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1 **A novel benzodiazepine derivative that suppresses microtubules dynamics and impairs**
2 **mitotic progression**

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22 **Running title:** A novel anti-mitotic benzodiazepine derivative

23 **Key words:** microtubules, mitosis, anti-mitotic drug, microtubule dynamics, SAC

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26
27 **Summary Statement:** identification of a novel promising antimitotic drug with the unique
28 properties altering microtubules growth and mitotic spindle organization.
29

30 **Abstract**

31 A novel 2,3-benzodiazepine-4 derivative, named 1g, has recently been shown to function as
32 an anti-proliferative compound. We now show that it perturbs the formation of a functional
33 mitotic spindle, inducing a spindle assembly checkpoint (SAC)-dependent arrest in human
34 cells. Live analysis of individual microtubules indicates that 1g promotes a rapid and
35 reversible reduction in microtubule growth. Unlike most anti-mitotic compounds, 1g does not
36 interfere directly with tubulin, nor perturbs microtubules assembly *in vitro*. The observation
37 that 1g also triggers a SAC-dependent mitotic delay associated with chromosome segregation
38 in *Drosophila* neural stem cells, suggests it targets a conserved microtubules regulation
39 module in human and flies. Altogether, our results indicate that 1g is a novel promising
40 antimitotic drug with the unique properties altering microtubules growth and mitotic spindle
41 organization.

42

43

44 **Introduction**

45 Microtubules are composed of α - and β -tubulin heterodimers whose assembly is highly
46 dynamic, undergoing stochastic phases of growth and shrinkage, a process called dynamic
47 instability (Mitchison and Kirschner, 1984). The microtubule network serves diverse and
48 specific functions in differentiated cells such as scaffold and cargo transport. As the cell
49 proceeds through mitosis, the microtubule network undergoes a dramatic reorganization to
50 build a mitotic spindle used to equally segregate chromatids in the two daughter cells. *In vivo*,
51 microtubules nucleation, organization as well as their intrinsic dynamic instability properties
52 are regulated by microtubule-associated proteins (MAPS). The influence of MAPs on the
53 dynamic assembly of microtubules contributes to the microtubules assembly patterns required
54 for the proper formation of the mitotic spindle (Desai and Mitchison, 1997; Prosser and
55 Pelletier, 2017).

56 The dynamic properties of mitotic spindle microtubules are required for fast and
57 amphitelic attachment of sister kinetochores to the opposite spindle poles, an event essential
58 to avoid missegregation of chromatids during anaphase and subsequent aneuploidy. In case of
59 erroneous attachments of chromosomes to spindle microtubules, the spindle assembly
60 checkpoint (SAC) remains unsatisfied and inhibits anaphase onset through inhibition of the
61 Anaphase Promoting Complex/Cyclosome (APC/C). Cells are then delayed in mitosis until
62 all defective attachments have been corrected (Khodjakov and Rieder, 2009).

63 The central role of microtubules in orchestrating cell division together with the property of
64 SAC-dependent mitotic delay have made microtubules a target of choice for cancer
65 chemotherapeutic agents aiming to block cell division (Janssen and Medema, 2011).
66 Microtubules binding compounds that block microtubule dynamic, such as Vinka alkaloid
67 and taxanes, have emerged as efficient anti-tumor agents and are currently used in therapeutic
68 treatments. However, their use leads to severe side effects including neuropathy. Moreover,
69 between tumors a variability in the response to the treatment with these compounds is
70 observed. The identification of new anti-mitotic drugs with alternative modes of actions thus
71 remains a priority for anti-cancer research (Dumontet and Jordan, 2010).

72 Activation of the AMPA receptor by glutamate has long been known to enhance cell
73 proliferation (Stepulak et al., 2014). A recent screen to identify new anti-proliferative
74 compounds has highlighted the non-competitive AMPA receptor agonist derivate 1-(4-amino-
75 3, 5-dimethylphenyl)-3,5-dihydro-7,8-ethylenedioxy-4 h-2,3-benzodiazepin-4-one (called
76 here after *Ig*) as a potent novel growth inhibitor (Parenti et al., 2016). Upon treatment with
77 *Ig*, human leukemia T cells accumulate with a 2N DNA content, suggesting an arrest of cells

78 at the G2/M phase of the cell cycle. In the current study, we have thus evaluated the potential
79 of 1g as a novel promising and efficient anti-mitotic drug with a unique effect on [microtubule](#)
80 [growth](#).

81

82 **Results**

83 ***1g* arrests cells in mitosis**

84 A previous study has indicated that 1g induces an accumulation of Jurkat cells at the
85 G2/M phase of the cell cycle (Parenti et al., 2016). To confirm the effect of 1g on the
86 proliferation of transformed human epithelial cells, we followed an asynchronous population
87 of HeLa cells by phase contrast time-lapse microscopy for 20hrs (Fig. 1A; Movies S1 and S2).
88 Control cells progressively rounded up to enter mitosis, divided and spread back down over
89 this period of time, increasing the total number of cells. On the contrary, cells treated with 1g
90 similarly rounded up, but no cell division was observed, suggesting that cells remained
91 arrested in prometaphase and were unable to progress into anaphase and complete their
92 division. This progressive accumulation of rounded cells was observable in a dose dependent
93 manner with an optimal 1g concentration of 2.5 μ M (Fig. 1B,C).

94 To evaluate whether cells treated with 1g display a cell division defect, we synchronized
95 HeLa cells at the G2/M transition using the CDK1 kinase inhibitor RO3306 (Vassilev, 2006),
96 then released them in presence of DMSO (control) or 1g and followed their progression
97 through mitosis (Fig. 1D,E). Their mitotic stage was defined by staining the cells for tubulin
98 and DNA. As expected, control cells reached the metaphase-anaphase transition within 45
99 min and 79% of the cells were in telophase at 90 min post-release. In contrast, only 6% of
100 1 μ M 1g treated cells had passed the metaphase-anaphase transition by 90 min and none of the
101 dividing cells exposed to 2.5 μ M and 5 μ M 1g had reached anaphase. Furthermore, dividing
102 cells treated with 2.5 μ M and 5 μ M 1g remained in prometaphase, displaying an abnormal
103 mitotic spindle (Fig. 1D-lower right panel). Whereas control cells were able to fully congress
104 their chromosomes to the metaphase plate within 45 min, cells treated with 1g still displayed
105 major congression defects at 90 min (Figs 1D,F, 2B,D). This failure to establish a metaphase
106 plate was associated with the detection of the SAC component BUBR1 on the kinetochore in
107 agreement with an activation of the spindle assembly checkpoint (Fig. 1F). The drastic
108 increase in the mitotic index (Fig. 1C) of the asynchronous cell population after 16 hrs
109 exposure to 1g implies that at high drug concentration, cells remain blocked in mitosis thru
110 time (82 \pm 5 % in 2.5 μ M 1g vs 7.4 \pm 0.81 % in DMSO). Instead, the more modest increase in

111 mitotic index (26.8 ± 0.8 % in $1 \mu\text{M}$ 1g vs 82.0 ± 5.0 % in $2.5 \mu\text{M}$ 1g) when cells are exposed to
112 $1 \mu\text{M}$ 1g suggests that at suboptimal 1g concentrations, cells can progress through mitosis but
113 with extensive delay. Indeed, anaphase figures could be observed 4 hrs post-released in cells
114 treated with $1 \mu\text{M}$ 1g, but not with higher concentrations (data not shown).

115 To confirm that the accumulation of rounded cells observed in the asynchronous cell
116 population treated with 1g (Fig. 1A) was indeed due to the activation of the SAC making the
117 cells unable to exit mitosis, HeLa cells were treated with 1g alone or in combination with the
118 SAC inhibitor AZ3146 (Tipton et al., 2013), and followed by videomicroscopy. In presence
119 of AZ3146, 1g treated cells progressively rounded up, but unlike cells treated with 1g alone,
120 proceeded through a defective anaphase-telophase and spread back down. It is interesting to
121 note that these cells displayed no cytosolic sign of cell death throughout the 16hrs of the
122 treatment with both inhibitors (Fig. 1G and Movies S3 and S4). This data indicate that 1g
123 impacts cell proliferation via mitotic arrest and not direct cell death. However, prolong
124 mitotic arrest has been shown to result in subsequent cell death (Brito and Rieder, 2006;
125 Stanton et al., 2011) explaining the previously described apoptotic effect of the 1g
126 compound observed after 48hrs of treatment (Parenti et al., 2016). All together these
127 experiments demonstrate that treatment of proliferating HeLa cells with 1g activates the SAC,
128 arresting the cells in prometaphase.

129 **1g disrupts the formation of the mitotic spindle**

130 The disorganized mitotic spindle observed in 1g treated cells, prompted us to further
131 characterize the dynamics of the mitotic spindle assembly. To that purpose, we imaged by
132 spinning microscopy live HeLa cells expressing GFP-tubulin after their release from the
133 CDK1 inhibitor induced G2/M arrest (Fig. 2A,B movies S5, S6 and S7). Control cells treated
134 with DMSO rapidly separated their spindle poles and 100% of the cells formed a bipolar
135 spindle within 15 ± 4 min (Fig. 2C). All cells achieved full chromosome congression into a
136 metaphase plate and progressed into anaphase in an average time of 55 ± 7 min (Fig. 2B,D).
137 When treated with low 1g concentrations ($1 \mu\text{M}$), 90% of the cells formed a bipolar spindle
138 within 28 ± 13 min. However, at 80 min post NEB, 90% of $1 \mu\text{M}$ treated cells still displayed
139 unaligned chromosomes and thus had not satisfied the SAC (arrow head fig 2B). At higher 1g
140 concentrations (2.5 - $5 \mu\text{M}$), several microtubule nucleation sites could be observed and the
141 microtubule growth from centrosomes was strongly reduced (Fig. 2A -arrow heads). Albeit
142 clustering of the microtubule asters could be observed, in 70% of the cells exposed to $2.5 \mu\text{M}$

144 1g and in 100% of those treated with 5 μ M, 3 asters or more were present and strong
145 chromosome congression defects were observed at 80min post NEB (Fig. 2B arrows, D, E).
146 No bipolar mitotic spindles were formed at those concentrations (Fig. 2C). Visualization of
147 the centrosomes, using HeLa cells expressing the centriolar protein centrin-GFP, confirmed
148 that microtubule nucleation emanated from the two centrosomes. However, the additional
149 small asters observed did not contain centrin-GFP, indicating that 1g did not trigger
150 centrosome amplification (Fig. 2G). In addition, a decrease of 25% (1 μ M 1g) and 37% (2 μ M
151 1g) in spindle poles distance compared to control cells was observed (Fig. 2F). The defect in
152 centrosome separation was further accentuated at 5 μ M, with 64% decrease in distance
153 between centrosomes in cells treated with 5 μ M 1g as compared to DMSO (Fig. 2H). The
154 formation of shorter microtubules observed in presence of 1g most likely accounts for the
155 reduced centrosome separation, since centrosome separation is a microtubule dependent
156 process occurring in prophase and prometaphase (Wittmann et al., 2001).

157

158 **1g alters microtubule growth in cells**

159 The dynamic properties of microtubules are crucial for the extensive remodeling of
160 interphase arrays of microtubules into a mitotic bipolar spindle (Desai and Mitchison, 1997).
161 We thus investigated whether 1g interferes with microtubule polymerization. We first
162 performed a depolymerization-repolymerization type of assay (Fig. 3A). The microtubule
163 network of interphase cells was depolymerized at 4 $^{\circ}$ C in presence or absence of 1g. We then
164 followed the dynamics of microtubules regrowth as the samples were returned to 37 $^{\circ}$ C. Long
165 newly nucleated microtubules could be observed 5 min after the temperature switch in control
166 cells, and extensive microtubule repolymerization was present after 10 min. Presence of 1g
167 did not affect cold induced depolymerization of microtubules, indicating that 1g does not act
168 as a microtubule stabilizing agent (Fig. 3A $t=0$ and G). Indeed, in presence of stabilizing
169 agents, such as taxan, cold resistant microtubules bundles can be detected (Stanton et al.,
170 2011). However, 1g markedly slowed down the dynamics of the microtubule network
171 reformation (Fig 3A $t=5,10$ and 15min). Only short and fragmented microtubules were
172 observed in 1g treated cells in the first 10 min, and it required 30 min to polymerize a
173 microtubule network equivalent to the one formed after 15 min in control cells.

174 The growing microtubule +tip protein EB1 has been commonly used to image microtubule
175 + ends and to quantify their dynamics (Matov et al., 2010). To further investigate and
176 quantify the effect of 1g on microtubule dynamics, we imaged HeLa cells expressing EB1-

177 GFP by spinning disk confocal microscopy at 1 sec time intervals before and immediately
178 after addition of *Ig* or DMSO (Fig. 3B and movies S8, S9). In control HeLa cells,
179 measurements of EB1 comets velocity indicated a microtubules growth speed of 16.6 ± 0.83
180 $\mu\text{m}\cdot\text{min}^{-1}$. Whereas addition of DMSO in control cells did not significantly alter microtubule
181 growth speed, *Ig* treatment resulted in 25% decrease in mean growth rate (from 14.2 ± 0.3
182 before to $10.6 \pm 0.3 \mu\text{m}\cdot\text{min}^{-1}$ *Ig* $2.5\mu\text{M}$) (Fig. 3C,D) without affecting the median growth
183 lifetime. Furthermore, the initial microtubule growth pattern (before treatment, Fig. 3B,C)
184 was recovered shortly after drug removal (washout, Fig. 3B,C) indicating that the action of *Ig*
185 is reversible ($15.3 \pm 1.1 \mu\text{m}\cdot\text{min}^{-1}$ after washout)(Fig. 3B,C, panels and Movies S8, S9). *Ig*
186 treatment did not significantly alter the number of nucleation events (Fig. 3E), nor the number
187 or duration of growth pauses (Fig. 3F). In control cells, we could infer a shrinkage rate of
188 $32.9 \pm 2.2 \mu\text{m}\cdot\text{min}^{-1}$, which was not statistically different in DMSO treated cells (34.5 ± 1.7
189 $\mu\text{m}\cdot\text{min}^{-1}$). In presence of *Ig*, no shrinkage events were detected. When tracking EB1 comets
190 shrinkage can only be inferred when followed by significant EB1 labelled microtubule
191 regrowth (Matov et al., 2010). The absence of detectable shrinkages in presence of *Ig* could
192 be explained by the fact that the main events occurring were terminal shortenings and
193 shrinkage followed by short or slow growth phases that did not produce detectable EB1-
194 comets within the temporal window analyzed. We thus clearly observed a slowdown in
195 microtubules polymerization in interphase cells. Altogether, these data indicate that *Ig* is able
196 to promote a fast and reversible inhibition of microtubules growth during interphase and
197 mitosis.

198 To assess whether the *Ig* compound targets directly tubulin polymerization, we tested the
199 impact of *Ig* on microtubule self-assembly *in vitro*. We performed turbidity assays classically
200 used to analyze the effect of drugs or MAPs on microtubule assembly, including microtubule
201 nucleation and elongation (Gallaud et al., 2014)(Fig. 3G). Absorbance at 350 nm, which is
202 directly proportional to the amount of microtubule polymers formed, revealed no significant
203 difference when purified tubulin was incubated in polymerizing buffer at 37°C in presence of
204 a range of *Ig* concentrations or DMSO. More specifically, we did not observe any effect of
205 the drug on nucleation (same lag-phases at different concentrations), on elongation
206 (sigmoid) phase, nor on the plateau of polymerization (total mass of microtubules assembled).
207 In addition, no significant formation of aggregates was detected when the temperature was
208 shifted back to 0°C , the average absorbance going back to the base line for all samples. The

209 absence of significant alteration in tubulin polymer assembly, or on formation of aggregates
210 indicates that 1g does not interfere with microtubule self-assembly directly.

211

212 **1g interferes with cell division in tissue**

213 During the last decades, *Drosophila Melanogaster* has emerged as an interesting model to
214 identify new genes required for cell division as well as for cancer research (Gonzalez, 2013).
215 Therefore, we have examined the impact of the compound on neural stem cells (neuroblasts)
216 division, in the developing brain of *Drosophila* larvae. Third instar larval brain expressing
217 RFP- α -tubulin and H2A-GFP were dissected and cultured in Schneider medium containing
218 DMSO, or various concentrations of 1g. For comparison, we used in parallel 20 μ M Taxol and
219 10 μ M Nocodazole treatments (Fig. 4A, S1). Control neuroblasts formed bipolar spindles and
220 started to segregate their chromosomes 5.9 \pm 0.4 min after the nuclear envelope breakdown
221 (NEB). As expected, following prolonged mitotic arrest, neuroblasts treated with Taxol or
222 Nocodazole underwent slippage (Fig. S1D,E). In presence of 1g, mitosis duration was
223 significantly increased in a dose-dependent manner (6.1 \pm 0.6 min, 8.2 \pm 0.6 min and 10.9 \pm 3.1
224 min for 5 μ M, 10 μ M and 20 μ M treatment respectively) (Fig 4D), suggesting an activation of
225 the SAC. The delay observed was in the range of the one observed in Msps-depleted
226 neuroblasts (15.5 \pm 0.8 min, $n=44$) (Fig. S1D), a MAP whose down regulation has been
227 described to severely disrupt the integrity of the mitotic spindle (Cullen et al., 1999).
228 Furthermore, in agreement with SAC activation, 1g treated neuroblasts exhibited dose-
229 dependent mitotic spindle-assembly defects (5 μ M: 35,3%, 10 μ M: 100% of neuroblasts) (Fig.
230 4B). Lagging chromatids were frequently observed in 31,3% ($n=6/17$) and 68,4 % ($n=13/19$)
231 of the neuroblasts treated respectively with 10 and 20 μ M of 1g (Fig. 4C). In 79% ($n=42$) of
232 the cells, we observed the presence of tripolar spindles and the formation of two central
233 spindles (Fig. 4A, 1g 8,9,13min, Fig. 4E,F). The outcome of mitosis was variable. Whereas
234 some cells proceed to double cytokineses leading to the formation of three daughter cells
235 (Fig.4A, 1g-dotted lines 13min), in some instances, regression of the initial double
236 cytokinesis furrow could also be observed. In either case, following defective division 1g
237 treated neuroblast progenies were able to polymerize microtubules from their interphase
238 centrosome (Fig. 4G,H).

239

240 **Discussion**

241 We here show that the anti-proliferative effect of 1g in cancer cell lines observed
242 previously (Parenti et al., 2016) is caused by interference with microtubule polymerization
243 and defective mitotic spindle assembly, leading consequently to SAC activation.
244 Noteworthy, the 1g concentrations used in cell treatments (5 μ M or less) are 20 times inferior
245 to the Kd for the AMPA receptor (Kd>100 μ M (Micale et al., 2008)), indicating that the
246 strong effects of 1g on cell division are independent of the AMPA receptor signaling
247 pathway.

248
249 Our *in vitro* assays with pure tubulin indicate that 1g does not directly target the intrinsic
250 microtubule polymerization. When used *in cellulo* or *in vivo*, we did not observe the massive
251 effects on the microtubule cytoskeleton that are triggered with microtubule binding agents
252 such as taxol or nocodazole. Instead, under 1g treatment, the overall architecture of HeLa
253 cells interphase microtubules network remains intact. Moreover, *Drosophila* neuroblasts are
254 still able to nucleate *de novo* microtubules from the daughter centrosome following division.
255 As cells proceed from interphase to mitosis a 10 fold increase in the turnover rate of
256 microtubules is required for the reorganization of the microtubule network into a mitotic
257 spindle and for the capture of chromosomes (Desai and Mitchison, 1997; Prosser and
258 Pelletier, 2017). This change in microtubule turnover rate implies that a drug that moderately
259 impedes microtubule dynamics in interphase is expected to trigger more drastic effects on
260 mitotic microtubules. Indeed, the main effect of 1g was observed during cell division in fly
261 brain neuroblasts and in HeLa cells.

262 In both systems exposed to 1g, SAC activation is observed resulting either in a mitotic
263 delay in fly neuroblasts or mitotic arrest in mammalian cells. While neuroblasts treated with
264 taxol or nocodazole that severely impairs spindle assembly remained arrested and underwent
265 mitotic slippage, the mitotic delay in fly neuroblasts treated with 1g, did not exceed 3 times
266 the duration of cell division. The presence of kinetochores unattached by the microtubules of
267 the mitotic spindle is responsible for SAC activation and mitotic arrest. In 1g treated
268 neuroblast, the observation of a mitotic delay rather than a mitotic arrest, suggests that albeit
269 displaying a tripolar shape the spindle microtubules still manage to grow and correctly attach
270 kinetochores. However, the altered microtubule growth result in a time delay to perform that
271 task and satisfy the SAC. On the other hand, HeLa cells remained arrested following 1g
272 treatment. The difference in genome size could account for this difference in 1g drug
273 response. Fly cells harbors only four chromosomes making it easier to achieve kinetochore
274 attachment under defective MT polymerization and thus satisfy the SAC and exit mitosis. By

275 contrast, SAC satisfaction is likely more problematic for HeLa cells which requires the
276 correct attachment of 70-82 chromosomes (Landry et al., 2013).

277 Nevertheless, the mitotic phenotypes are similar between neuroblasts and HeLa cells: the
278 centrosomal microtubule asters are present, but they only nucleate short microtubules.
279 Moreover, additional microtubule asters are detected, that fails to coalesce into a bipolar
280 structure. This inability to form a bipolar spindle suggests that not only the growth of MT is
281 altered but also the structure of the metaphase spindle, such as the organization/bundling of
282 the interpolar microtubules. Altogether our results support the hypothesis that 1g targets a
283 MAP involved in the regulation of microtubule growth during interphase and mitosis and
284 which is essential for mitotic spindle assembly. The similarities in the mitotic phenotypes
285 observed in HeLa cells and fly neural stem cells indicate that the 1g target and its functional
286 motifs are likely conserved between human and flies. Further studies will be needed to
287 identify the direct cellular target of 1g in order to obtain further insight on its mechanism of
288 action.

289 Microtubules targeting agents are widely used in chemotherapy, but their lack of specificity
290 for dividing tumor cells is a limitation. Indeed, their toxicity for the neural, immunological
291 and gastric systems, due to their profound effect on interphase microtubules functions as well
292 as tumor resistance, foster a need for development of new agents (Dumontet and Jordan,
293 2010; Stanton et al., 2011). Target of choice are cellular microtubules regulators that
294 modulates microtubule dynamics and organization specifically in proliferating cells. As
295 discussed above the 1g compound may comply with the requirement of new pharmaceuticals
296 compounds with a more specific mode of action.

297

298 **Materials and methods**

299 **Cell Culture**

300 HeLa Kyoto cells were grown in Dulbecco's modified Eagle's medium Glutamax (Gibco)
301 supplemented with 10% fetal calf serum (PAA), 100U/ml penicillin and 100ug/ml
302 streptomycin. For synchronization experiments, cells were treated for 16 hrs with 5 μ M RO-
303 3306 to arrest cells in G2/M, cells were then washed in complete medium and released for the
304 indicated times.

305 pEGFP-Tub (BD bioscience), pGFP- EB1 (gift from P. Chavrier, Institut Curie, Fr) and were
306 used respectively to generate, Tubulin-GFP and EB1-GFP HeLa stable cell line.

307 **Small Molecules Inhibitors**

308 Ig was synthesized by R. Ettari (University of Messina, Italy) (Parenti et al., 2016) and its
309 purity verified by ¹H NMR and ¹³C NMR. Absence of contaminants was also subsequently
310 confirmed by HPLS-MS-MS. Ig was dissolved in DMSO at a stock concentration of 50 mM.
311 Further dilutions were performed in tissue culture medium. DMSO control treatment is
312 equivalent to the amount of DMSO present in the highest Ig treatment concentration used in
313 the experiment. The final DMSO concentrations in the assays were therefore inferior or equal
314 to 0.01% in HeLa cell experiments and 0.04% for drosophila tissue experiments.

315 Mps1 inhibitor AZ3146 was from Calbiochem, the CDK1 inhibitor RO-3306 from Merck,
316 Taxol and Nocodazol from Sigma Aldrich.

317 **Antibodies, Immunoblotting and Immunofluorescence**

318 The following commercial antibodies were used: α -tubulin (clone YL1/2, Millipore; 1:1000),
319 anti-BUBRI (clone 9, BD bioscience; 1:500), anti-GFP (clone 7.1 and 13.1, Roche; 1:1000),
320 anti-Phospho Histone H3 (clone CMA312, Millipore; 1:1000). DNA was stained with
321 Hoechst 33342 or To-Pro-3 iodide (Invitrogen). For microtubule immunofluorescence
322 staining, cells were grown on glass coverslips and fixed with methanol at -20°C (tubulin). For
323 BubR1 staining, cells were first permeabilized with 0,5 % Triton in PHEM (60mM PIPES, 25
324 mM HEPES, 10 mM EGTA, 4mM MgSO₄·7H₂O) for 5 minutes at room temperature and then
325 fixed with PFA in PHEM for 10 minutes. Antibody staining was then performed as described
326 previously (Benaud et al., 2004). Brain from wandering third instar larvae were dissected and
327 maintained in 100 μ l of Schneider media supplemented with DMSO or 20 μ M Ig for 1 h at
328 25°C before fixation and immunostaining as described previously (Gallaud et al., 2014).

329 **Drosophila stocks**

330 Flies were maintained under standard conditions at 25°C. *w¹¹¹⁸* flies were used as controls for
331 immunostaining experiments. Flies expressing H2A-GFP (Clarkson and Saint, 1999) and the
332 recombinant Insc-Gal4, UAS-Cherry- α -tubulin as well as the flies *UAS-*
333 *ChRFP::Tubulin* (BDSC 25774) and *Insc-Gal4* (BDSC 8751) were obtained from the
334 Bloomington Drosophila Stock Center (Indiana University, #5941 and #25773 respectively).
335 The flies expressing ubiquitously Tubulin tagged with RFP (RFP-tub) is a gift from Renata
336 Basto (Institut Curie, Paris, France). The cell membrane marker (Ubi-PH(PLC γ)-GFP
337 (Gervais et al., 2008) is a gift from Antoine Guichet (Institut Jaccques Monod, Paris, France).
338 The *Msp*s RNAi line (ref. GD21982) was purchased from the Vienna Drosophila RNAi
339 Center. The knock down of *Msp*s in central brain neuroblasts was driven by Insc-Gal4.

340 **Live Cell Imaging and Microscopy**

341 For live imaging, *Drosophila* brains expressing H2A-GFP and Cherry- α -tubulin were
342 dissected in Schneider's *Drosophila* medium containing 10% FCS. Following 10 min pre-
343 incubation with DMSO or the indicated concentration of chemical compounds, isolated
344 brains were loaded and mounted on stainless steel slides. The preparations were sealed
345 with mineral oil (Sigma) as previously described (Gallaud et al., 2014). Hela cells were
346 grown in Lab-Tek I chambered cover-glasses (Nunc). Bright field images of asynchronous
347 dividing Hela cells were acquired every 5 min with a 20x objective on a DMRIBE inverted
348 microscope (Leica) equipped with CO₂ heated incubator chamber and a CoolSNAP ES BW
349 camera (Roper scientific). Live fluorescent images were acquired on a spinning disk
350 microscope using a Plan Apo 60x/1.4 NA objective on an Eclipse Ti-E microscope (Nikon)
351 equipped with a spinning disk (CSU-X1; Yokogawa), a thermostatic chamber (Life Imaging
352 Service), Z Piezo stage (Marzhauser), and a charge-coupled device camera (CoolSNAP
353 HQ2; Roper Scientific). *Drosophila* live images were alternatively acquired with a spinning
354 disk system consisting of a DMI8 microscope (Leica) equipped with a 63X/1.4NA oil
355 objective, a CSU-X1 spinning disk unit (Yokogawa) and an Evolve EMCCD camera
356 (Photometrics). The microscope is controlled by the Inscoper Imaging Suite and the dedicated
357 software (Inscoper).

358 For Hela cells, time-lapse Tubulin-GFP images were acquired every 1min for the 45min time
359 lapse or every 5min for the 90min time lapse, and EB1GFP comets every 0.5sec using
360 Metamorph Software (Universal imaging). For *drosophila* neuroblasts, Z-series were
361 acquired every 30 or 60 seconds. Immunofluorescence images of fixed samples were
362 acquired with SP5 confocal microscope (Leica), or with an API DeltaVision microscope
363 equipped with a coolSnapHQ camera (Princeton instruments) using the SoftWorX software.
364 Image acquisition was coupled to deconvolution when indicated. Images were processed and
365 measurements performed using Fiji software (<http://fiji.sc/>). Analysis of EB1comets were
366 performed using the Matlab based open source u-track particle tracking (version2.0) software
367 (Danuser Lab, UT Southwern Medical Center).

368 **Turbidimetry assay**

369 Commercial lyophilised tubulin (PurSolutions, Nashville, USA) was reconstituted at 500 μ M
370 in distilled water according to the manufacturer instructions. Tubulin was diluted at 50 μ M in
371 10 % glycerol, 1 mM GTP, 0.02 % DMSO in BRB80 buffer (80 mM K-Pipes, 1 mM EGTA,
372 1mM MgCl₂, pH 6.8 with KOH), and in the presence of 0 μ M, 5 μ M or 10 μ M *Ig*. Control
373 samples contained the same amount of DMSO as *Ig* samples. Suspensions were centrifuged

374 at 33,000 g at 4 °C for 5 min before polymerisation. Samples were transferred into 100 µl
375 quartz cuvettes (Hellma), and measurements at 350 nm were performed in a UVIKON XS
376 spectrophotometer thermostated at 35 °C to stimulate tubulin polymerisation. After 30 min of
377 polymerisation, depolymerisation was induced by a cold temperature shift at 4 °C to assess
378 the presence of aggregates.

379

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384

385 **Conflict of interest**

386 The content of this manuscript is included in a current patent application

387

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396

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398

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- 460

461 **Figure Legends**

462 **Figure 1: 1g arrests proliferating cells in prometaphase and activates the spindle**

463 **assembly checkpoint. A-** Proliferation of HeLa cells treated with DMSO or 1g (5 μ M)

464 monitored by imaging asynchronous cells for 16 and 20hrs. scale bar=30 μ m (full sequence in

465 Movies S1 and S2). **B-** Dose response effect of 1g on cell proliferation. Phase contrast images

466 of HeLa cells treated with the indicated concentration of 1g for 16hrs. scale bar=30 μ m. **C-**

467 Mitotic index (% mitotic cells/total cells) of HeLa cells treated with DMSO or the indicated

468 concentration of 1g for 16hrs (n \geq 150 cells). Student t-test, ****P<0.0001. **D-**Cells

469 synchronized at the G2-M transition were released in presence of DMSO or the indicated

470 concentration of 1g for 45min (metaphase in control cells) or 90min (telophase in control).

471 Cells are stained for α -tubulin in green and DNA in magenta. scale bar=10 μ m. **E-**

472 Quantification of mitotic cells repartition in the different phases of mitosis in presence of

473 increasing concentration of 1g (n \geq 80 cells). **F-** HeLa cells synchronized at the G2/M

474 transition were released in presence of DMSO or 5 μ M 1g for 45 min. Z stack projections of

475 cells stained for the presence at the kinetochore of the SAC protein BUBR1 and DNA. scale

476 bar=5 μ m. **G-** Activation of the SAC arrests 1g treated cells in prometaphase. Live phase

477 contrast imaging of proliferating asynchronous HeLa cells treated with 5 μ M 1g alone or in

478 combination with 2 μ M AZ3146. scale bar=30 μ m. (full sequence in Movies S3 and S4).

479

480 **Figure 2: 1g interferes with mitotic spindle assembly in dividing cells. A-**Spinning disk

481 images (Max projection) of HeLa tubulin-GFP cells undergoing mitosis in presence of

482 DMSO, 1 μ M or 2,5 μ M 1g. Scale bar=10 μ m. (full sequence in Movies S5, S6 and S7). **B-**

483 HeLa expressing tubulin-GFP (green) and H2B-mcherry (red) imaged for 90min following

484 G2/M released in presence of DMSO or the indicated concentration of 1g. t₀= nuclear

485 envelop breakdown (NEB); Arrows points to microtubule asters and arrow head to unaligned

486 chromosomes. Scale bar=10 μ m. **C-**Quantification of % cells treated with the indicated

487 concentration of 1g forming a bipolar spindle by 80 min. n \geq 15. **D-** Quantification of % cells

488 congressing their chromosomes into a metaphase plate by 80 min. n \geq 15. **E-** Quantification of

489 the number of microtubules asters in cells treated with the indicated concentration of 1g at

490 t=80min. n \geq 18. **F-**Analysis of the distance between spindle poles, 45 min after the G2/M

491 block release in cells treated with DMSO or 1 μ M and 2 μ M of 1g. n \geq 40 cells. **G-** HeLa

492 centrin-GFP cells synchronized at the G2/M transition were released for 45 min in presence of

493 DMSO or 5 μ M 1g and stained for tubulin, GFP and DNA. DeltaVision deconvoluted

494 projections, scale bar=5 μ m. **H-** Analysis of centrosome separation in cells treated with
495 DMSO or 5 μ M 1g. n \geq 49 cells. Student t-test, ****P<0.0001.

496

497 **Figure 3: 1g alters microtubules growth in cells.** **A-**Microtubules of HeLa cells treated with
498 DMSO or 5 μ M 1g were depolymerized at 4°C (t0) and allowed to repolymerize by switching
499 back the cells to 37°C for the indicated times. Z stack projection images of cells stained for
500 α -tubulin. **B-** Spinning disk time lapse images of HeLa EB1-GFP cells (1 image/sec) before
501 treatment, in presence of DMSO or 2,5 μ M 1g, and after washing out the drug (control: Movie
502 S8; 1g: Movie S9). Time projection of EB1-GFP over 1 min are presented for the different
503 conditions. **C-**Kymograph (time (sec) vs distance) representations of the EB1 tracks over 1
504 min. Scale bar=1 μ m. Quantification of EB1 comets velocity (**D-**) nucleation events (**E-**) and
505 inferred number of pauses (**F-**). For each condition n \geq 700 tracks in 10 (control) to 15 (1g)
506 independent measurements. ****P<.0001, Student's t-test. **G-** *In vitro* microtubule
507 polymerization assay. DMSO (ctl) or 5 and 10 μ M 1g was added to purified tubulin in
508 polymerization buffer at 4°C. The formation of microtubule polymers was monitored by the
509 absorbance at 350nm as the temperature was increased to 35°C. Formation of stable
510 aggregates are visualized as temperature is shifted back to 4°C.

511

512 **Figure 4: Effect of 1g on *Drosophila* neuroblasts proliferation.**

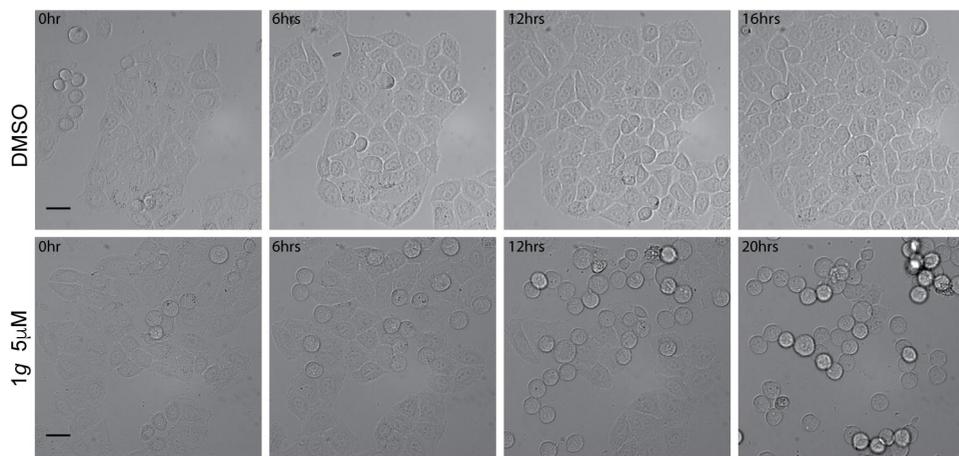
513 Dividing neural stem cells of drosophila brains expressing tubulin-RFP and Histone H2B-
514 GFP imaged by spinning disk confocal microscopy. **A-** Selected frames of dividing
515 *Drosophila* larval neuroblasts incubated with DMSO or 1g. Time 00:00 (min:sec) = NEB.
516 Dotted circle outlines the neuroblast cell contour (t5-t9) and the neuroblast progeny (t12):
517 neuroblast (large dotted circle) and ganglion mother cell GMC (small dotted circle). Scale
518 bar: 10 μ m. **B-**Analysis of the percentage of mitotic spindle assembly defects in neuroblasts
519 treated with DMSO or 1g. n \geq 17 cells for each condition. **C-** Analysis of chromosomes
520 segregation defects corresponding to lagging chromosomes in DMSO and 1g treated brains.
521 n \geq 17 cells. **D-** Analysis of mitosis duration measured from NEB to anaphase onset in
522 neuroblasts exposed to DMSO (05:87 \pm 0.1, n=43), 5 μ M (06:14 \pm 0.19, n=17), 10 μ M
523 (08:24 \pm 0.19, n=17), 20 μ M (10:60 \pm 0.5, n=42) 1g. **P<0.0012; ***P<1.3x10⁻⁴ (Wilcoxon
524 test). **E-** Analysis of the frequency of formation of tripolar spindle in neuroblast treated
525 with DMSO (0%, n=44) or 20 μ M 1g (87%, n=42). **F-** Z stack image of prometaphase
526 neuroblasts incubated for 1hr in DMSO or 1g and stained for α -tubulin (green) and DNA

527 (magenta). Arrow heads points to tripolar asters. scale bar=10 μ m. **G,H**- Selected frames
528 illustrating interphase aster reformation following mitosis of *Drosophila* larval neuroblasts
529 incubated with DMSO or 1g. Arrow points to interphase aster. Scale bar=10 μ m.

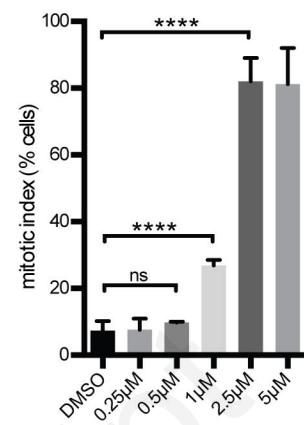
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Figure 1

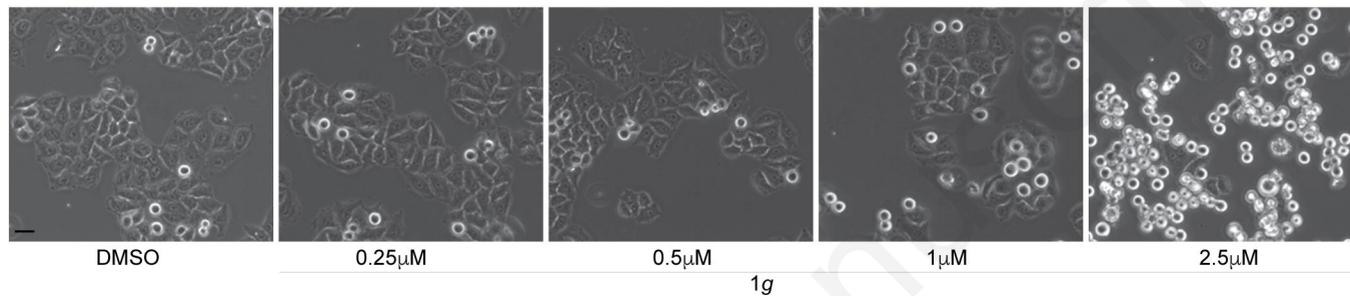
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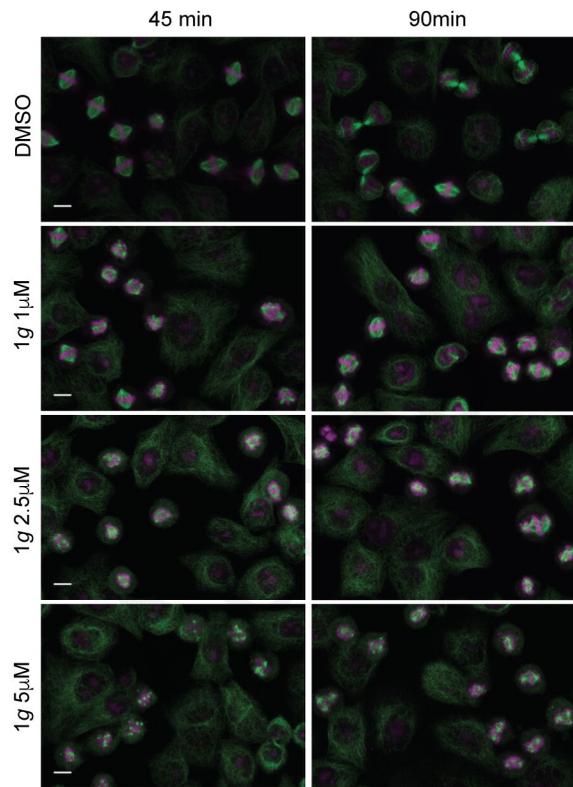
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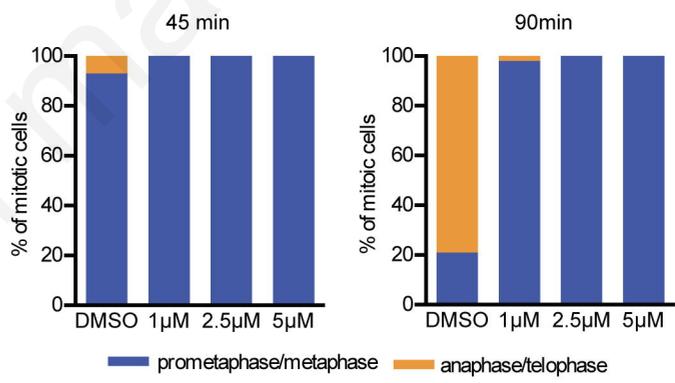
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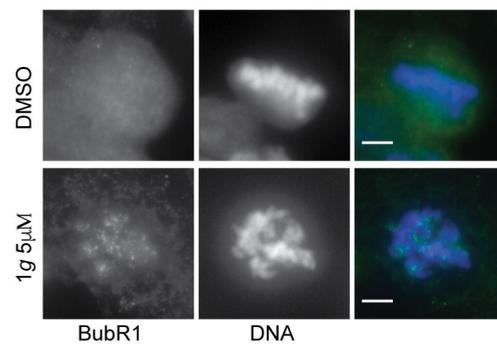
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E-



F-



G-

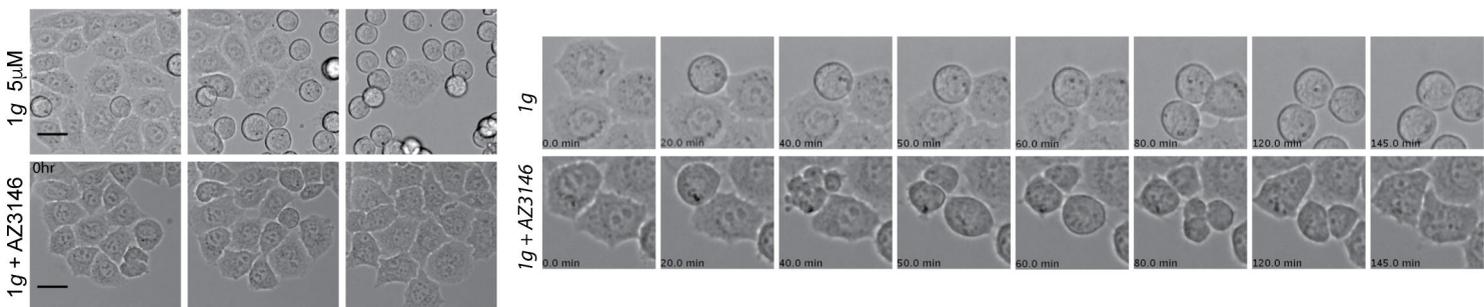


Figure 2

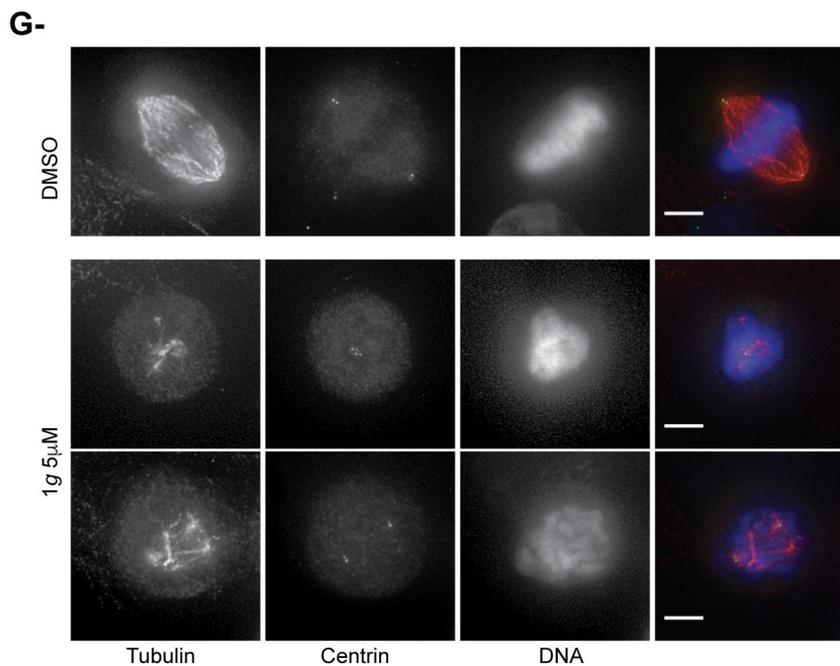
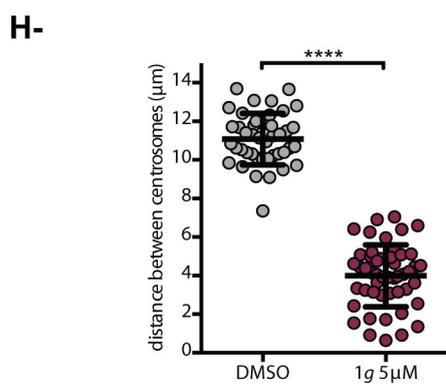
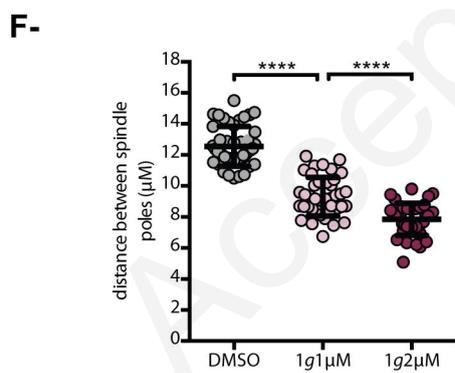
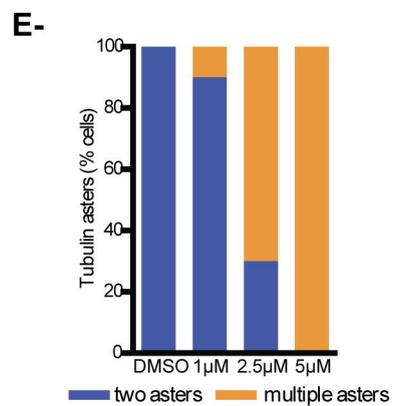
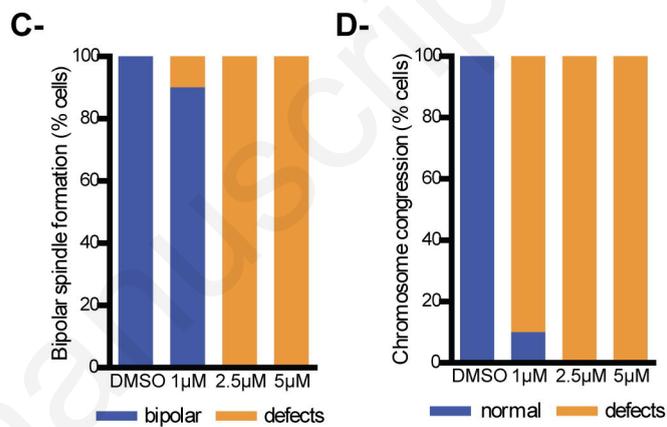
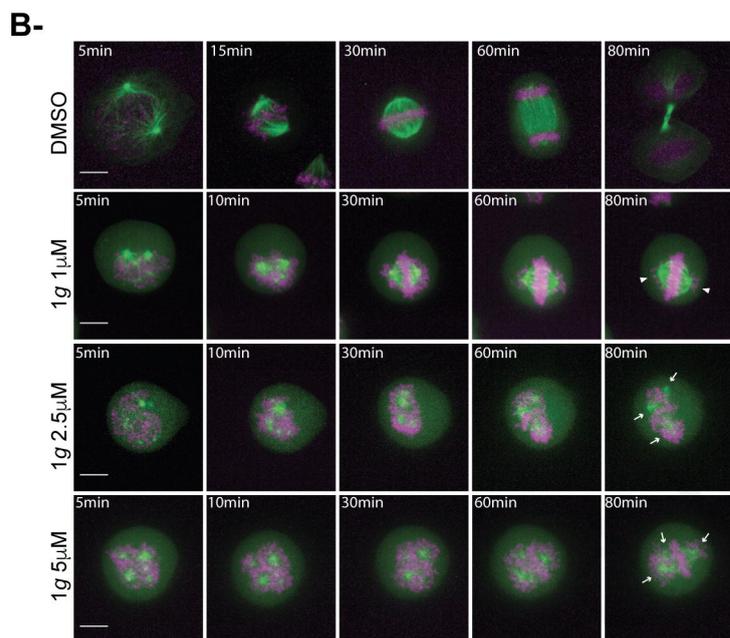
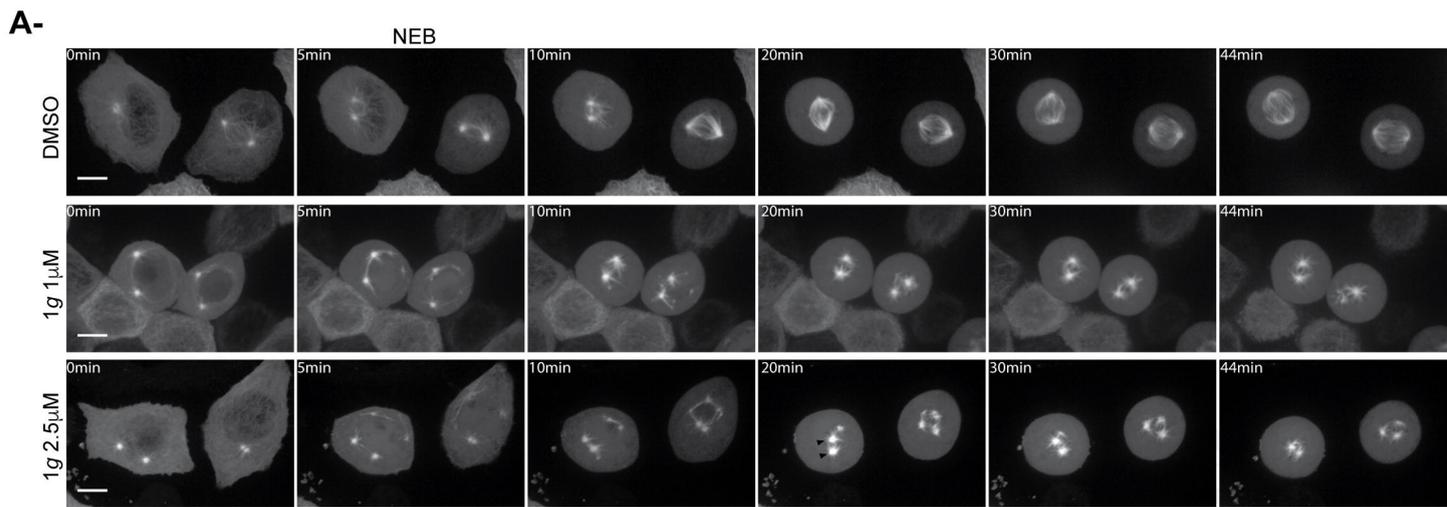
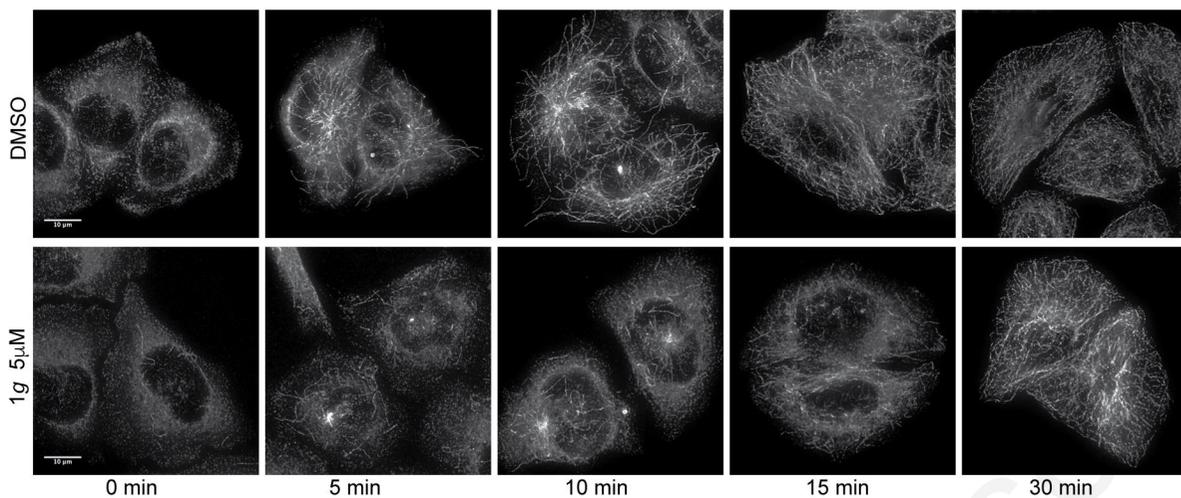


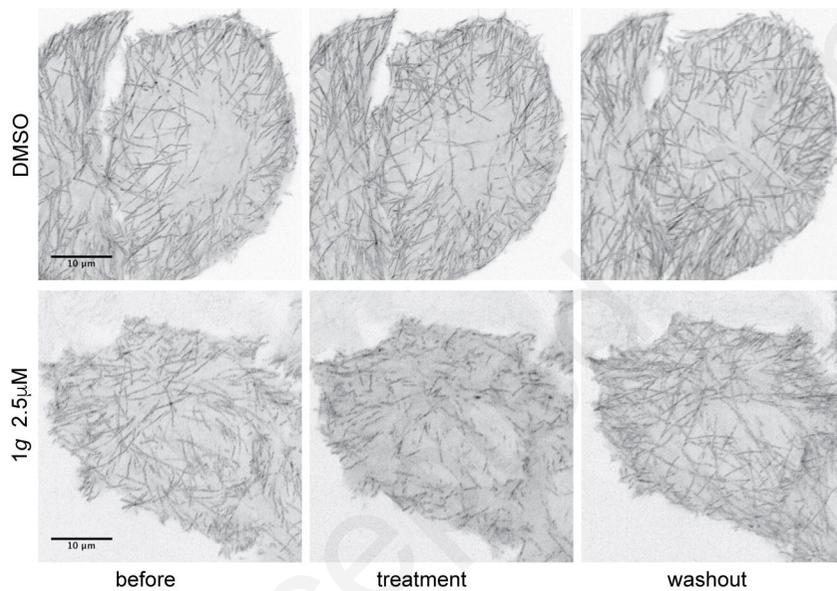
Figure 3

A-



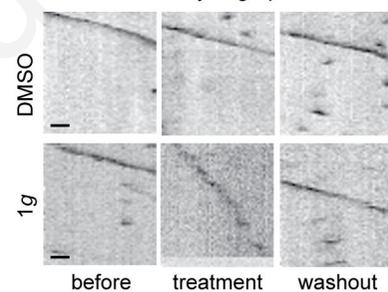
B-

EB1 Tracks

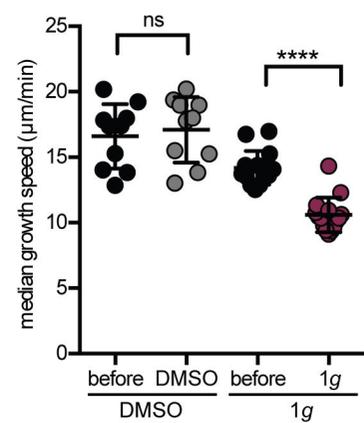


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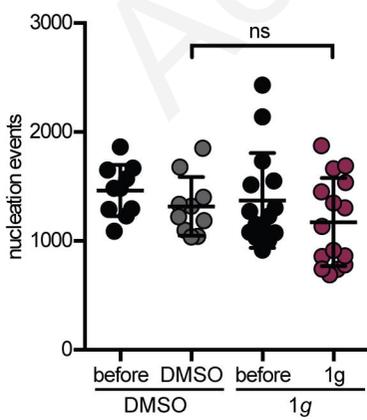
EB1 kymograph



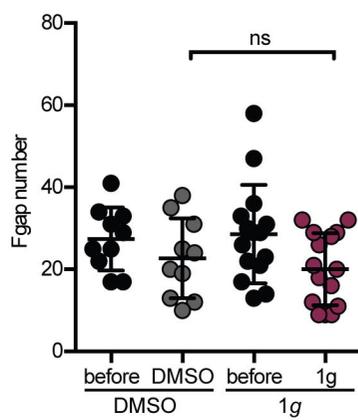
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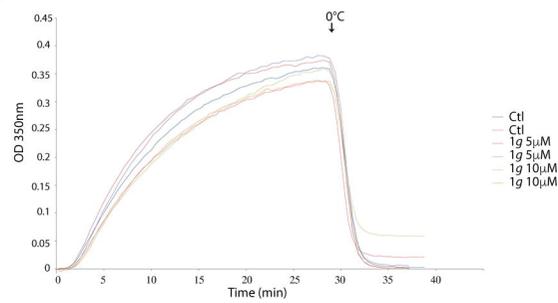


Figure 4

