Signaling compartment at the ciliary tip is formed and maintained by intraflagellar transport and functions as sensitive salt detector

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Abstract

Primary cilia are ubiquitous antenna-like organelles that mediate cellular signaling and represent hotspots for human diseases termed ciliopathies. How signaling subcompartments are established within the microtubule-based organelle, and for example support Hedgehog or cGMP signal transduction pathways, remains a central question. Here we show that a *C. elegans* salt-sensing receptor type guanylate cyclase, GCY-22, accumulates at a high concentration within the distal region of the cilium. This receptor uses DAF-25 (Ankmy2 in mammals) to cross the transition zone (TZ) membrane diffusion barrier in the proximal-most region of the ciliary axoneme. Targeting of GCY-22 to the ciliary tip is dynamic, requiring the cargo-mobilizing intraflagellar transport (IFT) system. Disruption of transit across the TZ barrier or IFT trafficking causes GCY-22 protein mislocalization and defects in the formation, maintenance, and function of the ciliary tip compartment required for chemotaxis to low NaCl concentrations. Together, our findings reveal how a previously undescribed cilium tip cGMP signaling compartment is established and contributes to the physiological function of a primary cilium.

Introduction

The primary cilium is a specialized signaling organelle used by metazoan cells to transduce environmental cues. Signaling proteins important for cilia function must reach and maintain their correct sub-ciliary position and concentration. Failure to do so results in various diseases[1]. Our understanding of the regulation of protein localization in cilia, and their relevance in creating functional signaling domains, remains limited, however.

Cilia use two mechanisms, a trafficking system and a diffusion barrier, that function together to regulate the trafficking of proteins into, within, and out of cilia. The main ciliary trafficking machinery, intraflagellar transport (IFT), facilitates bidirectional transport of cargo, including signaling proteins, from the base/foundation (basal body) to the tip of the axoneme[2]. Anterograde IFT to the tip relies on kinesins, and cytoplasmic dynein enables retrograde transport back[3,4]. Two IFT modules, subcomplexes-A and -B[5,6], together with another module containing BBS proteins (BBSome) that is thought to bridge the subcomplexes, play essential roles in cargo transport[7,8]. The best-known IFT cargos are axoneme structure components, including tubulin[9,10], but signaling proteins, like the TRPV channel subunits OSM-9 and OCR-2 in the nematode *C. elegans*, are also transported[11]. Additionally, several mammalian ciliary signaling proteins, namely the GPCR SSTR3 and Hedgehog signaling component SMO, traverse the cilium by both IFT and diffusion[12].

To help confine proteins to cilia, a subdomain immediately distal to the basal body, called the transition zone (TZ), acts as a diffusion barrier for both membrane and soluble proteins[13–15]. How the TZ acts with IFT or other trafficking systems to regulate the composition of the sensory organelle is not well understood[16,17].

Signaling proteins can have different sub-ciliary localizations, including the proximal or distal segments, or ciliary tip. For example, the *C. elegans* cyclic nucleotide gated channel TAX-2 localizes to the proximal region adjoining the TZ[18], while OSM-9 and OCR-2[11] and several GPCRs[18–20] localize along the length of the cilium. In mammals, the kinesin-like protein KIF7 and Hedgehog signaling components SUFU and GLI2 localize at the cilium tip[21–23]. Mislocalization of ciliary proteins can impair signaling and development. For example, mislocalization of PDE6 and GRK1 can cause retinitis pigmentosa[24] and *BBS2* mutant mice display defects presumably caused by mislocalized rhodopsin[25]. Despite their importance, the mechanisms that govern how signaling components concentrate along specific ciliary subdomains remain largely unknown.

To explore the molecular mechanisms underlying ciliary signaling domain formation, maintenance and function, we use the cilium-dependent NaCl response of *C. elegans* as a model system. Its attraction to NaCl is mediated by two bilateral chemosensory ASE head neurons that express receptor-type guanylate cyclases, including GCY-22 (ASER), required for responding to Cl^-, and GCY-14 (ASEL), for the Na^+ response[26–29].

By endogenously tagging GCY-22 with GFP, we discovered that the guanylate cyclase exists at a high concentration in a cilium tip compartment. This localization depends on the IFT machinery, including the BBS complex. We further show that DAF-25 (mammalian Ankmy2 ortholog), is required for GCY-22 ciliary entry. Structure-function studies uncovered GCY-22 protein domains needed for entry and tip localization. Disrupting receptor localization at the tip compartment hinders the ability of *C. elegans* to detect low concentrations of NaCl. Together, our findings provide mechanistic insights into the formation, maintenance and function of a novel ciliary subdomain essential for cGMP-signaling.

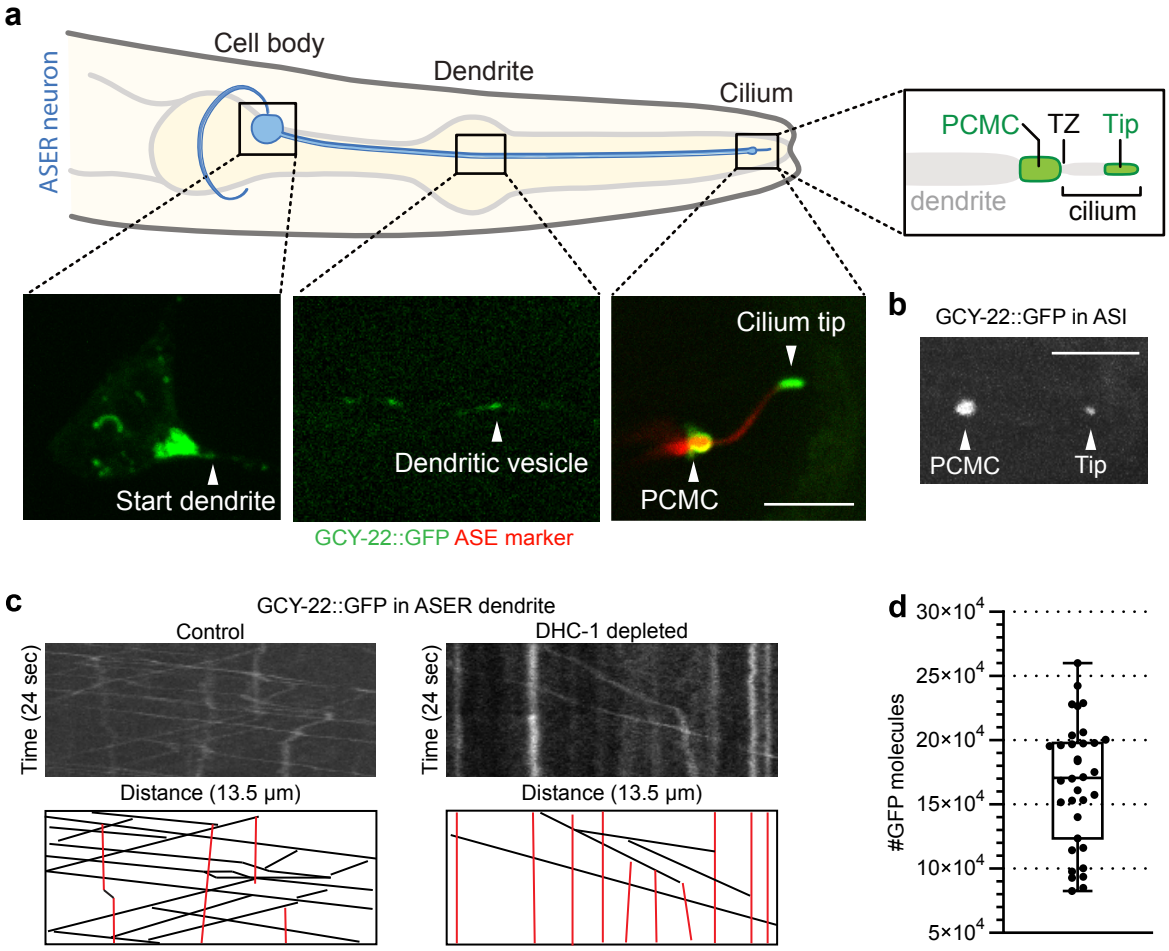
RESULTS

GCY-22::GFP is highly concentrated at the ASER ciliary tip and periciliary membrane compartment

We used CRISPR/Cas9 to tag the *gcy-22* gene with *GFP* and determine the sub-cellular localization of this receptor in the ASER neuron. Confocal microscopy and co-labeling with markers for the ER (TRAM-1) and trans Golgi (APT-9) revealed that GCY-22::GFP is present within both of these cell body compartments (**Fig. 1a**, **Supplementary Fig. 1a,b**). In the dendrite, time-lapse imaging revealed anterograde and retrograde transport of GCY-22::GFP (**Fig. 1c**), reminiscent of kinesin and dynein-mediated vesicle transport[30]. Auxin inducible degradation of the dynein heavy chain subunit DHC-1[31] resulted in stationary particles, showing that GCY-22::GFP dendritic transport is DHC-1-dependent

87 (**Fig. 1c**). At the dendritic terminus, GCY-22::GFP is abundant at the periciliary membrane
88 compartment (PCMC), a vestibule of the cilium (**Fig. 1a**).

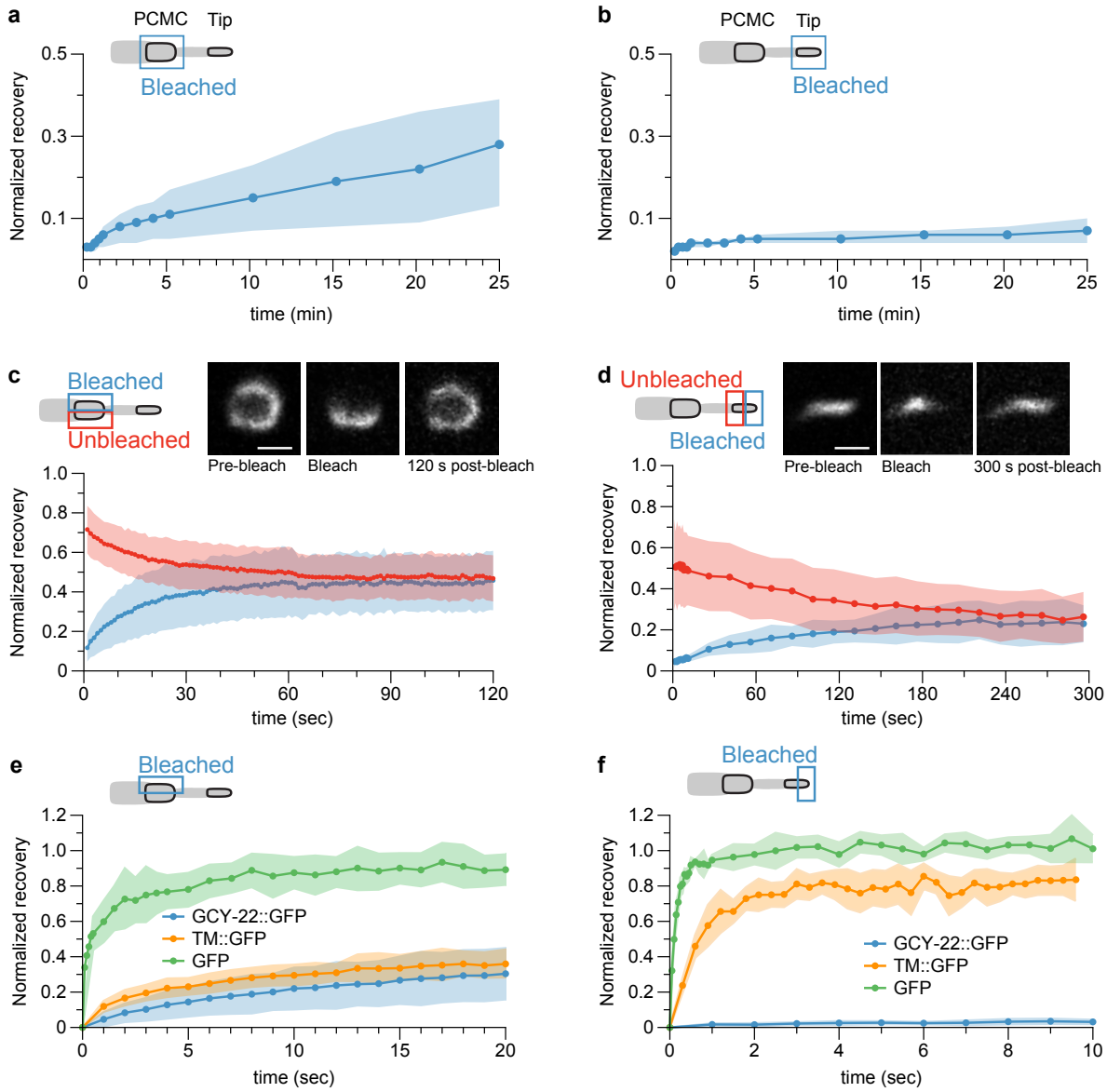
Figure 1



89
90 The most striking localization of GCY-22::GFP is its strong enrichment at the ciliary tip (**Fig. 1a**), but
91 not along the axoneme. GCY-22::GFP ectopically expresssed in ASI localized to the PCMC and
92 ciliary tip, although in low amounts (**Fig. 1b**), suggesting a localization mechanism that is not cell
93 specific. Using purified GFP as reference, we estimated the ASE cilium tip concentration of GCY-
94 22::GFP to be 2.77 mM, reflecting an average density of 106,571 (±31,016) molecules/µm^2 (**Fig. 1d**).
95 Only rhodopsin in mammalian photoreceptor cells has a similar density, of up to 48,300
96 molecules/µm^2, which enables photoreceptors to respond to single photons[32–34]. This suggests that
97 the ASE neuron ciliary tip compartment could function as a highly sensitive salt detector.
98
99 **GCY-22::GFP exists in stable pools at the periciliary membrane and ciliary tip**
100 To determine how GCY-22::GFP pools at the PCMC and ciliary tip are replenished, we used
101 Fluorescent Recovery after Photobleaching (FRAP). Recovery of the entire PCMC (28% after 25
102 minutes) was modest, potentially reflecting continuous transport of GCY-22::GFP from the cell body to

103 the dendritic terminus (**Fig. 2a**). Recovery of the entire ciliary tip was slower (7% after 25 minutes),
104 indicating little or slow transport towards the tip (**Fig. 2b**).
105
106 We photobleached half the PCMC or tip to assess GCY-22::GFP motility *within* these compartments.
107 GCY-22::GFP fully redistributed within the PCMC in 60 seconds (**Fig. 2c**) whereas tip recovery was
108 slower at 3-4 minutes (**Fig. 2d**). These experiments demonstrate lateral diffusion, and no bound
109 fraction, within the PCMC and tip compartments.

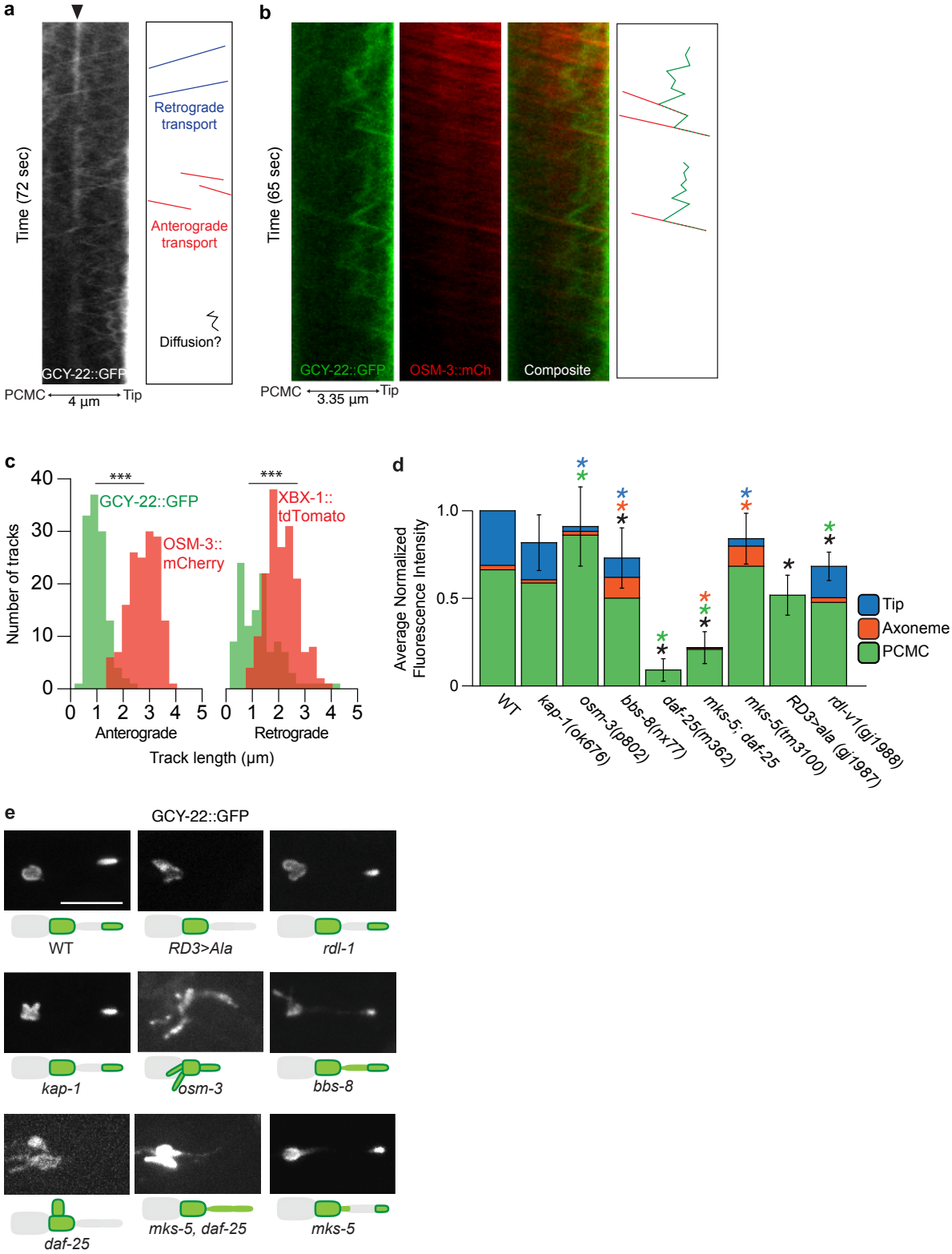
Figure 2



110
111 To place these results in context, we measured the redistribution of free GFP and of GFP fused to the
112 transmembrane domain of GCY-22 (TM::GFP). After photobleaching half the PCMC, GFP
113 fluorescence recovered quickly (~90% after 8 seconds). Recovery of TM::GFP was much slower

114 (~35% after 20 seconds) and comparable to GCY-22::GFP recovery, possibly due to protein crowding
115 within the PCMC (**Fig. 2e**).
116
117 After photobleaching half of the cilium, free GFP fluorescence recovered very quicly (~100% after 2
118 seconds). TM::GFP recovery was slightly slower (~80% after 3 seconds), reflecting slower diffusion of
119 transmembrane proteins. In contrast, GCY-22::GFP recovered minimally 10 seconds after
120 photobleaching, indicating the presence of a distinct membrane compartment limiting GCY-22::GFP
121 motility (**Fig. 2f**).
122
123 **Ciliary tip compartment of GCY-22::GFP depends on active IFT**
124 Because the proximal region of the axoneme (middle segment) and part of the distal segment
125 seemed void of GCY-22::GFP, we hypothesized that it might be actively transported by IFT. In
126 *C. elegans*, anterograde IFT relies on kinesin-II and OSM-3 (ortholog of mammalian KIF17) in the
127 middle segment and OSM-3 alone in the distal segment[3,4,16,35].
128
129 Using time-lapse microscopy and kymogram analysis, we identified GCY-22::GFP particles moving
130 between the PCMC and ciliary tip (**Fig. 3a**). Two-color imaging of GCY-22::GFP and an mCherry-
131 tagged OSM-3 showed overlapping anterograde tracks (**Fig. 3b**). On average, we observed 37.75 (±
132 8.23) anterograde OSM-3::mCherry tracks/minute compared to 5.93 anterograde GCY-22::GFP
133 tracks/minute, suggesting that a subset of IFT particles transport GCY-22::GFP (**Table 1,**
134 **Supplementary Table 1**). Anterograde OSM-3::mCherry particles often move along the full length of
135 the cilium (average track length 2.75 ± 0.54 µm); in contrast, GCY-22::GFP tracks spanned half the
136 cilium or shorter (average length 1.0 ± 0.4 µm, $P < 0.001$, **Fig. 3c**). Retrograde GCY-22::GFP particles
137 displayed short tracks and occasionally tracks spanning the entire axoneme (average length 1.3 ±0.8
138 µm), significantly shorter than retrograde tracks of the tdTomato tagged dynein subunit XBX-1
139 (average length 2.12 ± 0.59 µm, $P < 0.001$) (**Fig. 3c**).
140
141 In many animals, stationary fluorescence signals that indicate paused particles are observed, likely at
142 the transition between the middle and distal segments (**Fig. 3a**). Interestingly, GCY-22::GFP tracks
143 frequently reversed from retrograde to anterograde, docking with an anterograde OSM-3::mCherry
144 track and moving towards the tip (**Fig. 3b**). Notably, this behavior was not observed for IFT proteins.
145 These data suggest that GCY-22::GFP particles moving away from the tip are transported back by
146 anterograde IFT, keeping GCY-22::GFP concentrated at the tip.

Figure 3



147

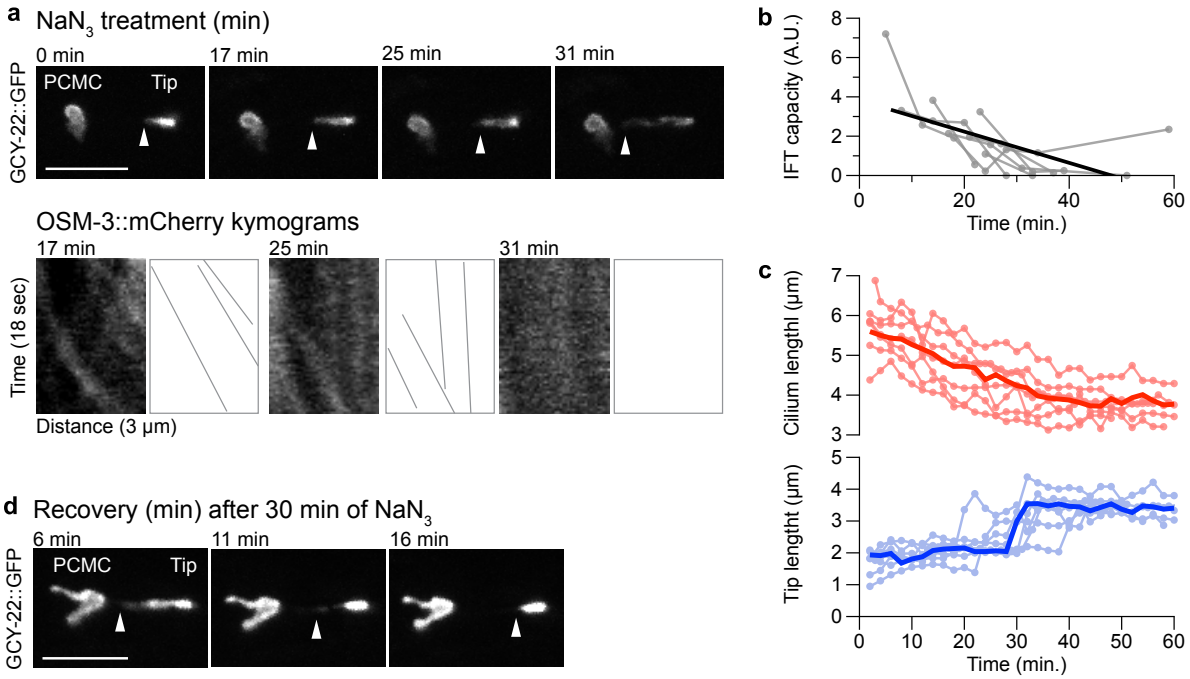
148 To study the contribution of IFT to cilium tip localization, we visualized the localization of GCY-
149 22::GFP in different IFT mutant backgrounds. Disrupting the kinesin-II subunit KAP-1, which plays a
150 role in IFT particle entry into the cilium but does not affect cilium length[3,16,35], did not influence ciliary

151 GCY-22::GFP levels or localization (**Fig. 3d,e**). In contrast, *osm-3* mutants lacking a distal segment
152 showed increased levels of GCY-22::GFP in the PCMC and along the remaining axoneme but no
153 GCY-22::GFP tip compartment (**Fig. 3d,e**). Disruption of the BBSome subunit BBS-8[36] reduced
154 overall ciliary levels of GCY-22::GFP, including at the tip, while increasing its localization along the
155 length of the cilium (**Fig. 3d,e**). These changes in GCY-22::GFP localization correlate with a decrease
156 in GCY-22::GFP tracks (**Table 1**). Taken together, our results show that kinesin-II is not required for
157 import of GCY-22::GFP into the cilium, and that both OSM-3-mediated IFT and the BBSome are
158 important for transport into the cilium and enrichment at the tip.
159
160 To test if IFT is responsible for the ciliary tip localization of GCY-22::GFP, we used NaN_3 to stop ATP
161 production, and thus IFT, over time[37]. OSM-3::mCherry particles slowed down and became less
162 frequent after 10 minutes of treatment (**Fig. 4a,b**). IFT capacity, defined as the number of IFT tracks
163 multiplied by speed, reached 0 after 30-50 minutes (**Fig. 4b**). During treatment, cilium length steadily
164 shortened, while the GCY-22::GFP tip compartment extended into the cilium as early as 10 minutes in
165 some animals and completely collapsed after 20-30 minutes (**Fig. 4a,c**). Importantly, loss of cilium tip
166 localization after 30 minutes of NaN_3 treatment was reversible, with complete recovery within 16
167 minutes (**Fig. 4d**). These experiments suggest that a minimal IFT capacity is needed to maintain
168 GCY-22::GFP tip localization, although we cannot exclude an IFT-independent mechanism caused by
169 the NaN_3 treatment.
170
171 Taken together, these experiments show that IFT plays an important role in actively maintaining the
172 high GCY-22 concentration at the ciliary tip.
173
174 **Table 1.** Quantification of GCY-22::GFP tracks in the cilium in different mutant backgrounds.

Genotype	# animals with tracks (n)	*P*-value[a]	Total min. analyzed	Total anterograde tracks (tracks/min)	Total retrograde tracks (tracks/min)
Wild type	17(18)	-	22.75	135 (5.93)	146 (6.42)
kap-1	16(18)	1	18	179 (9.94)	127 (7.06)
osm-3	7(13)	0.026	19.5	33 (1.7)	18 (0.9)
bbs-8	12(17)	0.094	28.5	65 (2.3)	25 (0.9)
daf-25	0(18)	<0.001	-	-	-
mks-5	5(14)	<0.001	21	35 (1.7)	9 (0.4)
mks-5; daf-25	4(21)	<0.001	17.5	22 (1.3)	6 (0.3)
gcy-22(RD3>ala)	0(16)	<0.001	-	-	-
rdl-1	9(13)	0.136	19.5	52 (2.7)	63 (3.2)

[a] χ^2-test was used to compare distributions of animals with and without GCY-22::GFP tracks to WT.

Figure 4

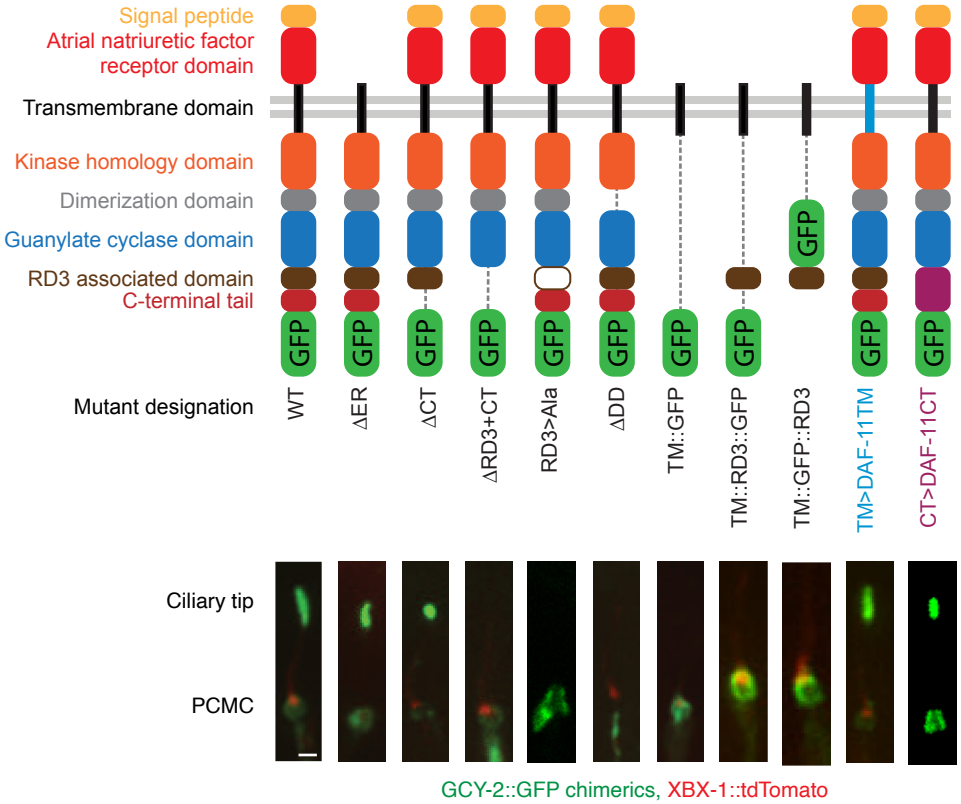


175

176 **GCY-22 trafficking and cilium import is regulated by the transition zone and requires DAF-25**
177 The accumulation of GCY-22::GFP at the PCMC suggests that it is prevented from readily diffusing
178 into the cilium. We therefore tested whether disruption of the TZ influences its ciliary entry.
179 Surprisingly, loss of MKS-5 (mammalian RPGRIP1/RPGRIP1L ortholog), which removes all known
180 proteins and characteristic Y-link structures from the TZ[13,14,38], has a relatively subtle effect on GCY-
181 22::GFP localization—it mislocalizes at the TZ and base of the axoneme, and displays a reduced
182 amount at the cilium tip (**Fig. 3d,e**). The lack of increased entry suggested a mechanism other than
183 the TZ in regulating GCY-22::GFP ciliary entry.
184
185 The Ankmy2 protein DAF-25 is known to be required for ciliary localization of guanylate cyclases[38–40],
186 cyclic nucleotide gated channels and GPCRs[18,38], but not for IFT[39]. In *daf-25* mutants, GCY-22::GFP
187 did not enter the cilium, and no IFT tracks of GCY-22::GFP or ciliary tip localization was observed
188 (**Fig. 3d,e**, **Table 1**). Interestingly, *daf-25* animals showed lower levels of GCY-22::GFP at the PCMC,
189 very few moving vesicles and diffuse fluorescence in the dendrite, and more diffuse localization in the
190 cell body, which did not overlap with the trans-Golgi marker APT-9::mCherry (**Fig. 3e**,
191 **Supplementary Fig. 1-3**). To test whether DAF-25 functions together with the TZ to traffic GCY-
192 22::GFP into the cilium, we generated a *daf-25; mks-5* mutant strain. In this double mutant, GCY-
193 22::GFP was present at low levels in the cilium but not at the tip (**Fig. 3d,e**). Very few IFT tracks of
194 GCY-22::GFP were visible, potentially explaining the lack of cilium tip localization (**Table 1**). Because
195 OSM-3-mediated IFT is not affected in *daf-25; mks-5* animals (**Supplementary Table 1**), MKS-5 and

196 DAF-25 may be required to link GCY-22::GFP to the IFT machinery, allowing for the formation of the
197 cilium tip compartment.
198
199 Together, our data reveals that DAF-25 is required for GCY-22::GFP import across the TZ and into
200 the cilium, and together with the core TZ scaffolding protein MKS-5 is involved in loading GCY-
201 22::GFP as IFT cargo.
202
203 **GCY-22::GFP localization requires its dimerization and RD3-associated domains**
204 To identify protein domains required for GCY-22::GFP ciliary trafficking, we generated a series of
205 deletion constructs. Several domains can be recognized in GCY-22 (**Fig. 5**), including an extracellular
206 receptor domain which possibly provides specificity for Cl^- ions[28,29]. These experiments showed that
207 its dimerization and RD3 domains are required for cilium entry (**Fig. 5**).

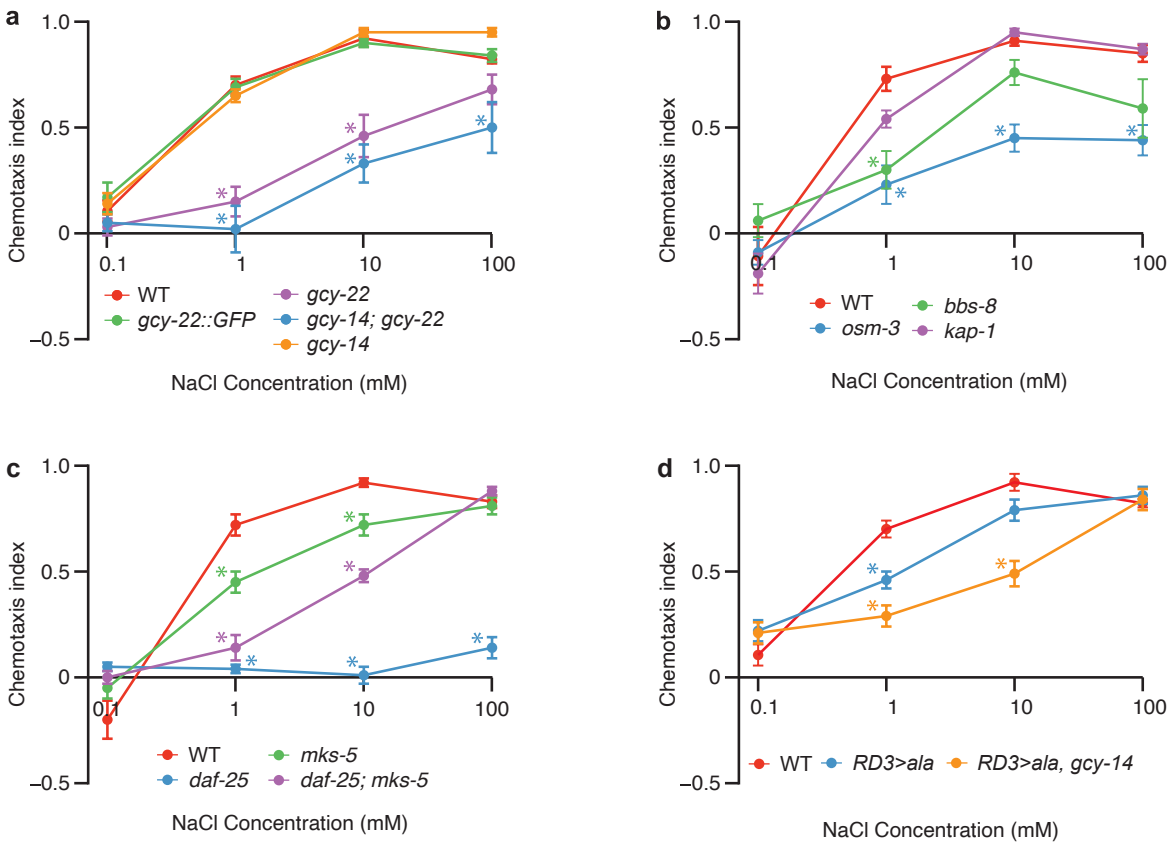
Figure 5



208
209 The RD3 domain shows homology to the RD3-binding domain identified in the mammalian guanylate
210 cyclase GUCY2D/GC1[41,42]. In a *gcy-22 loss-of-function* background, GCY-22(ΔRD3+CT)::GFP
211 localized at the PCMC but did not enter the cilium (**Fig. 5**). To confirm that the RD3 domain is
212 required for cilium import, we replaced 8 residues (W1042-I1049) of this domain with alanines using
213 CRISPR/Cas9, to create GCY-22(RD3>Ala)::GFP. This mutation completely abolished ciliary entry

214 (**Fig. 3d,e**). More diffuse fluorescence in the dendrite and cell body was also observed, reflecting a
215 potential trafficking defect of GCY-22(RD3>Ala)::GFP (**Supplementary Fig. 3**).
216
217 Next, we tested if the RD3 domain is *sufficient* for cilium entry by generating two RD3::GFP constructs
218 fused with the GCY-22 transmembrane (TM) domain (TM::GFP::RD3 and TM::RD3::GFP). These
219 proteins showed diffuse localization in the cell body, some dendritic transport, PCMC localization, and
220 a very weak ciliary signal with no evidence of IFT transport (**Fig. 5**, **Supplementary Fig. 4**). These
221 results suggest that the RD3 domain is not sufficient for correct routing and import in the cilium.
222
223 The *C. elegans* RD3 orthologue, RDL-1, influences the trafficking of GCYs to the PCMC and cilium[43].
224 Mutating *rdl-1* reduced GCY-22::GFP levels in the PCMC but not cilium tip (**Fig. 3d,e**). In addition,
225 GCY-22::GFP was more diffuse in the cell body and dendrite (**Supplementary Fig. 1,3**), in
226 agreement with RDL-1 regulating an early trafficking pathway for GCYs[43].
227
228 In contrast to GCY-22::GFP, the guanylate cyclase DAF-11 localizes along the entire cilium in ASI,
229 ASJ and ASK neurons[39,44]. We swapped the GCY-22 TM domain or C-terminal end, starting at the
230 highly conserved W residue at the start of the RD3 domain, with those of DAF-11 (TM>DAF-11TM
231 and CT>DAF-11CT, respectively). Both chimeric proteins localized to the PCMC and cilium tip,
232 suggesting that the TM domains and C-termini of GCY-22 and DAF-11 do not regulate their sub-
233 ciliary localization (**Fig. 5**). In addition, these results suggest that the C-terminal region of these
234 guanylate cyclases functions cell independently in cilium import.
235
236 Together, our data show that GCY-22 ciliary entry and tip localization requires the dimerization and
237 RD3 domains, and likely involve the combined action of more than one domain.
238
239 **GCY-22::GFP cilium tip compartment is required for high NaCl sensitivity**
240 ASE neurons express several receptor-type guanylate cyclases, GCY-22 and GCY-14 appearing to
241 be most important for detecting NaCl[26,28,29]. We tested *gcy-22* and *gcy-14* loss-of-function mutants
242 and a *gcy-14; gcy-22* double mutant in a NaCl chemotaxis quadrant assay. Animals are tested for
243 their preference for a particular NaCl concentration versus no NaCl[45,46]. Deleting *gcy-22*, but not *gcy-*
244 *14*, significantly affected chemotaxis to NaCl (**Fig. 6a**). Chemotaxis by the double mutant was not
245 significantly different from the *gcy-22* single mutant, suggesting that GCY-22 is more important than
246 GCY-14 in our assay (**Fig. 6a**).

Figure 6.



247

248 To determine if GCY-22 cilium tip localization is important for *C. elegans'* sensitivity to NaCl, we first
249 tested IFT mutants for their chemotaxis response. *kap-1* mutant animals showed a wild-type response
250 to NaCl (**Fig. 6b**), consistent with the normal tip localization of GCY-22::GFP. *bbs-8* mutant animals
251 showed reduced chemotaxis to 1 mM NaCl ($P < 0.01$; **Fig. 6b**), which correlates with the reduced
252 cilium tip levels of GCY-22::GFP. *osm-3* mutant animals, which have short cilia where GCY-22::GFP
253 localizes along its entire length, showed the strongest chemotaxis defect at all NaCl concentrations
254 tested ($P<0.01$; **Fig. 6b**).

255

256 Next, we tested *daf-25* mutant animals, which lack ciliary GCY-22::GFP. These animals showed a
257 strong chemotaxis defect, with only a modest response to 100 mM NaCl (**Fig. 6c**). This suggests that
258 *daf-25* is required for ciliary import of proteins essential for chemotaxis, and consistent with the
259 mislocalization of guanylate cyclases and CNGs[18,39,40]. Surprisingly, disrupting the TZ in the *daf-25*
260 mutant (*daf-25; mks-5* double mutant) resulted in a partially-restored response to 10 mM NaCl, and a
261 wild-type response to 100 mM (**Fig. 6c**). These responses roughly correlate with the levels of ciliary
262 GCY-22::GFP, where the increased amount in the double mutant appears sufficient for detecting high

263 NaCl concentrations. However, the possibility remains that other proteins that play a role in NaCl
264 sensory transduction are also affected.
265
266 To specifically test the contribution of GCY-22::GFP ciliary tip localization to NaCl sensation, we
267 tested the RD3>Ala mutant animals for their chemotaxis response. These animals showed a mild
268 chemotaxis defect at 1 mM NaCl (P=0.023), but wild-type responses to 10 and 100 mM NaCl
269 (**Fig. 6d**), suggesting that a small amount of GCY-22 could still be present in the cilium. Additionally, a
270 chemotaxis defect in these animals could be masked by functional redundancy. To test this latter
271 possibility, we made a *gcy-14; gcy-22(RD3>Ala)* double mutant and observed a stronger chemotaxis
272 defect at 10 mM NaCl (P=0.020) compared to *gcy-22(RD3>Ala)* single mutants (**Fig. 6d**).
273 Interestingly, *gcy-14; gcy-22(RD3>Ala)* animals showed stronger chemotaxis to 100 mM NaCl than
274 *gcy-14; gcy-22* animals (P=0.004), indicating that the GCY-22::RD3>Ala protein is functional in
275 detecting NaCl.
276
277 Together these experiments show that a high level of GCY-22 is required at the cilium tip for
278 detecting, and efficient chemotaxis to, low NaCl concentrations.
279
280 **Discussion**
281 Some signaling proteins localize to specific ciliary subdomains, whereas others distribute along the
282 length of the cilium, suggesting that their specific localization patterns are functionally important. Our
283 understanding of how the signaling protein localization within the cilium is regulated remains limited.
284 Here, we identified mechanisms involved in the localization of the putative Cl^- receptor GCY-22 to a
285 unique ciliary tip domain in the ASER neuron of *C. elegans* and provide evidence that this specific
286 localization is essential for its function as a highly sensitive NaCl sensor.
287
288 Our results suggest that IFT is the primary driving force behind GCY-22 localization to the cilium tip.
289 First, we identified DAF-25 as essential for ciliary import. DAF-25 functions together with the TZ, by
290 way of MKS-5, to load GCY-22 as cargo onto IFT particles. Second, dual-color imaging showed co-
291 localization of GCY-22::GFP with OSM-3-kinesin in a subset of IFT particles, suggesting association
292 between GCY-22::GFP and certain IFT particles or 'trains'. Strikingly, many GCY-22::GFP particles
293 moving towards the ciliary base were picked up by anterograde IFT trains and relocalized to the tip.
294 This behavior, not previously observed for other IFT-associated proteins, maintains a high density of
295 receptor molecules within the tip compartment. How this is regulated—for example, whether this
296 depends on differential affinity for anterograde or retrograde IFT machinery—remains to be
297 determined. Finally, interfering with IFT diminishes the accumulation of GCY-22::GFP at the tip. This
298 indicates that IFT plays a crucial role in actively maintaining a specialized signaling compartment at
299 the cilium tip.
300

301 Interestingly, our FRAP assays indicate the presence of a distinct tip membrane compartment with
302 properties different from the rest of the cilium. GCY-22 might therefore bind other, yet-to-be-identified
303 protein(s) at the cilium tip. Similarly, how Hedgehog signaling components remain confined to the
304 cilium tip is unknown[47]. However, we found a high density of GCY-22::GFP molecules at the cilium tip,
305 which slows lateral diffusion[48]. This may explain, independent of tethering by other proteins, the slow
306 redistribution of GCY-22::GFP in the tip compartment.
307
308 The high concentration of GCY-22 at the ciliary tip suggests that it forms a highly sensitive detection
309 apparatus, reminiscent of the high densities of rhodopsin in mammalian photoreceptor cells, which
310 enable the detection of single photons[34,49]. Indeed, mislocalization of GCY-22::RD3>Ala::GFP
311 affected chemotaxis to 1 mM NaCl most, suggesting that the ciliary tip compartment is required to
312 navigate small differences in NaCl concentrations, likely important for *C. elegans* in its natural habitat.
313
314 In conclusion, our study revealed mechanisms for generating and maintaining a specialized ciliary tip
315 domain that is analogous to the cGMP signaling domain of mammalian ciliary photoreceptors, and
316 ciliary tip domain of the Hedgehog signaling cascade. Our findings suggest that such domains may be
317 broadly used as signaling compartments and should be sought and analysed in mammalian cilia in
318 the context of different signaling pathways, human physiology and disease.
319
320 **Methods**
321 *Strains and Constructs*
322 Strains were cultured using standard methods[50]. Alleles used in this research were: *kap-1(ok676),*
323 *osm-3(p802), bbs-8(nx77), daf-25(m362), mks-5(tm3100), gcy-14 (pe1102), gcy-22(tm2364), dhc-*
324 *1(ie28[dhc-1::degron::GFP]), ieSi57[eft-3p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)].*
325
326 The $p_{rab\text{-}3}$*::mCherry::apt-9* construct was (gift from A. Pasparaki) and used to generate $p_{rab\text{-}3}$*::mCherry::tram-1.* A 1.5 kb genomic DNA fragment containing *tram-1* was amplified with primers
328 #2581 and #2582 and used to replace *apt-9.* $p_{gpa\text{-}4}$*::gcy-22::GFP* was generated by inserting two PCR
329 fragments of 2.5 kb and 2 kb, amplified with primers #3152 and #3154, and primers #3153 and
330 #3151, together containing the genomic *gcy-22* locus, into pGJ325[46]. To generate the p_{U6}*::osm-*
331 *3_sgRNA* vector we cloned an *osm-3* guide into the p_{U6}*::unc-119::sgRNA* vector[51]. The $p_{flp\text{-}6}$*::mCherry*
332 construct was generated by inserting a 2 kb *flp-6* promoter sequence, amplified with primers #2867
333 and #2869, into a pPD95.77 (gift from A. Fire) backbone containing *mCherry*. The *osm-3::mCherry*
334 template construct was generated by inserting *mCherry* and two 1.5 kb homology arms, amplified
335 from genomic DNA using primers #2679 and #2643 and primers #2660 and #2646, into the backbone
336 of p_{U6}*::unc-119::sgRNA.* The $p_{gcy\text{-}5}$*::xbx-1::tdTomato* construct was generated by inserting a 325 bp
337 promoter from *gcy-5*, amplified using primers 355 and 356, and a 2.2 kb genomic fragment of *xbx-1*,

338 amplified using primers 326 and 327, into a pPD95.81 vector containing *tdTomato*. The $p_{gcy\text{-}5}$*::gcy-*
339 *22::GFP* construct was generated by PCR fusion[52]. First, a 4.6 kb genomic sequence of *gcy-22*,
340 amplified using primers #221 and #248, and a 325 bp genomic fragment of the *gcy-5* promoter,
341 amplified using primers #219 and #222, were fused using primers #443 and #215. Subsequently, this
342 product was fused upstream of the GFP-coding cassette, including the *unc-54* 3'-UTR, from
343 pPD95.67 (gift from A. Fire) using primers #220 and #609. The final fusion product was cloned into
344 pGEMT easy.
345
346 The ΔER strain was generated by fusing the 426 bp *gcy-5* promoter, amplified with primers #218 and
347 #458, and a 2.6 kb ΔER fragment, amplified from genomic DNA using primers #457 and #248, using
348 primers #219 and #215. This product was fused to *GFP*::*unc-54-3'-UTR* from pPD95.67 using primers
349 #220 and #609. The ΔCT strain was generated by amplifying from $p_{gcy\text{-}5}$*::gcy-22::GFP,* a $p_{gcy\text{-}5}$*::gcy-*
350 *22::ΔCT* fragment, using primers M13Fwd and #433, and *GFP* using primers #432 and M13Rev, and
351 fusing these products using primers #220 and #609. The ΔRD3+CT strain was generated by fusing a
352 *gcy-22* fragment upstream of the RD3 domain, amplififed using primers #434 and #435, and a
353 fragment containing GFP, amplified using primers #152 and #434, using primers #220 and #609. The
354 ΔDD strain was generated by amplifying fragments upstream and downstream of the DD from $p_{gcy\text{-}}$
355 $_{5}$*::gcy-22::GFP* using primers #470 and #219, and primers #469 and #609, and fusing these products
356 using primers #220 and #609. The *TM>DAF-11TM* strain was generated by amplifying $p_{gcy\text{-}5}$*::gcy-*
357 *22::GFP* fragments up and downstream of the *TM*, using primers #440 and #443, and primers #441
358 and #215. Primers #441 and #440 contained overlapping regions of the *daf-11 TM*. Primers #443 and
359 #215 were used to fuse these two fragments.
360
361 The TM::GFP strain was generated by fusing a *gcy-22 TM* fragment, amplified from ΔER using
362 primers #219 and #454, to *GFP* using primers #220 and #609. The *TM::RD3::GFP* strain was
363 generated by amplifying *a* $p_{gcy\text{-}5}$*::gcy-22(TM)* fragment, amplified from $p_{gcy\text{-}5}$*::gcy-22(TM)::GFP* using
364 primers #220 and #607. Next, a *gcy-22(RD3)* fragment, amplified from genomic DNA using primers
365 #606 and #607, was fused to *GFP::unc-54-3'-UTR* pPD95.77, using primers #608 and #609. Finally,
366 the $p_{gcy\text{-}5}$*::gcy-22(TM)* and *RD3::GFP::unc-54-3'-UTR* fragments were fused using primers #220 and
367 #609. *TM::GFP::RD3* strain was generated by amplifying a $p_{gcy\text{-}5}$*::gcy-22(TM)::GFP* fragment without
368 *unc-54* 3'-UTR from $p_{gcy\text{-}5}$*::gcy-22(TM)::GFP* using primers #220 and #601, and *a gcy-22(RD3)*
369 fragment from genomic DNA using primers #602 and #603. Subsequently, these two fragments were
370 fused using primers #220 and #604.
371
372 PCR fusion products were injected with $p_{gcy\text{-}5}$*::xbx-1::tdTomato*, and pRF4::*rol-6(su1006)*[53] or $p_{unc\text{-}}$
373 $_{122}$*::GFP*[54] to generate transgenic strains.
374

375 *Microinjections*

376 Microinjections were performed using standard methods[55].

377

378 *CRISPR/Cas9*

379 To generate the *osm-3::mCherry* allele, animals were injected with a mixture containing p_{U6}*::osm-*
380 *3_sgRNA* (45 ng/µl), $p_{eft\text{-}3}$*::cas9-SV40_NLS::tbb-2* (50 ng/µl), pRF4::*rol-6(su1006)* (50 ng/µl), and a
381 plasmid containing an *mCherry* repair template with 1500 bp homology arms (20 ng/µl). Animals were
382 injected and placed on separate 6 cm NGM plates. Three days later, F1 offspring was picked, allowed
383 to self-reproduce, and screened by PCR. We used a *dpy-10* based co-CRISPR method and a PCR-
384 generated repair template of GFP, amplified with primers #3120 and #3121, with 35 bp homology
385 arms to generate the *gcy-22::GFP(gj1976)* allele. We used ssODN repair template #3393, with 35 bp
386 homology arms, and guide g10, to generate the *gcy-22(RD3>Ala)::GFP(gj1987)* allele. The *rdl-*
387 *1(gj1989)* deletion allele was generated using two guides, g14 and g15, and no template.

388

389 We used the CRISPR/Cas9 method as described by Dokshin *et al.*[56] to generate the *gj2113[gcy-*
390 *22::CT>daf-11CT::GFP]* allele. Briefly, a gBlock (IDT) containing the *daf-11CT* repair template was
391 cloned into a pGEM vector. Subsequently, the repair template was amplified using primers #3404 and
392 #3405 with 35 bp homology arms. The repair template, guide (g10), and pRF-4::*rol-6(su1006)* were
393 injected into GJ3452 according to protocol.

394

395 A list of strains, primers, and guides used in this research can be found in Supplementary Table 3.

396

397 *Microscopy*

398 Animals were immobilized on a 6% agarose pad, using 0.10 µm polystyrene microspheres (Polybead,
399 Polysciences Inc.) and 10 mM Levamisole (Sigma) as an anesthetic in M9 buffer, unless stated
400 otherwise. Fluorescence images were taken using a spinning disc confocal microscope (Nikon Ti-
401 eclipse) with an EM CCD camera (QuantEM512C, Photometrics) and Metamorph Imaging software,
402 unless stated otherwise. Images were analyzed using FIJI software (version 2.0.0).

403

404 For FRAP experiments, a FRAP3D unit (ROPER) was used. Pre-bleach, 10 images were taken at 1
405 second intervals. Post-bleach the following images were taken: for the entire PCMC or tip 4 images at
406 15 sec. intervals, 4 images at 60 sec. intervals, and 4 images at 5 min. intervals; for half of the PCMC
407 120 images at 1 sec. intervals ; for half of the cilium tip 10 images at 1 sec. intervals and 18 images at
408 15 sec. intervals; for GFP 200 images at 50 ms intervals (a subset was plotted in **Fig. 2f**); for
409 TM::GFP in the PCMC 20 images at 1 sec. intervals; for TM::GFP in the cilium tip 33 images at 300
410 ms intervals. For the time-lapse images, animals were immobilized on a 6% agarose pad and M9
411 containing 10 mM Levamisole. Images were taken at 300 ms intervals and kymograms were

412 generated using the KymographClear 1.0. ImageJ plugin[57]. Kymograms were analyzed using a
413 custom ImageJ plugin written by I. Smal. Dual color time-lapse images were taken using a DV2 beam
414 splitter (MAG Biosystems).
415
416 Quantification of GCY-22::GFP fluorescence in different mutant backgrounds, and of GFP molecules
417 at the cilium tip, was performed on a laser scanning confocal microscope (SP5 AOBS, Leica). A
418 dilution series of purified GFP in PBS was used to generate a calibration curve for the GFP
419 fluorescence. Assuming a confocal volume of 0.1 femtoliter the fluorescence intensity of the
420 calibration curve was converted into number of GFP molecules. The integrated fluorescence intensity
421 of the cilia was measured and converted to number of GFP molecules. The membrane density of
422 GFP molecules in the cilium tip was calculated, assuming a membrane surface of 1.56 μm^2.
423
424 *NaN_3 treatment*
425 To stop ATP production, NaN_3 (Sigma) was added to a 6% agarose pad at a concentration of 20 mM.
426 Animals were shortly incubated in a drop of M9 containing 10 mM Levamisole (Sigma) before
427 immobilization. Imaging started 10 min after immobilization. To assess the recovery of the cilium tip,
428 animals were incubated in M9 buffer containing 20 mM NaN_3 for 30 min and subsequently
429 immobilized for imaging without NaN_3.
430
431 *Auxin inducible degradation*
432 For the auxin inducible degradation of DHC-1::GFP::degron, animals were cultured on NMG plates
433 containing 1 mM IAA (Sigma) for 48 hrs prior to imaging.
434
435 *Chemotaxis assays*
436 The quadrant assay used to asses chemotaxis to NaCl was adapted from Wicks *et al.* and Jansen *et*
437 *al.*[45,46]. Briefly, two diagonally opposite quadrants of a sectional petri dish (Star Dish, Phoenix
438 Biomedical) were filled with 13.5 mL buffered agar (1.7% Agar, 5 mM K_2HPO_4/KH_2PO_4 pH 6, 1 mM
439 $CaCl_2$ and 1 mM $MgSO_4$) containing NaCl and two diagonally opposite quadrants with 13.5 mL
440 buffered agar without NaCl. Immediately before the assay, the plastic dividers between the quadrants
441 were covered with a thin layer of agar. Age synchronized *C. elegans* populations were washed 3
442 times for 5 min with CTX buffer (5 mM K_2HPO_4/KH_2PO_4 pH 6, 1 mM $CaCl_2$ and 1 mM $MgSO_4$).
443 Approximately 100 animals were placed in the middle of a sectional dish. After 10 min, animals on
444 each quadrant were counted and a chemotaxis index (CI) was calculated for each plate (CI = (#
445 animals on NaCl – # animals not on NaCl)/ total # animals). To determine the CI of a strain, 2 assays
446 per day were performed on at least 3 days.
447

Statistics

Statistical analyses were performed using R software, version 3.6.0. IFT track lengths were compared using a Mann-Whitney U test. Comparisons of the chemotaxis indexes, florescence intensities, and OSM-3::mCherry track counts and speeds were performed with a one-way ANOVA, followed by a pairwise t-test with Holm correction. Distributions of animals with and without GCY-22::GFP IFT-like tracks were compared using a Chi^2-test.

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Author Contributions

S.N.B., M.R.L., and G.J. conceived the study. S.N.B. and S.R. generated strains, performed experiments and analyzed the data. J.J. and C.L. generated strains, performed experiments and analyzed the data for the construct-based domain deletion experiments. S.R. and G.K. designed and performed the experiments and analysed the data for the GFP quantification experiment. S.R. and S.N.B. designed experiments, S.R. performed the experiments, and S.R., S.N.B., and A.B.H. analysed the data for the FRAP experiments. S.N.B., M.R.L. and G.J. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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476

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610

611 **Figure Legends**

612 **Figure 1.** GCY-22::GFP traffics along the dendrite from the cell body to the base of the cilium and
613 concentrates at the ciliary tip in the salt-sensing ASER neuron.
614 **(a)** Schematic of the head of the animal showing the ASER cell body, dendrite and cilium. Inserts:
615 schematic of the cilium depicting the periciliary membrane compartment (PCMC), transition zone
616 (TZ), and cilium tip, and fluorescence images with GCY-22::GFP (green) and mCherry (red) showing
617 localization in the cell body, vesicles in the dendrite and localization to the PCMC and tip of the cilium.
618 **(b)** Ectopic expression of GCY-22::GFP in the ASI neuron showing localization at the PCMC and
619 cilium tip. **(c)** Kymograms showing dendritic transport tracks of GCY-22::GFP in wild-type background
620 and reduced transport in a *dhc-1::GFP::degron* background in the presence of auxin (IAA). Black lines
621 indicate moving and red lines indicate stationary vesicles. **(d)** Quantification of GFP molecules at the
622 cilium tip of ASE neurons. Scale bars indicate 5 µm.
623

624 **Figure 2.** GCY-22::GFP is present in stable pools at the PCMC and cilium tip.
625 **(a)** Fluorescence recovery after photobleaching (FRAP) of the entire PCMC (n=7). **(b)** FRAP of the
626 entire cilium tip compartment (n=8). **(c)** FRAP of half of the PCMC (n=19). Insert: Fluorescence
627 images of the PCMC pre- and post-bleach. **(d)** FRAP of half of the tip compartment (n=6). Insert:
628 fluorescence images of the tip pre- and post-bleach. **(e)** Fluorescence recovery of GCY-22::GFP
629 (blue, n=19), TM::GFP (orange, n=11), and GFP (green, n=7) after photobleaching half of the PCMC.
630 **(f)** Fluorescence recovery of GCY-22::GFP (blue, n=6), TM::GFP (orange, n=7), and GFP (green,
631 n=6) after photobleaching half of the tip compartment. Scale bars represent 1 µm. Colored areas
632 indicate SD.
633
634 **Figure 3.** The GCY-22-containing cilium tip compartment is actively maintained by IFT.
635 **(a)** Kymogram of GCY-22::GFP showing anterograde and retrograde ciliary transport and possible
636 diffusion tracks. Black arrowhead indicates stationary signal. **(b)** Kymograms showing partial overlap
637 of IFT tracks of GCY-22::GFP (green) and of the mCherry-tagged anterograde IFT motor protein
638 OSM-3 (red). **(c)** Quantification of track length of GCY-22::GFP (green, n=13 cilia), OSM-3::mCherry
639 (left graph, red, n=7 cilia), and tdTomato-tagged retrograde IFT motor protein XBX-1 (right graph, red,
640 n=24 cilia). Asterisks indicate significant difference in length (*P*-value: * <0.05, ** <0.01, *** <0.001,
641 two-tailed Mann-Whitney U test). **(d)** Quantification of normalized fluorescence intensity in different
642 mutant backgrounds of GCY-22::GFP in the PCMC, axoneme and cilium tip. Distribution of
643 fluorescence between the PCMC (green), axoneme (orange) and cilium tip (blue). Significant
644 differences (P-value <0.05) are indicated by colored asterisks (black is total cilium fluorescence,
645 ANOVA followed by pairwise t-test with Holm correction), n≥5. **(e)** Representative fluorescence
646 images and schematics showing localization of GCY-22::GFP in PCMC and cilium in different mutant
647 backgrounds. Scale bar represents 5 µm.
648
649 **Figure 4.** Maintenance of the GCY-22-containing cilium tip compartment is ATP dependent.
650 **(a)** Fluorescence images of GCY-22::GFP showing cilium tip collapse and kymograms of OSM-
651 3::mCherry showing IFT arrest during NaN_3 treatment. Asterisk indicates PCMC, arrowheads indicate
652 proximal end of tip compartment. **(b)** Quantification of distal segment IFT capacity (number of tracks
653 multiplied by speed) during NaN_3 treatment (n=7) and linear regression (black line, R^2=0.37). **(c)**
654 Quantification of cilium length (red) and cilium tip (blue) during NaN_3 treatment. Darker lines show
655 time-normalized, average result (n=7). **(d)** Representative fluorescence images showing recovery of
656 the cilium tip compartment after 30 min of NaN_3 treatment (n=5). Arrowheads indicate proximal end of
657 tip compartment. Scale bars represent 5 µm.
658
659 **Figure 5.** Cilium tip localization of GCY-22::GFP requires the dimerization and RD3-associated
660 domains. Schematic of GCY-22::GFP wild-type and different chimeric proteins showing deleted
661 domains: extracellular receptor domain (ΔER), C-terminal tail (ΔCT), RD3-associated domain

662 (ΔRD3), dimerization domain (ΔDD), transmembrane domain only (TM::GFP), transmembrane
663 domain and RD3-associated domain fused to GFP in two orientations (TM::RD3::GFP and
664 TM::GFP::RD3) and chimeric proteins with GCY-22 domains replaced with corresponding DAF-11
665 domains, transmembrane domain (TM>DAF-11TM) and CRISPR/Cas9 based C-terminal tail
666 (CT>DAF-11CT). Fluorescence images showing corresponding localization of GCY-22::GFP versions
667 (green) and XBX-1::tdTomato (red).
668
669 **Figure 6.** Cilium tip localization of GCY-22 is required for sensitive detection of NaCl.
670 **(a)** Chemotaxis indexes of different mutants showing wild type response of *gcy-22::GFP* animals and
671 involvement of GCY-22. **(b)** Chemotaxis to 1, 10 and 100 mM NaCl requires the IFT anterograde
672 motor OSM-3, and the BBSome subunit BBS-8 for chemotaxis to 1 mM. **(c)** Chemotaxis to 1, 10, and
673 100 mM NaCl requires the Ankmy2 protein DAF-25, and the TZ component MKS-5 for chemotaxis to
674 1 and 10 mM. **(d)** Chemotaxis to 1 and 10 mM NaCl requires cilium tip localization of GCY-22 and
675 involves GCY-14. Asterisks indicate significant difference compared to wild type. ANOVA followed by
676 pairwise t-test with Holm correction, n ≥ 6 assays. Full statistical analysis can be found in
677 Supplementary table 2.
678
679 **Supplementary Figure 1**. GCY-22::GFP colocalizes with TRAM-1 and APT-9 positive vesicles in the
680 ASER cell bodies of wild-type animals. (a) Fluorescence images of ASER cell bodies showing GCY-
681 22::GFP (green) and the ER-marker TRAM-1::mCherry (red), and colocalization (composite). (b)
682 Fluorescence images of ASER cell bodies showing GCY-22::GFP (green) and the Golgi-marker APT-
683 9::mCherry (red), and colocalization (composite). (c) Fluorescence images showing more diffuse
684 localization of GCY-22::GFP (green) in *daf-25* and *rdl-1* mutant animals, APT-9::mCherry positive
685 vesicles (red) and no colocalization in *daf-25* animals and partial colocalization *rdl-1* animals
686 (composite). Scale bars represent 5 µm.
687
688 **Supplementary Figure 2.** Kymograms of dendritic transport of GCY-22::GFP showing vesicular
689 transport in wild-type animals and reduced transport in different mutant backgrounds. Examples of
690 transport (arrowheads) and stationary signal (asterisks) are indicated. Scale bars represent 9 seconds
691 (vertical) and 2 µm (horizontal).
692
693 **Supplementary Figure 3.** GCY-22::GFP localization in the cell body, dendrite and cilium of the ASER
694 neurons in wild-type and different mutant backgrounds.
695
696 **Supplementary Figure 4.** Fluorescence images of chimeric proteins containing the transmembrane
697 domain and RD3-associated domain (RD3) and GFP in the cell body, dendrite, and cilium of ASER
698 neurons. Kymograms of the dendrite showing diffuse signal and occasional vesicular transport and of
699 the cilium showing diffuse signal only. Asterisks indicate PCMC, scale bar indicates 5 µm.

700

701 **Supplementary Figure 5.** Genomic sequences of alleles generated in this research using

702 CRISPR/Cas9.