The sweet and sour sides of trypanosome social motility

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Abstract

We recently showed that the formation of elegant geometric patterns by communities of *Trypanosoma brucei* on semi-solid surfaces, dubbed social motility (SoMo) by its discoverers, is a manifestation of pH taxis. This is caused by procyclic forms generating and responding to pH gradients through glucose metabolism and cyclic AMP signalling. These findings established that trypanosomes can sense and manipulate gradients, potentially helping them navigate through host tissues. At the same time, the host itself and bystanders such as endosymbionts have the potential to shape the environment and influence the chances of successful transmission. We postulate that the ability to sense and contribute to the gradient landscape may also underlie the tissue tropism and migration of other parasites in their hosts.

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27 **Parasites on the move**

28 Parasite life cycles are traditionally depicted as a static series of distinct morphological
29 forms, with emphasis placed on the names of these different forms and the cells or tissues
30 where they are found. Such depictions pay little or no attention to the fact that life cycles are
31 dynamic: with few exceptions, parasites need to migrate from one tissue to the next and
32 undergo several rounds of differentiation before they can be transmitted. In the case of
33 *Trypanosoma brucei* in its insect host, the tsetse fly, the parasite follows a tortuous route that
34 requires it to overcome physical barriers before it can be transmitted to a mammalian host. To
35 what extent is the parasite a passive traveller with no control over its destination? Or can it
36 sense where it is and determine what route to take? In addition, can parasite populations
37 manipulate their environment to improve their chances of survival and transmission? Our
38 recent work has shown that *T. brucei* can both create and react to gradients and that
39 perception of these gradients requires specific **adenylate cyclases** (ACs; see Glossary) and at
40 least one cyclic AMP response protein (CARP). Rather than being a simple bidirectional
41 interaction between the parasite and its host, however, this seems to be a three-way
42 conversation that also involves parasites communicating with each other, and possibly with
43 other resident microorganisms in the fly.
44

45 **Social motility and cyclic AMP signalling**

46 More than a decade ago, it was shown that procyclic culture forms, which correspond to
47 tsetse midgut forms, formed multicellular communities when inoculated on agarose plates.
48 Parasites initially stayed at the inoculation site, then groups of parasites started migrating
49 outwards in finger-like radial projections. This enigmatic coordinated movement, named **social**
50 **motility** (SoMo), was of great interest to our laboratory since its discovery by Kent Hill's group
51 at UCLA [1]. Not all laboratories could reproduce this phenomenon when it was first reported,
52 however. The reason for this discrepancy became clear when our laboratory demonstrated that
53 SoMo was a property of **early procyclic forms**, but not of **late procyclic forms** [2]. These two
54 life-cycle stages are morphologically indistinguishable in culture, but >70 transcripts are
55 differentially expressed [3]. The most obvious difference between the two forms is that early

56 procyclic forms express a surface protein known as **GPEET procyclin**, allowing these cells to be
57 identified with ease [4]. Although this solved the issue of reproducibility in different settings, it
58 did not explain why trypanosomes performed SoMo on plates, nor did it address the question
59 of whether it was biologically relevant.
60 In the years that followed, the Hill group began to elucidate the mechanisms behind
61 SoMo. They manipulated intracellular **cyclic AMP** (cAMP) levels by knocking down genes that
62 either generate cAMP (adenylate cyclases) or hydrolyse it (**phosphodiesterases**) or by using
63 chemical inhibitors. Perturbing intracellular cAMP levels could either make the parasites non-
64 responsive (no migration) or very sensitive (hyper SoMo)[5–7] **(Figure 1A)**. This led them to
65 propose that compartmentalised cAMP microdomains could elicit different types of behaviour
66 [5–7]. Later, a collaboration between our groups showed that flagellar phosphodiesterase B1
67 (PDEB1) was not only required for SoMo, but also for trypanosomes to move from the **midgut**
68 **lumen** of the tsetse to the **ectoperitrophic space** [8] **(Figure 2)**. This suggested that localised
69 cAMP signalling polarised cells and conferred a sense of direction **(Figure 1B)**.
70
71 **Identifying the migration signals**
72 Something that intrigued us right from the start was the ability of a migrating population
73 to sense other communities on a plate and to reorient so that they did not collide. Our initial
74 thought was that there ought to be extracellular mediators, possibly exosomes or proteins, that
75 were released by the parasites themselves. We therefore exposed migrating projections to
76 concentrated medium from a dense liquid culture (conditioned medium) or, as a control,
77 concentrated, fresh medium. These initial experiments showed two important reactions by the
78 parasites. Projections close to the site where conditioned medium was spotted were repelled
79 and reoriented away from it. By contrast, projections that were exposed to fresh medium
80 changed direction to move towards it [9] **(Figure 1A)**. An obvious difference between fresh and
81 conditioned medium was the colour: the medium contains a pH indicator, and it is well known
82 that trypanosomes acidify their medium as they grow. Could pH be a driver of migration? This
83 seemed almost too simplistic. We took it a step further and measured the pH at different
84 locations on plates with migrating populations. Not too surprisingly, the parasites acidified their

85 surroundings, resulting in pH gradients on the plates, but this was still not enough to prove that
86 these caused SoMo. To test this, we took one molar solutions of hydrochloric acid and sodium
87 hydroxide and spotted these close to migrating projections. The parasites responded very
88 strongly to both solutions, although not by dying as one of us predicted! Sodium hydroxide
89 acted as an attractant, making the projections reroute towards it, while hydrochloric acid acted
90 as a repellent, with projections reorienting to avoid it **(Figure 1A)**. These first experiments were
91 performed with early (SoMo-competent) procyclic forms, but how would late procyclic forms
92 react to pH? Interestingly, these were also attracted to basic solutions, but were indifferent to
93 acidic solutions. These differences in their responses explain the self-organising properties of
94 mixtures of early and late procyclic forms in SoMo communities, with early procyclic forms
95 forming the projections while late procyclic forms remain at the inoculation site **(Figure 1A)** [9].
96 In parallel with these experiments, our laboratory performed RNA-Seq to see if there
97 were differences in expression between cells at the tip of a projection and cells at the root, the
98 position where the radial projection emerges from the central community [9]. This showed
99 that cells at the tip upregulated transcripts for high affinity hexose transporters and glycolytic
100 enzymes. Exposing the trypanosomes to varying concentrations of glucose revealed that it was
101 required to generate the pH gradient and that this correlated with their ability to perform
102 SoMo. RNA-seq also identified other transcripts that were differentially expressed between the
103 tip and the root, including several ACs. If cAMP signalling was involved in SoMo, would it also
104 mediate the response to pH? Interestingly, when a PDEB1 null mutant was exposed to alkali or
105 acid, it showed no pH response at all [9]. Another protein that we implicated in the reaction to
106 pH was cyclic AMP response protein 3 (CARP3), one of four proteins previously identified in a
107 genome-wide RNAi screen of bloodstream forms for resistance to an inhibitor of PDEB1 [10]. In
108 vitro, a CARP3 null mutant showed a major SoMo defect and, although it was still attracted to
109 alkali, it did not react to acid on plates.
110 *T. brucei*, which has the most complex life cycle of all the African trypanosomes [11,12],
111 has an expanded set of more than seventy ACs, and many of these are stage-regulated [3,5,13].
112 Immunoprecipitation of CARP3 from early procyclic forms showed that it was physically
113 associated with several ACs, predominantly with ACP3 and ACP5, two enzymes localised to the

114 flagellar tip [5,9]. Furthermore, ACP3 coprecipitated ACP5 and vice versa, and both pulled
115 down CARP3. To complete the circle, a single knockout of ACP5 gave the same phenotypes *in*
116 *vitro* as CARP3 – a SoMo defect and a lack of response to acid. We were not able to generate a
117 double knockout of ACP5, however, nor could we delete ACP3 using CRISPR/Cas9. This suggests
118 that these two ACs have additional, essential functions in procyclic forms that are independent
119 of CARP3 [9].

120 As is often the case, we were not the only people to be interested in how cAMP signals
121 are transduced in trypanosomes. Daniel Vélez-Ramirez and members of the Hill laboratory used
122 proximity labelling by ACP1 to detect proteins in its vicinity. This led to the identification of five
123 other adenylate cyclases (ACP2-ACP6), as well as calpain-like protein 1.3, CARP3 and the
124 flagellar protein FLAM8 [14]. Very recently, Sabine Bachmaier and coworkers published a study
125 on cAMP signalling emanating from the flagellar tip and implicated FLAM8 in signal
126 transduction in SoMo [15]. FLAM8 is localised to the tip of the flagellar microtubules [16] and
127 anchors a pool of CARP3 at this position. It is not known whether FLAM8 is sufficient for this or
128 if additional proteins are required.

129 Both our group and Bachmaier and colleagues realised that CARP3 can be N-
130 myristoylated at its N-terminus, which would enable it to attach to the inner leaflet of the
131 plasma membrane [17], and therefore tagged the protein at the C-terminus so as not to disturb
132 this modification. This led to the identification of a common set of proteins: in addition to
133 multiple ACs and calpain 1.3, these included proteins not expected to be at the flagellar tip,
134 such as components of the **flagellar attachment zone** and the **axoneme**. Other proteins
135 identified in CARP3 pulldowns are not localised to the flagellum; these include vacuolar protein
136 pump subunit B, a component of the acidocalcisome, and mitochondrial ATP synthase subunits
137 (TrypTag.org [18]). The latter is compatible with a report of CARP3 in the mitochondrial
138 proteome of procyclic forms [19].

139 Since CARP3 appears to act as a node between the ACs in the flagellar membrane and
140 downstream effectors of signalling, it was logical to test the behaviour of knockouts *in vivo*.
141 Three independent CARP3 null mutants were generated and tested in tsetse flies [9,15]. These
142 gave varying results, some of which might be attributable to strain differences, but it is also

143 plausible that the different infection protocols affected the outcomes to some extent. The
144 CARP3 knockout that we generated in *T. brucei* Lister 427 (Bern) was defective at colonising the
145 midgut of tsetse and did not give rise to infections of the **proventriculus** [9]. Thus, in common
146 with PDEB1, CARP3 is required for the early phase of fly infection. The wild-type stock normally
147 results in heavy infections of the proventriculus, but does not colonise the salivary glands, so no
148 conclusions could be drawn about a requirement for CARP3 later in the life cycle. Bachmaier
149 and colleagues generated CARP3 knockouts in two different stocks of AnTat 1.1, which can
150 colonise the glands. In a first experiment they included glutathione in the infective bloodmeal;
151 this is an additive that results in 100% midgut infections, while not affecting the proportion of
152 midgut infections that gives rise to salivary gland infections [15]. Glutathione is an antioxidant,
153 but it is not known whether it exerts its effect solely by inhibiting parasite killing by reactive
154 oxygen species, or whether it also affects the structure or barrier function of the peritrophic
155 matrix. In this experiment only the midgut and the salivary glands were examined. Although
156 this knockout gave comparable rates and intensities of midgut infections as its parental line, it
157 failed to colonise the salivary glands. There was no information, however, on whether the
158 block occurred between the midgut and the proventriculus or between the proventriculus and
159 the glands. In another experiment, performed with their second knockout (without
160 glutathione), they examined the posterior and anterior midgut and the proventriculus. The
161 knockout was able to colonise the proventriculus, although both the prevalence and the
162 intensity of infection were reduced. Although not identical, this was in line with our findings. In
163 these experiments as well, however, there were no salivary gland infections. This implies that
164 trypanosomes also require CARP3 and, by extension, sensing by AC, to find their way from the
165 proventriculus to the salivary glands. A FLAM8 knockout was also tested *in vivo*. Consistent
166 with its role in positioning CARP3 at the flagellar tip, this mutant was unable to perform SoMo
167 and had a phenotype in flies that was strongly reminiscent of our CARP3 and PDEB1 knockouts
168 [15].

169 Taken together, the combined results from the two groups showed that that all
170 knockouts resulting in SoMo defects impact negatively on colonisation of the midgut and/or
171 proventriculus [8,9,15,20]. CARP3 and PDEB1 are required for the parasites to perform SoMo

172 and to react to a drop in pH on plates, confirming their crucial role in **pH taxis**. Whether FLAM8
173 is required for sensing pH is currently unknown. While CARP3 is clearly an important player in
174 migration to or survival in the salivary glands, we suspect that its role is AC-dependent, but
175 SoMo-independent (see below).
176
177 **Where to go next?**
178 It has long been speculated that trypanosomes use ACs to survey their environment and
179 to respond to external stimuli (**Figure 3**). We have now shown that this is the case for SoMo,
180 which is pH sensing by early procyclic forms [9], and that it is highly likely to pertain to other
181 environmental signals as well. For example, it may apply to the positive **chemotaxis** exhibited
182 by procyclic culture forms in response to bacterial colonies [21] or negative chemotaxis in
183 response to exosomes shed by stressed parasites [22]. At present, we do not know how ACs
184 sense pH, but one possibility is that alterations in amino acid charges lead to conformational
185 changes. These might shift the balance between AC monomers and dimers (enzyme activity
186 requires dimers [5]) or result in changes in dimerisation partners. Conformational changes
187 might also modulate interactions with downstream signalling factors, including CARP3. By
188 controlling AC activity and creating a local pool of cAMP at the flagellar tip, a wild-type cell
189 would become polarised and thus be able to move along external gradients [7]. This, in turn,
190 would result in increased cAMP production and maintain directed motility. In contrast, a cell
191 lacking flagellar PDEB1 is likely to be flooded with cAMP, while CARP3/FLAM8 knockouts, with
192 impaired signalling, are less likely to produce cAMP at the flagellar tip in response to the
193 stimulus. In both these cases, a cell would not be polarised and would not orient itself in
194 response to external cues. Given the trypanosomes' small size and the fact that the ACs are
195 tightly localised at the tip, sensing might operate temporally rather than spatially, in much the
196 same way that bacteria sense gradients in their environment [23]. Once ACs are activated, it is
197 not clear how signals at the tip are translated into directed motion or increased ability to
198 overcome barriers. Down-regulating CARP3 in bloodstream forms leads to altered steady state
199 levels of several ACs [15], which complicates the analysis of its function. Moreover, CARP3 does
200 not seem to bind cAMP [15], a result that we confirmed independently (A. Naguleswaran and

201 IR, unpublished data). It is possible, however, that transient binding is enough to trigger a
202 cascade of events (**Figure 3A**). Alternatively, cAMP might bind to one or more proteins
203 associated with CARP3 and exert its effects indirectly (**Figure 3B**). It was previously reported
204 that orthologues of CARP3 were readily identifiable in other *Trypanosoma* spp., including *T.*
205 *cruzi*, with 48% amino acid identity [10]. In contrast, the closest relatives in *Leishmania* spp.,
206 with approximately 17% amino acid identity, were members of a multigene family associated
207 with stibogluconate resistance [24]. Proteins from this family have the potential to be N-
208 myristoylated [25] and, given the striking structural similarities predicted by Alphafold [26,27]
209 (**Figure 4**), it is possible that at least some of them fulfil the same function(s) as CARP3 in
210 *Leishmania* spp.
211 How might SoMo, and the discovery that it is pH taxis, be relevant to trypanosome and
212 tsetse physiology? It was shown almost twenty years ago that there is a marked difference in
213 pH from the midgut (pH 8) to the proventriculus (pH>10) [28]. To date, the pH of the salivary
214 glands has not been measured. It would be instructive to measure pH differences over shorter
215 distances too, for example between the midgut lumen and the ectoperitrophic space, and to
216 determine the effect of trypanosome infections. Furthermore, it has been reported that
217 commensals can have an impact on pH and midgut colonisation [29]. We have shown that
218 trypanosomes can generate pH gradients *in vitro*, and that this will trigger their migration on
219 plates when the pH in the centre of the colony drops by about 0.2 pH units [9]. Might
220 something similar help drive early procyclic forms from the lumen to the ectoperitrophic space?
221 In a laboratory setting, tsetse flies receive bloodmeals at regular intervals of 2-3 days
222 [8,9,15,30]. Might these relatively frequent feeds, intended to optimise fly survival and
223 infection rates, have the unintended effect of buffering pH differences and impeding migration
224 during the early phase of infection? It is known that late procyclic forms are attracted to alkali,
225 which would be consistent with their subsequent movement towards the proventriculus, but
226 there is no information on whether post-mesocyclic/proventricular forms are responsive to pH
227 gradients. It is therefore premature to assume that migration to the salivary glands is SoMo/pH
228 taxis. Given the multiplicity of ACs associated with CARP3 [9,14,15], and the fact that more
229 than a dozen ACs are specifically upregulated in the proventriculus [13], we consider it probable

230 that the trypanosomes moving from the proventriculus to the salivary glands are responding to
231 different environmental signals at this stage in their life cycle. It is also worth noting that the
232 ability to colonise the salivary glands and to perform SoMo are not inextricably linked. As we
233 showed previously, MAP kinase kinase 1 and the surface protein PSSA2 are both required for
234 this step in vivo, but their knockouts are still SoMo-competent [2,31,32].
235 Cells at the tips of SoMo projections and midgut forms 3 days post infection both
236 upregulate transcripts for high-affinity transporters and glycolytic enzymes. It is often assumed,
237 and stated in reviews, that tsetse flies do not provide a source of glucose for trypanosomes;
238 surprisingly, however, there is no primary literature on this topic. Glucose is present in blood
239 meals and could also be generated through gluconeogenesis by the tsetse, by trypanosomes
240 themselves or by endosymbionts. Alternatively, glycerol or glucose-containing disaccharides
241 might be present under certain conditions. Glycerol metabolism by trypanosomes also results in
242 acidification of their external environment and substituting glycerol for glucose in plates
243 supports SoMo [9]. Here too, an analysis of the metabolomes of different tissues in uninfected
244 and infected flies could be informative.
245

246 **Concluding remarks**

247 The concept that cells form gradients that they themselves can respond to is steadily
248 gaining traction and there are examples of bacteria, unicellular eukaryotes and mammalian
249 cells that are capable of this [33]. This endows motile cells with a degree of autonomy, allowing
250 them both to set a path and follow it. Perception of gradients by *T. brucei* may also play a role
251 in the mammalian host (see **Outstanding Questions**). The findings that the parasites colonise
252 skin and adipose tissue, as well as the bloodstream and central nervous system [34,35], raises
253 the question of how they recognise and home into these organs. If sensing of metabolite
254 gradients by ACs also plays a role in tissue tropism, and ultimately pathology, this might present
255 new opportunities for targeted therapies, not only for the diseases caused by the African
256 trypanosomes, but also for different forms of Leishmaniasis and Chagas Disease.
257 In addition to the example that we have presented here, it has been shown previously
258 that *Entamoeba histolytica* can produce and respond chemotactically to ethanol, a product of

259 anaerobic glycolysis [36]. We suspect that there will be many more examples of parasites that
260 both create and respond to gradients, thereby improving their chances of survival and
261 transmission.
262
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269
270 **Declaration of Interests**
271 The authors declare no competing interests.
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367

368 **Figure legends**

369 **Figure 1. SoMo in response to autonomous and exogenous pH gradients. A** Community lifts of
370 SoMo plates, incubated with anti-EP procyclin (green) and anti-GPEET procyclin (red)
371 antibodies. Upper panel: Overview of different SoMo phenotypes upon knockdown or knockout
372 of genes involved in cAMP signaling. Lower panel: Reorientation of projections after exposure
373 to conditioned medium, alkali or acid. **B** Illustration of parasites that are polarised by cAMP
374 (WT, red flagellar tip) or show a defect in polarisation. Figure created with BioRender.com.
375

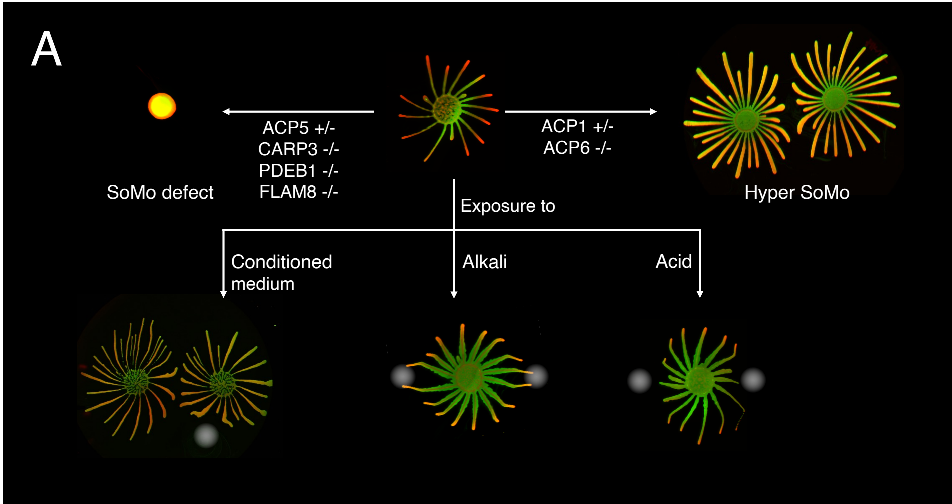
376 **Figure 2. The insect host and anatomical context. A** Side-view of a tsetse fly after a blood meal.
377 Image credit to Dr. Simon Imhof. **B** Schematic of a tsetse fly (adapted from [8]. Left panel,
378 isolated tsetse fly midgut (midgut lumen: visualised by staining the peritrophic matrix with
379 fluorescein-tagged wheat germ agglutinin. Ectoperitrophic space: visualised by staining the
380 nuclei of epithelial cells with Hoechst dye in blue). Right panel, an isolated tsetse fly
381 proventriculus stained with Hoechst dye to visualise nuclei. Scale bar: 100 microns.
382

383 **Figure 3. Alternative models for cAMP signaling. A** An unknown factor (x) associated with
384 CARP3 binds cAMP that is produced locally by the activated AC in the complex. cAMP binding
385 induces a conformational change releasing the factor alone, or together with CARP3, to exert its
386 downstream effects. **B** Factor x is activated by binding cAMP and is now able to associate with
387 CARP3. This either acts as a docking platform for additional factors (y) or, alternatively,
388 dislodges CARP3 from the AC. Releasing CARP3 from the complex would enable it to interact
389 with proteins outside of the flagellar tip. Depending on the stimulus, movement could be
390 towards or away from the source. Figure created with BioRender.com.
391

392 **Figure 4. Structural comparison between *T. brucei* CARP3 (green) and putative orthologues in**
393 **other trypanosomatids. A** and **B** are predictions that were calculated with Alphafold [26,27]. **A**
394 *T. cruzi* hypothetical protein, conserved, *Tc00.1047053506825.40* (orange); Z-score 30.5; RSMD
395 score 5.3. **B** *Leishmania* infantum stibogluconate resistance protein, *LinJ_31_3380* (orange); Z-

396 score 23.1; RSMD score 6.5. According to Holm [37], structures with Z-scores >20 are definitely
397 homologous.

Figure 1



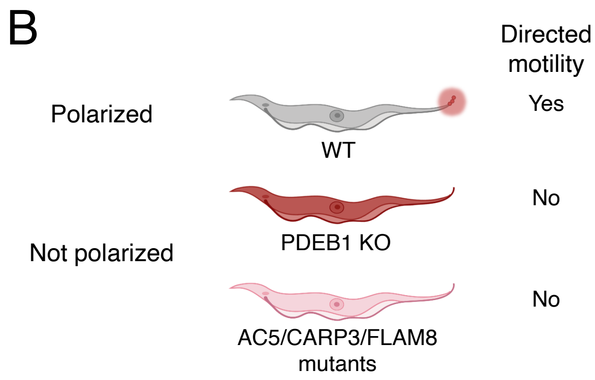
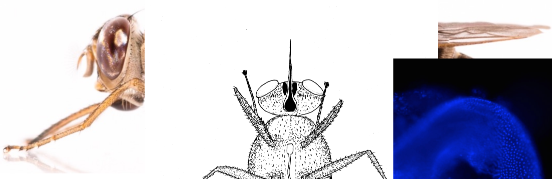


Figure 2

A



B

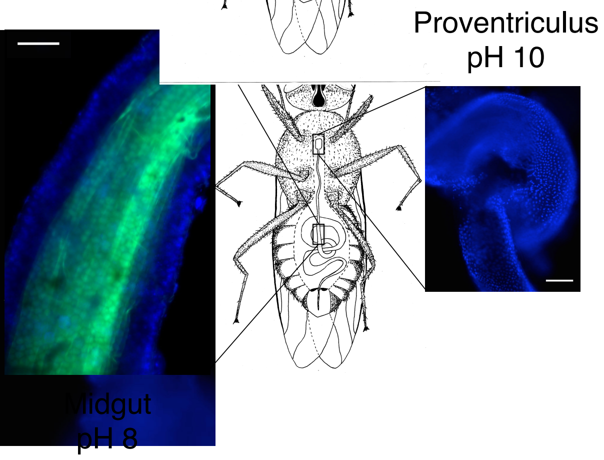


Figure 3

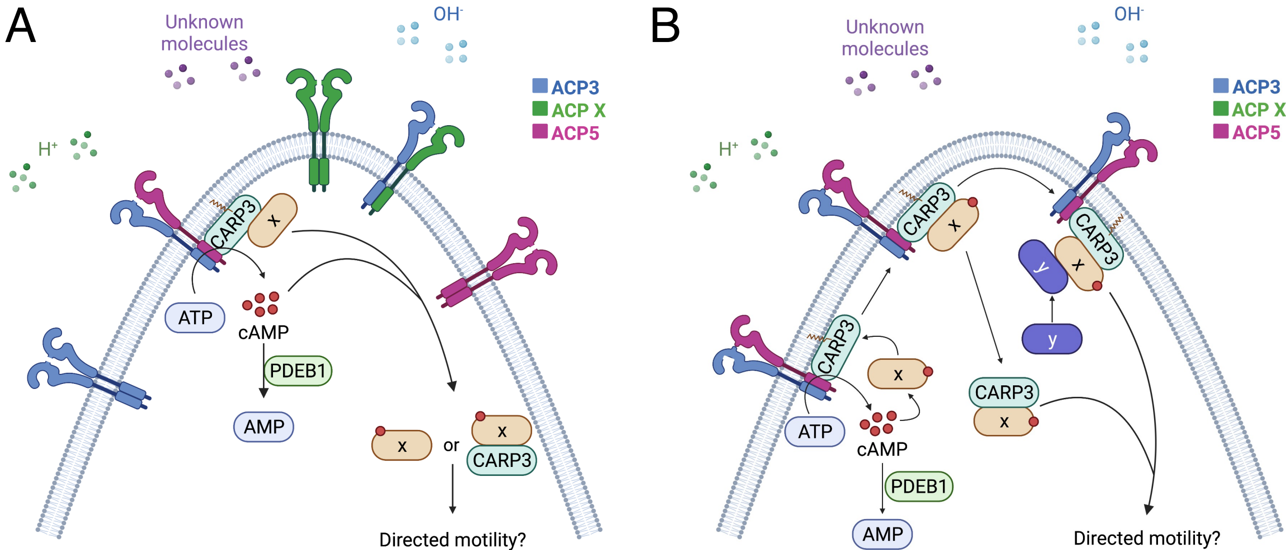
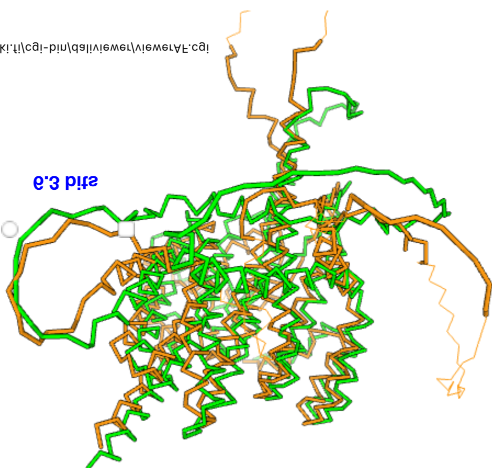


Figure 4

A



B