Data supporting the findings of this study were contained within this paper and the supplementary files.

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## References

- Abd-El-Barr, M.M., K. Sykoudis, S. Andrabi, E.R. Eichers, M.E. Pennesi, P.L. Tan, J.H. Wilson, N. Katsanis, J.R. Lupski, and S.M. Wu. 2007. Impaired photoreceptor protein transport and synaptic transmission in a mouse model of Bardet-Biedl syndrome. *Vision Res.* 47:3394-3407.
- Amor, J.C., D.H. Harrison, R.A. Kahn, and D. Ringe. 1994. Structure of the human ADP-ribosylation factor 1 complexed with GDP. *Nature.* 372:704-708.
- Avidor-Reiss, T., A.M. Maer, E. Koundakjian, A. Polyanovsky, T. Keil, S. Subramaniam, and C.S. Zuker. 2004. Decoding cilia function: defining specialized genes required for compartmentalized cilia biogenesis. *Cell.* 117:527-539.
- Awasthi, M., P. Ranjan, K. Sharma, S.K. Veetil, and S. Kateriya. 2016. The trafficking of bacterial type rhodopsins into the Chlamydomonas eyespot and flagella is IFT mediated. *Sci Rep.* 6:34646.
- Berthold, P., S.P. Tsunoda, O.P. Ernst, W. Mages, D. Gradmann, and P. Hegemann. 2008. Channelrhodopsin-1 initiates phototaxis and photophobic responses in chlamydomonas by immediate light-induced depolarization. *The Plant cell.* 20:1665-1677.
- Cuvillier, A., F. Redon, J.C. Antoine, P. Chardin, T. DeVos, and G. Merlin. 2000. LdARL-3A, a Leishmania promastigote-specific ADP-ribosylation factor-like protein, is essential for flagellum integrity. *J Cell Sci.* 113 ( Pt 11):2065-2074.
- Dateyama, I., Y. Sugihara, S. Chiba, R. Ota, R. Nakagawa, T. Kobayashi, and H. Itoh. 2019. RABL2 positively controls localization of GPCRs in mammalian primary cilia. *J Cell Sci.* 132.
- Dong, B., S. Wu, J. Wang, Y.X. Liu, Z. Peng, D.M. Meng, K. Huang, M. Wu, and Z.C. Fan. 2017. Chlamydomonas IFT25 is dispensable for flagellar assembly but required to export the BBSome from flagella. *Biol Open.* 6:1680-1691.
- Duan, S., H. Li, Y. Zhang, S. Yang, Y. Chen, B. Qiu, C. Huang, J. Wang, J. Li, X. Zhu, and X. Yan. 2021. Rabl2 GTP hydrolysis licenses BBSome-mediated export to fine-tune ciliary signaling. *EMBO J.* 40:e105499.
- Efimenko, E., K. Bubb, H.Y. Mak, T. Holzman, M.R. Leroux, G. Ruvkun, J.H. Thomas, and P. Swoboda. 2005. Analysis of xbx genes in C. elegans. *Development.* 132:1923-1934.
- ElMaghloob, Y., B. Sot, M.J. McIlwraith, E. Garcia, T. Yelland, and S. Ismail. 2021. ARL3 activation requires the co-GEF BART and effector-mediated turnover. *Elife.* 10:e64624.
- Fan, Z.-C., R.H. Behal, S. Geimer, Z. Wang, S.M. Williamson, H. Zhang, D.G. Cole, and H. Qin. 2010. Chlamydomonas IFT70/CrDYF-1 Is a Core Component of IFT Particle Complex B and Is Required for Flagellar Assembly. *Mol Biol Cell.* 21:2696-2706.
- Fansa, E.K., S.K. Kosling, E. Zent, A. Wittinghofer, and S. Ismail. 2016. PDE6delta-mediated sorting of INPP5E into the cilium is determined by cargo-carrier affinity. *Nat Commun.* 7:11366.
- Fu, L., Y. Li, S. Yao, Q. Guo, Y. You, X. Zhu, and B. Lei. 2021. Autosomal Recessive Rod-Cone Dystrophy Associated With Compound Heterozygous Variants in ARL3 Gene. *Frontiers in Cell and Developmental Biology.* 9.
- Fujisawa, S., H. Qiu, S. Nozaki, S. Chiba, Y. Katoh, and K. Nakayama. 2021. ARL3 and ARL13B GTPases participate in distinct steps of INPP5E targeting to the ciliary membrane. *Biol Open.*
- Gillingham, A.K., and S. Munro. 2007. The Small G Proteins of the Arf Family and Their Regulators. *Annual Review of Cell and Developmental Biology.* 23:579-611.
- Goetz, S.C., and K.V. Anderson. 2010. The primary cilium: a signalling centre during vertebrate development. *Nat Rev Genet.* 11:331-344.
- Gotthardt, K., M. Lokaj, C. Koerner, N. Falk, A. Giessl, and A. Wittinghofer. 2015. A G-protein activation cascade from Arl13B to Arl3 and implications for ciliary targeting of lipidated proteins. *Elife.* 4.
- Hanke-Gogokhia, C., Z. Wu, C.D. Gerstner, J.M. Frederick, H. Zhang, and W. Baehr. 2016. Arf-like Protein 3 (ARL3) Regulates Protein Trafficking and Ciliogenesis in Mouse Photoreceptors. *Journal of Biological Chemistry.* 291:7142-7155.
- Hildebrandt, F., T. Benzing, and N. Katsanis. 2011. Ciliopathies. *N Engl J Med.* 364:1533-1543.

- 778 Hu, J., X. Deng, N. Shao, G. Wang, and K. Huang. 2014. Rapid construction and screening of artificial  
779 microRNA systems in *Chlamydomonas reinhardtii*. *Plant J.* 79:1052-1064.
- 780 Ismail, S.A., Y.X. Chen, M. Miertzschke, I.R. Vetter, C. Koerner, and A. Wittinghofer. 2012. Structural  
781 basis for Arl3-specific release of myristoylated ciliary cargo from UNC119. *EMBO J.* 31:4085-  
782 4094.
- 783 Jin, H., S.R. White, T. Shida, S. Schulz, M. Aguiar, S.P. Gygi, J.F. Bazan, and M.V. Nachury. 2010. The  
784 conserved Bardet-Biedl syndrome proteins assemble a coat that traffics membrane proteins to  
785 cilia. *Cell.* 141:1208-1219.
- 786 Lechtreck, K.F., J.M. Brown, J.L. Sampaio, J.M. Craft, A. Shevchenko, J.E. Evans, and G.B. Witman.  
787 2013. Cycling of the signaling protein phospholipase D through cilia requires the BBSome only  
788 for the export phase. *J Cell Biol.* 201:249-261.
- 789 Lechtreck, K.F., E.C. Johnson, T. Sakai, D. Cochran, B.A. Ballif, J. Rush, G.J. Pazour, M. Ikebe, and G.B.  
790 Witman. 2009. The *Chlamydomonas reinhardtii* BBSome is an IFT cargo required for export of  
791 specific signaling proteins from flagella. *J Cell Biol.* 187:1117-1132.
- 792 Li, N., and W. Baehr. 1998. Expression and characterization of human PDEdelta and its *Caenorhabditis*  
793 *elegans* ortholog CEdelta. *FEBS Lett.* 440:454-457.
- 794 Li, Y., Q. Wei, Y. Zhang, K. Ling, and J. Hu. 2010. The small GTPases ARL-13 and ARL-3 coordinate  
795 intraflagellar transport and ciliogenesis. *J Cell Biol.* 189:1039-1051.
- 796 Liew, G.M., F. Ye, A.R. Nager, J.P. Murphy, J.S. Lee, M. Aguiar, D.K. Breslow, S.P. Gygi, and M.V.  
797 Nachury. 2014. The intraflagellar transport protein IFT27 promotes BBSome exit from cilia  
798 through the GTPase ARL6/BBS3. *Dev Cell.* 31:265-278.
- 799 Linari, M., M. Hanzal-Bayer, and J. Becker. 1999. The delta subunit of rod specific cyclic GMP  
800 phosphodiesterase, PDE delta, interacts with the Arf-like protein Arl3 in a GTP specific manner.  
801 *FEBS Lett.* 458:55-59.
- 802 Liu, P., and K.F. Lechtreck. 2018. The Bardet-Biedl syndrome protein complex is an adapter expanding  
803 the cargo range of intraflagellar transport trains for ciliary export. *Proc Natl Acad Sci U S A.*  
804 115:E934-E943.
- 805 Liu, P., X. Lou, J.L. Wingfield, J. Lin, D. Nicastro, and K. Lechtreck. 2020. *Chlamydomonas* PKD2  
806 organizes mastigonemes, hair-like glycoprotein polymers on cilia. *J Cell Biol.* 219.
- 807 Liu, Y., R.A. Kahn, and J.H. Prestegard. 2010. Dynamic structure of membrane-anchored Arf\*GTP. *Nat*  
808 *Struct Mol Biol.* 17:876-881.
- 809 Liu, Y.-X., B. Xue, W.-Y. Sun, J.L. Wingfield, J. Sun, M. Wu, K.F. Lechtreck, Z. Wu, and Z.-C. Fan.  
810 2021. Bardet-Biedl syndrome 3 protein promotes ciliary exit of the signaling protein  
811 phospholipase D via the BBSome. *Elife.* 10:e59119.
- 812 Lokaj, M., S.K. Kosling, C. Koerner, S.M. Lange, S.E. van Beersum, J. van Reeuwijk, R. Roepman, N.  
813 Horn, M. Ueffing, K. Boldt, and A. Wittinghofer. 2015. The Interaction of CCDC104/BARTL1  
814 with Arl3 and Implications for Ciliary Function. *Structure.* 23:2122-2132.
- 815 Loktev, A.V., Q. Zhang, J.S. Beck, C.C. Searby, T.E. Scheetz, J.F. Bazan, D.C. Slusarski, V.C. Sheffield,  
816 P.K. Jackson, and M.V. Nachury. 2008. A BBSome subunit links ciliogenesis, microtubule  
817 stability, and acetylation. *Dev Cell.* 15:854-865.
- 818 Lv, B., L. Wan, M. Taschner, X. Cheng, E. Lorentzen, and K. Huang. 2017. Intraflagellar transport  
819 protein IFT52 recruits IFT46 to the basal body and flagella. *J Cell Sci.* 130:1662-1674.
- 820 Mangeol, P., B. Prevo, and E.J. Peterman. 2016. KymographClear and KymographDirect: two tools for  
821 the automated quantitative analysis of molecular and cellular dynamics using kymographs. *Mol*  
822 *Biol Cell.* 27:1948-1957.
- 823 Mourão, A., A.R. Nager, M.V. Nachury, and E. Lorentzen. 2014. Structural basis for membrane targeting  
824 of the BBSome by ARL6. *Nat Struct Mol Biol.* 21:1035.
- 825 Nachury, M.V. 2018. The molecular machines that traffic signaling receptors into and out of cilia. *Curr*  
826 *Opin Cell Biol.* 51:124-131.

- 827 Nachury, M.V., A.V. Loktev, Q. Zhang, C.J. Westlake, J. Peranen, A. Merdes, D.C. Slusarski, R.H.  
828 Scheller, J.F. Bazan, V.C. Sheffield, and P.K. Jackson. 2007. A core complex of BBS proteins  
829 cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. *Cell*. 129:1201-1213.
- 830 Nachury, M.V., and D.U. Mick. 2019. Establishing and regulating the composition of cilia for signal  
831 transduction. *Nat Rev Mol Cell Biol*. 20:389-405.
- 832 Nagel, G., D. Ollig, M. Fuhrmann, S. Kateriya, A.M. Musti, E. Bamberg, and P. Hegemann. 2002.  
833 Channelrhodopsin-1: a light-gated proton channel in green algae. *Science*. 296:2395-2398.
- 834 Nagel, G., T. Szellas, W. Huhn, S. Kateriya, N. Adeishvili, P. Berthold, D. Ollig, P. Hegemann, and E.  
835 Bamberg. 2003. Channelrhodopsin-2, a directly light-gated cation-selective membrane channel.  
836 *Proceedings of the National Academy of Sciences of the United States of America*. 100:13940-  
837 13945.
- 838 Nakayama, K., and Y. Katoh. 2020. Architecture of the IFT ciliary trafficking machinery and interplay  
839 between its components. *Critical Reviews in Biochemistry and Molecular Biology*:1-18.
- 840 Nishimura, D.Y., M. Fath, R.F. Mullins, C. Searby, M. Andrews, R. Davis, J.L. Andorf, K. Mykytyn, R.E.  
841 Swiderski, B. Yang, R. Carmi, E.M. Stone, and V.C. Sheffield. 2004. Bbs2-null mice have  
842 neurosensory deficits, a defect in social dominance, and retinopathy associated with  
843 mislocalization of rhodopsin. *Proc Natl Acad Sci U S A*. 101:16588-16593.
- 844 Nozaki, S., R.F. Castro Araya, Y. Katoh, and K. Nakayama. 2019. Requirement of IFT-B-BBSome  
845 complex interaction in export of GPR161 from cilia. *Biol Open*.
- 846 Pazour, G.J., N. Agrin, J. Leszyk, and G.B. Witman. 2005. Proteomic analysis of a eukaryotic cilium. *J*  
847 *Cell Biol*. 170:103-113.
- 848 Ratnapriya, R., S.G. Jacobson, A.V. Cideciyan, M.A. English, A.J. Roman, A. Sumaroka, R. Sheplock,  
849 and A. Swaroop. 2021. A Novel ARL3 Gene Mutation Associated With Autosomal Dominant  
850 Retinal Degeneration. *Frontiers in Cell and Developmental Biology*. 9.
- 851 Sahin, A., B. Espiau, E. Tetaud, A. Cuvillier, L. Lartigue, A. Ambit, D. Robinson, and G. Merlin. 2008.  
852 The Leishmania ARL-1 and Golgi traffic. *PLoS one*. 3:e1620.
- 853 Schneider, L., C.A. Clement, S.C. Teilmann, G.J. Pazour, E.K. Hoffmann, P. Satir, and S.T. Christensen.  
854 2005. PDGFRalpha signaling is regulated through the primary cilium in fibroblasts. *Curr*  
855 *Biol*. 15:1861-1866.
- 856 Schrick, J.J., P. Vogel, A. Abuin, B. Hampton, and D.S. Rice. 2006. ADP-ribosylation factor-like 3 is  
857 involved in kidney and photoreceptor development. *Am J Pathol*. 168:1288-1298.
- 858 Singla, V., and J.F. Reiter. 2006. The primary cilium as the cell's antenna: signaling at a sensory organelle.  
859 *Science*. 313:629-633.
- 860 Su, X., K. Driscoll, G. Yao, A. Raed, M. Wu, P.L. Beales, and J. Zhou. 2014. Bardet-Biedl syndrome  
861 proteins 1 and 3 regulate the ciliary trafficking of polycystic kidney disease 1 protein. *Hum Mol*  
862 *Genet*. 23:5441-5451.
- 863 Sun, L., and J. Pan. 2019. Bardet-Biedl syndrome protein-8 is involved in flagellar membrane protein  
864 transport in *Chlamydomonas reinhardtii*. *Sheng Wu Gong Cheng Xue Bao*. 35:133-141.
- 865 Sun, W.-Y., B. Xue, Y.-X. Liu, R.-K. Zhang, R.-C. Li, W. Xin, M. Wu, and Z.-C. Fan. 2021.  
866 *Chlamydomonas* LZTFL1 mediates phototaxis via controlling BBSome recruitment to the basal  
867 body and its reassembly at the ciliary tip. *Proceedings of the National Academy of Sciences*.  
868 118:e2101590118.
- 869 Taschner, M., and E. Lorentzen. 2016. The Intraflagellar Transport Machinery. *Cold Spring Harb*  
870 *Perspect Biol*. 8.
- 871 Thomas, S., K.J. Wright, S. Le Corre, A. Micalizzi, M. Romani, A. Abhyankar, J. Saada, I. Perrault, J.  
872 Amiel, J. Litzler, E. Filhol, N. Elkhartoufi, M. Kwong, J.L. Casanova, N. Boddart, W. Baehr, S.  
873 Lyonnet, A. Munnich, L. Burglen, N. Chassaing, F. Encha-Ravazi, M. Vekemans, J.G. Gleeson,  
874 E.M. Valente, P.K. Jackson, I.A. Drummond, S. Saunier, and T. Attie-Bitach. 2014. A  
875 homozygous PDE6D mutation in Joubert syndrome impairs targeting of farnesylated INPP5E  
876 protein to the primary cilium. *Hum Mutat*. 35:137-146.

- 877 Vaughan, M., and J. Moss. 1997. Activation of toxin ADP-ribosyltransferases by the family of ADP-  
878 ribosylation factors. *Adv Exp Med Biol.* 419:315-320.
- 879 Veltel, S., A. Kravchenko, S. Ismail, and A. Wittinghofer. 2008. Specificity of Arl2/Arl3 signaling is  
880 mediated by a ternary Arl3-effector-GAP complex. *FEBS Lett.* 582:2501-2507.
- 881 Wang, Z., Z.-C. Fan, S.M. Williamson, and H. Qin. 2009. Intraflagellar transport (IFT) protein IFT25 is a  
882 phosphoprotein component of IFT complex B and physically interacts with IFT27 in  
883 *Chlamydomonas*. *PLoS One.* 4:e5384.
- 884 Watzlich, D., I. Vetter, K. Gotthardt, M. Miertzschke, Y.X. Chen, A. Wittinghofer, and S. Ismail. 2013.  
885 The interplay between RPGR, PDEdelta and Arl2/3 regulate the ciliary targeting of farnesylated  
886 cargo. *EMBO Rep.* 14:465-472.
- 887 Wright, K.J., L.M. Baye, A. Olivier-Mason, S. Mukhopadhyay, L. Sang, M. Kwong, W. Wang, P.R.  
888 Pretorius, V.C. Sheffield, P. Sengupta, D.C. Slusarski, and P.K. Jackson. 2011. An ARL3-  
889 UNC119-RP2 GTPase cycle targets myristoylated NPHP3 to the primary cilium. *Genes &*  
890 *Development.* 25:2347-2360.
- 891 Xue, B., Y.-X. Liu, B. Dong, J.L. Wingfield, M. Wu, J. Sun, K.F. Lechtreck, and Z.-C. Fan. 2020.  
892 Intraflagellar transport protein RABL5/IFT22 recruits the BBSome to the basal body through the  
893 GTPase ARL6/BBS3. *Proc Natl Acad Sci U S A.* 117:2496-2505.
- 894 Ye, F., D.K. Breslow, E.F. Koslover, A.J. Spakowitz, W.J. Nelson, and M.V. Nachury. 2013. Single  
895 molecule imaging reveals a major role for diffusion in the exploration of ciliary space by  
896 signaling receptors. *Elife.* 2:e00654.
- 897 Ye, F., A.R. Nager, and M.V. Nachury. 2018. BBSome trains remove activated GPCRs from cilia by  
898 enabling passage through the transition zone. *J Cell Biol.* 217:1847-1868.
- 899 Yeh, C., A. Li, J.Z. Chuang, M. Saito, A. Caceres, and C.H. Sung. 2013. IGF-1 activates a cilium-  
900 localized noncanonical Gbetagamma signaling pathway that regulates cell-cycle progression. *Dev*  
901 *Cell.* 26:358-368.
- 902 Zhang, H., R. Constantine, S. Vorobiev, Y. Chen, J. Seetharaman, Y.J. Huang, R. Xiao, G.T. Montelione,  
903 C.D. Gerstner, M.W. Davis, G. Inana, F.G. Whitby, E.M. Jorgensen, C.P. Hill, L. Tong, and W.  
904 Baehr. 2011a. UNC119 is required for G protein trafficking in sensory neurons. *Nat Neurosci.*  
905 14:874-880.
- 906 Zhang, H., S. Li, T. Doan, F. Rieke, P.B. Detwiler, J.M. Frederick, and W. Baehr. 2007. Deletion of  
907 PrBP/delta impedes transport of GRK1 and PDE6 catalytic subunits to photoreceptor outer  
908 segments. *Proc Natl Acad Sci U S A.* 104:8857-8862.
- 909 Zhang, H., X.H. Liu, K. Zhang, C.K. Chen, J.M. Frederick, G.D. Prestwich, and W. Baehr. 2004.  
910 Photoreceptor cGMP phosphodiesterase delta subunit (PDEdelta) functions as a prenyl-binding  
911 protein. *J Biol Chem.* 279:407-413.
- 912 Zhang, Q., J. Hu, and K. Ling. 2013. Molecular views of Arf-like small GTPases in cilia and ciliopathies.  
913 *Exp Cell Res.* 319:2316-2322.
- 914 Zhang, Q., Y. Li, Y. Zhang, V.E. Torres, P.C. Harris, K. Ling, and J. Hu. 2016. GTP-binding of ARL-3 is  
915 activated by ARL-13 as a GEF and stabilized by UNC-119. *Sci Rep.* 6:24534.
- 916 Zhang, Q., D. Nishimura, S. Seo, T. Vogel, D.A. Morgan, C. Searby, K. Bugge, E.M. Stone, K.  
917 Rahmouni, and V.C. Sheffield. 2011b. Bardet-Biedl syndrome 3 (Bbs3) knockout mouse model  
918 reveals common BBS-associated phenotypes and Bbs3 unique phenotypes. *Proc Natl Acad Sci U*  
919 *S A.* 108:20678-20683.
- 920

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926

927 **Author contributions**

928 Z-C.F. conceived and directed the project. Y-X.L. performed major experiments. Y-X.L. and W-Y.S.  
929 performed immunostaining assays. B.X. screened the *bbs8::BBS8::YFP* cell. R-K.Z. produced the ARL3  
930 antibody. W-J.L. assisted in isolating and purifying cilia. Y-X.L., X.X. and Z-C.F. analyzed the data. Z-  
931 C.F. interpreted and wrote the paper.

932

933 **Competing interests**

934 The authors declare no competing interests.

935

936 **Figure legends**

937

938 **Figure 1. ARL3 diffuses into cilia.** (A). Immunoblots of whole cell extracts (WCE) and ciliary extracts  
939 (CE) of CC-5325 and *arl3* cells probed for IFT43 and IFT139 (IFT-A), IFT22 and IFT70 (IFT-B1), and  
940 IFT38 and IFT57 (IFT-B2). (B). Immunoblots of CE of three cell groups including CC-125, CC-  
941 125::IFT43::HA::YFP, and *arl3*::IFT43::HA::YFP cells; CC-125, CC-125::IFT22::HA::YFP, and  
942 *arl3*::IFT22::HA::YFP cells; and CC-125, CC-125::IFT38::YFP, and *arl3*::IFT38::YFP cells probed with  
943  $\alpha$ -IFT43,  $\alpha$ -IFT22, and  $\alpha$ -IFT38, respectively. (C). TIRF images and corresponding kymograms of three  
944 cell groups including CC-125::IFT43::HA::YFP and *arl3*::IFT43::HA::YFP cells; CC-  
945 125::IFT22::HA::YFP and *arl3*::IFT22::HA::YFP cells; and CC-125::IFT38::YFP and *arl3*::IFT38::YFP  
946 cells. The time and transport lengths are indicated on the right and on the bottom, respectively. The ciliary  
947 base (base) and tip (tip) were shown. Velocities and frequencies of YFP- and HA::YFP-labeled proteins  
948 to traffic inside cilia were shown as graphs. Error bar indicates S.D. n: number of cilia analyzed. n.s.: non-  
949 significance. (D). Velocities and frequencies of YFP- and HA::YFP-tagged proteins to traffic inside cilia  
950 in panel C were shown as numbers. (E). Immunoblots of CE of CC-5325, *arl3*, and  
951 *arl3*::ARL3::HA::YFP cells probed with  $\alpha$ -ARL3. (F). TIRF image and corresponding kymogram of the  
952 *arl3*::ARL3::HA::YFP cell (Movie S1, 15 fps). For panels A, B, and E,  $\alpha$ -tubulin and acetylated (Ac)-  
953 tubulin were used to adjust the loading of WCE and CE, respectively. For panels B and E, MW:  
954 molecular weight. For panels C and D, Ant. and Ret. represent anterograde and retrograde, respectively.

955

956 **Figure 2. ARL3<sup>GDP</sup> requires its N-terminal 15 amino acids and G2 residue for membrane**  
957 **association and diffusion into cilia.** (A). Schematic presentation of bacterially expressed ARL3 and its  
958 variants. (B). SDS-PAGE visualization of bacterially expressed ARL3 and its variants indicated on the  
959 top and immunoblots of liposome-incubated ARL3 and its variants in the presence of GTP $\gamma$ S or GDP  
960 probed with  $\alpha$ -ARL3. MW: molecular weight. (C). Schematic presentation of ARL3::HA::YFP and its  
961 variants. (D). Immunoblots of WCE and CE of cells indicated on the top probed with  $\alpha$ -ARL3. Alpha-  
962 tubulin and acetylated (Ac)-tubulin were used to adjust the loading of WCE and CE, respectively. MW:  
963 molecular weight. (E). Immunoblots of ciliary fractions of cells indicated on the left probed with  $\alpha$ -HA,  
964  $\alpha$ -IFT57 (ciliary matrix marker),  $\alpha$ -PLD (ciliary membrane marker) and Ac-tubulin (axoneme marker).  
965 (F). TIRF images and corresponding kymograms of cells indicated on the top (Movies S2-S5, 15 fps).  
966 The time and transport lengths are indicated on the right and on the bottom, respectively. The ciliary base  
967 (base) and tip (tip) were shown. (G). Cells indicated on the left stained with  $\alpha$ -CEP290 (red) and  $\alpha$ -YFP  
968 (green). Phase contrast (PC) images of cells were shown. Inset shows the proximal ciliary region. Scale  
969 bars: 10  $\mu$ m. For panels A and C,  $\Delta$ N15 stands for the N-terminal 15 amino acids of ARL3 deleted.

970 **Figure 3. ARL3<sup>GTP</sup> is required for the BBSome to move cross the TZ for ciliary retrieval. (A and B).**

971 Immunoblots of WCE (A) and CE (B) of cells indicated on the top probed for the BBSome subunits  
972 BBS1, BBS4, BBS5, BBS7, and BBS8. (C). Immunoblots of sucrose density gradient of CE of cells  
973 indicated on the left probed for the BBSome subunits BBS1, BBS4, BBS5, BBS7, and BBS8. (D).  
974 Immunoblots of WCE and CE of cells indicated on the top probed with  $\alpha$ -BBS8,  $\alpha$ -ARL3,  $\alpha$ -BBS1,  $\alpha$ -  
975 BBS4,  $\alpha$ -BBS5, and  $\alpha$ -BBS7. MW: molecular weight. (E). TIRF images and corresponding kymograms  
976 of *bbs8::BBS8::YFP* and *arl3-bbs8::BBS8::YFP* cells (Movies S6-S7, 15 fps). The time and transport  
977 lengths are indicated on the right and on the bottom, respectively. The ciliary base (base) and tip (tip)  
978 were shown. Velocities and frequencies of fluorescent proteins to traffic inside cilia were shown as graphs.  
979 Error bar indicates S.D. n: number of cilia analyzed. n.s.: non-significance. (F). Velocities and  
980 frequencies of BBS8::YFP to traffic inside cilia of *bbs8::BBS8::YFP* and *arl3-bbs8::BBS8::YFP* cells in  
981 panel E were shown as numbers. (G). CC-125, *bbs8::BBS8::YFP*, and *arl3-bbs8::BBS8::YFP* cells  
982 stained with  $\alpha$ -CEP290 (red) and  $\alpha$ -YFP (green). Phase contrast (PC) images of cells are shown. Inset  
983 shows the proximal ciliary region. Scale bars: 10  $\mu$ m. H. Schematic representation of how loss of ARL3  
984 blocks outward diffusion of the BBSome through the TZ for ciliary retrieval. For panels A, B, and D,  
985 alpha-tubulin and acetylated (Ac)-tubulin were used to adjust the loading of WCE and CE, respectively.  
986 For panels E and F, Ant. and Ret. represent anterograde and retrograde, respectively.

987  
988 **Figure 4. The BBSome is a major ARL3 effector at the proximal ciliary region but not in the cell**

989 **body. (A).** *arl3::ARL3::HA::YFP*, *arl3::ARL3<sup>Q70L</sup>::HA::YFP*, and *arl3::ARL3<sup>T30N</sup>::HA::YFP* cells  
990 stained with  $\alpha$ -BBS8 (red) and  $\alpha$ -YFP (green). Phase contrast (PC) images of cells were also shown. Inset  
991 shows the proximal ciliary region and the basal bodies. Scale bars: 10  $\mu$ m. (B). Immunoblots of sucrose  
992 density gradient of CE of *arl3::ARL3::HA::YFP*, *arl3::ARL3<sup>Q70L</sup>::HA::YFP*, and  
993 *arl3::ARL3<sup>T30N</sup>::HA::YFP* cells probed with  $\alpha$ -BBS1,  $\alpha$ -BBS4,  $\alpha$ -BBS5,  $\alpha$ -BBS7,  $\alpha$ -BBS8, and  $\alpha$ -ARL3.  
994 (C). Immunoblots of  $\alpha$ -YFP-captured proteins from CE of HR-YFP (HA::YFP-expressing CC-125 cells),  
995 *arl3::ARL3::HA::YFP* (in the presence of GTP $\gamma$ S or GDP), *arl3::ARL3<sup>Q70L</sup>::HA::YFP*, and  
996 *arl3::ARL3<sup>T30N</sup>::HA::YFP* cells probed for the IFT-B1 subunits IFT22 and IFT70 and the BBSome  
997 subunits BBS1, BBS4, BBS5, BBS7, and BBS8. Input was quantified with  $\alpha$ -YFP by immunoblotting. A  
998 schematic representation of how IFT-detached BBSome exists independently of the IFT-B1/BBSome  
999 entity in HMEKN buffer was shown on the right. (D and E). Immunoblots of  $\alpha$ -YFP-captured proteins  
1000 from cell body extracts (CBE) of HR-YFP (HA::YFP-expressing CC-125 cells), *arl3::ARL3::HA::YFP*  
1001 (in the presence of GTP $\gamma$ S or GDP), *arl3::ARL3<sup>Q70L</sup>::HA::YFP*, and *arl3::ARL3<sup>T30N</sup>::HA::YFP* cells  
1002 probed for the IFT-B1 subunits IFT22 and IFT70 and the BBSome subunits BBS1, BBS4, BBS5, BBS7,  
1003 and BBS8 in the absence of DTT (D) and in the presence of DTT (E). For both panels, input was

1004 quantified with  $\alpha$ -YFP by immunoblotting. MW stands for molecular weight. **(F)**. Bacterially expressed  
1005 MBP, MBP::BBS1, MBP::BBS2, MBP::BBS4, MBP::BBS5, MBP::BBS7, MBP::BBS8, and  
1006 MBP::BBS9 (upper left) were mixed with ARL3<sup>Q70L</sup> or ARL3<sup>T30N</sup> (middle left) and complexes recovered  
1007 on amylose beads were resolved by SDS-PAGE followed by Coomassie blue staining and  
1008 immunoblotting with  $\alpha$ -ARL3 (right). A schematic representation of direct interactions of ARL3<sup>Q70L</sup> but  
1009 not ARL3<sup>T30N</sup> with BBS1 and BBS5 of the BBSome was shown (lower left). MW, molecular weight.

1010

1011 **Figure 5. ARL3<sup>GTP</sup> recruits the BBSome for diffusing through the TZ for ciliary retrieval. (A).**  
1012 Immunoblots of WCE and CE of cells indicated on the top probed with  $\alpha$ -ARL3,  $\alpha$ -BBS8,  $\alpha$ -BBS1,  $\alpha$ -  
1013 BBS4,  $\alpha$ -BBS5, and  $\alpha$ -BBS7. Alpha-tubulin and acetylated (Ac)-tubulin were used to adjust the loading  
1014 of WCE and CE, respectively. MW: molecular weight. **(B)**. Immunoblots of ciliary fractions of *arl3-*  
1015 *bbs8::ARL3::HA::YFP*, *arl3-bbs8::ARL3<sup>Q70L</sup>::HA::YFP*, and *arl3-bbs8::ARL3<sup>T30N</sup>::HA::YFP* cells  
1016 probed with  $\alpha$ -HA,  $\alpha$ -IFT57 (ciliary matrix marker),  $\alpha$ -PLD (ciliary membrane marker) and Ac-tubulin  
1017 (axoneme marker). **(C)**. TIRF images and corresponding kymograms of *arl3-bbs8::ARL3::HA::YFP*,  
1018 *arl3-bbs8::ARL3<sup>Q70L</sup>::HA::YFP*, and *arl3-bbs8::ARL3<sup>T30N</sup>::HA::YFP* cells. The time and transport  
1019 lengths are indicated on the right and on the bottom, respectively. The ciliary base (base) and tip (tip)  
1020 were shown. **(D)**. Schematic representation of how IFT trains and the BBSome cycle between basal body  
1021 and cilia. BBSome diffusion through the TZ for ciliary retrieval was shown. **(E)**. TIRF images and  
1022 corresponding kymograms of *ift46::IFT46::YFP*, *bbs8::BBS8::YFP*, and *arl3-bbs8::BBS8::YFP* cells  
1023 ([Movies S8-S10](#), 15 fps). The time was indicated on the bottom. The ciliary base (base) and tip (tip), the  
1024 transition zone (TZ) and the basal body (BB) were shown. The corresponding schematic representation of  
1025 how IFT46::YFP and BBS8::YFP cycle between the basal body and cilia was shown.

1026

1027 **Figure 6. ARL3<sup>GTP</sup> recruits PLD-laden BBSomes to move cross the TZ for ciliary retrieval. (A).**  
1028 Immunoblots of WCE and CE of CC-5325, *arl3*, *arl3::ARL3::HA::YFP*, *arl3::ARL3<sup>Q70L</sup>::HA::YFP*, and  
1029 *arl3::ARL3<sup>T30N</sup>::HA::YFP* cells probed for PLD. Alpha-tubulin and acetylated- $\alpha$ -tubulin (Ac-tubulin)  
1030 were used as a loading control for WCE and CE, respectively. **(B)**. CC-5325, *arl3*, *arl3::ARL3::HA::YFP*,  
1031 *arl3::ARL3<sup>Q70L</sup>::HA::YFP*, and *arl3::ARL3<sup>T30N</sup>::HA::YFP* cells were stained with  $\alpha$ -PLD (red) and  $\alpha$ -  
1032 IFT81 (green). Phase contrast (PC) images of cells were shown. Inset shows the proximal ciliary region  
1033 and the basal bodies. Scale bars: 10  $\mu$ m. **(C)**. Immunoblots of  $\alpha$ -YFP-captured proteins from CE of HR-  
1034 YFP (HA::YFP-expressing CC-125 cells), *arl3::ARL3::HA::YFP* (in the presence of GTP $\gamma$ S or GDP),  
1035 *arl3::ARL3<sup>Q70L</sup>::HA::YFP*, and *arl3::ARL3::HA::YFP* cells probed for the IFT-B1 subunits IFT22 and  
1036 IFT70, the IFT-B2 subunits IFT38 and IFT57, the IFT-A subunits IFT43 and IFT139, the BBSome



1037 subunits BBS1 and BBS4, and PLD. Input was quantified with  $\alpha$ -YFP by immunoblotting. MW stands for  
1038 molecular weight.

1039

1040 **Figure 7. ARL3 mediates phototaxis through controlling BBSome ciliary retrieval. (A and B).**

1041 Single-cell motion assay (A) and population phototaxis assay (B) of CC-125, CC-5325, and *arl3* cells.

1042 (C). Immunoblots of WCE and CE of cells indicated on the top probed with  $\alpha$ -ARL3,  $\alpha$ -BBS1,  $\alpha$ -BBS4,

1043  $\alpha$ -BBS5,  $\alpha$ -BBS7, and  $\alpha$ -BBS8. Alpha-tubulin and acetylated (Ac)-tubulin were used to adjust the loading

1044 of WCE and CE, respectively. MW: molecular weight. (D). Cells indicated on the left were stained with

1045  $\alpha$ -BBS8 (red) and  $\alpha$ -IFT81 (green). Phase contrast (PC) images of cells were also shown. Inset shows the

1046 proximal ciliary region right and the basal bodies. Scale bars: 10  $\mu$ m. (E and F). Single-cell motion assay

1047 (E) and population phototaxis assay (F) of CC-125, ARL3<sup>miRNA</sup>, ARL3<sup>Res-WT</sup>, ARL3<sup>Res-Q70L</sup>, and ARL3<sup>Res-</sup>

1048 T30N cells. For panels A and E, the direction of light is indicated as green arrows and the radial histograms

1049 show the percentage of cells moving in a particular direction relative to the light (six bins of 60° each).

1050 Composite micrographs show the tracks of single cells. Each of the five merged frames was assigned a

1051 different color (blue is frame 1 and red is frame 5, corresponding to a travel time of 1.5 s). Scale bar: 10

1052  $\mu$ m. For panels B and F, the direction of light is indicated as white arrows.

1053

1054 **Figure 8. Hypothetical model for how ARL3 promotes outward PLD-laden BBSome diffusion**

1055 **through the TZ.** ARL3<sup>GDP</sup> diffuses into cilia and is activated to become ARL3<sup>GTP</sup> by an unknown

1056 mechanism (?). Following the transportation from the ciliary tip to base, cargo (PLD) laden BBSomes

1057 shed from the retrograde IFT train at the proximal ciliary region right above the TZ and is bound to

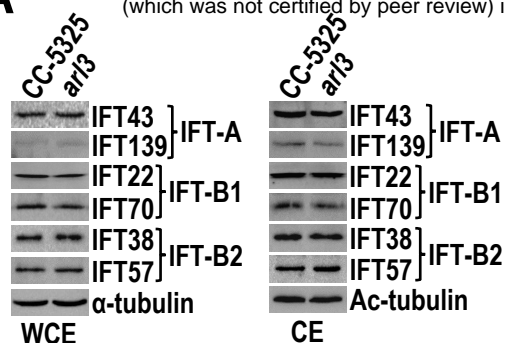
1058 ARL3<sup>GTP</sup> as a ARL3 effector. ARL3<sup>GTP</sup> then recruits the cargo (PLD) laden BBSome to diffuse through

1059 the TZ for ciliary retrieval.

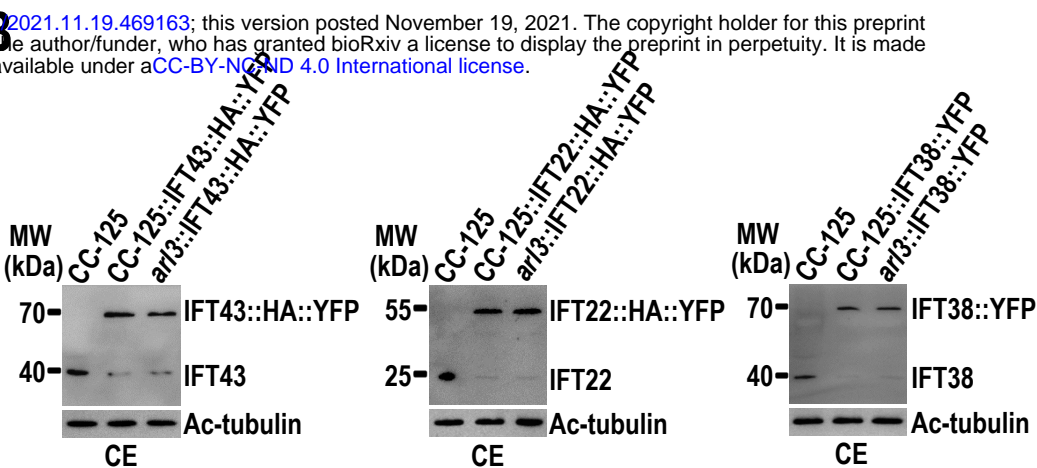
# Figure 1

**A**

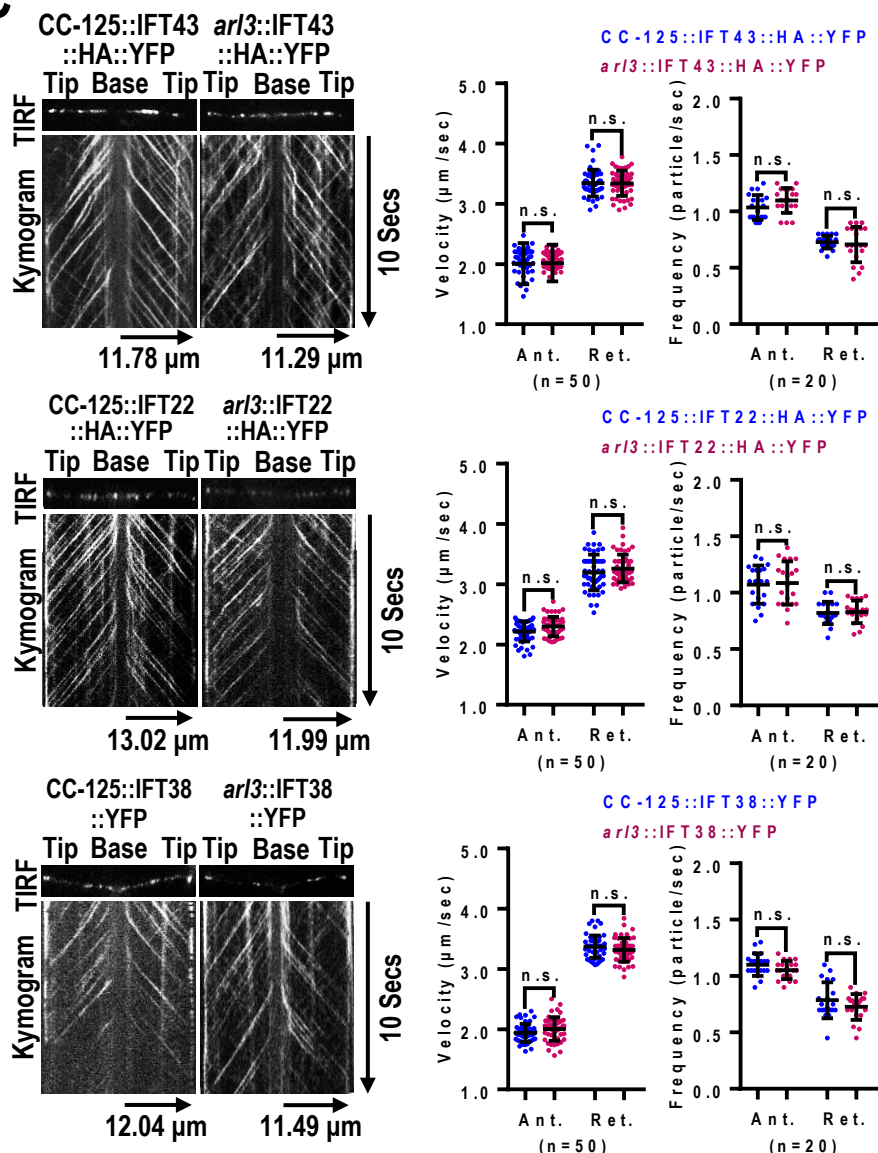
bioRxiv preprint doi: <https://doi.org/10.1101/2021.11.19.469163>; this version posted November 19, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



**B**



**C**



**D**

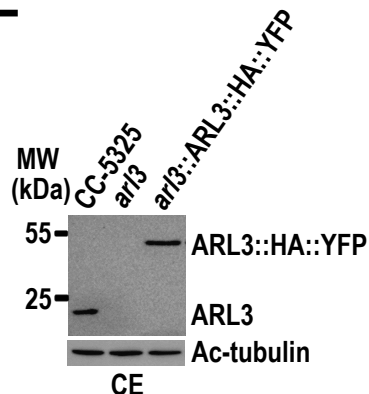
## Particle frequency and velocity measurement

Strain	Frequency (particle/sec)	
	Ant.	Ret.
CC-125::IFT43::HA::YFP	1.04±0.11	0.73±0.06
<i>arl3::IFT43::HA::YFP</i>	1.10±0.11	0.71±0.16
CC-125::IFT22::HA::YFP	1.07±0.17	0.82±0.10
<i>arl3::IFT22::HA::YFP</i>	1.09±0.19	0.83±0.10
CC-125::IFT38::YFP	1.10±0.10	0.79±0.16
<i>arl3::IFT38::YFP</i>	1.05±0.08	0.73±0.11

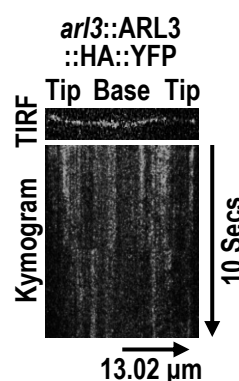
  

Strain	Velocity ( $\mu\text{m}/\text{sec}$ )	
	Ant.	Ret.
CC-125::IFT43::HA::YFP	2.00±0.34	3.34±0.22
<i>arl3::IFT43::HA::YFP</i>	2.02±0.30	3.34±0.21
CC-125::IFT22::HA::YFP	2.21±0.17	3.20±0.29
<i>arl3::IFT22::HA::YFP</i>	2.30±0.16	3.26±0.23
CC-125::IFT38::YFP	1.94±0.15	3.37±0.19
<i>arl3::IFT38::YFP</i>	2.00±0.19	3.32±0.19

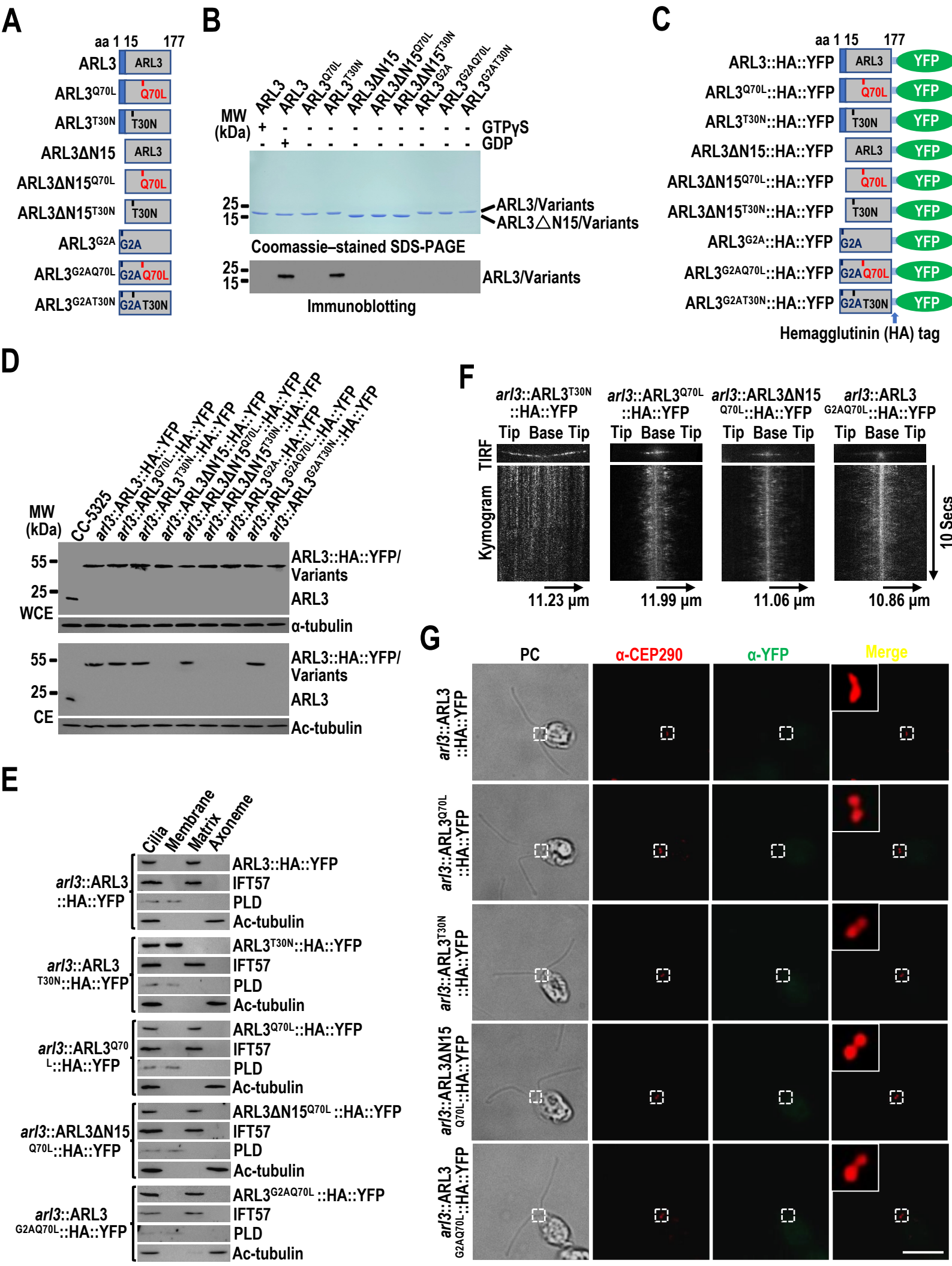
**E**



**F**

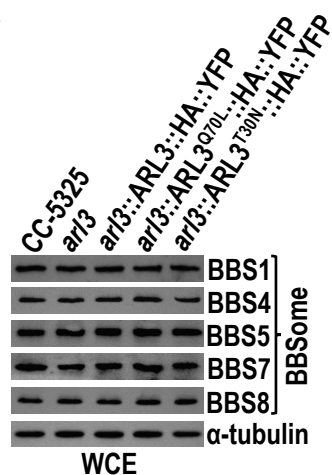


# Figure 2

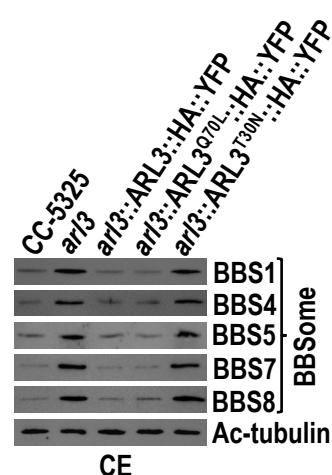


**Figure 3**

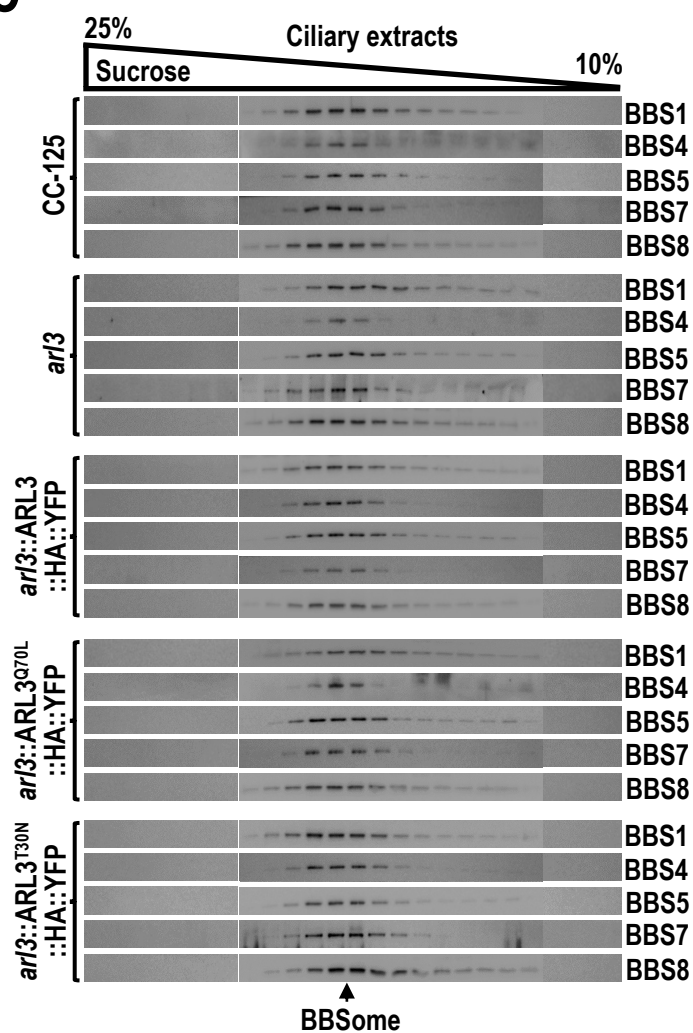
**A**



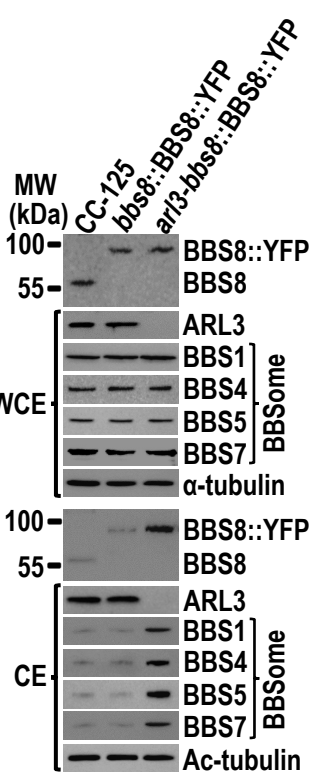
**B**



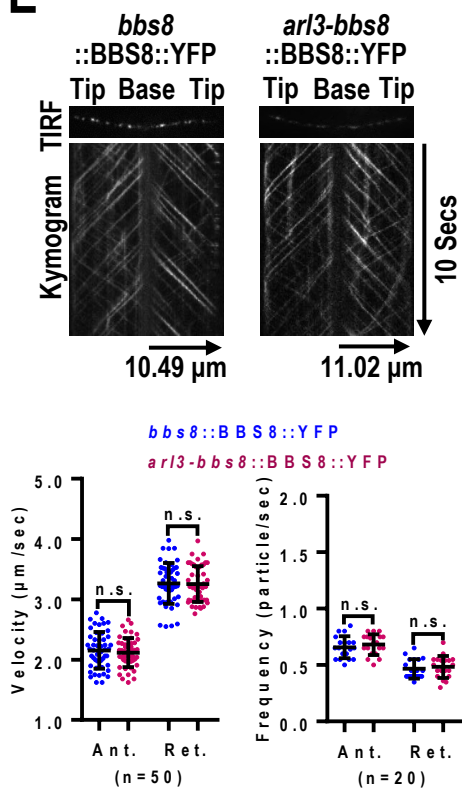
**C**



**D**



**E**

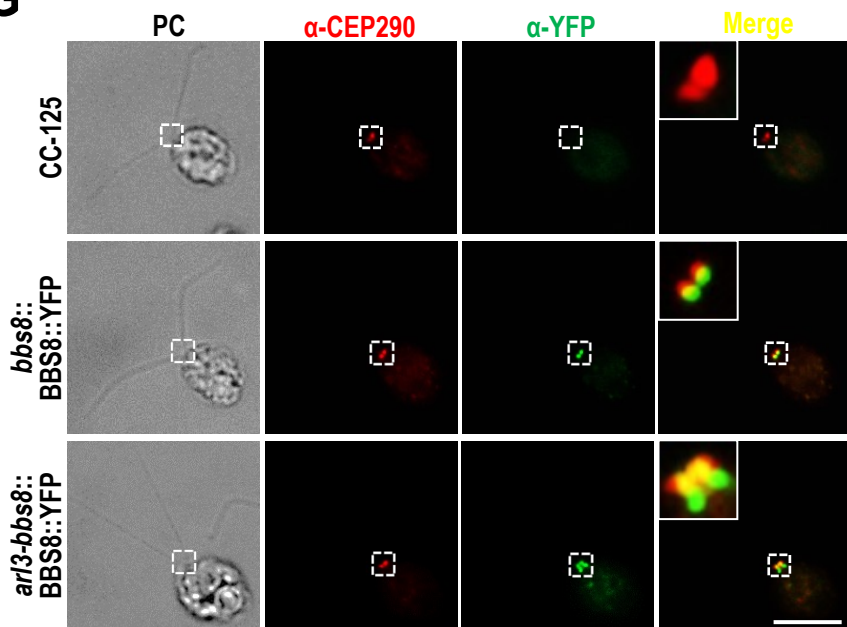


**F**

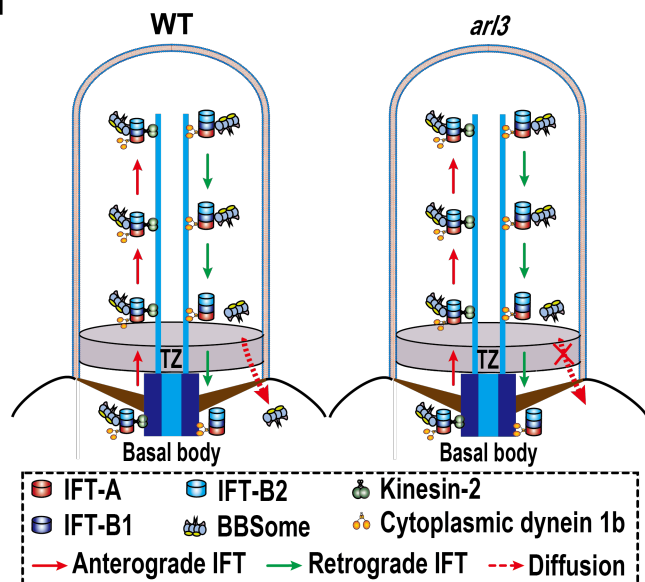
**BBS8::YFP frequency and velocity measurement**

Strain	Frequency (particle/sec)		Velocity (μm/sec)	
	Ant.	Ret.	Ant.	Ret.
<i>bbs8::BBS8::YFP</i>	0.66±0.10	0.47±0.09	2.15±0.30	3.26±0.34
<i>arl3-bbs8::BBS8::YFP</i>	0.68±0.09	0.48±0.10	2.12±0.24	3.25±0.30

**G**



**H**



**Figure 4**

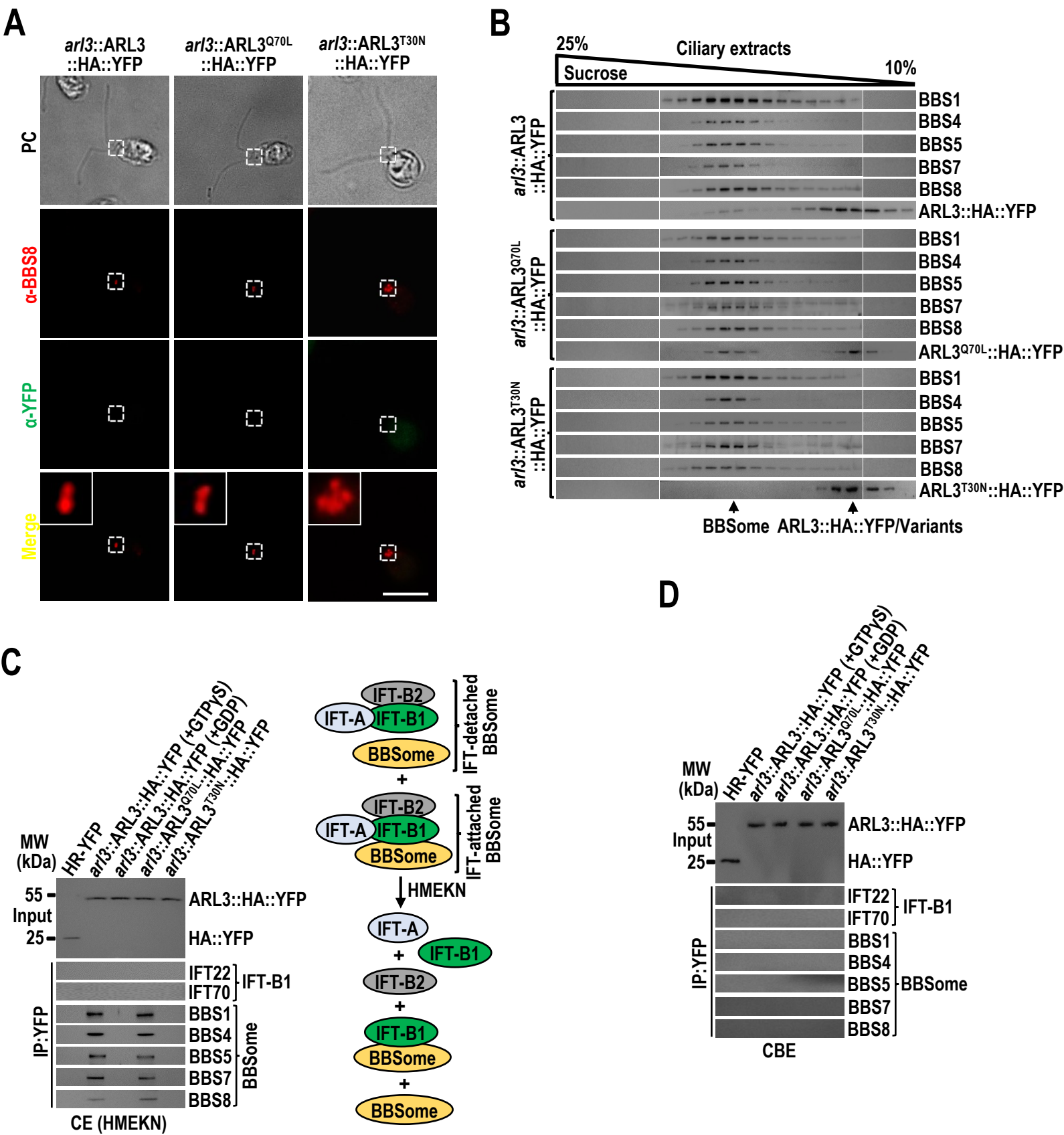
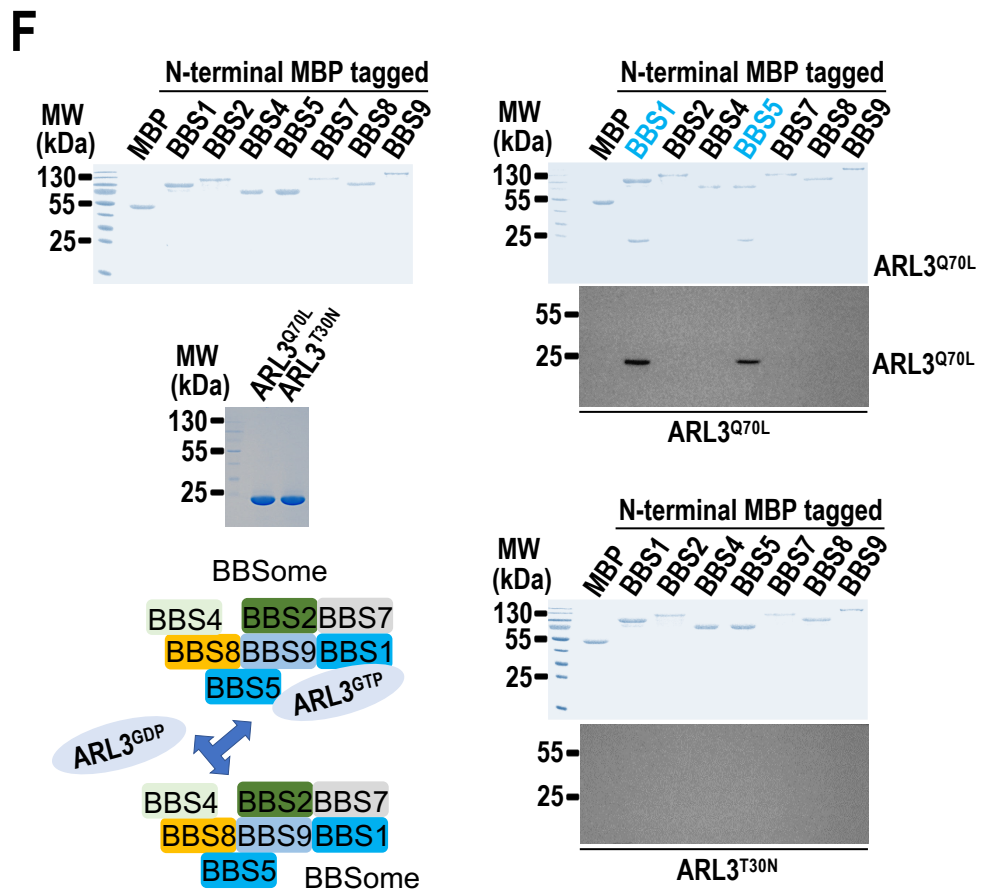
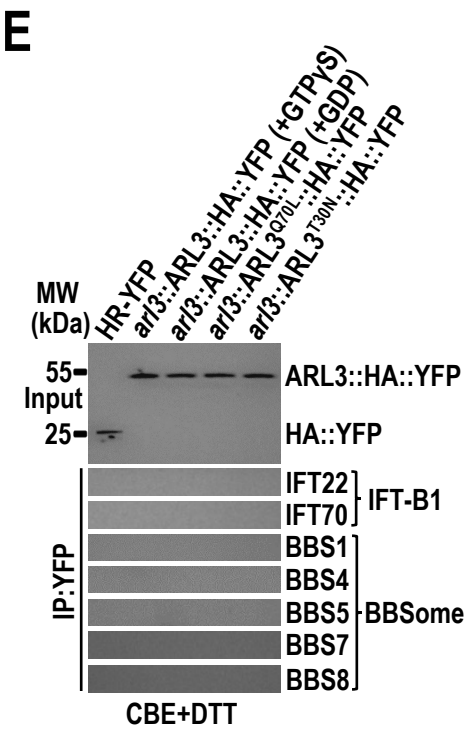
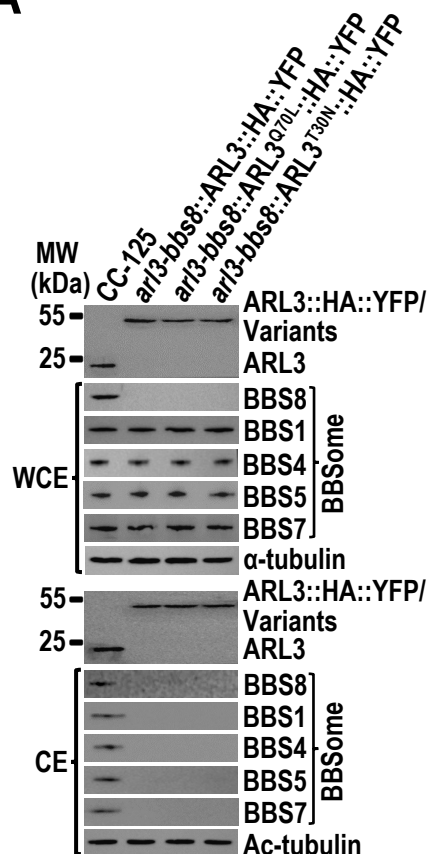


Figure 4 (Cont')

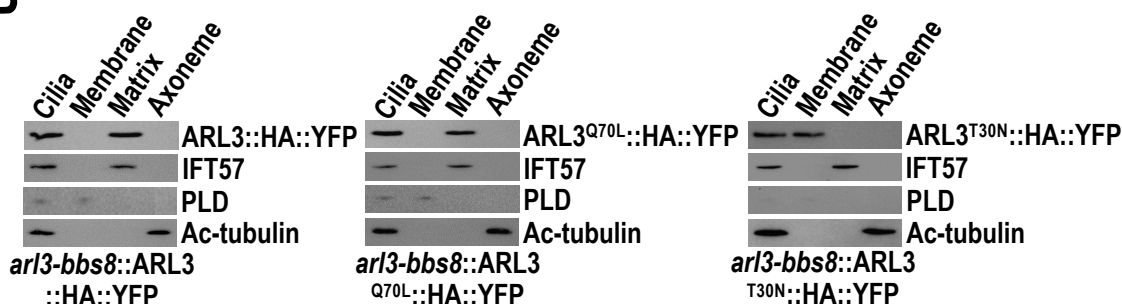


**Figure 5**

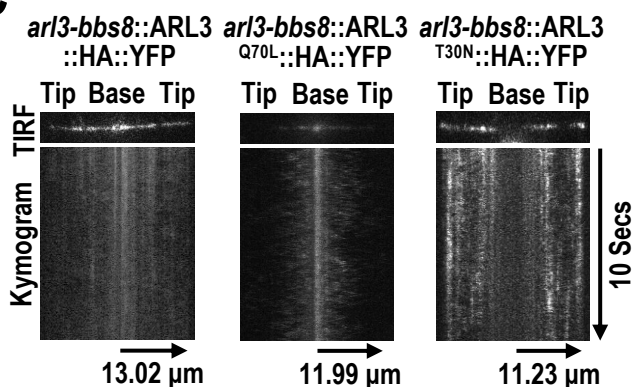
**A**



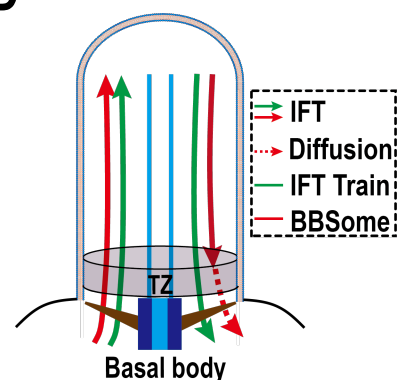
**B**



**C**



**D**



**E**

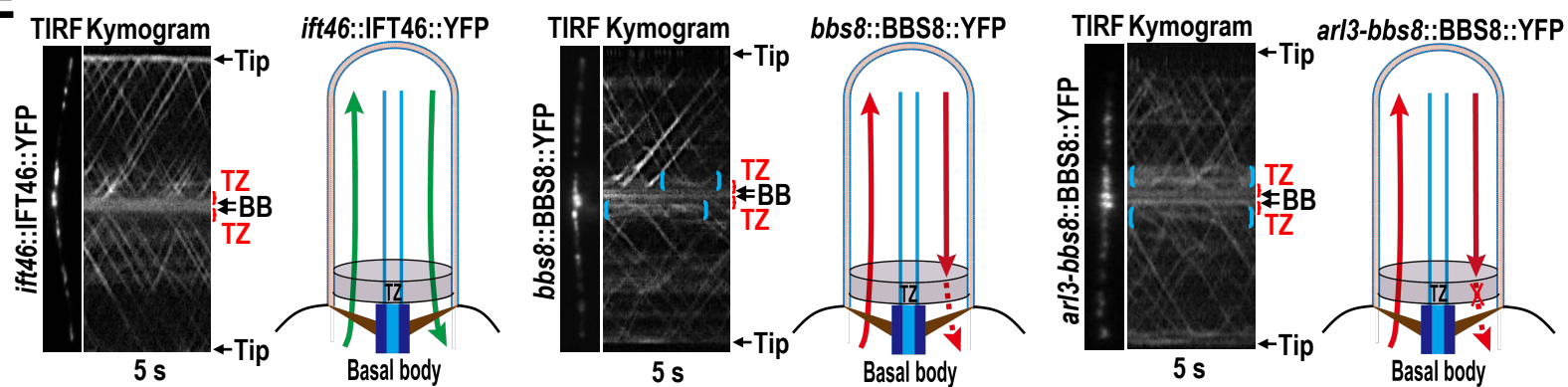
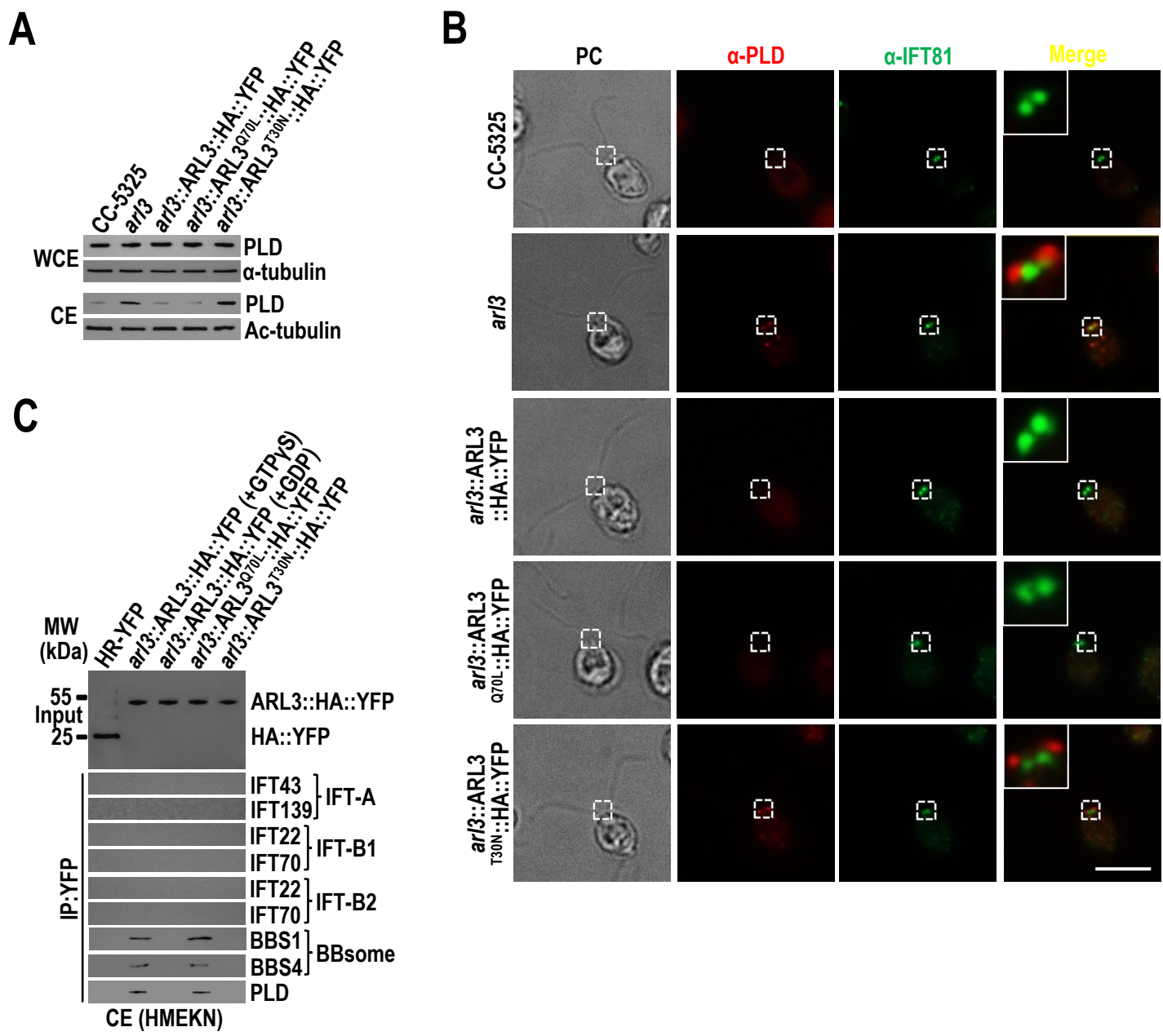


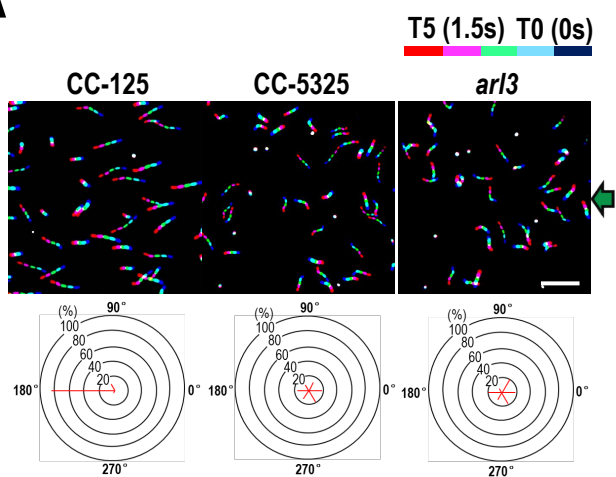
Figure 6



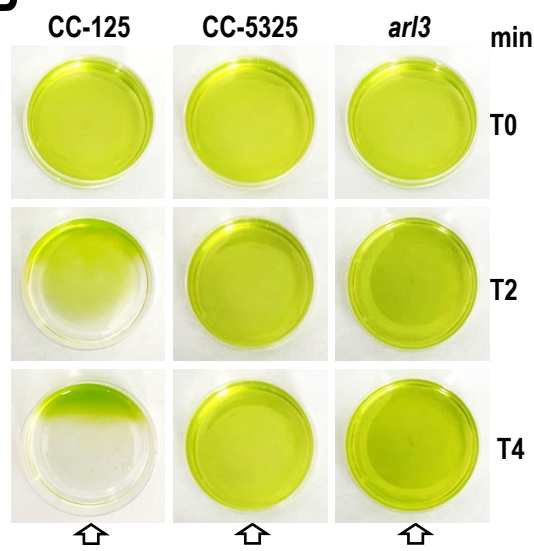


# Figure 7

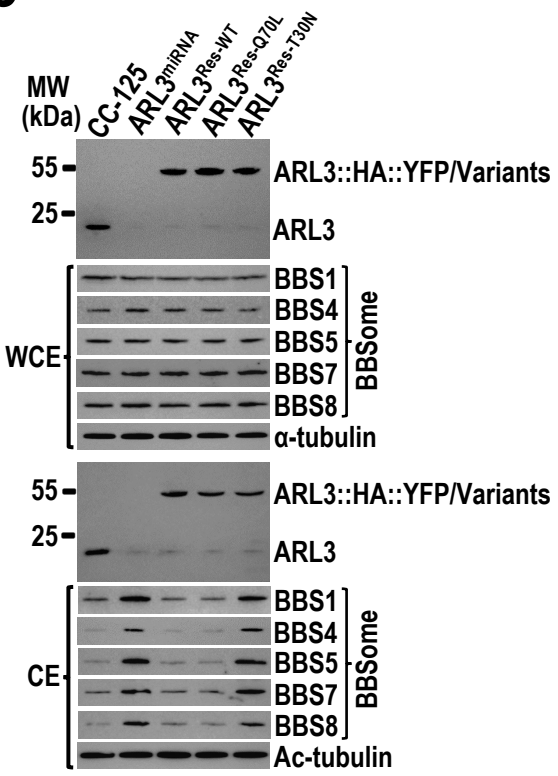
## A



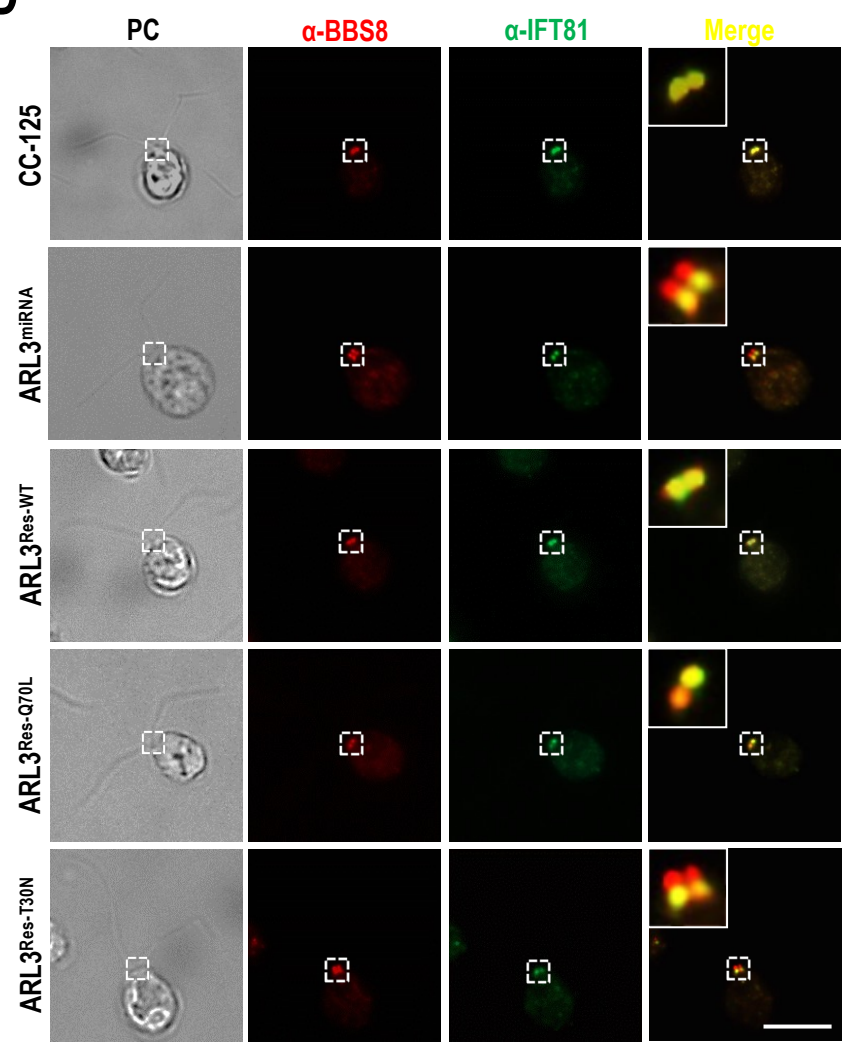
## B



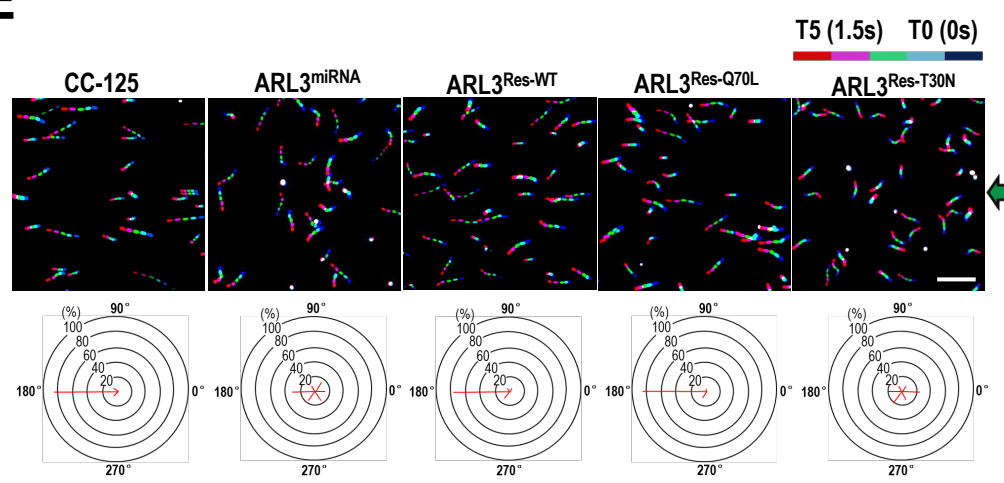
## C



## D



## E



## F

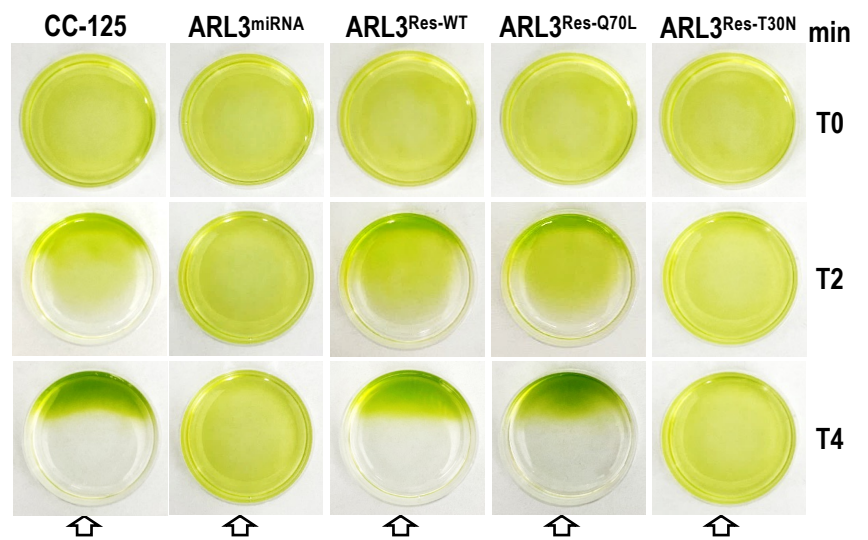


Figure 8

