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The aerotaxis of *Dictyostelium discoideum* is independent of mitochondria, nitric oxide and oxidative stress

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21 **Keywords:** *Dictyostelium discoideum*, Aerotaxis, Hypoxia, Oxidative stress, Nitrosative stress,
22 Mitochondria, Microfluidics

23 **Abstract**

24 Spatial and temporal variations of oxygen environments affect the behaviors of various cells and are
25 involved in physiological and pathological events. Our previous studies with *Dictyostelium*
26 *discoideum* as a model of cell motility have demonstrated that aerotaxis toward an oxygen-rich
27 region occurs below 2% O₂. However, while the aerotaxis of *Dictyostelium* seems to be an effective
28 strategy to search for what is essential for survival, the mechanism underlying this phenomenon is
29 still largely unclear. One hypothesis is that an oxygen concentration gradient generates a secondary
30 oxidative stress gradient that would direct cell migration towards higher oxygen concentration. Such
31 mechanism was inferred but not fully demonstrated to explain the aerotaxis of human tumor cells.
32 Here, we investigated the role on aerotaxis of flavohemoglobins, proteins that can both act as

33 potential oxygen sensors and modulators of nitric oxide and oxidative stress. The migratory
34 behaviors of *Dictyostelium* cells were observed under both self-generated and imposed oxygen
35 gradients. Furthermore, their changes by chemicals generating or preventing oxidative stress were
36 tested. The trajectories of the cells were then analyzed through time-lapse phase-contrast microscopic
37 images. The results indicate that both oxidative and nitrosative stresses are not involved in the
38 aerotaxis of *Dictyostelium* but cause cytotoxic effects that are enhanced upon hypoxia.

39 1 Introduction

40 Oxygen is vital for many living organisms (Hsia et al, 2013). The oxygen concentration inside
41 multicellular organisms is lower than that in the atmosphere due to oxygen consumption by cells, and
42 changes spatially and temporally (Vaupel et al, 1991). The variations of oxygen environment affect
43 various cell behaviors physiologically and pathologically (Eltzschig & Carmeliet, 2011; Semenza,
44 2000). Aerotaxis, the motion of cells toward optimal oxygen levels, is well known in bacteria (Taylor
45 et al, 1999), but the characteristics and mechanisms of aerotaxis in eukaryotic cells are poorly
46 elucidated. Although intracellular protein hypoxia-inducible factors (HIF) generally express their
47 functions by transcriptionally activating genes in the nuclei (Semenza, 2007), they do not seem to
48 induce a rapid directional motility change. Recently, an *in vitro* study reported a clear oxygen-
49 directed migration of both epithelial and breast tumor cells under a self-generated hypoxic
50 environment caused by cellular oxygen consumption in a confined environment (Deygas et al, 2018;
51 Mikaelian et al, 2022). Such oxygen-directed migration was also observed even after the knockout of
52 HIF in the cells, suggesting a HIF-independent aerotaxis. An oxidative gradient seems to overlap the
53 oxygen gradient and is required to direct aerotactic motility through activation of the epithelial
54 growth factor receptor (EGFR). The cysteine 797 located near the ATP binding site of EGFR was
55 shown to be a target of redox regulation (Truong et al, 2016) and might be the target of the hypoxia
56 induced oxidation that potentiate its tyrosine kinase activity. Most of the dioxygen is metabolized in
57 mitochondria to produce ATP as part of respiration processes (Spinelli & Haigis, 2018).
58 Mitochondria generate a burst of oxidant molecules, both reactive oxygen species (ROS) and reactive
59 nitrogen species (RNS) during hypoxia (Castello et al, 2006; Doorly & Graciet, 2021; Poyton et al,
60 2009), and inactivation of mitochondrial respiration prevents aerotaxis (Mikaelian et al, 2022). It was
61 thus hypothesized that in hypoxic condition an oxygen gradient will cause cells to generate a
62 secondary redox gradient that would modulate EGFR activity to direct cell migration toward higher
63 oxygen concentrations (Mikaelian et al, 2022).

64 *Dictyostelium discoideum* vegetative amoeba also shows collective migration toward an
65 oxygen-rich region. In a confined spot assay, *Dictyostelium* cells exhibited long-lasting and highly
66 stable migration towards oxygen-rich regions (i.e., aerotaxis) guided by a self-generated oxygen
67 gradient, forming a stable ring of cells to escape from the hypoxic region (Biondo et al, 2021;
68 Cochet-Escartin et al, 2021). Moreover, the aerotaxis of *Dictyostelium* was successfully confirmed
69 under controlled oxygen gradient generated by microfluidic devices with gas channels for supply of
70 gas mixture of nitrogen and oxygen (Hirose et al, 2022). Under hypoxic conditions, below 2% O₂,
71 which corresponds to 25 μM O₂ in the culture medium, the migration of *Dictyostelium* cells was
72 enhanced (aerokinesis), and the oxygen gradient guided the cells toward the oxygen-rich region
73 (aerotaxis). A catalase-deficient strain of *Dictyostelium* demonstrated an aerotactic ring formation
74 quicker than the wild type, suggesting that hydrogen peroxide (H₂O₂) could play a role in the
75 aerotaxis of *Dictyostelium* (Biondo et al, 2021). How cells sense an oxygen gradient and integrate it
76 to direct motility is not clear as this phenomenon is little studied contrary to response to hypoxia. It is
77 possible that aerotaxis uses some of the hypoxia sensing mechanisms. In eukaryotes, oxygen is
78 mainly used to generate energy through oxidative phosphorylation occurring in mitochondria.

79 Hypoxia can thus be detected indirectly through the lack of energy and increase in free radicals (ROS
80 and RNS) resulting from impaired mitochondrial functions (Castello et al, 2006; Hernansanz-Agustín
81 et al, 2017). A more direct way to detect hypoxia involves oxygen-dependent enzymes that will
82 regulate downstream responses. The only well-documented oxygen sensing system in *Dictyostelium*
83 is PhyA, a member of the oxygen-dependent prolyl-hydroxylase domain (PHD) proteins also found
84 in mammals and plants (West et al, 2007; Zhang et al, 2012). PhyA is a prolyl hydroxylase that acts
85 as an oxygen sensor to control the terminal phase of *Dictyostelium* multicellular development that
86 results into the differentiation of spore and stalk cells. Since PhyA present a low affinity of O₂, its
87 activity is restricted under 21% O₂. In presence of normal level of oxygen, PhyA hydroxylates a
88 proline on Skp1, an adaptor subunit of the Skp1–Cul1–F-box (SCF) class of E3 ubiquitin triggering
89 its glycosylation. The modified Skp1 will in turn associate with its targets to trigger their
90 polyubiquitination and degradation by the proteasome (West & Blader, 2015). PhyA activity is
91 reduced under 10% O₂, resulting into the accumulation of unmodified Skp1, preventing terminal
92 differentiation from proceeding. PhyA is expressed during *Dictyostelium* multicellular development
93 and control the culmination phase, such that it happens in a place with normal oxygen level such as
94 the soil surface rather than underground (West et al, 2007).

95 Other candidates include flavohemoglobins, oxygen-dependent enzymes that neutralize nitric
96 oxide (NO), a source of RNS that also acts as a signaling molecule regulating the hypoxic response in
97 many organisms (Castello et al, 2006). Flavohemoglobins are found in many bacteria, fungi and
98 protists where it oxidizes NO into nitrate (Cánovas et al, 2016) to maintain NO homeostasis. While
99 NO is produced at low level as part of nitrogen metabolism, high level can occur during hypoxia due
100 to dysfunction of mitochondrial cytochrome c oxidase (Castello et al, 2006; Gupta et al, 2005;
101 Planchet et al, 2005; Tischner et al, 2004) but can also results from fermentation of nitrite in acidic
102 pH or be released by plants or animals as part of antibacterial defense. The oxidation of NO by
103 flavohemoglobin is very efficient in saturation oxygen concentration but the reaction can also occur
104 in hypoxic condition when oxygen is in micromolar range (Poole et al, 1996). In anaerobic condition,
105 flavohemoglobins appear able to detoxify NO at a slow rate through an alternate reaction (Kim et al,
106 1999). As such, flavohemoglobins were shown to protect organisms from NO toxic effects both in
107 high and low oxygen conditions (Forrester & Foster, 2012). *Dictyostelium* genome encodes two
108 flavohemoglobins genes, *fhbA* and *fhbB*, that were successfully inactivated (Iijima et al, 2000).
109 *Dictyostelium* flavohemoglobin proteins share well conserved amino-acid sequences and structure
110 with the characterized bacterial and fungal homologs shown to act as NO dioxygenases (Dröge et al,
111 2014). While the enzymatic activity of FhbA and FhbB has not been tested, the double null mutant
112 *fhbA⁻fhbB⁻* displays poor viability when challenged with NO generating chemicals, consistent with their
113 expected NO dioxygenases functions (Iijima et al, 2000). The intracellular localization of
114 *Dictyostelium* flavohemoglobins has been not investigated but in organisms that also possess two
115 flavohemoglobin genes, such as filamentous fungi, one isomer is at least partly located in the
116 mitochondrial matrix despite lacking a classical targeting sequence while the other isomer is mostly
117 cytoplasmic (Forrester & Foster, 2012). In yeast, the YHB1 flavohemoglobin is distributed between
118 the cytosol and mitochondrial matrix in normoxia but relocates fully to mitochondria in anaerobic
119 condition (Cassanova et al, 2005). *Dictyostelium* cells generates NO at the onset of multicellular
120 development to regulate the initiation of cAMP pulses (Tao et al, 1997). This NO generation
121 coincides with the strong downregulation of *fhbA* and *fhbB* expression observed by RNA-seq
122 (Rosengarten et al, 2015). *fhbA* and *fhbB* are re-expressed at low level when aggregates are formed.
123 cAMP is secreted by *Dictyostelium* to act as a chemoattractant that directs cell motility during the
124 aggregation phase (Pal et al, 2019). It is thus possible that flavohemoglobins act as oxygen sensor
125 that would regulate NO level produced during hypoxia. NO could then act as a secondary signal to
126 direct cellular motility upward oxygen gradients.

127 In this context, available mutants in genes encoding for potential oxygen sensors or
128 signaling intermediates were tested for their ability to form aerotactic rings of cells when a micro-
129 colony (spot of cells) was covered with a glass coverslip. Out of the mutants tested, including
130 members of the PhyA-Skp1 pathway, only one displayed an abnormal phenotype during the spot
131 assay. A null mutant of the flavohemoglobin gene *fhbB* presented a poorly defined ring that expanded
132 at a slower rate than wild-type controls. Therefore, we investigated the role of flavohemoglobins and
133 the effects of oxidative stress on aerotaxis. The migratory behaviors of wild type and mutant
134 *Dictyostelium* cells were observed both with the spot assay and with microfluidic devices generating
135 an oxygen gradient. Chemicals were used to inhibit mitochondria functions, source of free radical or
136 anti-oxidant. The trajectories of the cells were then analyzed with time-lapse phase-contrast
137 microscopic images to draw the aerokinetic and aerotactic features of *Dictyostelium* cells under these
138 pharmacogenetic treatments. Thanks to complementary cell respiration and proliferation experiments,
139 micro-colony experiments were modeled with a Go-or-Grow mean field model previously introduced
140 (Cochet-Escartin et al, 2021) in order to confirm the existence of a true aerotactic response in all
141 analyzed experiments. This complementary experimental and theoretical approach indicates that
142 neither flavohemoglobins nor oxidative stress are involved in the aerotaxis of *Dictyostelium* contrary
143 to expectations. Instead, our data indicates that hypoxia increases the toxic effects of oxidative stress
144 on cell motility, leading indirectly to an impaired aerotactic motility. Surprisingly, we observed that
145 for *Dictyostelium*, mitochondrial respiration is dispensable both for generation of an oxygen gradient
146 and for aerotactic response.

147 **2 Methods**

148 **2.1 Cells and strains**

149 The wild types AX2 and AX3, and the flavohemoglobin null mutants *fhbA*⁻, *fhbB*⁻ and *fhbA*⁻*B*⁻
150 strains of *Dictyostelium* cells were provided by the National BioResource Project (NBRP Nenkin,
151 Japan) and cultured axenically with HL5 medium (HLF2; Formedium, UK) containing 100 mM
152 glucose and 1/1000 penicillin-streptomycin (P/S) (15140-122; Gibco, USA) on cell culture dishes at
153 22°C or under agitation. PhyA- strain is a kind gift from C. West (University of Georgia, USA).

154 **2.2 Chemicals and reagents**

155 Sodium nitroprusside dihydrate (SNP) (71778; Sigma-Aldrich, USA), which releases NO, was
156 added at 10 – 100 µM to the culture medium to investigate the effect of RNS on the aerotaxis. H₂O₂
157 (18084-00; Kanto Chemical, Japan) was added at 100 – 500 µM to investigate the effect of ROS on
158 the aerotaxis. Ethidium bromide (EtBr) (E1510, Sigma-Aldrich), which inhibits mitochondria DNA
159 replication, was added to the culture medium 24 h before experiments at 30 µg/ml for pretreatment of
160 the cells. Oligomycin (O4876, Sigma-Aldrich) was used at 10 µg/ml, and antimycin A (A8674,
161 Sigma-Aldrich) was used at 37.5 µM. Tiron (4,5 dihydroxy-1,3-benzene-disulphonic acid; D7389,
162 Sigma-Aldrich) was used at 10 mM.

163 **2.3 Microfluidic Devices**

164 A double-layer microfluidic device with oxygen-controllability was used (Fig. S1) (Hirose et
165 al, 2022). The device was 30×30 mm square, and the thickness was 4 mm. Inside the device, two gas
166 channels in a 1-mm interval were located at 0.5 mm from the bottom surface, perpendicularly
167 crossing above three parallel media channels on the bottom (Fig. S1A). The width and height were
168 2 mm and 0.15 mm for all channels, respectively. The device mainly consisted of
169 polydimethylsiloxane (PDMS, Sylgard 184 Silicone Elastomer Kit, The Dow Chemical Company,

USA) and contained a polycarbonate film to prevent gas infusion from the atmosphere. The x and y -axes were defined to be parallel and perpendicular to the media channels, respectively, and the z -axis was in the vertical direction. The origin was set at the center of the device. The oxygen concentration in the device was controlled by gas exchange between the channels, supplying gas mixtures at predefined oxygen concentrations to the gas channels. The oxygen concentration in the device was computed by three-dimensional numerical simulation using commercial multiphysics software (COMSOL Multiphysics ver. 5.5, COMSOL AB, Sweden) and verified using an oxygen-sensing film which emitted phosphorescence quenched by oxygen (Hirose et al, 2021). The computational and experimental results showed almost the same tendency especially for the minimum value of oxygen concentration, showing an oxygen concentration gradient from 0.4% to 21% O₂ was generated along each media channel in the x -direction when the gas mixtures containing 0% and 21% O₂ were supplied to the left and right gas channels, respectively (gray solid line in Figs. S1B-F). At the most hypoxic location at $x = -1.6$ mm, the slope of the oxygen concentration gradient inverted.

Dictyostelium cells were harvested from the culture dishes and introduced into the media channels in the microfluidic device at 2×10^6 cells/ml (400 cells/mm²) with the HL5 culture medium. After incubation for 20 min to adhere *Dictyostelium* cells to the bottom surface of the media channels, the HL5 culture medium was replaced by fresh one. Here, depending on the experimental conditions, reagents were added to the HL5 culture medium. For accurate comparisons, each device was set to have channels for control condition and each of another two conditions at the same time. The device was placed in a stage incubator (TP-CHSQ-C, Tokai Hit, Japan) mounted on an inverted microscope (IX83, Olympus, Japan), and controlled at 22°C. Just after gas mixtures of oxygen and nitrogen were supplied at 30 ml/min to the left and right gas channels, time-lapse imaging was started.

A second type of microfluidic device, with a Y-shape channel was used to generate a chemical gradient of soluble molecules (Fig. 3A). The device was placed in the same stage incubator mounted on the microscope, and controlled at 22°C. The x and y -axes were defined to be perpendicular and parallel to the merged channel, respectively. The origin was set at the junction of the channels from the two inlets. *Dictyostelium* cells were first allowed to adhere on the microchannel in HL5 culture medium for 20 min. HL5 culture media without and with a pharmacological substance at a concentration C_0 (SNP at 100 μM or H₂O₂ at 500 μM) were injected from the left and right inlets, respectively, at 1 ml/h, and time-lapse imaging was then started. The average velocity was $U_0 = 0.37$ mm/s in the merged channel inducing a wall-shear stress around 4.4 mPa low enough not to affect cell migration and adhesion on the bottom glass (Rupprecht et al, 2012). The profile of chemical concentration $C(x, y)$ at a given position (x, y) was calculated by the following equation:

$$C(x, y) = \frac{C_0}{2} \left[1 + \operatorname{erf} \left(\frac{x}{2} \sqrt{\frac{U_0}{Dy}} \right) \right], \quad (1)$$

where D is the diffusion coefficient of the chemical substance in water. For the H₂O₂ gradient we used $D = 1.4 \times 10^{-3}$ mm²/s (van Stroey-Biezen et al, 1993). Addition of 100 μM SNP leads to rapid increase of NO in the media to reach a prolonged plateau after 25 min (Bradley & Steinert, 2015). As the experimental set-up require 30 min, we expect a constant level of NO for the duration of the assay, hence for the SNP gradient experiment. We used the diffusion constant of NO, $D = 2.2 \times 10^{-3}$ mm²/s (Zacharia & Deen, 2005), to compute the relative NO concentration profile. Cells were imaged and tracked between 1 h and 2 h after starting the media flow.

212 2.4 Imaging and analyzing cell migration in microfluidic devices

213 Phase-contrast microscopic images of the central part of each media channel including the
 214 region of interest (ROI) displayed in Figs. S1A and were taken by the microscope with a 4× objective
 215 lens (UPLFLN4XPH, Olympus) every 30 s for 2 h. The positions of *Dictyostelium* cells were
 216 detected at each time step by analyzing the images using ImageJ (National Institutes of Health, USA)
 217 with its built-in plugin, Find Maxima. Then, the individual cells were tracked using MATLAB
 218 (MathWorks, USA) by minimizing the total of squared displacements with a published tracking code
 219 (Blair & Dufresne). All trajectory data were analyzed using in-house MATLAB programs. The
 220 migration distance in 1 min between the time steps t_i and t_{i+1} was defined as dl_i , which was
 221 decomposed into the x and y -directional displacements $dl_{x,i}$ and $dl_{y,i}$, respectively ($dl_i^2 = dl_{x,i}^2 + dl_{y,i}^2$),
 222 where the x -direction is defined as the gradient direction for both devices. The average speed $v = \overline{dl_i}$
 223 and the average x -directional velocity $v_x = \overline{dl_{x,i}}$ were averaged from all 1-min cell displacements in
 224 $\Delta x = 200 \mu\text{m}$ wide slabs as a function of x -position. The aerotactic index was defined as
 225 $AI = \overline{dl_{x,i}/dl_i}$ referring to a chemotactic index normally used as a metric for chemotaxis (Amselem
 226 et al, 2012). The maximal AI_{max} was measured in each individual experiment as the maximum value
 227 of AI on the positive side ($x > -1.6 \text{ mm}$) since the oxygen gradient was better controlled than on the
 228 negative side. A chemotaxis index $CI = \overline{dl_{x,i}/dl_i}$ was computed to analyze the data by experiments
 229 with the Y-shaped microfluidic devices.

230 2.5 Spot assay and ring speed kymographs

231 The confined spot assay offers a quick and robust way to test whether cells can generate
 232 oxygen gradients and move aerotactically (Biondo et al, 2021; Cochet-Escartin et al, 2021; Deygas et
 233 al, 2018). A drop of either 2, 3 or 5 μL of a cell suspension containing 2000, 3000 or 5000 cells
 234 respectively was carefully deposited on a dish. The drop was incubated for 30 min in humid
 235 atmosphere at 22°C before adding 2 ml of HL5 culture medium. An 18 mm or 22 mm diameter round
 236 glass coverslip cleaned with ethanol then thoroughly rinsed in HL5 was gently deposited on top of it.
 237 Image acquisition was performed using a lens-free imaging device (Cytonote 6W, Iprasense, France)
 238 or the aforementioned inverted microscope. Images were taken every 5 to 15 min for 24 h. The
 239 software Horus (Iprasense, France) was used to process and reconstruct the Cytonote images, and a
 240 homemade ImageJ macro based on the polar transformer plug-in was used to construct kymographs
 241 from the original images by polar transformation and z -stacking of frames. The ring speed was
 242 determined by calculating the slope of the front density band associated with ring propagation on
 243 these kymographs between 16 h and 24 h. To improve signal to noise ratio in kymographs, images
 244 were filtered from their background by using the Find-Edges function of ImageJ before applying the
 245 polar transformation.

246 2.6 Oxygen consumption and proliferation experiments

247 For consumption measurements of *fhb* null mutant and for those of the respiratory inhibitors
 248 oligomycin and antimycin A (Fig. S6C), about 10^6 total cells suspended in HL5 culture medium were
 249 placed in a respirometer (Oxygraphs 2k high-resolution respirometers, Oroboros Instruments,
 250 Austria) to determine oxygen concentration changes over time. After the measurement of the basal
 251 respiration, oligomycin (10 $\mu\text{g/ml}$) or antimycin A (37.5 μM) were added successively. Oxygen
 252 consumption were calculated using the change of oxygen level over time and normalized per cell. For
 253 consumption measurements of EtBr treated cells or in presence of SNP or H_2O_2 (Fig. S6D), a 6-mL
 254 glass vial was filled with 2×10^7 cells suspended in HL5, and closed it with a cap through which an
 255 optical oxygen probe was plunged. The cap was carefully sealed around the probe to avoid external

256 oxygen entering the bottle. We measured the change of oxygen level over time with this probe
 257 coupled to its oxymeter (OXROB3 robust probe and Firesting oxymeter, Pyroscience, Aachen,
 258 Germany).

259 For proliferation experiments, cells were plated at about 10^4 cells/cm² in 6-well plates, and
 260 imaged and counted with the lens-free imaging device or the inverted microscope every 30 min for
 261 24 h.

262 2.7 RT-qPCR

263 Four plates containing 2.3×10^6 AX3 cells in 1.5 ml HL5 culture medium were prepared with
 264 either no chemicals, 100 μ M SNP or 100 μ M H₂O₂. The two plates containing SNP or H₂O₂ and a
 265 plate without chemical were placed at 22°C and 21% O₂. The last plate containing cells in HL5
 266 culture medium was incubated at 22°C and 0.4% O₂. After 3 h, total RNA was extracted (RNeasy
 267 Plus Mini Kit, 74134, Qiagen, Germany), quantified and adjusted at 1 μ g to synthesise cDNA (First
 268 Strand cDNA Synthesis Kit for RT-PCR, 11483188001, Roche, Switzerland). Then a qPCR was
 269 performed (DyNAmo Flash SYBR Green qPCR Kit, F415L, Thermo Fisher Scientific, USA) using
 270 the following primers: *rmlA*-F (5'-GCACCTCGATGTCGGCTTAA-3'), *rmlA*-R (5'-
 271 CACCCCAACCCTTGAAACT-3'), *cxgE*-F (5'-ATGTCCCACGCATTACCAG-3'), *cxgE*-R (5'-
 272 CATAGGCAGCGTAAAAATCTTCTC-3'), *cxgS*-F (5'-AAGTTGTTAAATCTCAACTC-3'), *cxgS*-
 273 R (5'-TTTATCAACGCCATATTTAA-3'), *fhbA*-F (5'-GAACTCACATCAGACATTAC-3'), *fhbA*-
 274 R (5'-CTACATAGTCACCAGCTGGA-3'), *fhbB*-F (5'-GATGGTAAAGAGATAGCTAC-3'), and
 275 *fhbB*-R (5'-CTGATAAACTGTAATGTCTGAC-3'). The experiments were performed in duplicate
 276 for each gene. The relative gene expression was determined using the $2^{-\Delta\Delta C_t}$ method (Livak &
 277 Schmittgen, 2001) using *rmlA* as a housekeeping gene.

278 2.8 Statistical test

279 For each measurement type, experiments were repeated three to six times. Significant
 280 differences between three or more conditions were evaluated by one-way ANOVA or two-way
 281 ANOVA followed by a post-Tukey test for multiple comparisons. In each test, statistical significance
 282 and the tendency toward significance were inferred at $*p < 0.05$ and $\dagger p < 0.1$, respectively.

283 2.9 Mean-field 'Go-or-Grow' model

284 The simulations were performed using a numerical mean-field 'Go-or-Grow' model coded in
 285 the Python language, which we recently introduced to explain ring propagation in confined spot
 286 experiments (Cochet-Escartin et al, 2021). The mean field model used for the aerotactic dispersion
 287 consisted of coupled reaction-diffusion equations for both oxygen and cell concentrations
 288 respectively:

$$289 \quad \frac{\partial C}{\partial t} = D_{O_2} \Delta C - b_0(C) \rho \quad (2)$$

$$290 \quad \frac{\partial \rho}{\partial t} = D_{\text{cells}}(C) \Delta \rho + r_0(C) \rho - \vec{\nabla} \cdot \vec{J}_{\text{aero}} \quad (3)$$

292 where C is the oxygen concentration, t is the time, D_{O_2} is the oxygen diffusion constant, b_0 is the cell
 293 oxygen consumption, ρ is the cell density, D_{cells} is the cellular diffusion constant, r_0 is the cell
 294 proliferation rate, and \vec{J}_{aero} is the advection term. From our recent observations that the aerotactic

295 displacements are more correlated to the relative oxygen gradient $\left(\frac{\vec{v}C}{C}\right)$ than to the absolute gradient
 296 $\vec{v}C$ (Hirose et al, 2022)), we propose a slight modification to the advection term in the shape of
 297 $\vec{J}_{\text{aero}} = \rho \vec{v}_{\text{aero}}$, with:

$$298 \quad \vec{v}_{\text{aero}} = \chi l \left(\frac{\vec{v}C}{C}\right) \frac{1}{1+e^{-(C-C_0)/s}} \quad (4)$$

299 χ is a proportionality constant called aerotactic bias (unique for each experimental condition, see
 300 below), $l = 10 \mu\text{m}$ is the approximate cell size, and the latter two terms comprise the response
 301 function to oxygen relative gradient. We choose an initial cell distribution and a boundary condition
 302 for oxygen $C(L = 9 \text{ mm}) = 21\% \text{ O}_2$ mimicking the experimental situation. Mesh sizes of $\Delta x = 10 \mu\text{m}$
 303 and $\Delta t = 0.05 \text{ min}$ were used. To avoid negative O_2 values a threshold $C_1 = 0.5\% \text{ O}_2$ was chosen
 304 below which cells consume oxygen relative to the local ambient oxygen concentrations. We also used
 305 a proliferation threshold below which cells stop dividing $C_2 = 0.2\% \text{ O}_2$ as recently measured (Carrère
 306 et al, 2023). This is also a slight modification to our initial Go-or-Grow model for which $C_0 = C_2$
 307 (Cochet-Escartin et al, 2021).

308 The aerotactic bias, the oxygen threshold, and the steepness factor in Eq. (4) were respectively
 309 updated to $\chi_0 = 30 \mu\text{m}/\text{min}$, $C_0 = 0.4\% \text{ O}_2$ and $s = 0.1\% \text{ O}_2$ upon further analysis of density
 310 accumulation of cells and maximum aerotactic displacement along the gradient perceived in the
 311 microfluidic device experiments with the AX2 in HL5 control condition (Ghazi et al., in preparation).
 312 For other conditions, C_0 and s were kept constant as we never observed any shift in the range of
 313 oxygen concentrations triggering an aerotactic response, but χ was set proportional to the measured
 314 aerotactic x -directional velocity v_x (Fig. S1C and Supplementary Table S1), as $\chi = \chi_0 \frac{v_x}{v_{x0}}$ where v_{x0} is
 315 the aerotactic speed of the control condition reported in Fig. 2E or 2H. The cellular diffusion constant
 316 D_{cells} plays a minor role on the ring propagation speed or ring formation time (Ghazi et al., in
 317 preparation). It was not systematically measured but we used a scaling relation motivated by former
 318 studies (Golé et al, 2011), $\sqrt{\frac{D_{\text{cells}}}{D_0}} = \frac{v}{v_0}$ where $D_0 = 30 \mu\text{m}^2/\text{min}$ (Cochet-Escartin et al, 2021) and
 319 $v_0 = 5 \mu\text{m}/\text{min}$ (Fig. S3D) are the diffusion constant and average speed of the AX2 control condition
 320 in the ring region. All parameter values, cell oxygen consumption b_0 , aerotactic x -directional velocity
 321 v_x , absolute displacement speed v , cell proliferation rate r_0 , were taken directly from experimental
 322 observations discussed in this article and summarized in Supplementary Table S1. Oxygen diffusion
 323 constant in HL5 medium at 22°C was taken from the one in pure water at 20°C : $D_{\text{O}_2} = 2,000 \mu\text{m}^2/\text{s}$
 324 (Cochet-Escartin et al, 2021). On one hand, salt and sugar present in HL5 decrease D_{O_2} by about
 325 $100 \mu\text{m}^2/\text{s}$, but on the other hand, temperature increases D_{O_2} by about $50 \mu\text{m}^2/\text{s}/^\circ\text{C}$ (Goldstick et al,
 326 1976; Jamnongwong et al, 2010).

327

328 **3 Results**

329 **3.1 Abnormal motility of flavohemoglobin B null mutant upon hypoxia**

330 Micro-colonies of various strains were confined with a glass coverslip and observed by
 331 videomicroscopy. The behavior of the PhyA null mutant or of the one of the wild type AX3 cells
 332 were similar to the one previously described with AX2 cells (Figs. 1A-D and S2). After less than an
 333 hour, the oxygen depletion at the center of the colony induced an increase of aerokinetic motility and

334 the formation of an expanding ring of aerotactic cells. This resulted in a sharp density profile, the ring
335 being two to three times of higher density than the interior of the colony. All of *fhb* null mutants
336 presented a much slower and rougher ring of cells (Figs. 1E-P) than the AX3 strain, the slowing
337 down of the ring being especially more marked and significant for the *fhbB*⁻ and *fhbA*⁻*B*⁻ (Fig. S3C).
338 Furthermore, small aggregates of cells were frequently observed inside the mutant colonies while
339 they were very rare for the wild type.

340 In order to test solely the aerotaxis of *fhb* null mutants in imposed oxygen gradients, and not
341 the intermingled response to self-generated gradients with oxygen consumption and cell proliferation,
342 we used a double-layer microfluidic device generating a 0.4% to 21% O₂ gradient (Fig. S1A). In such
343 a device, at the most hypoxic location where the slope of the oxygen concentration gradient was
344 inverted, the directionality of the cell migration was changed from the $-x$ direction for $x < -1.6$ mm to
345 $+x$ direction for $x > -1.6$ mm, implying migration toward higher oxygen levels (Fig. S1B with the
346 already reported AX2 strain (Hirose et al, 2022)). The existence of such an inversion even when the
347 signal-to-noise in cell displacement is low is a signature of an aerotactic response. Cells migrated
348 also in the y -direction but the average of the y -directional displacements were almost zero regardless
349 of the position (Fig. S1B). The AX2 strain migrated faster in the hypoxic region than in the normoxic
350 region (positive aerokinesis, Fig. S1C).

351 The parental AX3 strain presented a higher non-directional motility than the mutants in all
352 oxygen concentrations (Fig. 2A) and displayed a strong aerotactic bias when the oxygen level was
353 under 1.5% O₂ (Fig. 2B), resulting in a maximal aerotactic index AI_{\max} of 0.13 (Fig. 2C). The average
354 speed of all *fhb* null mutants was significantly reduced (Fig. 2A), but all exhibited an aerotactic
355 displacement. Even if for the *fhbB*⁻ and *fhbA*⁻*B*⁻ strains, it was just measurable (see inset of Fig. 2B).
356 As a result, the AI_{\max} was slightly reduced in *fhbA*⁻ strain comparatively to wild type, but reduced by
357 more than half for the *fhbB*⁻ and *fhbA*⁻*B*⁻ strains, indicating a strong reduction in the ability of the
358 cells to properly sense or respond to oxygen gradients in these mutants. We performed a comparative
359 random motility assay in plastic dishes at 21% and 0.4% O₂ (Fig. S4). The AX3 strain exhibited a
360 higher motility in the hypoxic condition confirming the results of Fig. 2A and the flavohemoglobin
361 mutants motility was normal in normoxia but slightly reduced by hypoxia (Fig. S4).

362 In many organisms, flavohemoglobins expression is induced by NO, while induction upon
363 hypoxia is uncommon (reviewed in Forrester and Foster, 2012). Therefore, the level of expression of
364 *fhbA* and *fhbB* genes in wild type cells were tested by RT-qPCR in response to hypoxia and SNP, a
365 chemical source of NO or H₂O₂ as a generic source of ROS (Table 1). Both flavohemoglobins
366 expression were strongly induced after 3 h in 0.4% O₂. Strong induction was also observed for *cxgS*,
367 an optional subunit of cytochrome c oxidase induced by hypoxia, used here as positive control
368 (Bisson et al, 1997). As expected, *cxgE*, the alternate optional subunit of cytochrome c oxidase
369 expressed only during growth in normoxia was sharply down-regulated at 0.4% O₂ (Bisson et al,
370 1997). On the other hand, treatment with SNP, a classical source of NO, did not induce *fhbB*
371 expression and rather slightly repressed *fhbA*. As mitochondria also release ROS, H₂O₂ effect was
372 tested but didn't affect the flavohemoglobins expression. Thus, low oxygen concentration appears to
373 be key to induce the expression of flavohemoglobin genes, reinforcing the possibility that the
374 proteins are important in this condition required for aerotaxis to appear.

375

376 3.2 Oxidative stress does not affect aerotaxis

377 Micro-colonies of wild type AX2 cells were confined in HL5 supplemented with either
378 H₂O₂ as a source of ROS or with SNP as a source of RNS by generating NO in the media. Neither
379 product prevented the formation of the expanding ring of cells even though the ring speed was
380 reduced with SNP or H₂O₂ (Figs. S3A and S5). It was not possible to use much higher concentrations
381 of these chemicals as they prevented cell adhesions or killed the cells at 1 mM. As an alternate way
382 to change the potential effect of ROS, we used Tiron, a free radical scavenger that had previously
383 been used to prevent oxidative stress in *Dictyostelium* (Bloomfield & Pears, 2003). Addition of
384 10 mM Tiron to confined wild type cell had no visible effect on ring formation appearance or
385 migration speed (Fig. S5).

386 To detect eventual fine effect of free radical, SNP and H₂O₂ were applied to wild type cells
387 placed in the microfluidic device previously described. However, lower concentration had to be used
388 as they impair cell adhesion to the glass bottom of the device. The 100 μM H₂O₂ did not affect
389 migration speed or response to oxygen gradient of the cells (Figs. 2D-F). On the other hand, the
390 100 μM SNP reduced only slightly the cell speed at 21% O₂ (3.5 μm/min versus 4.5 μm/min) but
391 dramatically at 0.4% O₂ (1.5 μm/min versus 5 μm/min) (Fig. S3D). Despite this strong speed
392 reduction, the SNP-treated cells still responded to oxygen gradients and the maximum aerotactic
393 index remained similar to that of untreated cells (Fig. 2F).

394 To further exclude a role of ROS and RNS in controlling aerotaxis, wild type cells were
395 placed in a Y-shaped microfluidic device generating a chemical gradient of soluble molecules: either
396 SNP or H₂O₂. Higher levels of NO (Fig. 3B) or of H₂O₂ (Fig. 3D) induce a lower motility of
397 *Dictyostelium* cells (negative chemokinesis) but cells did not show specific chemotaxis towards or
398 away from either product, up to 100 μM for SNP and 500 μM for H₂O₂ as exemplified by the
399 chemotactic index (Figs. 3C and E). These experiments unambiguously indicate that cells do not
400 respond to direct ROS or RNS gradients. As ROS/RNS are not implicated in the observed aerotactic
401 responses, we investigate whether mitochondria are involved in any other way.

402 3.3 Mitochondrial role during aerotaxis

403 As mitochondria are major oxygen sinks and are potentially involved in direct or indirect
404 oxygen sensing for aerotaxis, three different mitochondrial inhibitors were used on confined wild
405 type cells. Treatment with 10 μg/ml of the mitochondria replication inhibitor EtBr for 5 days was
406 shown to inhibit mitochondrial respiration in *Dictyostelium* cells by 75% and to prevent cell division
407 (Kobilinsky & Beattie, 1977). For our experiments, a pretreatment of 24 h with 30 μg/ml EtBr was
408 used as it results into a strong reduction of cell growth (Chida et al, 2004). This resulted into a
409 reduction of total respiration by 35% comparatively to their untreated control (Figs. S6D). The
410 confined colony of EtBr-treated cells formed an expanding ring (Figs. 4E-H) but with a decreasing
411 migration speed with respect to the non-treated AX2 cells tested (Fig. S3B) and a slight delay of ring
412 formation time (compared Figs. 4D and 4H). Although it was not measured, cell growth was
413 expected to be strongly reduced since the media contained EtBr during the confinement assays. The
414 EtBr-treated cells were also tested for their aerotactic response in an oxygen gradient (Figs. 2D-F).
415 There was no reduction of the aerotactic response and the aerotactic index was similar to untreated
416 cells. Interestingly, on the normoxic side, the addition of EtBr markedly reduced the migration speed
417 of the cells but with no significant reduction on the hypoxic side (Fig. 2D).

418 Oligomycin and antimycin are fast acting inhibitors of mitochondrial respiration that reduce
419 considerably oxygen consumption: by nearly 60% with oligomycin, and 85% with antimycin as
420 measured in this study (Fig. S6C). As expected, these drugs also reduce considerably cell division

421 (Fig. S6B). Despite this strong inhibition, an expanding ring of cells was formed in the confined spot
422 assay, but with a 5 h (oligomycin) to 6 h delay (antimycin) as shown with the kymographs of
423 Figs. 4D, 4L and 4P. In addition, the ring expansion speed was slower than for the untreated AX2
424 cell colony (control) especially for the antimycin treatment (Figs. 4M-P and S3B). In the microfluidic
425 assay, oligomycin treated cells (red curves in Figs. 2G-H) responded as did the EtBr-treated cells
426 (green curves in Figs. 2D-E) with a similar aerotactic displacement along the direction of the gradient
427 but a slightly reduced migration speed on the normoxic side. On the other hand, both migration speed
428 and aerotactic response were reduced by the antimycin treatment (blue curves in Figs. 2G-H). Such
429 general reduction of motility is not unexpected as antimycin prevents the cytochrome c oxidase to
430 generate ATP which is required for motility (Wilson et al, 1992).

431 3.4 Modeling of the ring formation and speed of expansion in the different condition tested

432 To interpret experiments, we used the numerical mean-field version of the Go-or-Grow model
433 (Cochet-Escartin et al, 2021). Briefly, the rapid oxygen consumption by cells in the confined area and
434 the very selective aerotactic cell response are the two key ingredients for ring formation and
435 propagation. Ring formation may occur even in the absence of proliferation (Biondo et al, 2021;
436 Cochet-Escartin et al, 2021). However, proliferation is necessary to sustain a stable long-lived ring
437 (Rieu et al, 2022). The ring speed depends mostly on the aerotactic speed and slightly on the
438 proliferation rate. Here, we simulated the experiments with various O_2 diffusion constants values
439 taken from literature and found that the changes in the ring speed between the various extreme
440 literature values were negligible (Fig. S7 and its caption). The ring speed also did not depend on
441 initial cell density, only the formation time depended on it: the denser, the quicker the ring formation
442 and the more it appeared in the peripheral position of the initial spot (Fig. S8). After adjusting the
443 aerotactic response from microfluidic experiments, and the initial cell density profile and other
444 parameters from experiments, the model predicted a ring formation within an hour for AX2 and AX3
445 cell spots (Figs. 5C and 5A) in agreement with experiments (Figs. 4D and 1D). The simulated ring
446 propagated at constant speeds of ~ 1.1 and $1.2 \mu\text{m}/\text{min}$ for AX2 and AX3, respectively. These values
447 were in good agreement with the range of measured ring speeds (Figs. S3A-C), given the
448 experimental variability.

449 All other cell lines tested in this study presented an aerotactic response (i.e., $v_x > 0$) in the
450 region of 0 – 2% O_2 (Figs. 2B, 2E, and 2H). Simulated rings of *fhbB*⁻ mutant (Fig. 5B) were 50%
451 slower and much wider than those of the parent cell line AX3 (Fig. 5A) as the x -directional velocity
452 v_x was only 37% of their parent one. But, the rings appeared as quickly as their parent did as the
453 cellular oxygen consumption or proliferation was not impaired. The rings of AX2 treated with
454 100 μM SNP (Fig. 5D) were 21% slower than those of non-treated cell line (Fig. 5C) as the x -
455 directional velocity v_x was also reduced by 21% with respect to their parent. They were also wider
456 but not significantly delayed as the oxygen consumption of this condition was only slightly decreased
457 with respect to the control. These findings were in very good agreement with experimental
458 observations (Figs. 1D, 1L, S5D, and S5H).

459 Oligomycin and antimycin treatments corresponded to a very different set of parameters. The
460 aerotactic directional migration speed v_x of oligomycin-treated cells was similar to the non-treated
461 AX2 (Fig. 2H) but they did not proliferate at all (Fig. S6B). As a result, once initiated the ring
462 expanded poorly (Fig. 5E) and even stopped after a while. The ultra-low oxygen consumption of
463 antimycin-treated cells made the ring appear at very late simulation times (about 7 h in Fig. 5F)
464 comparable to experimental observations (Fig. 4P).

465 One can conclude from these simulations that:

- 466 (i) Rings formed only in presence of aerotaxis.
- 467 (ii) Ring speed mostly correlated with the aerotactic strength (x -directional velocity proportional
468 to aerotactic bias).
- 469 (iii) Ring formation time did not correlate with the aerotactic strength but with the overall oxygen
470 consumption by the cells in the confined area.

471 4 Discussion

472 *Dictyostelium* motility towards extracellular cAMP has been described extensively and served
473 as a model for chemotaxis in other organisms (Pal et al, 2019). cAMP acts through G protein-coupled
474 receptors (GPCR) to regulate actin polymerization and cell motility. Spatial gradients of cAMP
475 between the front and the back of the cells are detected and amplified intracellularly through second
476 messengers to direct motility. *Dictyostelium* uses similar signaling mechanisms to respond to a wide
477 variety of signals such as temperature (Poff & Skokut, 1977), light (Fisher, 1997; Marée et al, 1999),
478 electric current (Sato et al, 2007; Zhao et al, 2002), and chemical substances such as folate (Pan et al,
479 2016). In all these characterized situations, cells perceive differences of the signal levels on the
480 plasma membrane, usually using a receptor, and integrate them to become polarized and direct their
481 motility (Devreotes & Horwitz, 2015). In the present case, the initial signal is oxygen, a molecule
482 normally present in high amount in the environment and constantly used for basic metabolism. There
483 is no known cell surface receptor for oxygen and its hydrophobic properties allow its free diffusion
484 through membranes before being metabolized. Inactivation of the G-beta protein required for
485 transducing most GPCR signal does not prevent aerotaxis (Biondo et al, 2021).

486 To identify the signaling mechanism, we tested mutants in gene-coding potential oxygen-
487 sensing proteins using a confined spot assay to observe an aerotactic ring, and then further dissected
488 this phenomenon to model and distinguish what aspect of the cell properties was affected by the
489 mutation. The same strategy was then used when challenging cells with various chemicals. Among
490 tested mutants, the *fhbB*⁻ and *fhbA*⁻*B*⁻ strains were the one presenting a somewhat altered ring
491 formation with the micro-colony confining test. However, the fact that a ring was formed and
492 expanded, even if irregular and at lower speed, indicates that the cells still present some aerotaxis,
493 which was confirmed when imposing an oxygen gradient. The strong reduction but not complete
494 abolition of the aerotactic index (AI_{max} , Fig. 2C) could indicate a direct role for flavohemoglobins in
495 sensing oxygen gradients, but can also be an undirect effect that mask the cell aerotaxis. Our further
496 assays with imposed oxygen or chemical gradients and simulation argue for an undirect role of
497 flavohemoglobins and NO. *Dictyostelium* cells did not move towards or away from NO generated by
498 SNP in the microfluidic RNS gradient experiment (Fig. 3), excluding a role for NO to act as
499 secondary gradient to orient cell motility. If NO was used as a signal to orient cell or even to fine
500 tune aerotaxis, swamping the cells with 500 μ M SNP should reduce their aerotaxis, which was not
501 the case. Instead, SNP impairs general motility by reducing the average cell speed, this effect being
502 more pronounced in hypoxic conditions (Fig. S3D). As SNP reduces more cell motility in hypoxic
503 condition and flavohemoglobins expression is induced at low oxygen (Table 1), it suggests that those
504 enzymes main function is to protect cells from toxic effects of NO generated upon hypoxia. The
505 weak aerotactic response of the *fhbB*⁻ cells cannot be fully explained by the motility reduction caused
506 by acute exposure to NO in hypoxic condition. It seems that the strains present other defects, maybe
507 due to adaptation to chronic exposure to self-generated NO. In most microorganisms, low level NO is
508 produced as part of nitrogen metabolism, and flavohemoglobins regulate NO homeostasis (Cánovas
509 et al, 2016). It is thus likely that inactivation of *fhbB* results into elevated levels of NO even during

510 normal growth that would cause the mutants' phenotypes. The observation that *fhbB*⁻ cells were
511 bigger than the wild type without being polynucleated and more sensitive to toxicity when exposed to
512 high glucose concentration suggests a broad metabolic unbalance (Iijima et al, 2000). Differences
513 between short-term and long-term exposure to NO have been observed when incubating
514 *Dictyostelium* with SNP over prolonged period (Taminato et al, 2002). Wild type cells' growth is
515 little affected during the first 20 h but protein kinase A (PKA) becomes transiently activated after
516 12 h of SNP treatment through the *yakA* pathway as part of a stress response that then leads to
517 reduced cell growth. The protein kinase, *yakA*, regulates cell division, actin filament polymerization,
518 transition from growth to development and the arrest of growth in response to stresses, including
519 exposure to NO (Mantzouranis et al, 2010). The *fhbB*⁻ cells might thus present a low-level activation
520 of the *yakA* pathway in response to a constant stress due to elevated exposure to RNS, leading to
521 abnormal cell size and motility. Thus, the *fhbB*⁻ mutant appears to be affected in aspects unrelated to
522 the rapid adaptation to hypoxia that characterize aerotaxis. As such, the data obtained with SNP
523 treatment appear more significant than the one with the *fhbB*⁻ strain and lead to the conclusion that
524 aerotaxis is not directly controlled by NO signaling.

525 Based on our experimental data with various strains and conditions, we used a model allowing
526 us to explain many outcomes of ring formation and propagation in our confined spot assay. Cells first
527 need to generate an oxygen gradient through respiration. Changes in cell number or respiration
528 capability will mostly affect the timing to generate a sufficiently steep gradient for cells to detect. But
529 eventually, aerotactic motility occurs and a ring is formed as shown with the various mitochondrial
530 inhibitors that reduced but not fully abolished oxygen consumption. Surprisingly, the inhibition of
531 mitochondrial respiration did not block aerotactic ring formation in *Dictyostelium* contrary to
532 epithelial or breast tumor cells (Deygas et al, 2018; Mikaelian et al, 2022). The inhibition of
533 mitochondrial respiration by oligomycin or antimycin in human cell lines likely prevents the
534 formation of the steep oxygen gradient needed to direct the outward cell motility (Mikaelian et al,
535 2022). Like many protists, *Dictyostelium* possesses an alternate oxidase that consumes oxygen and is
536 not blocked by cyanides (Kimura et al, 2010), explaining how the cells retain a significant respiration
537 capability even in presence of antimycin or oligomycin. With *Dictyostelium*, our results allow to
538 distinguish the role of mitochondria as a sink for oxygen to generate a gradient from its putative
539 functions as oxygen sensor. As mitochondria are not necessary for aerotaxis, it excludes their direct
540 role as oxygen sensors for *Dictyostelium*. Mikaelian et al. have also shown that mitochondria are
541 dispensable for oxygen sensing in human cancer cells by showing that cells with impaired
542 mitochondria will follow the gradient generated by normal one. Since the main ATP biosynthesis
543 pathway is dispensable, a control of aerotaxis through a secondary signal based on energy depletion,
544 such as ADP/ATP ratio is also unlikely. Antimycin not only blocks ATP synthesis but also induces
545 ROS production in *Dictyostelium*'s mitochondria (Downs et al, 2021), yet aerotaxis remained
546 normal. Aerotaxis was also unaffected upon treatment with H₂O₂ or Tiron, a ROS scavenger. Taken
547 together, these data also exclude a role for ROS as secondary signal for aerotaxis in *Dictyostelium*.
548 Controversially, Biondo et al. suggested that *Dictyostelium* cells utilized intracellular H₂O₂ as an
549 activator or an enhancer of aerotactic migration (Biondo et al, 2021), similar to mammalian cells
550 (Deygas et al, 2018). They performed a confined spot assay with a catalase-deficient strain and
551 reported that its aerotactic ring formation was much quicker than the wild type. However, our
552 experiments and modeling of aerotactic ring shows that the timing of its formation is absolutely not
553 dependent upon the strength of aerotaxis, but is determined by the number of cells in the micro-
554 colony and their oxygen consumption. The presented data illustrate well these concepts: the
555 mitochondrial inhibitors that reduce oxygen consumption without influencing aerotaxis do delay the
556 timing of the ring formation, while the *fhbB*⁻ mutant with reduced aerotaxis but normal respiration
557 form a ring at the same time than the wild type. In mammalian cells, there are clear evidence that

558 aerotactic motility requires the modification of the receptor tyrosine kinase EGFR by oxidative stress
559 (Deygas et al, 2018; Mikaelian et al, 2022). As the *Dictyostelium* genome is devoid of any receptor
560 tyrosine kinase (Eichinger et al, 2005), it is not surprising that this specific mechanism is not
561 conserved. As some of the strains tested in Mikaelian et al. do not express EGFR, alternate oxygen
562 sensors might also exist in human. While the reported results didn't allow to identify the molecular
563 mechanism behind *Dictyostelium* aerotaxis, we were able to exclude the most expected contributors:
564 mitochondria or secondary signals linked to ROS and RNS.

565

566 **5 Data Availability Statement**

567 The data presented in this study are available upon reasonable request to the corresponding authors.

568 **6 Author Contributions**

569 Conceptualization, S.H., J.-P.R., C.A. and K.F.; methodology, S.H., J.-P.R. Y.V. and K.F.; software,
570 S.H. N.G and J.-P.R.; validation, S.H.; formal analysis, S.H., J.-P.R; investigation, S.H., J.H. and
571 D.R; resources, S.H., J.-P.R. and K.F.; data curation, S.H.; writing-original draft preparation, S.H.
572 and K.F.; writing-review and editing, S.H., J.-P.R., O.C.-E., C.A. and K.F.; visualization, S.H.;
573 supervision, C.A., J.-P.R. and K.F.; project administration, K.F.; funding acquisition, S.H., J.-P.R.
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742 **Figure 1:** *fhb* KO cells migrate toward oxygen in the confined spot assay. Snapshots right after
 743 (A,E,I,M), 6 h after (B,F,J,N) and 12 h after (C,G,K,O) covering the colony with a glass coverslip
 744 non permeable to O₂: cells quickly consume available O₂ and move outwardly with a characteristic
 745 ring front. (D, H, L, P) Kymographs of mean image intensity (horizontal axis: radial distance from
 746 spot center; vertical axis: time). (A-D) AX3 parent cell line, (E-H) *fhbA*⁻, (I-L) *fhbB*⁻, (M-P) *fhbA-B*⁻.
 747 Bars: 500 μm and 250 μm for snapshots and kymographs (horizontal axis) respectively.

748 **Figure 2:** Effect of various treatments on the aerotactic response of *Dictyostelium* to an imposed
 749 0.4% to 21% external oxygen gradient (left to right) generated by supplying pure nitrogen and air to
 750 the gas channels of a double-layer microfluidic device. (A,D,G) Average speed (*i.e.*, absolute value
 751 of the displacement per minute) *vs.* the position *x* along the gradient direction. (B,E,F) *x*-directional
 752 velocity (*i.e.*, displacement along gradient direction per minute) *vs.* the position *x* along the gradient
 753 direction. The computed profile of oxygen concentration *c* is overlapped (gray solid line) on these
 754 profiles showing the oxygen gradient from 0.4% O₂ to 21% O₂ (right axis). (C,F,I) Maximal
 755 (positive) value AI_{\max} of the average aerotactic index. The values and error bars on profiles and on
 756 AI_{\max} are means and standard deviations over independent experiments. Significant differences were
 757 assessed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.
 758 **p* < 0.05. The various experimental conditions are the following. (A-C) Response of *fhb* null
 759 mutants and associated AX3 parent cell line (*N* = 3 for *fhbA*⁻ and *fhbA-B*⁻, *N* = 6 for *fhbB*⁻ and AX3).

760 (D-F) Effect of SNP, H₂O₂ and EtBr on the AX2 control strain ($N = 3$ for SNP, H₂O₂ and EtBr
761 treated cells, $N = 9$ for the control AX2). (G-I) Effect of oligomycin or antimycin treatment on the
762 migration of the control AX2 cells ($N = 3$ for all conditions).

763 **Figure 3:** Gradients of SNP or H₂O₂ do not induce chemotaxis but reduce the migration speed. (A)
764 Schematic of the Y-shaped microchannel device. (B-C) HL5 culture medium without and with (B)
765 SNP at 100 μ M or (C) H₂O₂ at 500 μ M were injected from the left and right inlets, respectively, at
766 1 ml/h (i.e., the average velocity at 0.37 mm/s in the merged channel). The average absolute values of
767 the x -directional displacements in 1 min (left), and the average chemotactic index, $CI = \overline{dl_{x,t}/dl_t}$,
768 (right) under the chemical gradient are plotted as a function of the transverse direction x . The error
769 bars show standard deviation of independent experiments ($N = 3$). The calculated profile of chemical
770 concentration C is overlapped (red dashed line).

771 **Figure 4:** AX2 cells migrate toward oxygen in the confined spot assay even when their
772 mitochondrial activity is impaired. Snapshots (A,E,I,M) right after, (B,F) 6 h after, (C,G,J,N) 12 h
773 after and (K,O) 24 h after covering the colony with a glass coverslip non-permeable to O₂. (D, H, L,
774 P) Kymographs of mean image intensity (horizontal axis: radial distance from spot center; vertical
775 axis: time). (A-D) AX2 parent cell line (Control), (E-H) EtBr treated AX2, (I-L) oligomycin treated
776 cells, (M-P) antimycin treated cells. Bars: 1 mm and 500 μ m for snapshots and kymographs
777 (horizontal axis), respectively.

778 **Figure 5:** Simulated kymographs of cell density for the various experimental conditions presented in
779 this study: (A) AX3 control with 2000 cells initially plated; (B) *fhbB*⁻ cell line with 2000 initial cells;
780 (C) AX2 control cell line with 5000 initial cells; (D) AX2 cells treated with 100 μ M SNP
781 (2000 initial cells); (E) AX2 cells treated with oligomycin (5000 initial cells); (F) AX2 cells treated
782 with antimycin (5000 initial cells). The line in (B) is the slope of the ring-band of (A). The lines in
783 (E,F) are the slope of the ring-band of (C). The line in (D) comes from the corresponding control
784 AX2 at low initial density not shown. See Supplementary Table S1 for the experimental parameters
785 used.

786

787 **Table 1:** Changes in expression level of a series of genes in response to oxidative stress or hypoxia.
788 Wild type cells in petri dishes were incubated for 3 h with 100 μ M SNP as source of NO, 100 μ M
789 H₂O₂ or in a hypoxia chamber with 100% nitrogen, resulting in a final concentration of 0.4% O₂.
790 RNA was extracted and gene expression quantified by RT-qPCR using untreated cells as standard.

Gene	Conditions		
	H ₂ O ₂	SNP	Hypoxia
<i>cxgE</i>	0.95	1.09	0.10
<i>cxgS</i>	0.77	0.64	3.14
<i>fhbA</i>	0.84	0.44	6.02

fhbB



1.13

0.93

4.07

791