

1 H<sup>+</sup>- and Na<sup>+</sup>- elicited swift changes of the microtubule system in the biflagellated green alga  
2 *Chlamydomonas*  
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## 31 Abstract

32

33 The microtubule cytoskeletal system is integral to diverse cellular processes. Although  
34 microtubules are known for dynamic instability, the system is tightly controlled in typical  
35 interphase animal cells. In contrast, diverse evidence suggests that the system is mercurial in the  
36 unicellular fresh water green alga, *Chlamydomonas*, but intense autofluorescence from  
37 photosynthesis pigments has hindered the investigation. By expressing a bright fluorescent  
38 reporter protein at the endogenous level, we demonstrate in real time discreet sweeping changes  
39 in algal microtubules elicited by fluctuation of intracellular H<sup>+</sup> and Na<sup>+</sup>. These results suggest  
40 disparate sensitivity of this vital yet delicate system in diverse organisms; and illuminate how pH  
41 may drive crucial cellular processes; how plants respond to, and perhaps sense stresses; and how  
42 many species could be susceptible to accelerated changes in global environments.

43

## 44 Introduction

45

46 The microtubule (MT) cytoskeletal system is integral to many crucial processes in eukaryotic  
47 cells. The opposing ends of these cylindrical polymers exhibit distinct properties, with the plus  
48 end growing and shrinking stochastically. MTs establish the polarity of cells and serve as tracks  
49 for positioning and trafficking intracellular components. They could also form complex  
50 machineries. One example is the mitotic apparatus enabling accurate segregation of  
51 chromosomes during mitosis and meiosis. These actions require harnessing MT dynamic  
52 instability and the involvement of a wide array of accessory proteins and various post-  
53 translational modifications (reviewed by Hashimoto, 2015; Song and Brady, 2014). Not  
54 surprisingly, MTs are the target of natural toxins, such as Taxol (Weaver, 2014), and a number of  
55 herbicides (reviewed by Hashimoto, 2015). Notably, biotic and abiotic stresses alter plant MTs,  
56 while Taxol exacerbates stress-induced maldevelopment of seedlings (reviewed by Wang, et al.,  
57 2011; Hardham, 2013; Oda, 2015; Hepler, 2016). Yet the mechanisms underlying stress-induced  
58 changes of MTs and the broad implications remain elusive.

59 *Chlamydomonas reinhardtii* is uniquely suited for addressing these issues. The  
60 unicellular fresh water green alga has signature features of both animal and plant cells (Merchant  
61 et al., 2007). Like animal cells, it has motile flagella that contain stable MT bundles. Like typical  
62 plant cells, the alga is equipped with vacuoles, chloroplast, and dynamic cortical MTs that serve  
63 as tracks for the delivery of the enzymes which synthesize the cell wall (Paredes et al., 2006).  
64 Curiously, its MT system appears susceptible to environmental changes. Its flagella sever readily  
65 when the aqueous environment changes suddenly (Lefebvre et al., 1978; reviewed by Quarmby,  
66 2009). The best characterized stimulus is pH shock. Organic acid, such as acetic acid (HA)  
67 diffuses across the plasma membrane and then becomes ionized. The resulting acidification in  
68 the cytosol triggers nearly simultaneous influx of extracellular Ca<sup>2+</sup>, which signals flagella  
69 amputation (Quarmby et al., 1996; Wheeler et al., 2008; Hilton et al., 2016). Cortical MTs  
70 become fewer and shorter, detected by immunofluorescence and biochemistry 5 mins after pH  
71 shock (Wang et al., 2013).

72 While live cell imaging will be ideal for revealing these acid-induced responses with  
73 higher spatial and temporal resolution, autofluorescence from photosynthetic pigments in  
74 chloroplast obscures commonly used fluorescent reporters (Lang et al., 1991; Rasala, 2013).  
75 Recently, we succeeded in revealing dynamic cortical MTs by taking advantage of the new

76 fluorescent protein, NeonGreen (NG) that is 2.7 X brighter than EGFP (Shaner et al., 2013), and  
77 the relative abundant plus end-binding protein, EB1, as the NG carrier (Harris et al., 2016).

78 EB1 plays central roles in eukaryotes (Su et al., 1995; reviewed by Akhmanova and  
79 Steinmetz, 2010; Kumar and Wittmann, 2012). Its N-terminal domain preferentially binds to the  
80 lattice among tubulins at the plus end of MTs, whereas its C-terminal domain can associate with  
81 a wide array of proteins. The two domains operate in concert to accelerate MT dynamics (Rogers  
82 et al., 2002; Vitre et al., 2008; Maurer et al., 2014) and recruit various +TIP effector proteins that  
83 function at the plus end. In live cell imaging, fluorescent EB1 exhibits a comet pattern seemingly  
84 leading the plus end of nascent growing MTs, where tubulins transition from the GTP state to the  
85 GDP state (Maurer et al., 2012; Zanic et al., 2009; reviewed by Gardner et al., 2013). As such  
86 fluorescent EB1 has been commonly used to report newly generated growing MTs (Piehl et al.,  
87 2004; Matov et al., 2010). However, binding to the GDP zone increases in a number of scenarios  
88 (e.g. Tirnauer et al., 2002; Goldspink et al., 2013; Tortosa et al., 2013; Sayas and Avila, 2014).  
89 What causes the switch remains uncertain.

90 Using EB1-NG as a reporter, we captured in real time unexpected changes in EB1-NG  
91 patterns and MT dynamics signaled through H<sup>+</sup> and Na<sup>+</sup>. The remarkable sensitivity and the  
92 distinct responses in wild type (WT) cells and mutants shed critical insight on the divergence of  
93 the MT system, pH regulated processes and the vulnerability of organisms subjected to  
94 environmental stresses.

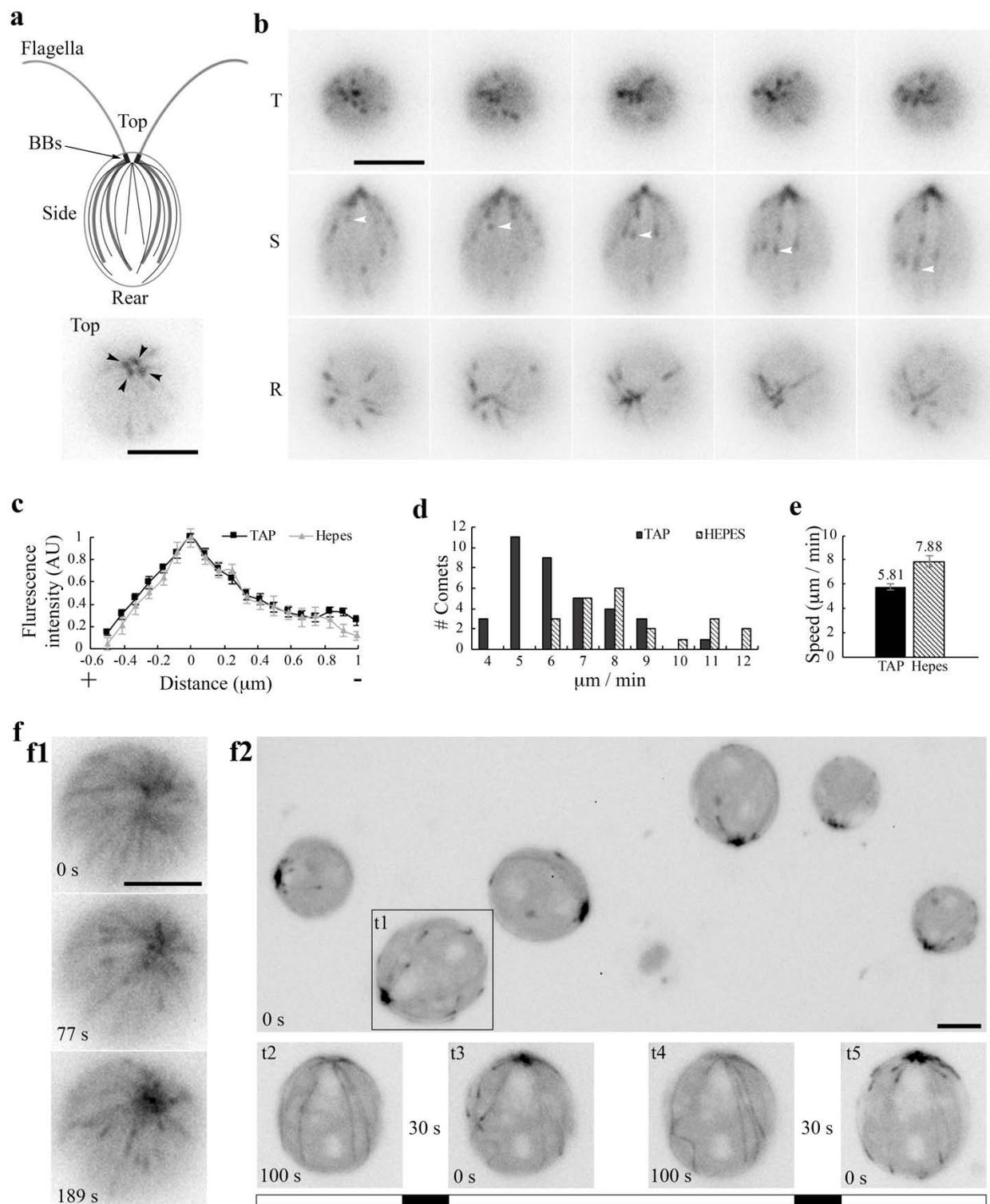
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## 96 **Results**

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### 98 ***EB1-NG reports remarkable sensitivity of the MT system in Chlamydomonas***

99 The MTOC in *Chlamydomonas* cells at interphase is comprised of two pairs of basal bodies  
100 (BBs) (Figure 1a, top panel). The mother BB nucleates the assembly of the axoneme, a MT-  
101 based scaffold that drives the rhythmic beating of the flagellum. Four rootlet MT bundles (thick  
102 lines) arrange in a cruciate pattern positioning BBs at the apical end and the other organelles  
103 (Mittelmeier et al., 2011; Picariello et al., 2014). These MT bundles consisting of more than two  
104 acetylated stable MTs. In contrast, cortical MTs (thin lines) are singular (Horst et al., 1999) and  
105 highly dynamic (Harris et al., 2016). Under widefield fluorescence microscopy EB1-NG  
106 expressed at the level of endogenous EB1 from a genomic construct does not reveal stable MTs  
107 except the flagellar tip where plus ends of axonemal MTs undergo turnover continuously (Harris  
108 et al., 2016). In addition, plus ends of growing cortical MTs appears like the typical comets  
109 observed in other eukaryotic cells. Comets emerged from four spots underneath flagella,  
110 corresponding to BBs (bottom panel) (Pedersen et al., 2003). As shown from the top, side and  
111 rear views of cells, comets of nascent cortical MTs travel along the contour of the cell body  
112 toward the posterior pole (Figure 1b, white arrowhead; Supplemental video 1-1). Near the pole  
113 comets vanish presumably when MTs stop growing or switch to the shrinking phase. The pattern  
114 appears similar in cells resuspended in the commonly used Tris-Acetate-Phosphate (TAP)  
115 culture medium or 10 mM HEPES buffer (with 5 mM Na<sup>+</sup>).



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**Figure 1.** EB1 in *Chlamydomonas*. **(a)** A schematic picture depicting flagella and the MT network in the cell body (top panel). Black dots, basal bodies (BB). Thick lines, four stable rootlet microtubule bundles. Thin lines in the cell body, the dynamic cortical MTs. A top view of EB1-NG transgenic cells reveal a pattern that resembles 4 BBs (bottom panel). **(b)** Time-lap fluorescent images were taken 10 sec apart from the top (T), side (S) and rear (R) of cells resuspended in the TAP culture medium. EB1-NG appeared like typical comets (arrowheads), emerging from the BB area, coursing along the contour of the cell body and then vanishing as approaching the rear end. The frame rate is 1 frame/sec. **(c)** Normalized line scans along the length of MT plus ends showed a similar EB1 intensity profile in the TAP medium (n = 18 comets from 6 cells) and the Na<sup>+</sup>/HEPES buffer (n=11 comets from 3 cells). The position with peak intensity was designated as 0. The value was negative toward plus end; positive toward BBs. AU, arbitrary unit of fluorescence intensity. **(d)** The distribution and **(e)** the mean and the SEM of EB1 comet speed in the TAP medium (n = 36 comets from 6 cells in 6 recordings) and 5 mM Na<sup>+</sup>/HEPES buffer (n = 22 comets from 3 cells in 3 recording) are significant different (Mann-Whitney U test, P < 0.001). **(f)** Altered MT patterns during fluorescence microscopy. The EB1 comet pattern occasionally switched to a bird cage pattern **(f1)**. Comets returned while the bird cage receded in ~ 1 min. In flattened cells that were compressed by the cover slip gradually, both MTs and comets became explicit **(f2, top panel)**. Comets disappeared after ~ 100 sec (bottom panel, t2), but returned after illumination was switched off for 30 sec (t3). The process was repeatable after another 100 sec illumination and then another light off period (t4 and t5). The alternate white and black bars illustrate the scheme of alternate illumination and dark periods. Scale bars, 5 μm.

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118 The birth of new comets from BBs appeared stochastic. We did not measure the birth  
119 rates, hindered by substantial fluctuations and the narrow apical area. Instead we analyzed comet  
120 length and speed from the side view. Line scans along the lengths of comets show the typical  
121 feature of EB1 comets - the brightest spot corresponds to the area where tubulins are primarily at  
122 the transitional state, slightly behind the leading edge of plus ends with GTP-tubulins (Figure  
123 1c). The distribution of comet speeds shows that MT growth rates varies nearly two folds (Figure  
124 1d). The dataset from cells in the TAP medium (black bars) skews toward the slow end relative  
125 to the Na<sup>+</sup>/HEPES dataset (hatched bars). The average velocities are significantly different (Mann-  
126 Whitney U test, p < 0.001), at  $5.8 \pm 0.26$  and  $7.9 \pm 0.42$  μm/sec respectively (Figure 1e), which  
127 are within the normal range measured in diverse eukaryotic cells (Harris et al., 2016).

128 Curiously, in some long recordings, comets suddenly gave way to a bird cage-like pattern  
129 (Figure 1f1, top panel; Supplemental video 1-2) as if all cortical MTs were revealed by anti-  
130 tubulin immunofluorescence (Horst et al., 1999; Dymek et al., 2006). Comets returned  
131 automatically after ~1 min (middle and bottom panels). This unpredictability suggests that this  
132 pattern is caused by fluctuated intracellular conditions. When cells gradually flattened by the  
133 coverslip, MTs also became visible as a broken bird cage with comets (1f2, top panel, t1;  
134 Supplemental video 1-3). However, comets disappeared after ~ 100 seconds (bottom panel, t2).  
135 Interestingly, after a 30-second dark period, comets returned upon excitation light was switched  
136 back (t3). Simply alternating the dark and light period replicated the disappearance and return of  
137 comets (t4 and t5). These observations demonstrate that *Chlamydomonas* MT system is highly  
138 mercurial; and suggests that excitation illumination creates a condition that is unfavorable for  
139 MT dynamics, but is reversed in the dark. As illumination opens channelrhodopsins that conduct

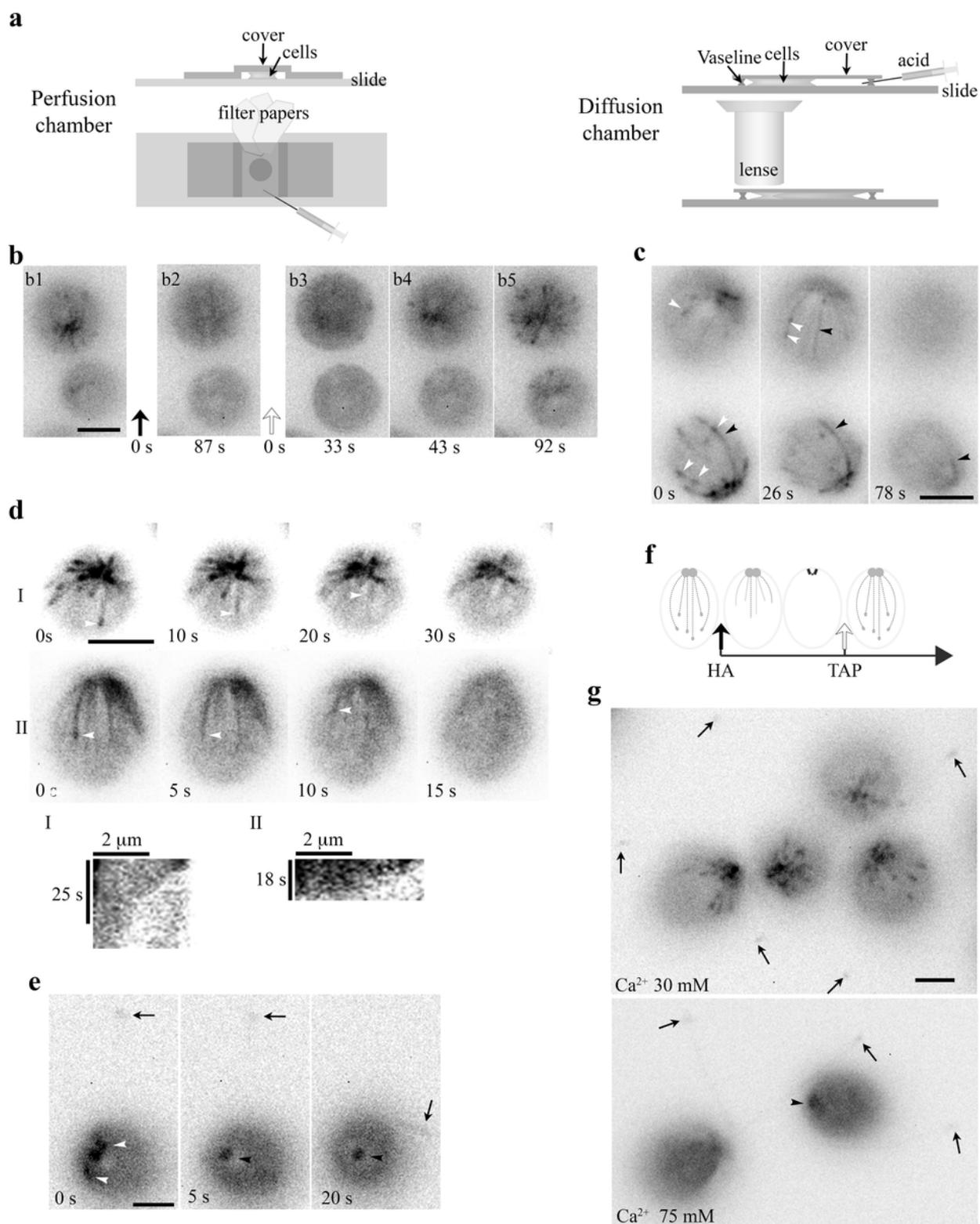
140 a number of cations and  $\text{Cl}^-$  (Nigel et al., 2002; 2003; reviewed by Hegemann and Berthold,  
141 2009), we hypothesize that fluctuations of electrolyte concentrations modulate the MT system in  
142 *Chlamydomonas*. Considering the light sensitivity, we elected to use wide field microscopy and  
143 minimal light intensity to test this.

144  
145 ***Sequential changes in the MT system elicited by a short HA pulse and subsequent wash***

146 We first used the well-defined pH shock, recording EB1-NG signals in cells exposed to HA in  
147 two complementary devices, perfusion chamber and diffusion chamber (Figure 2a). Recording of  
148 events in perfusion chambers started immediately before injection. Similar to pH shock, all cells  
149 were subjected to a swift change of environments as the injected solution was immediately  
150 pulled across the chamber by filter papers placed at the opposite end (Wheeler et al., 2008).  
151 However, the initial period was not decipherable due to flowing of injected fluid and unattached  
152 cells. For the diffusion chamber encircled by Vaseline, cells were placed at one side, underneath  
153 the coverslip and objective lens (right panel). Recording started after 100 mM HA was injected  
154 into the opposite side and cells stopped flowing. This design that decelerated the acidification  
155 process, captured the events as HA diffused across the field and into cells that were being  
156 recorded. However, the precise exposure time was uncertain; HA concentrations increased  
157 gradually, deflagellation was less effective; and not all cells were acidified to the same degree at  
158 the same time. Also the enclosed chamber cannot be washed.

159 Following the injection of 20 mM HA/TAP into the perfusion chamber, all comets  
160 vanished in the first discernable image taken at ~ 90 sec (Figure 2, b1-b2). After wash with 10  
161 mM pH7 HEPES, comets re-appeared at the BB area within ~ 45 sec and MT dynamics resumed  
162 (b3-b5). Thus HA exposure elicits the disappearance of EB patterns either by perturbing EB-MT  
163 interplays or causing cortical MTs to pause or disassemble. New dynamic MTs re-form rapidly  
164 after HA is washed away.

165 Diffusion chambers in which the acidification process occurs gradually allow us to  
166 capture another unexpected phenomenon before comets vanish. A broken bird cage pattern with  
167 a few MTs (Figure 2c, left and middle panel, black arrowhead) and comets (white arrowheads)  
168 appeared before the disappearance at a time designated as 78 second (right panel). To decipher  
169 the disappearance, we analyzed digitally enhanced recordings (Figure 2d, top panels). As shown  
170 in two representative cells, overall EB1 signals were fading with time, which could be due to  
171 photobleaching, pH sensitivity of fluorescent proteins, disassociation of EB1 or system-wide MT  
172 disassembly. Some MTs clearly underwent endwise resorption (arrowheads). Kymographs  
173 tracking plus ends of prominent MTs in two rare still cells show that, as expected, comets  
174 disappeared before the resorption of the respective MT. Measurements of the slopes show that  
175 the initial shortening speeds for these two events are 8 and 13  $\mu\text{m}/\text{min}$  but decelerate toward the  
176 BB area. These speeds revealed by EB1-NG are in line with the averaged  $10 \pm 3 \mu\text{m}/\text{min}$   
177 shortening speed of cortical MTs in cultured tobacco cells (Dixit and Cyr, 2004), faster than  
178 kinesin-13 catalyzed shortening (Helenius et al., 2006), and slower than the maximal shortening  
179 speed (30  $\mu\text{m}/\text{min}$ ) of MTs assembled from purified tubulins (O'Brien et al., 1997). Statistics of  
180 shortening speed was not analyzed because of few motionless cells and few shortening MTs with  
181 a definitive plus end. Tubulin reporters will be more appropriate for shortening analysis.



**Figure 2.** An HA pulse elicited swift sequential changes in the MT system. **(a)** Schematics depicting an open-ended perfusion chamber (left panels) and a diffusion chamber (right panels) for capturing the HA-induced rapid changes. **(b)** A 10-  $\mu$ l aliquot of cells resuspended in the TAP medium was placed in a perfusion chamber. The images **(b1, 2)** were captured before and after perfusion with 20 mM HA/TAP (pH 4.5,  $t=0$ , black arrow). The following recordings **(b3-5)** captured the events right after the TAP medium (pH 7) was injected to wash away HA ( $t=0$ , clear arrow). B3 is the first clear image after fluid and cells stopped flowing. Comets already disappeared within 87 seconds after HA perfusion. They started emerging 43 sec after wash. **(c)** The process preceding HA-induced disappearance of EB1 comets in diffusion chambers. A 40-  $\mu$ l aliquot of cells resuspended in HEPES was placed in a diffusion chamber encircled by Vaseline, under the coverslip and an objective lens. HA was injected to the other side of the chamber and diffused toward cells that were being imaged. During the gradual acidification process, both comets (white arrowheads) and shank binding MTs (black arrowheads) were evident first and then both patterns vanished. **(d)** Time lapse images and kymographs revealed endwise resorption of EB1-decorated MTs (white arrowheads). **(e)** Comets (white arrowheads) in the cell body vanished first before the excision of flagella (arrows). Following deflagellation, EB1 diffused away from the tip. EB1 signals remained at BBs but was static (black arrowhead). **(f)** A schematic depicting sequential changes in MTs upon exposure to HA and a subsequent wash with the TAP medium. Dotted lines with a comet, growing MTs. Dotted lines alone, shrinking MTs. Solid lines, shrinking MTs with EB1 shank binding. **(g)** Effects of  $[Ca^{2+}]_{ex}$  on MTs. In cells resuspended in 30 mM  $[Ca^{2+}]_{ex}$ , flagella remained attached (arrows in left panel), while comets were vibrant. In the 75 mM  $[Ca^{2+}]_{ex}$  group, comets disappeared and flagella were amputated (arrows in right panel). Static EB1 signals remained at BBs (arrowhead). Scale bars, 5  $\mu$ m.

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184 As for cells with EB1 signals detected in the cell body and flagella (Figure 2e)  
185 simultaneously, comets (white arrowheads in left panel) vanished first (middle panel) before  
186 flagella (arrow) were amputated (right panel). EB1 signals remained at the BB area but were  
187 static (black arrowhead). Contrary to deflagellation within seconds upon HA perfusion (Wheeler  
188 et al., 2008), the deflagellation in the diffusion chamber takes more than one minute due to  
189 gradual acidification. Thus when cells are exposed to HA, shank binding increases, comets  
190 disappear, endwise resorption becomes evident and then flagella become amputated. The  
191 sequential events occurring in the diffusion chamber are summarized in figure 2f.

192 Lowering intracellular pH elicits  $Ca^{2+}$  influx, whereas  $Ca^{2+}$  prevents MT formation and  
193 promotes MT disassembly (Weisenberg, 1972; O'Brien et al., 1997). To differentiate whether  
194 HA-induced changes are due to  $H^+$  or  $Ca^{2+}$ , we raised  $[Ca^{2+}]_{in}$  without adding HA. Calcium  
195 ionophore A23187 did not trigger deflagellation or evident changes in the MT system in our  
196 hands. This is not surprising since A23187 cannot elicit consistent effects in *Chlamydomonas*  
197 (Bloodgood and Levin, 1983). So we simply raised  $[Ca^{2+}]_{ex}$ . Perfusions of either HA or  $CaCl_2$   
198 solution elicit  $Ca^{2+}$  influx, leading to deflagellation, although the latter is less efficient (Wheeler  
199 et al., 2008). WT cells resuspended in 30 mM  $Ca^{2+}$ /HEPES appeared agitated, suggesting entry  
200 of  $Ca^{2+}$  (Figure 2g, top panel). However, flagella remained attached (arrows) and comet activity  
201 was robust. When cells were resuspended in 75 mM  $Ca^{2+}$ /HEPES solution (right panel), cells  
202 shed flagella (arrows). Comets already disappeared, while static EB1 signals remained at the BB

203 area (arrowhead). Thus the outcomes elicited by high  $[Ca^{2+}]_{ex}$  and HA are similar. The  
204 experiments testing the effects of  $Ca^{2+}$  and hypertonicity will be described in later sessions.

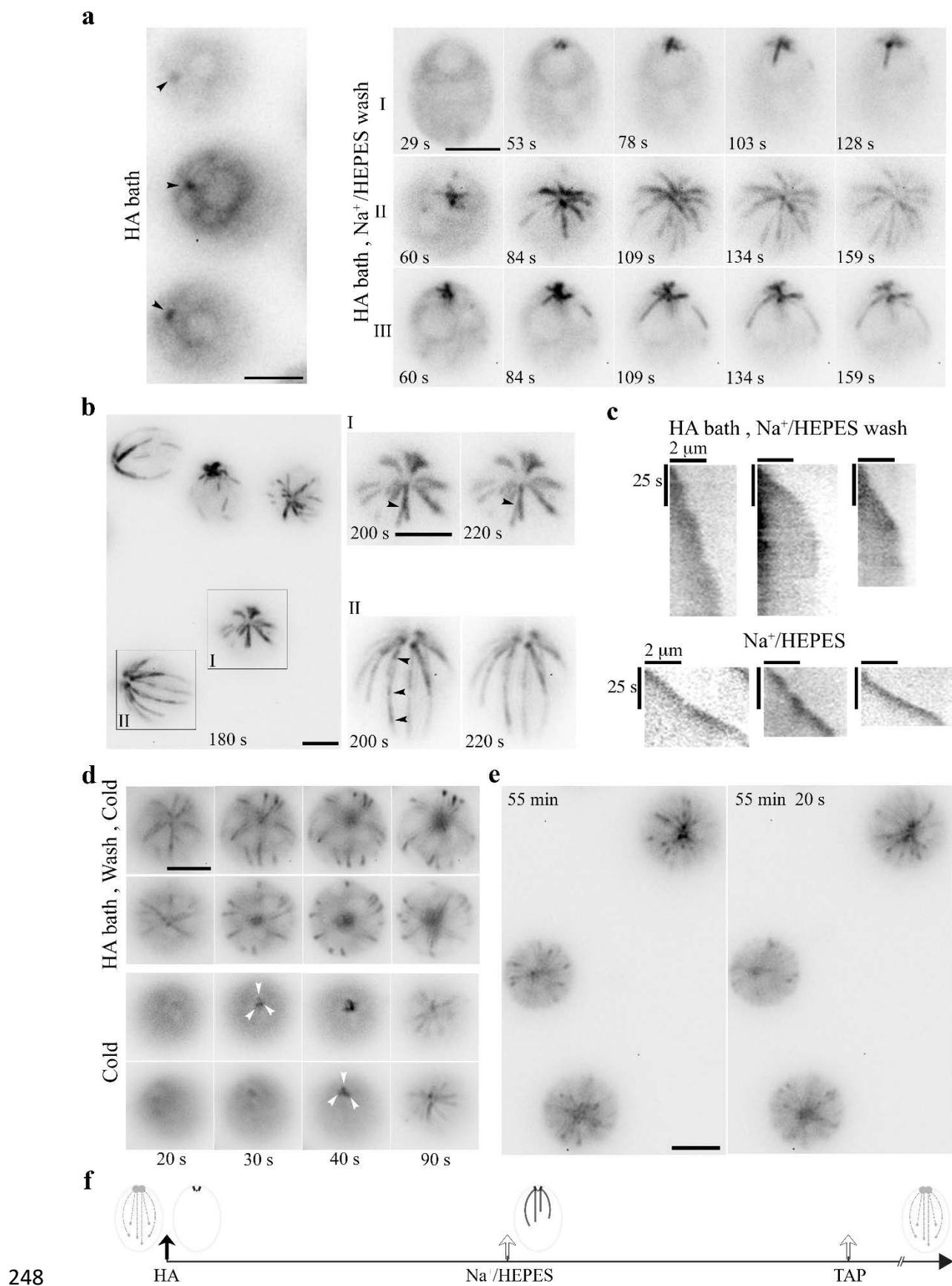
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### 206 ***Formation of cold-resistant thick MT fibers in the recovery phase after HA bath***

207 *Chlamydomonas* expresses  $Na^+/H^+$  exchangers (Pittman et al., 2009), as well as various channels  
208 and pumps at different locations (Fujiu et al., 2011; Taylor et al., 2012) to maintain electrolyte  
209 homeostasis. We reasoned that altering HA treatment might also change other cations such as  
210  $Na^+$  or  $K^+$ . To test this, we extended HA exposure - resuspending cells in pH3, 10 mM  
211 HA/double distilled water (ddw) for 5 mins. As expected, EB1 patterns were absent except for  
212 the static signal at the BB area (Figure 3a, left panel), mimicking the final outcome of HA  
213 exposure in the diffusion chamber (Figure 2e). In contrast, resuspension in pH3, 10 mM HCl did  
214 not alter comet activity. Thus, as with deflagellation, HA-elicited changes in cortical MTs are  
215 due to intracellular rather than extracellular acidification. Images were taken as cells were  
216 washed with  $Na^+$ /HEPES in a perfusion chamber. After washing, dynamic EB1 signals emerged  
217 at the BB area after ~50 sec (Figure 3a, cell I in right panel). Interestingly, nascent MTs were not  
218 adorned with a typical comet. Compared to the bird cage pattern, they appeared thicker, fewer  
219 and nearly uniformly decorated, as if plus end tracking EB1 stayed behind growing MTs. In cells  
220 recorded at a later period (cell II and III, between 60 – 159 sec; Supplemental video 3), MT  
221 growth decelerated, especially between 109-159 sec. In cells recorded after 180 sec, all growth  
222 stopped (Figure 3b, left panel). This is further illustrated by two nearly identical images taken 20  
223 sec apart of two representative cells (cell I and II, right panels). Notably, some static fibers split,  
224 or had more than one comet aligned in tandem (arrowheads). Taken together, these observations  
225 strongly suggest that nascent MTs generated after HA bath and wash are abnormal, perhaps with  
226 a propensity to nucleate ectopically, branching or growing on top of the other piggy-backing as  
227 bundles. Kymograms comparing three representative MTs (Figure 3c, top panels) with growing  
228 MTs in the untreated control cells (bottom panels) confirm that EB1 signals extend further  
229 toward the BB area in cells recovering from HA bath. MT growth rates, shown by the slopes,  
230 fluctuate but were mostly slower than those in untreated control cells. Overall the rates decline  
231 until MT growth stops. Thus contrary to thin, dim, transient MT fibers in the acidification phase  
232 (Figure 2), MTs formed in the recovery phase after HA bath were thick, short, long-lived and  
233 bright.

234 MTs are cold labile. Cold treatment induces MT endwise resorption *in vitro* (Muller-  
235 Reichert et al., 1998). To test the stability of these thick MTs formed in the recovery phase, glass  
236 slides with a droplet of cells after HA bath and wash were placed on ice for 3 min. Images were  
237 taken using the microscope at the room temperature (RT) immediately, about 20 seconds after  
238 slides were removed from ice. Unexpectedly, most cells were imaged from the apical end and the  
239 focal planes drifted continuously, indicating that cells oriented toward the objective lens and  
240 floated gradually during this warm up period. As shown in two representative cells, EB1-  
241 decorated MTs after HA bath and wash remained after the removal from ice amidst the drift of  
242 focal planes (Figure 3d, top panels). Thus the thick static MTs formed after HA bath and wash  
243 are cold stable. The pattern remained for the subsequent 70-second recording period. In contrast,  
244 for the control without HA bath, EB1 patterns were undetectable initially (bottom panels),  
245 indicating cold lability. Dynamic EB1 signals gradually re-emerged at BBs after 30 second  
246 (white arrowheads).

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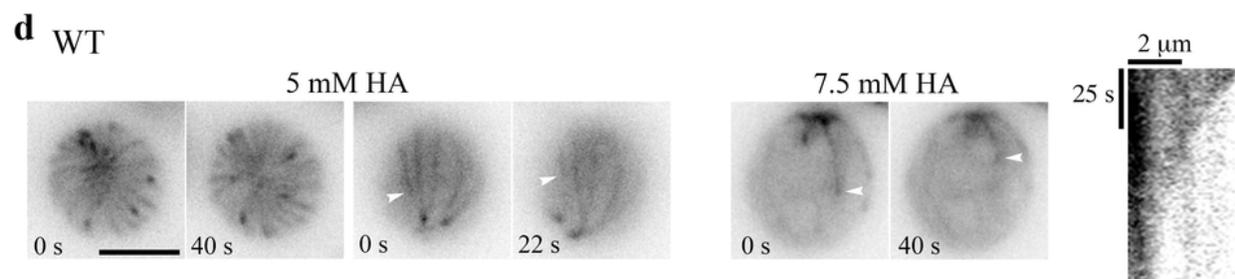
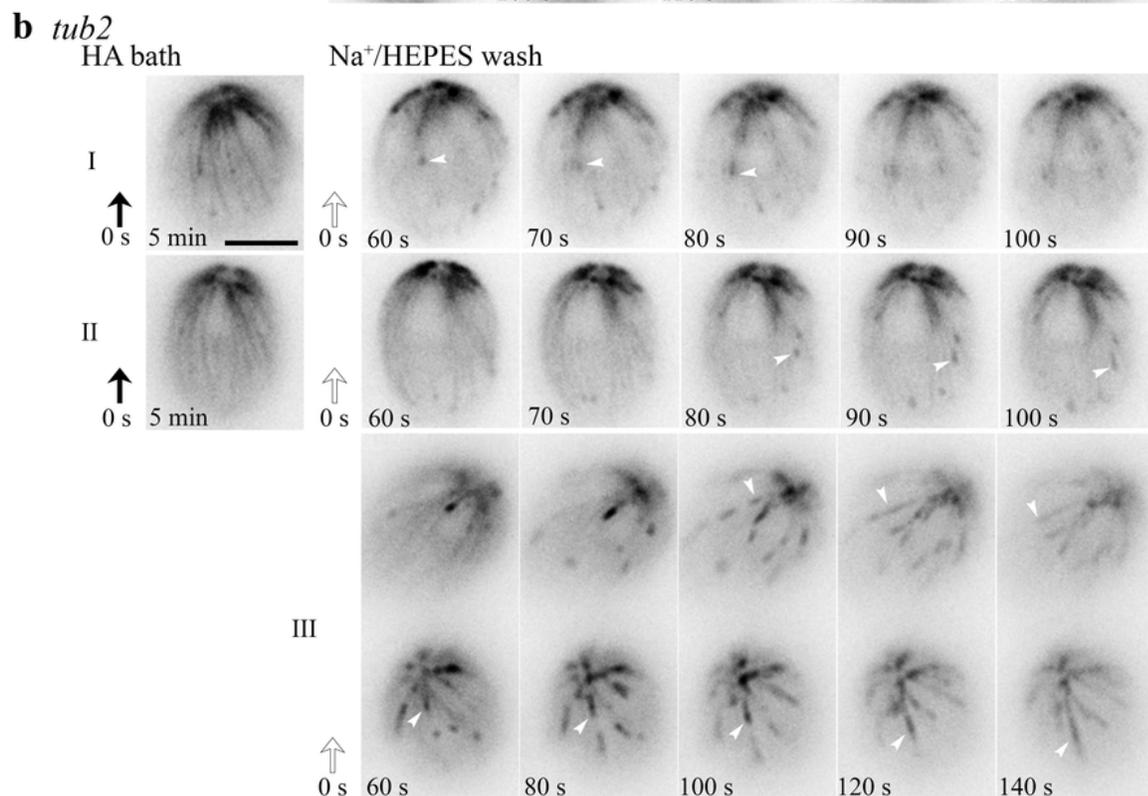
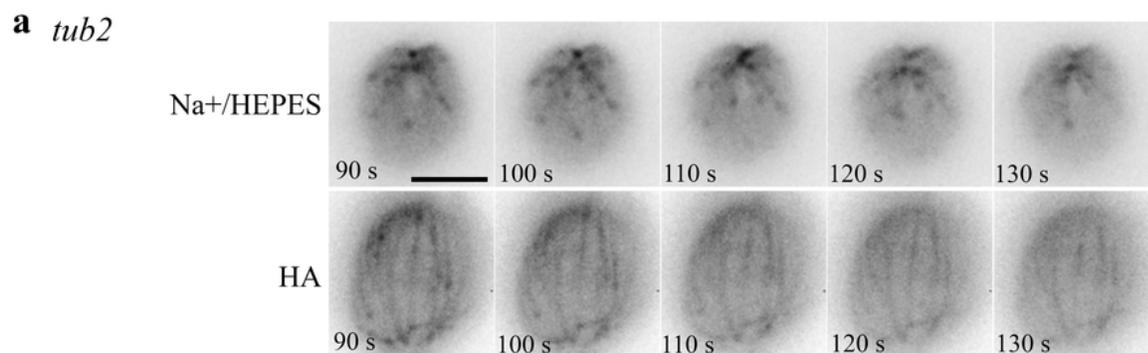
**Figure 3.** HA bath and a subsequent wash induced long-lived yet reversible changes to the MT system. **(a)** Static EB1 signals remained at BBs after cells were resuspended in 10 mM pH3 HA for 5 min (left panel). After replacing HA with the HEPES buffer, EB1 signal at the BB area intensified within 1 min (black arrowheads). But newly formed MTs were thick and prominent, lacking the typical comet (right panel, cell I). In cells recorded 60 sec after wash (cell II and III), MT elongation slowed down gradually. **(b)** In cells imaged ~180 sec after wash, EB1-decorated MTs in all cells stopped growing (top panel), as highlighted in two additional images of two representative cells captured 20 sec apart. In addition, a MT fiber in cell I split into two (arrowhead), while a fiber in cell II had multiple comets aligned in tandem (arrowheads), as if new MTs nucleated or grew on older ones. **(c)** Kymograms comparing the growth of three representative MTs in cells pretreated with 5-min HA bath and then the wash buffer (top panels) and in control cells in the HEPES wash buffer (lower panels). Comets in the latter manifested as intense spot at the plus end. The sharper slopes in the former indicated slower growth and eventually unchanged as the growth paused. **(d)** HA-induced long-lived MTs formed after HA bath and wash were cold resistant (top panels). As shown in two representative cells, frozen MTs remained in the image captured 20 sec after 3 min on ice. The changes in subsequent images were due to drift of focal planes as cells were floating gradually as warming up. In control cells without previous HA exposure (bottom panels), EB1 signals were absent initially (20 sec), consistent with cold lability. They gradually emerged at the BB area (white arrowheads) afterwards. **(e)** Although the MT system froze within minutes after HA bath and HEPES wash ( $t=0$  min), comets resumed 55 mins in cells recovered in TAP media as shown in two images captured 20 sec apart. Scale bars, 5  $\mu\text{m}$ . **(f)** A schematic summarizing the sequence of MT changes induced by HA bath and wash.

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To learn if frozen thick MTs after HA bath and wash were reversible, we continued imaging cells in the recovery phase. To prepare for the long recovery, wash buffer was replaced with TAP media. Thawing signs emerged gradually. Compared to frozen thick MTs (Figure 3e, left panel, black arrowheads), at 55 mins post wash, comet (white arrowheads in right panel) activity was vibrant as if the freeze had never occurred. The sequential events occurring after HA bath and wash are summarized in Figure 3f.

### 257 **Testing HA-induced phenomena by using a tubulin mutant and by reducing HA** 258 **concentrations**

259 In an attempt to decelerate pH-induced resorption in the acidification phase, we took advantage  
260 of a tubulin mutant, *tub2*. A missense mutation near the colchicine binding site in  $\beta$ -tubulin  
261 increases MT stability since *tub2* cells are colchicine-resistant, and have more acetylated MTs  
262 (Schibler and Huang, 1991). In the EB1-NG transgenic *tub2* cells, the comet pattern (Figure 4a,  
263 top panels) appeared indistinguishable from that in WT transgenic cells (Figure 1b). Thus our  
264 assay is not sensitive enough to report increased MT stability. Interestingly though, in the image  
265 taken immediately after resuspension in 10 mM HA, the bird cage pattern with a few comet-like  
266 spots already formed in every *tub2* cell (bottom panels). The pattern was rather stable, although  
267 some MTs seemed to be out of focus intermittently (bottom right panel), suggesting detachment  
268 from the plasma membrane. As shown in two representative cells, similar frozen bird cage  
269 pattern remained even after 5-min HA bath (Figure 4b; Supplemental Figure 4-1).



**Figure 4.** Tempered HA-induced changes by a  $\beta$ -tubulin mutation (**a-c**) and by reduced HA concentrations (**d**). **(a)** Time-lapse images at 10 sec intervals showed normal comets in *tub2* cells resuspended in 5 mM pH 7.4 Na<sup>+</sup>/HEPES (top panels). In contrast, a bird cage pattern with fine MT fibers was present in *tub2* cells resuspended in 10 mM, pH 3 HA (bottom panels). Most MTs froze but some appeared in and out of focus. **(b)** Frozen bird cage remains after 5-min HA bath as shown in two representative cells (left panel). After wash, comets (white arrowheads) reemerged at the plus end of existing fine MTs, instead of the BB area, while the bird cage pattern was fading. MTs were growing but comets were lengthening and the pace was very slow. In videos recorded only after wash, the recovery was faster. Some comets already emerged from the BB area. Some moved along old MTs and lengthened (panel III, white arrowheads). **(c)** A schematic summary of HA-induced changes in *tub2*. **(d)** Concentration-dependent effects of HA on MTs in WT cells. WT cells resuspended in 5 mM HA exhibited a dynamic bird cage pattern with dynamic comets (left, top and side view). Shrinking MTs were visible in the two side view images taken 22 sec apart (white arrowhead). Most patterns were absent in cells resuspended in 7.5 mM HA, except a few shrinking MTs (right). A kymograph revealed the endwise resorption. Scale bars, 5  $\mu$ m.

271  
272 After wash, comets returned (panel I and II) and lengthened as in WT cells. However,  
273 comets (white arrowheads) appeared at the plus end of existing MTs first rather than at BBs  
274 where nascent MTs emerged. The bird cage pattern gradually faded concomitantly. For images  
275 taken 60 seconds and later after wash, comets formed and moved along existing MTs (Panel III,  
276 Supplemental video 4-1). This strengthens the interpreted bundling propensity of MTs in WT  
277 cells recovered from HA bath (Figure 3b). These collective results indicate that the mutation in  
278 *tub2* hinders endwise resorption elicited by HA exposure, as such the bird cage pattern with  
279 cortical MTs uniformly decorated with EB1 is not vanishing; the other responses in *tub2* and WT  
280 cells are similar; and old MTs resorb to make room for new ones to maintain the number of  
281 cortical MTs. The HA responses of *tub2* cells are summarized in Figure 4c.

282 We further tested whether we could replicate the bird cage pattern in WT cells with less  
283 concentrated HA. As shown in two top view images taken 40 seconds apart immediately after  
284 resuspension in 5 mM HA (Figure 4d, left panel; Supplemental video 4-2), all WT cells had  
285 motile flagella and had a dynamic, rather than still, bird cage pattern in which MTs shrink or  
286 grow with a comet, as if the entire MT system were revealed by fluorescent tubulins and  
287 fluorescent EB1 simultaneously. The side view images recorded 22 seconds apart revealed  
288 endwise resorption of a shrinking MT (arrowhead). Only a few resorbing MTs were captured in  
289 cells resuspended in 7.5 mM HA (Figure 4d, right panel). This still cell allows us to plot the  
290 kymograph, which shows a tapered endwise resorption with the initial shortening speed of 4  
291  $\mu$ m/min. All MT patterns and comets disappeared in cells resuspended in 10 mM HA. Therefore,  
292 low [HA] increases EB1 shank-binding, rendering the bird-cage pattern. As HA concentrations  
293 increase, MTs stop growing and comets are lost. As resorption continues, perhaps even at a  
294 hastening pace, comet and bird cage patterns vanish. The 5 mM HA experiment consistently  
295 replicates the light-induced sporadic transient appearance of the bird cage pattern in WT cells  
296 (Figure 1, f1). Changes elicited by 7.5 mM HA partially mimics HA-induced responses in  
297 diffusion chambers (Figure 2b-e).

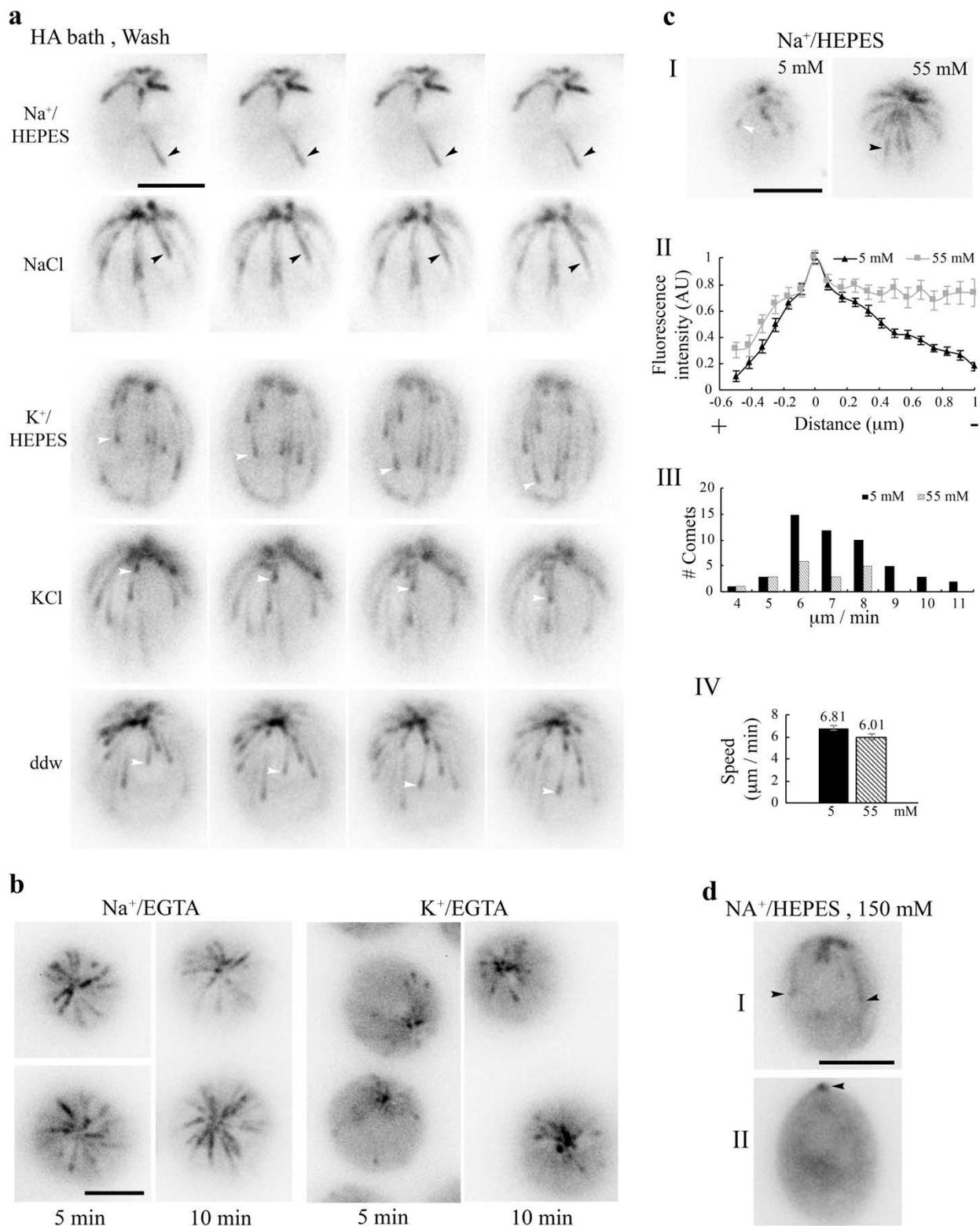
298 ***The long-lived EB1-decorated MTs are due to the rise of intracellular [Na<sup>+</sup>] but not [K<sup>+</sup>]***  
299 To identify the ion causing the formation of static thick EB1-decorated MT bundles in cells  
300 recovering from HA bath, HA-bathed cells were washed with different solutions. Interestingly,  
301 as shown by time-lapse images separated by a 10-second interval, EB1-decorated MT fibers  
302 formed only if the wash solution contained Na<sup>+</sup>, such as the NaOH-buffered HEPES (5 mM Na<sup>+</sup>)  
303 or 5 mM NaCl in ddw (Figure 5a, top two panels). On the other hand, comets resumed profusely  
304 if the wash solution lacked Na<sup>+</sup>, such as the KOH-buffered HEPES buffer, 5 mM KCl in ddw or  
305 plain ddw (bottom three panels). Therefore, Na<sup>+</sup> accounts for the reformation of thick, long-lived  
306 static MTs in the recovery phase of HA-bathed cells.

307 Since Na<sup>+</sup> has low permeability compared to K<sup>+</sup> (Ronkin and Buretz, 1960), we predict  
308 that Na<sup>+</sup> ions from the wash buffer are entering the cytosol when Na<sup>+</sup>/H<sup>+</sup> exchangers are  
309 removing H<sup>+</sup>. To test this, we resuspended WT cells in 10 mM, pH 8 EGTA buffered by NaOH  
310 (final ~21 mM Na<sup>+</sup>). Concentrated EGTA and EDTA are classical tools for creating permeable  
311 cell models perhaps by Ca<sup>2+</sup> chelation or other mechanisms (Miller, 1979; Tazawa and Shimmen,  
312 1983; Arikawa and Suzuki, 2002; Prachayasittiku et al., 2007). However, EGTA at this  
313 concentration does not influence polymerization of MTs from purified tubulins *in vitro* (Olmsted  
314 and Borisy, 1975). As expected, thick EB1-decorated MTs formed after cells were resuspended  
315 in Na<sup>+</sup>/EGTA for 5 min (Figure 5b, left panels). Thick MTs appeared static after 10 mins. In  
316 contrast, cells resuspended in KOH-buffered EGTA had vibrant comet activities (right panels).  
317 The static EB1-binding MTs in Na<sup>+</sup>/EGTA treated cells were also cold-resistant.

318 As K<sup>+</sup>/EGTA did not affect comet activity, we depleted [Ca<sup>2+</sup>]<sub>ex</sub> with K<sup>+</sup>/EGTA rather  
319 than Na<sup>+</sup>/EGTA to test if Ca<sup>2+</sup> is required for the changes elicited by 10 mM HA. After 5 min in  
320 K<sup>+</sup>/EGTA, 10 mM HA resuspension still elicited the bird cage pattern in *tub2* cells, and caused  
321 comets to vanish in WT cells. However, cells quickly burst, indicating perturbed plasma  
322 membrane. Thus HA-elicited changes in MTs do not require extracellular Ca<sup>2+</sup>.

323 Despite low Na<sup>+</sup> permeability of the plasma member, we further tested if boosting Na<sup>+</sup> in  
324 HEPES buffer to 55 mM with NaCl was sufficient to change EB1 patterns in WT transgenic  
325 cells. Indeed, compared to the growing MTs with typical comets in the control cells in the 5 mM  
326 group, in the 55 mM group comets were evidently but MTs were growing (panel I). This is  
327 reflected by the modest tapering of comet intensity in the linescan plot (panel II). Therefore, high  
328 [Na<sup>+</sup>]<sub>ex</sub> still could raise [Na<sup>+</sup>]<sub>in</sub>, increasing the time EB1 spent at the plus end and thus comet  
329 lengths. Despite the long comets, the distribution of comet velocity (panel III) and the average  
330 velocity (panel IV) show that [Na<sup>+</sup>]<sub>in</sub> at this level only slightly reduces MT growth rate (two-  
331 tailed p-value = 0.0497, < 0.05). Thus both comet length and MT growth are sensitive to [Na<sup>+</sup>],  
332 albeit the former has a higher sensitivity.

333 To test the effect of hypertonicity, WT transgenic cells were resuspended in 150 and 200  
334 mM [Na<sup>+</sup>]<sub>ex</sub> for 5 min before imaging. At these concentrations, flagellar motility ceased. As in 75  
335 mM [Ca<sup>2+</sup>]<sub>ex</sub> solution (Figure 2g), most comets vanished, although static EB1 signals remained at  
336 the BB area (Figure 5d). However, some cells still contain thick fibers (top panel), a signature of  
337 Na<sup>+</sup>-induced changes. Together, these results show that low Na<sup>+</sup> and high Na<sup>+</sup> elicit disparate  
338 MT responses. The modest increase of [Na<sup>+</sup>]<sub>in</sub> from 55 mM [Na<sup>+</sup>]<sub>ex</sub> alone promotes long comets  
339 but is insufficient to stop MT growth. The fact that both responses are triggered by HA bath  
340 followed by 5 mM Na<sup>+</sup>/HEPES wash or by 21 mM Na<sup>+</sup>/EGTA strongly suggests [Na<sup>+</sup>]<sub>in</sub> rises  
341 further to a level under 21 mM due to H<sup>+</sup>/Na<sup>+</sup> exchange or passive diffusion through EGTA-  
342 permeabilized membrane. However, most EB1 signal vanished, suggesting MT disassembly at  
343 the concentrations that deem hypertonic for *Chlamydomonas* either via [Na<sup>+</sup>]<sub>ex</sub> and/or [Ca<sup>2+</sup>]<sub>ex</sub>.



344

**Figure 5.** Na<sup>+</sup>-dependent changes of the MT system. **(a)** MTs in cells were largely frozen after 5-min 10 mM pH3 HA bath and 3 min in the wash solution, such as 5 mM pH7.4 Na<sup>+</sup>/HEPES buffer or 5 mM NaCl solution (black arrowheads). In contrast, growing MTs with a comet (white arrowheads) returned if the wash buffer lacked Na<sup>+</sup>, such as 5 mM K<sup>+</sup>/HEPES buffer, 5 mM KCl solution, or the double distilled water (ddw). **(b)** Thick MTs in cells resuspended in 21 mN Na<sup>+</sup>/EGTA for 5 min or 10 min (left panel), contrary to comets in cells in 21 mM K<sup>+</sup>/EGTA (right panel). Thick MTs were still growing after 5 min incubation but static after 10 min incubation **(c)** High [Na<sup>+</sup>]<sub>ex</sub>, without preexposure to HA, was sufficient to alter comet patterns. Contrary to typical comets in cells resuspended in the HEPES buffer with 5 mM Na<sup>+</sup>, long comets were thick in cells resuspended in 55 mM Na<sup>+</sup> for 5 min (panel I). Normalized linescans confirmed little tapered intensity (panel II, n=36 comets from 11 cells in 5 mM Na<sup>+</sup>; n=13 comets from 4 cells in 55 mM Na<sup>+</sup>). As shown in the range of speed (panel III) the long comets were moving, and the mean speeds of short and long comets were significantly different (panel IV, n=51 from 11 cells in 5 mM Na<sup>+</sup>; n=18 from 4 cells in 55 mM Na<sup>+</sup>) (P < 0.05). **(d)** Two representative cells after 5 min in 150 mM Na<sup>+</sup>/HEPES. Some cells still retained a few thick MTs (cell I). Some only had static EB1 signals at the BB area (cell II, arrowhead). Scale bars, 5 μm.

345

## 346 Discussion

347

348 Expression of EB1-NG as a reporter loosens the bottle neck posed by autofluorescence of  
349 *Chlamydomonas* and unleashes the potential of this MT model system. Contrary to the perceived  
350 stability of the MT system in typical interphase animal cells (Lieuvin et al., 1994), EB1-NG  
351 reports in real time the mercurial changes of algal MT system that are elicited by excitation  
352 illumination, compression, H<sup>+</sup> and Na<sup>+</sup>. We summarize the changes and discuss possible  
353 underlying mechanisms; and the new insight on the MT system, plant salinity responses and  
354 additional concerns of environmental stresses.

355

### 356 *Changes elicited by H<sup>+</sup>*

357 The changes of algal MT system elicited by intracellular acidification are swift, stunning and  
358 novel (Figure 2b-e, 3a and 4). Among heightened shank binding, diminished comet activity,  
359 paused MT growths, and MT shortening, the bird cage pattern of shank binding is the most  
360 sensitive, elicited reliably by 5 mM HA (Figure 4d). They are unlikely signaled through cell  
361 death pathways, since these changes are reversible, even after 5 min HA bath (Figure 2b, 3). The  
362 recovery occurs within a minute, either completely (Figure 2b) or protractedly (Figure 3e),  
363 depending on exposure and wash procedures. Extracellular Ca<sup>2+</sup> is not required for the HA-  
364 induced changes, since they still occur after K<sup>+</sup>/EGTA treatment. However, we cannot rule out  
365 the involvement of Ca<sup>2+</sup> released from intracellular storages and other signaling pathways.

366 Although the pH of [HA]<sub>ex</sub> that triggers these changes is ~3, we expect that the resulting  
367 intracellular pH is close to or higher than 6.3. HA-induced changes in diffusion chambers appear  
368 before deflagellation (Figure 2e) that occurs at pH 6.3 (Wheeler et al., 2008; Braun and  
369 Hegemann, 1999). Consistent with this, the bird cage pattern could be transiently triggered  
370 merely by illumination that may open H<sup>+</sup>-selective channelrhodopsin (Figure 1f1). Conversely,  
371 comets return to compressed cells when illumination is turned off for 30 seconds (Figure 1f2); or

372 return within ~ 45 sec after HA is washed away (Figure 2b). These observations strongly suggest  
373 that a slight imbalance of pH homeostasis is sufficient to elicit changes in algal MT system.

374 Although pH affects proteins' ionization and thus their functions and protein-protein  
375 interactions in general (Hepler, 2016), we speculate that the MT system is particularly sensitive  
376 to declining pH because of the acidic pI of tubulins and EB1. For example, the respective pI of  
377 *Chlamydomonas*  $\alpha$ -tubulin,  $\beta$ -tubulin and EB1 is 5.01, 4.82, and 5.7. A decrease of pH from the  
378 resting level will make these proteins less negatively charged, especially at their C-terminal  
379 acidic tails that are central to MT-accessory protein interplays and the targets of various post-  
380 translational modifications (reviewed by Song and Brady, 2014; Buey et al., 2011; Rovini et al.,  
381 2013). The resulting decreased repulsion could explain increased affinity of EB1 to GDP-tubulin,  
382 leading to nearly immediate appearance of the bird cage pattern in *tub2* cells (Figure 4a-b) or in  
383 WT cells exposed to 5 mM HA (Figure 4d). As pH descends further, additional changes in  
384 protein conformation may inhibit the growth of MTs and EB1 binding to plus ends, leading to  
385 comet reduction or ultimate disappearance.

386 These HA-induced changes explain long standing questions regarding pH variations. For  
387 example, the pollen tube tip has overlapping regions. The MT zone, in particular, lags behind the  
388 F-actin,  $\text{Ca}^{2+}$  and acid zone (Gibbon and Kropf, 1994; reviewed by Hepler, 2016). Likewise, a  
389 basic shift directs MT-supported fertilization processes of sea urchins, whereas depressing pH  
390 inhibits the processes and triggers MT disassembly (Schatten et al., 1985). This and an increase  
391 of 0.3-0.5 pH unit in mitosis inspired the pH clock hypothesis for cell cycle control (Gagliardi  
392 and Shain, 2013). In line with this, EB1 preferentially binds to MT plus ends in arrested mitotic  
393 phase extract of *Xenopus* oocytes, but uniformly decorates MTs in interphase extract (Tirnauer et  
394 al., 2002) perhaps with a lower pH, analogous to HA-induced bird cage pattern (Figure 4c).  
395 Given the role of EB1 in recruiting effector molecules and the swiftness of pH-induced changes  
396 in MTs and EB1 patterns, tuning pH may indeed control cell cycle at least for certain organisms.

397

### 398 **Changes elicited by $\text{Na}^+$**

399 The responses elicited by  $[\text{Na}^+]_{\text{in}}$  and  $[\text{H}^+]_{\text{in}}$  at low concentrations are distinct. Contrary to instant  
400 appearance of  $\text{H}^+$ -elicited bird cage pattern with fine, individual MTs (Figure 4d), as  $\text{Na}^+$   
401 continues rising, comets lengthen; cortical MTs undergo ectopic nucleation, splitting, bundling,  
402 decelerate and stop eventually (Figure 3 and 5). Although it takes longer to elicit  $\text{Na}^+$  responses,  
403 this is likely due to low  $\text{Na}^+$  permeability. Likewise,  $\text{Ca}^{2+}$  permeability is tightly controlled. As  
404 such  $[\text{Na}^+]_{\text{in}}$  and  $[\text{Ca}^{2+}]_{\text{in}}$  cannot be adjusted as nimbly as  $[\text{H}^+]_{\text{in}}$ .

405 The degree of changes correlates with  $[\text{Na}^+]_{\text{in}}$ . These changes aggravating with time in 21  
406 mM  $\text{Na}^+$ /EGTA (Figure 5b) indicates that they are occurring before  $[\text{Na}^+]_{\text{in}}$  reaches 21 mM,  
407 which is made possible by EGTA treatment. As EGTA chelates  $\text{Ca}^{2+}$ , this further rules out the  
408 involvement of extracellular  $\text{Ca}^{2+}$ . Similarly,  $\text{Na}^+$ -dependent responses emerge within ~45  
409 seconds once HA bath is replaced with various solutions containing only 5 mM  $\text{Na}^+$  (Figure 5a),  
410 suggesting accelerated rise of  $[\text{Na}^+]_{\text{in}}$  due to the activity of  $\text{Na}^+$ /  $\text{H}^+$  exchangers (Pittman et al.,  
411 2009). On the other hand, by simply relying on limited passive diffusion through the normal  
412 plasma membrane, 55 mM  $[\text{Na}^+]_{\text{ex}}$  is sufficient to change comet length but only slows down  
413 growth rate slightly (Figure 5c). Based on the incremented responses, rather than all-or-none  
414 responses to a threshold, we speculate that algal MT system is also sensitive to  $[\text{Na}^+]_{\text{in}}$ , perhaps  
415 in a linear manner. Contrary to  $[\text{Na}^+]_{\text{ex}}$ , raising  $[\text{K}^+]_{\text{ex}}$  had no evident effect (Figure 5 a-c). This is  
416 reasonable, given high  $[\text{K}^+]_{\text{in}}$ , ~ 70 mM in *Chlamydomonas* cells (Malhotra, 1995). It highlights  
417 the selective sensitivity of the algal MT system to  $\text{Na}^+$  and rules out mere ionic effects. One

418 interesting possibility is that  $\text{Na}^+$  binds to particular sites in algal tubulins, analogous to  $\text{Ca}^{2+}$   
419 binding sites in mammalian tubulins (Solomon, 1977; Serrano et al., 1986).

420

421 ***Common changes elicited by high extracellular HA,  $\text{Na}^+$  and  $\text{Ca}^{2+}$***

422 EB1 signals largely vanish at 150 mM  $[\text{Na}^+]_{\text{ex}}$  (Figure 5d), as in 10 mM HA (Figure 2c, 3a) and  
423 75 mM  $\text{Ca}^{2+}$  (Figure 2g) except residual static signals at the BB area. They are likely caused by  
424 synergistic disassembly and paused new growth, and an immotile EB1 population underneath  
425 BBs respectively (Yan et al., 2006; Pedersen et al., 2003). The similar outcomes caused by  
426 distinct ions and obvious shrinkage of the cell body at even higher concentrations of  $\text{Na}^+$  and  
427  $\text{Ca}^{2+}$  suggest that hypertonicity is involved. We envisage that high concentration responses could  
428 be caused by one cation exceeding a threshold concentration; and/or simultaneous rises of  
429 multiple electrolytes as  $\text{H}_2\text{O}$  moves out of cells. Hypertonicity may evoke additional pathways.

430 The capture of endwise resorption only in low concentration conditions (Figure 2d and  
431 4d) suggests that increased concentrations of these ions will heighten shortening- the incidence  
432 and/or speed. This is reminiscent to high  $\text{Ca}^{2+}$  effects. *In vitro*,  $\text{Ca}^{2+}$  blocks MT formation  
433 (Weisenberg, 1972), whereas 0.5-0.6 mM  $\text{Ca}^{2+}$  - in the absence of MAPs - could increase  
434 shortening incidence and accelerate shortening speed of MTs beyond 150  $\mu\text{m}/\text{min}$  (Karr et al.,  
435 1980; O'Brien et al., 1997). Although pH shock and high  $[\text{Ca}^{2+}]_{\text{ex}}$  only temporarily raise  $[\text{Ca}^{2+}]_{\text{in}}$   
436 up to 1  $\mu\text{M}$  (Wheeler et al., 2008), the lower concentration may be sufficient to heighten  
437 shortening. However, this possibility is weakened by the same result despite EGTA treatment.

438 Other proteins and signaling pathways are involved in the electrolyte-elicited responses.  
439 Kinesin-13 that catalyzes MT disassembly at a lower speed (Helenius et al., 2006) becomes  
440 phosphorylated within 5 min following pH shock; and knockdown of kinesin-13 ameliorated the  
441 pH shock-induced reduction in MT lengths and numbers (Wang et al., 2013). Although EB1  
442 patterns appear fully recovered 1 min after a pH pulse (Figure 2b), quantitative analysis and  
443 using similar reporters are needed to compare the results. Similarly, osmotic or salt stresses  
444 activate an atypical tubulin-kinase and phospholipase D to trigger disassembly or reorganization  
445 of plant MT system (Fujita et al., 2013; Dhonukshe et al., 2003). For yeast, sorbitol hypertonicity  
446 induces frozen MTs (Robertson and Hagan, 2008) and the recovery in 38 mins involves a stress-  
447 induced MAP kinase. Similar paradigms may be responsible for the resumption of MT dynamic  
448 in HA-bathed algae that takes  $\sim 55$  mins (Figure 3f).

449 The electrolyte sensitivity of algal MT system is contrary to the perceived stable MT  
450 system in interphase mammalian cells (Lieuvin et al., 1994) that have 140 mM  $[\text{Na}^+]_{\text{ex}}$ , and 140  
451 mM  $[\text{K}^+]_{\text{in}}$  (reviewed by Pohl et al., 2013). Consistent with this, we cannot elicit any obvious  
452 changes of EB1-EGFP patterns in mammalian epithelial cells by compression or illumination  
453 (Matov et al., 2010). Likewise, both  $\text{Na}^+$  and  $\text{K}^+$  promote tubulin polymerization, with 160 mM  
454  $\text{Na}^+$  as the optimal condition (Olmsted and Borisy, 1975). Different cation sensitivities could be  
455 due to sequence divergence. The other is the presence of different accessory proteins. MAPs  
456 obscure the cation sensitivity of mammalian MTs (Olmsted and Borisy, 1975; Wolff et al.,  
457 1996). Alternatively, signaling pathways or the capacity to maintain electrolyte homeostasis  
458 could differ. Thus while fundamental features of the MT system - likely dynamic instability and  
459 EB1 plus end tracking - are universal, electrolyte sensitivity and responses could diverge.

460 The sensitivity of algal MT system to  $\text{Na}^+$  is consistent with enlarged or clustered algal  
461 cells cultured in high salt media (Takouridis et al., 2015). This could be caused by anomalies in  
462 the MT-supported processes in the cell cycle, such as mitosis and trafficking-dependent release  
463 of hatching enzymes (Kubo et al., 2009). Yet this fresh water green alga has several strategies to

464 adapt to salinity (Perrineau et al., 2014), such as glycerol production (Husic and Tolbert, 1986),  
465 switches in gene expression (Gao et al., 2016), sexual reproduction and mutations (Takouridis et  
466 al., 2015). Salinity adaptation and the incredible  $H^+$  and  $Na^+$  sensitivity of algal MT system that  
467 bears semblance to that in both animal and plant cells demand a fresh look at how environmental  
468 stresses affect diverse organisms.

469 One is ocean acidification by anthropogenic  $CO_2$  (Raven et al., 2005). Hindered shell  
470 formation from the resulting extracellular acidification with pH declining by merely 0.1 unit has  
471 rightfully raised much alarm (Hoegh-Guldberg, et al., 2007; Waldbusser et al., 2013; Fitzer et al.,  
472 2016). Similarly, 5 mM and 7.5 mM HA differ 0.09 pH unit based on the  $pK_a$  of HA and the  
473 Henderson-Hasselbalch equation, but the intracellular acidification from this slight difference  
474 triggers distinct changes of *Chlamydomonas* MT (Figure 4d). Notably, dictated by  $CO_2$   
475 chemistry, the ratios of permeant  $CO_2$  and  $H_2CO_3$  to non-permeant ionic forms will increase  
476 further as solutions acidify, aggravating intracellular acidification. Therefore, intracellular  
477 acidification could be equally, if not more, insidious to marine species that are not equipped to  
478 cope with this stress; and may poise to shape aqueous landscapes and drive evolution (Cannon et  
479 al., 1985).

480 The other is salt stress and osmotic stress caused by draught, which is exacerbating due to  
481 climate changes or improper agriculture practices. The  $H^+$ -enhanced,  $Na^+$ -dependent responses  
482 of algal MTs (Figure 3a, 5) - bundling, cold-resistance and growth inhibition - resemble salinity-  
483 elicited changes in plants (e.g. Dhonukshe et al., 2003; Shoji et al., 2006; Wang et al., 2007;  
484 Zhang et al., 2012; Fujita et al., 2013; reviewed by Hardam, 2013; Hashimoto, 2015; Oda, 2015).  
485 The similarity comports with their common cortical MTs and a great homology of their proteins  
486 in the MT system (Dymek and Smith, 2006; Pedersen et al., 2003; review by Gardiner, 2013)  
487 except flagellar genes (Merchant et al., 2007). MT changes induced by various abiotic stresses,  
488 including salt, have prompted an interesting proposition that the MT system is an abiotic sensor  
489 of plant cells (Haswell and Verslues; 2015; Wang et al., 2011). Yet the involvement of signaling  
490 pathways and slow readouts in plant experiments question whether the changes of MTs are the  
491 consequence of salt stress instead. The EB1-reported, scaled responses that seem proportional to  
492  $[Na^+]_{in}$  and the speed of manifestations (Figure 4d and 5) in fresh water algae strengthen the  
493 possibility that plant MT system is an upstream player in the salinity signaling pathways, if not  
494 the very sensor. In line with this, channel-linked MTs are integral to osmolarity signaling  
495 transduction in mammalian osmosensory neurons (Prager-Khoutorsky et al., 2014). Using the  
496 experimental strategies developed in this study, it is possible to investigate quantitatively the  
497 diverged mechanisms of eukaryotic MT systems in sensing and responding to salt stresses.

498

## 499 **Materials and Methods**

500

### 501 ***Chlamydomonas* Strains and Culture**

502 The wild type strain CC-124 and a  $\beta$ -tubulin mutant *tub2-1* (formerly *col<sup>R4</sup>*) (Bolduc et al. 1988;  
503 Lee et al. 1990; Schibler et al. 1991) were from *Chlamydomonas* Resource Center  
504 (<http://www.chlamycollection.org/>). They were converted into EB1-NG transgenic strains as  
505 described (Harris et al. 2016). Cells were cultured in 300 ml pH7.0 standard  
506 tris/acetate/phosphate (TAP) liquid media with aeration at 25°C over a 14/10 light/dark cycle  
507 until reaching logarithmic phase of growth ( $5-10 \times 10^6$  cells/ml) (Sivadas et al., 2012). All  
508 experiments were completed at least 2 hours before the onset of the dark period.

509

## 510 **Solutions**

511 Glacial acetic acid (HA) was diluted with ddw to various concentrations ranging from 5 to 1000  
512 mM. The 10 mM HCl solution was titrated to pH3 with 1 M NaOH. The 1000 mM HA was  
513 added to the TAP medium to make 20 mM pH4.5 HA/TAP. For 5 mM Na<sup>+</sup>/HEPES and  
514 K<sup>+</sup>/HEPES, pH of 10 mM HEPES was adjusted to 7.4 with NaOH or KOH respectively. To  
515 make 21 mM Na<sup>+</sup>/EGTA and K<sup>+</sup>/EGTA solutions, 10 mM EGTA was titrated to pH8 with  
516 NaOH or KOH. The 5 mM NaCl and KCl solutions were made by dissolving the respective salt  
517 in ddw. The 5, 55, and 150 mM Na<sup>+</sup>/HEPES solutions were made by adding NaCl into 5 mM  
518 pH7.4 Na<sup>+</sup>/HEPES. The solutions of 30 and 75 mM Ca<sup>2+</sup>/HEPES were made by dissolving CaCl<sub>2</sub>  
519 in 5 mM pH7.4 Na<sup>+</sup>/HEPES.

520

## 521 **Live Cell Imaging and Treatments**

522 EB1-NG in live *Chlamydomonas* cells was imaged with Nikon Eclipse widefield microscope  
523 equipped with a short-arc mercury lamp, an FITC-HYQ optical filter set, a CoolSNAP-ES CCD  
524 camera and MetaMorph software. Each image was captured as a 16-bit grayscale file with 1  
525 second exposure. Streaming videos were recorded for 100 frames at a rate of 1 frame/second.

526 Typically cells were resuspended in solutions for 5 min unless indicated otherwise. An  
527 aliquot of 5  $\mu$ l cell suspension was placed on a slide and then covered by an 18 X 18-mm<sup>2</sup> cover  
528 slip. The edges were sealed with nail polish before imaging. For compression experiments, a 3  $\mu$ l  
529 aliquot of cell suspension was placed on a glass slide and then covered with a 22 X 22-mm<sup>2</sup>  
530 cover slip. Cells became gradually compressed by the coverslip as evident by flattened cell body.  
531 For pH pulse in a perfusion chamber, an aliquot of 10  $\mu$ l cells in the TAP medium was placed on  
532 a cover slip pre-coated with 5  $\mu$ l 0.001% poly-L-lysine. The cover slip was then inverted to  
533 assemble a perfusion chamber as shown in Figure 2a. The chamber was flushed with 200  $\mu$ l 20  
534 mM pH4.5 HA/TAP. Subsequently, HA/TAP was replaced by a flush of 200  $\mu$ l TAP. The entire  
535 process was recorded in two consecutive live-stream clips. For this long recording duration,  
536 excitation light intensity was reduced to 25% with a neutral density filter. For HA pulse in a  
537 diffusion chamber, 40  $\mu$ l cells in 5 mM pH7.4 Na<sup>+</sup>/HEPES was placed at one side of a diffusion  
538 chamber underneath a 40X objective lens (Figure 2a). A live-streaming video was recorded  
539 following the injection of 20  $\mu$ l 100 mM pH2.8 HA through the Vaseline wall to the opposite  
540 side of the chamber. For HA bath, a cell pellet from 50  $\mu$ l liquid culture was resuspended in 50  
541  $\mu$ l 10 mM pH3 HA. An aliquot of 10  $\mu$ l cell mixture was placed on a cover slip. The cell-loaded  
542 cover slip was inverted to create a perfusion chamber. After a total 5-min exposure to HA, HA  
543 was flushed away with an aliquot of 200  $\mu$ l-indicated fluid and then a video was recorded. To  
544 test MT cold lability after recovery from HA bath, a perfusion chamber with treated cells was  
545 chilled by ice for 3 minutes. A video was taken immediately afterwards, ~ 20 seconds after the  
546 chamber was removed from ice. Each treatment was repeated at least twice in each experiment.  
547 Individual experiments were repeated independently 3 times at least.

548

## 549 **Image Analysis**

550 To measure EB1 comet speed, a 40-second substack containing side views of cells were first  
551 made by the open source image process software, ImageJ (<https://imagej.nih.gov/ij/index.html>);  
552 and individual comets were analyzed with a Matlab-based particle tracking software,  
553 plusTipTracker (Applegate et al. 2011). In each cell that maintained completely quiescent for the  
554 tracking period, all tractable comets which transverse at least one third of the cell length were  
555 analyzed. The numbers of qualified cells and comets from numerous recordings were indicated.

556 To generate line scans of EB1 intensity at microtubule plus ends, a line tool in ImageJ was used  
557 to measure gray values along the length of comets. Relative fluorescence intensity was  
558 normalized after calculation by subtracting a background gray value measured next to the comet  
559 with the line tool. Histograms were generated with the Microsoft program, Excel. Kymographs  
560 were generated with an ImageJ plug-in multiple kymograph  
561 ([https://www.embl.de/eamnet/html/body\\_kymograph.html](https://www.embl.de/eamnet/html/body_kymograph.html)).

562

### 563 **Statistical Analysis**

564 All data are given as mean±SEM (standard error of the mean) and analyzed with Sigmaplot 13.0  
565 (Systat Software, Inc., San Jose, CA). Sample sizes for comet speed measurement are limited by  
566 the fact that few cells are entirely quiescent, which is necessary for digital tracking.

567

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570

### 571 **Competing Interests**

572 The authors declare that no competing interests exist.

573

### 574 **Supplemental data**

575 Video 1-1 (for Figure 1b) EB1-NG comets in WT cells.

576 Video 1-2 (for Figure 1f1) Transient bird-cage pattern in WT cells that occurred sporadically  
577 during imaging.

578 Video 1-3 (for Figure 1f2) Disappearance and return of comets in compressed cells following  
579 alternate periods of illumination and darkness.

580 Video 3 (for Figure 3a) WT cells in Na<sup>+</sup>/HEPES after 5-min HA bath.

581 Figure 4-1 (for Figure 4b) *tub2* cells in HA bath.

582 Video 4-1 (for Figure 4b) *tub2* cells in Na<sup>+</sup>/HEPES after 5-min HA bath.

583 Video 4-2 (for Figure 4d). WT cells treated with 5 mM HA.

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