

1 The RAC1 activator Tiam1 regulates centriole 2 duplication through controlling PLK4 levels

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9 **Key words**

10 Centriole, Centriole Duplication, PLK4, Tiam1, β TRCP, Aneuploidy

11 Summary

12 Centriole duplication is tightly controlled to maintain correct centriole number through the cell
13 cycle. A key component of this control is the regulated degradation of PLK4, the master
14 regulator of centriole duplication. Here we show that the Rac1 guanine nucleotide exchange
15 factor (GEF) Tiam1 localises to centrosomes during S-phase, where it is required for
16 maintenance of normal centriole number. Depletion of Tiam1 leads to an increase in
17 centrosomal PLK4, centriole overduplication and ultimately to lagging chromosomes at
18 anaphase and aneuploidy. The effects of Tiam1 depletion can be rescued by re-expression
19 of wild-type Tiam1 and catalytically inactive (GEF*) Tiam1, but not by Tiam1 mutants unable
20 to bind to the F-box protein β TRCP, implying that Tiam1 regulates PLK4 levels through
21 promoting β TRCP-mediated degradation.

22

23 Results

24 Centrioles are barrel-shaped, microtubule-rich organelles fundamental to cell division,
25 polarity and signalling. Centriole pairs recruit pericentriolar material (PCM) to form the
26 centrosome, the major microtubule organising centre [1]. In G1, a typical human cell
27 contains a single centrosome formed around two centrioles. At the G1/S transition, each of
28 the mother centrioles generates a single daughter centriole (see schematic, Supplementary
29 Figure 1a), which together recruit PCM to form one of the mitotic spindle poles [2].

30 Centriole number is tightly controlled. Increased centriole or centrosome number leads to
31 severe mitotic aberrations [2, 3]. During mitosis, cells with centrosome amplification form
32 transient multipolar spindles. Division can proceed in such cells through clustering of extra
33 centrosomes to pseudo-bipolar spindles. However, this gives rise to lagging chromosomes
34 and ultimately aneuploidy [4]. Moreover, even in cells with two centrosomes, uneven
35 centriole number at the mitotic poles can lead to lagging chromosomes and aneuploidy [5].
36 In turn, aneuploidy leads to transcriptional changes that can affect cell growth or cause
37 proteotoxic stress. Aneuploidy is also a feature of chromosomal instability (CIN), a driver of
38 cancer [3]. Indeed, centrosome amplification alone is sufficient for the initiation of
39 tumourigenesis [6].

40 To ensure correct centriole number, cells tightly regulate centriole production [2, 7]. PLK4, a
41 member of the polo-like kinase family, is the master regulator of centriole biogenesis [7].
42 During the G1-S transition, PLK4 phosphorylates its partner STIL, increasing recruitment of
43 SAS6, a key structural component of the new procentriole (Supplementary Figure 1a) [7].

44 This process is restricted by PLK4 degradation, which is mediated by the SCF E3 ligase
45 complex. PLK4 proteins homodimerise and trans-autophosphorylate a phospho-degron
46 recognised by β TRCP, an F-box protein that targets SCF to its substrates [8-10]. Thus, a
47 transient increase in PLK4 activity is tightly coupled to its destruction, efficiently terminating
48 its activity and preventing extra rounds of centriole duplication [9, 10]. Excess PLK4 has
49 been shown to lead to centriole overduplication across multiple species and cell types [8, 9,
50 11-13].

51 Tiam1, a guanine nucleotide exchange factor (GEF) for the small GTPase Rac1 has a wide
52 variety of functions including roles in cell adhesion, polarity, migration, transcription and
53 tumourigenesis [14-19]. We previously showed that Tiam1 localises to centrosomes in
54 prophase and prometaphase, where it activates Rac1 signalling to antagonise centrosome
55 separation [20]. Centrosomal Tiam1 is phosphorylated by CDK1 and this is required for
56 phosphorylation and consequently activation of the Rac1 effector PAK1/2 [21]. We decided
57 to investigate whether Tiam1 localises to centrosomes in other phases of the cell cycle. We
58 used our previously validated staining protocol [20] and stained U2OS cells with antibodies
59 against Tiam1, Pericentrin (as a centrosome marker) and PCNA which produces punctate
60 nuclear staining during S-phase [22]. We observed localisation of Tiam1 at centrosomes
61 during S-phase (Figure 1a), as well as throughout mitosis as we previously published [20]
62 (data not shown).

63 We hypothesized that Tiam1 plays an additional role in centrosome biology in S-phase,
64 when centriole duplication occurs [7]. To test this, we depleted Tiam1 from U2OS cells
65 using RNAi (Figure 1b). Control and knockdown cells were stained with antibodies against
66 Pericentrin and Centrin (to mark individual centrioles) (Figure 1c). Cells depleted of Tiam1
67 displayed an increase in centriole number, with many cells containing centrosomes with
68 additional Centrin puncta (i.e. greater than 2 centrioles per centrosome) (Figure 1c). Using
69 three independent siRNA sequences targeting Tiam1, we saw a significant increase in the
70 number of cells with centriole overduplication (Figure 1d).

71 To determine whether this effect could be observed in other cell lines, we used siRNA to
72 deplete Tiam1 in HCT116 colon cancer cells, which, like U2OS cells, have a normal centriole
73 number [5, 23]. Tiam1 depletion (Figure 1e) again resulted in excess centrioles compared
74 with control siRNA (Figure 1f and 1g). We also observed similar effects in the human breast
75 cancer cell line MCF7 (Supplementary Figure 1b-d). Together, this suggests a conserved
76 role for Tiam1 in centriole duplication across multiple cell types. To determine whether these
77 extra Centrin puncta represented true centrioles or rather centriolar satellites (which localise
78 around the centrosome and stain with Centrin antibody [24]), we placed control and Tiam1

79 knockdown cells on ice for 30 minutes to depolymerise microtubules (Supplementary Figure
80 1e). While this causes dispersion of centriolar satellites, true centrioles are unaffected [25,
81 26]. We observed a significant increase in Tiam1 knockdown cells with excess centrioles
82 following cold treatment (Supplementary Figure 1e and 1f), indicating that the extra Centrin
83 puncta are indeed centrioles. As a further confirmation, we also looked specifically at mitotic
84 U2OS cells and observed centriole overduplication at the mitotic poles following Tiam1
85 depletion, consistent with these aberrant centrioles forming functional centrosomes
86 (Supplementary Figure 1g-1i).

87 To demonstrate that the effects of Tiam1 depletion are on-target effects of siRNA
88 knockdown, we developed a system to restore wild-type Tiam1 levels using siRNA-resistant
89 Tiam1. We constructed HCT116 cells expressing mouse WT-Tiam1 [intrinsically resistant to
90 two siRNAs targeting the human Tiam1 sequence (Tiam1 KD#2 and Tiam2 KD#3)] under
91 the control of a doxycycline-inducible promoter, as in previous publications [17, 21]. We
92 could detect the exogenous WT-Tiam1 localising to centrosomes by immunofluorescence
93 following doxycycline treatment (Supplementary Figure 1j). As before, siRNA treatment
94 effectively depleted endogenous Tiam1; however, addition of doxycycline led to near-
95 endogenous expression of WT-Tiam1 protein (Figure 1h). Tiam1 depletion again led to an
96 increase in centriole number, which was effectively rescued by doxycycline-induced WT-
97 Tiam1 (representative images in Figure 1i, quantified in 1j). This demonstrates that Tiam1 is
98 required for normal centriole number.

99 To determine the mechanism by which Tiam1 maintains normal centriole number, we first
100 used flow cytometry to investigate whether Tiam1 knockdown affected cell cycle
101 progression, as increased S-phase duration can lead to centriole overduplication [27], while
102 failure to undergo cytokinesis results in centrosome amplification [28]. However, we saw no
103 difference in cell cycle progression nor evidence of polyploidy comparing control and Tiam1
104 knockdown cells (Supplementary Figure 2a-2c). Having excluded a cell cycle defect, we
105 decided to next examine whether Tiam1 knockdown affected PLK4 levels at centrosomes,
106 PLK4 being the key driver of centriole duplication [8, 9, 11-13]. We first confirmed that our
107 antibody detected centrosomal PLK4 by staining U2OS cells treated with either a control
108 siRNA or several PLK4 siRNAs (Supplementary Figure 2d). We calculated PLK4
109 centrosomal intensity (Supplementary Figure 2e) and saw a decrease in centrosomal PLK4
110 in all siRNA-treated cells compared with control cells (Supplementary Figure 2f).

111 Next we stained control or Tiam1-depleted U2OS cells for PLK4 and Pericentrin. We saw a
112 significant increase in centrosomal PLK4 in cells treated with Tiam1 siRNA compared with
113 control siRNA-treated cells (Figure 2a, quantified in 2b). The equivalent experiment with

114 HCT116 cells also demonstrated an increase in centrosomal PLK4 staining after Tiam1
115 depletion (Figure 2c, quantified in 2d).

116 To corroborate these immunofluorescence data, we biochemically measured PLK4 levels
117 following Tiam1 knockdown. We were unable to detect endogenous PLK4 by western blot
118 (similar to reports from other researchers, as the cellular levels of PLK4 are very low [10]),
119 and therefore over-expressed myc-tagged PLK4 in U2OS cells using a doxycycline-inducible
120 system. This induced centriole overduplication (Supplementary Figure 2g). We used anti-
121 Myc beads to immunoprecipitate exogenous PLK4, and saw that its levels increased
122 following Tiam1 knockdown (Figure 2e, quantified in 2f). Together, these experiments
123 indicate that Tiam1 limits PLK4 levels at the centrosome, thereby constraining centriole
124 duplication.

125 Given that Tiam1-depleted cells have elevated PLK4 levels and that high PLK4 drives
126 centriole overduplication, we hypothesised that low-level PLK4 inhibition in Tiam1 depleted
127 cells would restore their correct centriole number. We therefore treated HCT116 cells with
128 the specific PLK4 inhibitor Centrinone [29] at 20 nM, a concentration which had minimal
129 effects on centriole number in control siRNA treated cells (Figure 2g). Tiam1 knockdown in
130 the absence of the inhibitor led to an increase in centriole number (Figure 2g), whereas
131 knockdown cells treated with Centrinone did not display an increase in centriole number
132 compared to control-siRNA treated cells (Figure 2g). These findings show that the
133 production of extra centrioles in Tiam1-depleted cells requires increased PLK4 activity.

134 We next investigated the mechanism by which Tiam1 regulates PLK4 levels. Since Tiam1 is
135 best known for its ability to activate Rac1, we addressed whether the catalytic (GEF) activity
136 of Tiam1 was required for correctly regulating centriole numbers. We therefore expressed a
137 doxycycline-inducible siRNA-resistant 'GEF-dead' mutant (GEF*) Tiam1 (Figure 3a) in
138 HCT116 cells treated with either control siRNA or siRNA against endogenous Tiam1 (Figure
139 3b). GEF* Tiam1 localised to the centrosomes similarly to wild-type Tiam1 (Supplementary
140 Figure 3a) and its expression restored normal centriole number in cells depleted of
141 endogenous Tiam1 (Figure 3c, quantified in 3d). This indicates that Tiam1-dependent Rac1
142 activation is not required for normal centriole duplication nor its localisation to the
143 centrosome. This also suggests that this pathway is distinct from the one controlling
144 centrosome separation during prophase and prometaphase, which does rely on Tiam1-
145 mediated Rac1 activation [20, 21]. To further investigate the relationship between these two
146 centrosomal Tiam1 functions, we performed rescue experiments with Tiam1-1466A, a
147 CDK1-phosphorylation mutant version of Tiam1 (Supplementary Figure 3b) that is unable to
148 rescue centrosome separation in prophase despite correct localisation to centrosomes [21].

149 As with the GEF* mutant, expression of this mutant Tiam1 was able to restore normal
150 centriole number after Tiam1 knockdown (Supplementary Figure 3c and 3d), demonstrating
151 that this role of Tiam1 is distinct from that of Tiam1 in prophase.

152 As the function of Tiam1 in centriole duplication appeared to be independent of Rac1, some
153 other domain of Tiam1 must play a role in regulating centriole duplication. Indeed, Tiam1 is
154 a large multi-domain protein, which also acts as a molecular scaffold [30]. We therefore
155 performed our rescue experiment again with a well-characterised, N-terminally truncated
156 mutant of Tiam1, C1199 (Figure 3a). Interestingly, doxycycline-induced expression of
157 siRNA-resistant C1199 (Figure 3b) was unable to rescue centriole number in HCT116 cells
158 (Figure 3c, quantified in 3d). This suggests that a function performed by the N-terminus of
159 Tiam1 is required for normal centriole duplication. Significantly, this region of Tiam1
160 contains a phospho-degron required for binding of β TRCP [31], a component of the E3-
161 ligase complex also targeting centrosomal PLK4 for degradation during S-phase [8, 32].

162 We reasoned that the interaction between Tiam1 and β TRCP may be required for the ability
163 of Tiam1 to regulate PLK4 levels at the centrosome, and that the C1199 mutant may be
164 unable to substitute for endogenous Tiam1 because of its lack of β TRCP binding. To test
165 this, we produced a mutant version of full-length Tiam1 (referred to as Tiam1-AA; Figure 3a)
166 containing two point mutations in the β TRCP phospho-degron [31]. We confirmed that both
167 this mutant and C1199 were unable to bind to β TRCP by co-immunoprecipitation (Figure
168 3e). We also confirmed that Tiam1-AA was able to correctly localise to the centrosome in
169 the same way as wild-type Tiam1 (Supplementary Figure 3a), indicating that β TRCP binding
170 was not required for centrosomal localisation of Tiam1. We then performed Tiam1
171 knockdown and rescue experiments, inducibly expressing the siRNA-resistant Tiam1-AA
172 mutant to near-endogenous levels in cells depleted of Tiam1 (Figure 3f). In both uninduced
173 (-dox) and induced (+dox) cells there was a significant and indistinguishable increase in cells
174 with excess centrioles upon endogenous Tiam1 depletion compared with control-siRNA
175 treated cells (Figure 3g, 3h), showing that expression of Tiam1-AA is unable to compensate
176 for depletion of endogenous Tiam1. Therefore, we conclude that regulation of normal
177 centriole number requires the interaction between Tiam1 and β TRCP.

178 Centriole overduplication can lead to chromosome mis-segregation, aneuploidy and
179 chromosomal instability (CIN) [33], ultimately leading to tumourigenesis [6, 12, 34].
180 Chromosome mis-segregation can occur either through an imbalance of centriole numbers
181 at the two poles of the mitotic spindle [5] or via the formation of transient multipolar
182 intermediates arising from centrosome amplification [4]. Depletion of Tiam1, apart from
183 triggering centriole overduplication, also led to low levels of centrosome amplification

184 (Supplementary Figure 4a and 4b). Interestingly, Tiam1 depletion, while largely suppressing
185 tumour formation [14, 16], promotes malignant progression [14, 16] indicating a dual
186 oncogene/tumour suppressor role for Tiam1. As CIN is considered to drive the acquisition of
187 malignant hallmarks [35, 36], we investigated whether knockdown of Tiam1 could lead to an
188 increase in lagging chromosomes at anaphase, a widely used readout of chromosome mis-
189 segregation. We combined Hoechst staining with a centromere marker (CREST) to
190 distinguish true lagging chromosomes from acentromeric chromosome fragments and
191 chromosome bridges (Figure 4a). In the chromosomally-stable HCT116 cell line, we
192 observed a low level of lagging chromosomes at anaphase and early telophase in control-
193 siRNA treated cells (Figure 4a, quantified in 4b). Following Tiam1 knockdown, the number
194 of cells with lagging chromosomes significantly increased (Figure 4a, 4b). There was no
195 change in the number of chromosome bridges or acentric chromosomes (data not shown),
196 suggesting that this phenotype affects the segregation of whole chromosomes specifically.
197 We saw similar increases in lagging chromosomes in U2OS cells depleted for Tiam1
198 (Supplementary Figure 4c, quantified in 4d), indicating conservation of the role of Tiam1 in
199 chromosome segregation.

200 Given this increase in lagging chromosomes, and the previously reported chromosome
201 alignment defects arising from Tiam1 knockdown [20], we tested whether these would
202 translate into an increase in aneuploidy given longer periods of Tiam1 depletion. We grew
203 HCT116 cells for 8 days with two rounds of siRNA transfection (Figure 4c), and prepared
204 metaphase spreads. Chromosome number was determined by manual counting of Hoechst-
205 stained chromosomes (representative images in Figure 4d). While the range of
206 chromosome number found in control cells was relatively narrow (from 42 to 48
207 chromosomes), we found a much wider distribution of chromosome number in cells treated
208 with Tiam1 siRNA (from 29 to 60 for Tiam1 KD#1, and from 23 to 50 for Tiam1 KD#2),
209 indicating a significant increase in aneuploidy following Tiam1 depletion (Figure 4e). Our
210 data indicate that Tiam1 contributes to the control of chromosome segregation through
211 regulating centriole duplication.

212

213 Discussion

214 In this study, we identify Tiam1 as a new regulator of PLK4, the master regulator of centriole
215 duplication. PLK4 regulation involves a fine balance between a brief period of activity –
216 sufficient to initiate procentriole formation – and ubiquitin-mediated degradation to prevent
217 re-duplication. Too much activity leads to overduplication [1, 8, 9], too little to centriole loss

218 [29], but the full details of this precise spatio-temporal regulation remains to be determined
219 [7]. Defects in PLK4 levels can ultimately lead to defects in chromosome segregation [4, 6]
220 and other pro-oncogenic effects such as increased invasion [28, 37].

221 Our data indicate that Tiam1 acts as a modulator of PLK4 protein levels, with depletion of
222 Tiam1 leading to an increase in centrosomes containing more than 2 centrioles, and a small
223 increase in cells with centrosome amplification (see model in Figure 4f). While we have yet
224 to establish how precisely Tiam1 modulates PLK4 levels through β TRCP, in a previous
225 publication, we showed that the interaction between β TRCP and Tiam1 was necessary for
226 the degradation of TAZ, another β TRCP target protein [19]. Given our current findings, this
227 raises the possibility that Tiam1 is able to act as a scaffold for β TRCP more generally,
228 directing it to its targets, or enhancing its interaction with target proteins. As Tiam1 is itself a
229 target of β TRCP-mediated degradation, this could allow for precise temporal control of
230 β TRCP-target degradation, as once Tiam1 itself is degraded the targeting effect would be
231 removed.

232 The regulation of centriole duplication by Tiam1 appears distinct from its role in driving
233 centrosome separation during prophase [20], as, unlike the latter, the former does not
234 depend on activation of Rac1, nor does it require phosphorylation of Tiam1 at S1466. It is
235 interesting to speculate whether these two centrosomal functions of Tiam1 are temporally
236 segregated, and if so how. Perhaps distinct complexes of Tiam1 exist at centrosomes
237 (including either β TRCP and PLK4 or Rac1 and PAK1/2) dictated by mutually exclusive
238 intermolecular interactions. Alternatively, post-translational modification of Tiam1, such as by
239 CDK1 phosphorylation [21], might control a temporal switch between interacting partners.

240 With respect to the impact of Tiam1 depletion on chromosomal stability, we show here that
241 loss of Tiam1 leads to an increase in lagging chromosomes at anaphase and to aneuploidy.
242 The latter is likely due to a combination of the effects of centriole overduplication and
243 centrosome amplification demonstrated in this study, and chromosome congression defects
244 that arise from Tiam1 depletion, as we previously published [20, 21]. Together these
245 indicate a pathway by which Tiam1 depletion could enhance tumour progression. While
246 targeting the Tiam1-Rac pathway therapeutically remains a subject of ongoing investigation,
247 due to the dramatic reduction in tumour formation following loss of Tiam1 in animal models
248 [14, 16], this work highlights a need for more detailed understanding of Tiam1 signalling to
249 separate the pro- and anti-tumourigenic properties of Tiam1.

250 **Figures**

251

252 **Figure 1 – Tiam1 is required for maintenance of normal centriole number**

253

254 **Figure 2 – Tiam1 knockdown leads to an increase in centrosomal PLK4**

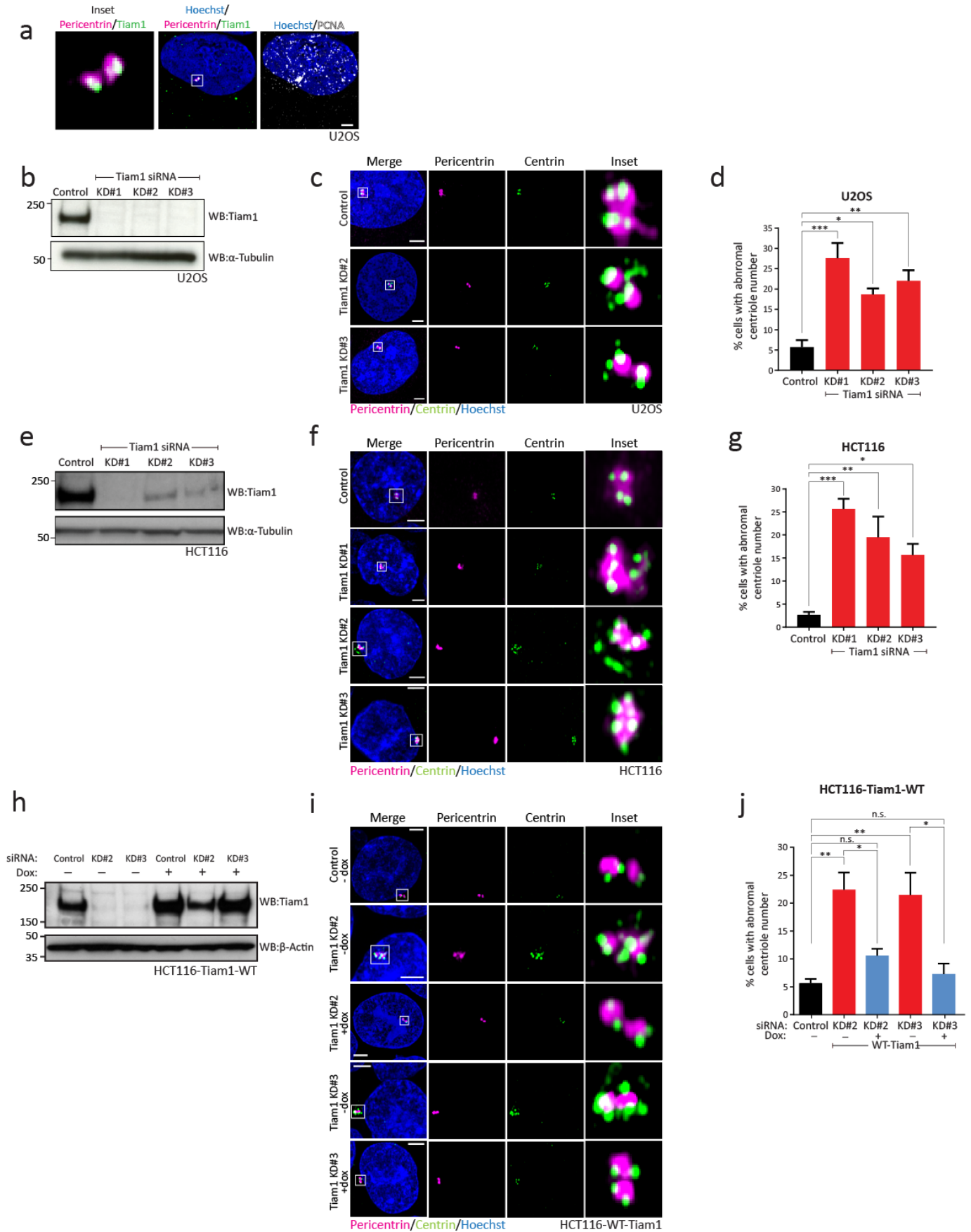
255

256 **Figure 3 – Tiam1 requires β TRCP binding to maintain normal centriole number**

257

258 **Figure 4 – Depletion of Tiam1 leads to lagging chromosomes at anaphase and early**
259 **telophase and aneuploidy**

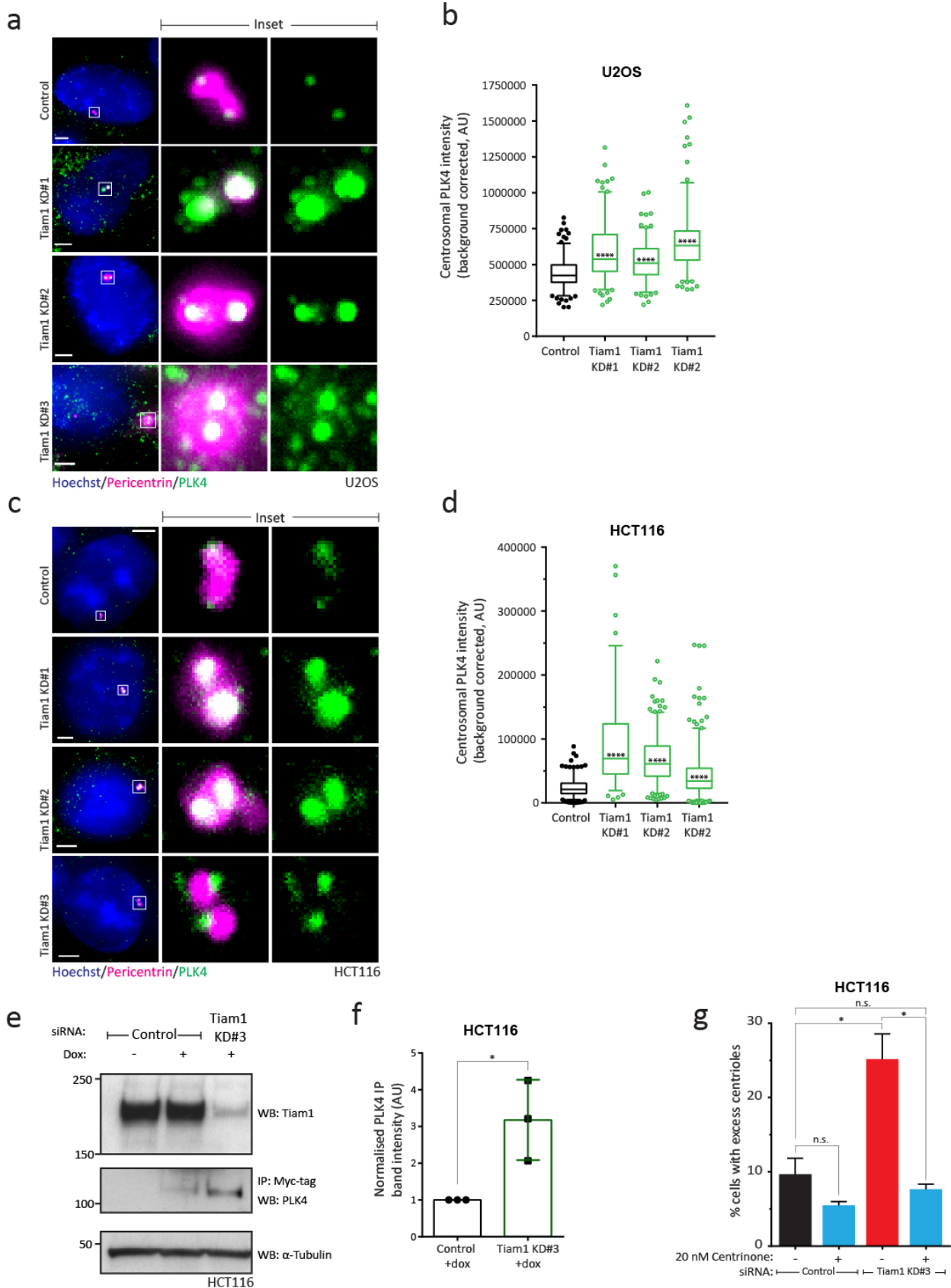
Figure 1



261 **Figure 1 – Tiam1 is required for maintenance of normal centriole number**

262 a) Endogenous Tiam1 (green) localises to centrosomes (marked by Pericentrin, magenta)
263 during S-phase (identified by punctate nuclear staining of PCNA, grey). Maximal z-
264 projection of planes containing Pericentrin staining. b) Western blot showing depletion of
265 endogenous Tiam1 in U2OS cells following transfection with three independent siRNAs. c)
266 Maximal z-projections of confocal images showing normal centriole number in U2OS cells
267 transfected with control siRNA, and excess centriole number in Tiam1-knockdown cells.
268 Individual centrioles marked with Centrin (green), centrosomes marked with Pericentrin
269 (magenta). d) Quantification of U2OS cells with abnormal centriole number from 3
270 independent experiments, quantified from images as in (c); more than 100 cells counted per
271 condition per experiment (one-way ANOVA, corrected for multiple comparisons.) e) Western
272 blot showing depletion of endogenous Tiam1 in HCT116 cells following transfection with
273 three independent siRNAs. In (b) and (e) α -Tubulin was used as a loading control. f)
274 Representative confocal microscopy images showing normal centriole number in HCT116
275 cells transfected with control siRNA, and excess centriole number in Tiam1-knockdown cells.
276 Individual centrioles marked with Centrin (green), centrosomes marked with Pericentrin
277 (magenta). g) Quantification of HCT116 cells with abnormal centriole number from 3
278 independent experiments (except Tiam1 KD#2, n=2), quantified from images as in (f); more
279 than 100 cells counted per condition per experiment (one-way ANOVA, corrected for multiple
280 comparisons.) h) Western blot from HCT116-Tiam1-WT cells showing depletion of
281 endogenous Tiam1 following siRNA transfection with Tiam1 KD#2 and KD#3, and
282 restoration of near-endogenous levels of Tiam1 following treatment with doxycycline (Dox) to
283 induce expression of siRNA-resistant wild-type (WT) Tiam1. β -Actin was used as a loading
284 control. i) Representative confocal images of control, Tiam1 knockdown and rescue cells
285 showing abnormal centriole number following Tiam1 knockdown and normal centriole
286 number in cells re-expressing wild-type Tiam1. j) Quantification of images as in (i) from 4
287 independent experiments; more than 50 cells counted per condition per experiment (one-
288 way ANOVA, corrected for multiple comparisons; t-test for comparing +/- dox cells treated
289 with the same siRNA.) For all quantification: *** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$ n.s. = not
290 significant; error bars show S.E.M. All scale bars 3 μ m.

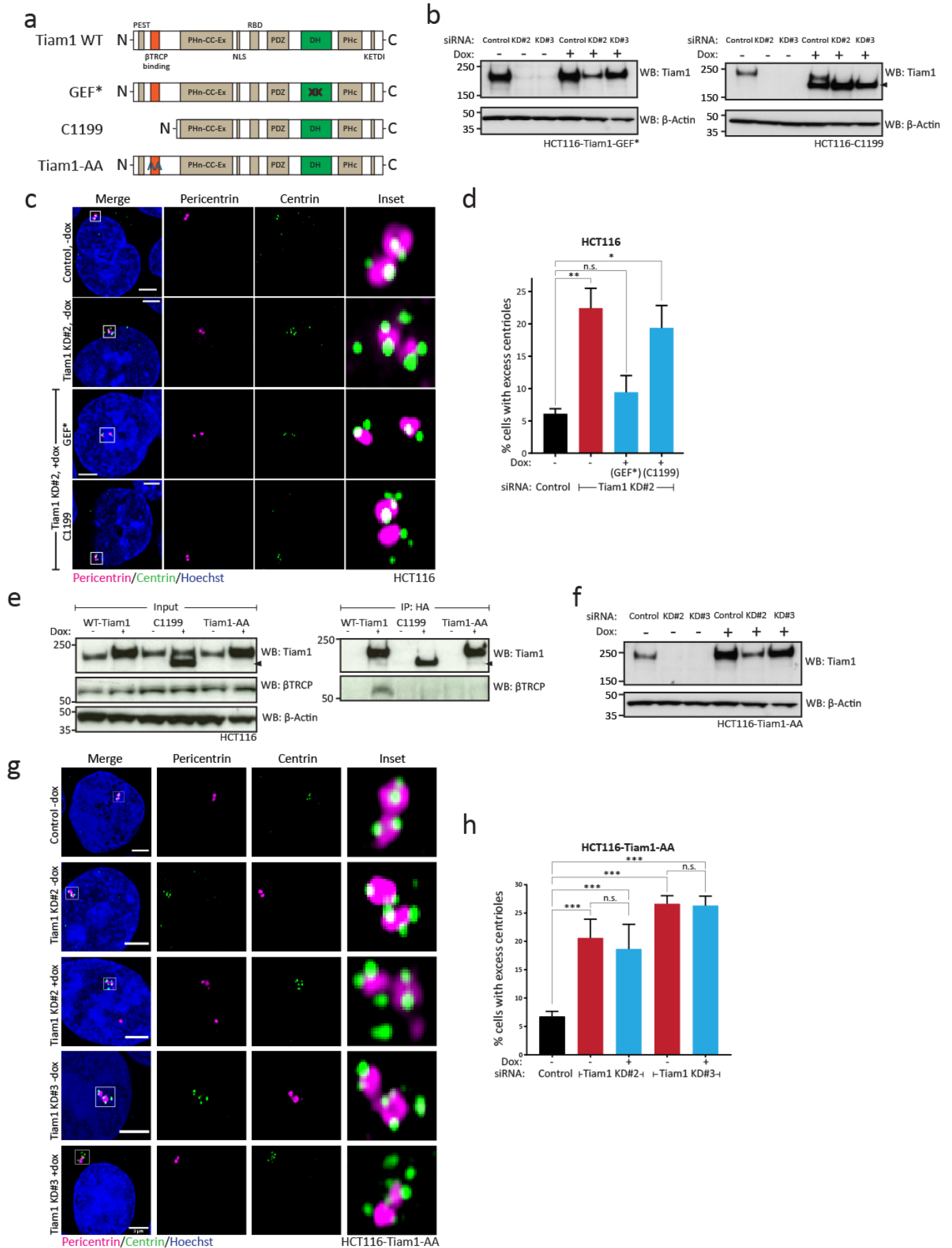
Figure 2



292 **Figure 2 – Tiam1 knockdown leads to an increase in centrosomal PLK4**

293 a) Maximal z-projections of U2OS cells stained for Pericentrin (magenta) as a marker of
294 centrosomes, and endogenous PLK4 (green), showing an increase in centrosomal PLK4
295 staining intensity in Tiam1-knockdown cells. b) Quantification of PLK4 intensity at
296 centrosomes in U2OS cells from three independent experiments from images as in (a) [total
297 number of cells: n=186 (control), n=175 (KD#1), n=165 (KD#2), n=170 (KD#3)]. Box shows
298 25th to 75th percentiles, whiskers show 5th to 95th percentiles; median is marked with a line.
299 One-way ANOVA, corrected for multiple comparisons. c) Maximal z-projections of HCT116
300 cells stained for Pericentrin (magenta) as a marker of centrosomes, and endogenous PLK4
301 (green), showing an increase in centrosomal PLK4 staining in Tiam1-knockdown cells. d)
302 Quantification of PLK4 intensity at centrosomes in HCT116 cells from two independent
303 experiments from images as in (c) [total number of cells: n=237 (control), n=97 (KD#1),
304 n=251 (KD#2), n=296 (KD#3)]. Box shows 25th to 75th percentiles, whiskers show 5th to
305 95th percentiles; median is marked with a line. One-way ANOVA, corrected for multiple
306 comparisons. e) Representative western blot showing an increase in expression of
307 exogenous myc-tagged PLK4 (as determined by immunoprecipitation of myc-tagged PLK4)
308 in U2OS cells following Tiam1 knockdown, compared with control transfected cells.
309 Dox=doxycycline, added to induce expression of exogenous myc-tagged PLK4. α -Tubulin
310 was used as a loading control. f) Quantification of increased PLK4 expression (as
311 determined by immunoprecipitation of myc-tagged PLK4) from control and Tiam1
312 knockdown cells (n=3 independent experiments, bars show mean +/- SEM, dots represent
313 each individual experiment). PLK4 pull-down levels normalised to Tubulin input. g)
314 Quantification of cells with abnormal centriole number in U2OS cells transfected with either
315 control siRNA or Tiam1 KD#3 siRNA, and treated with either vehicle or Centrinone (20 nM).
316 For all quantification: **** p<0.0001 * p<0.05. Scale bars are 3 μ m.

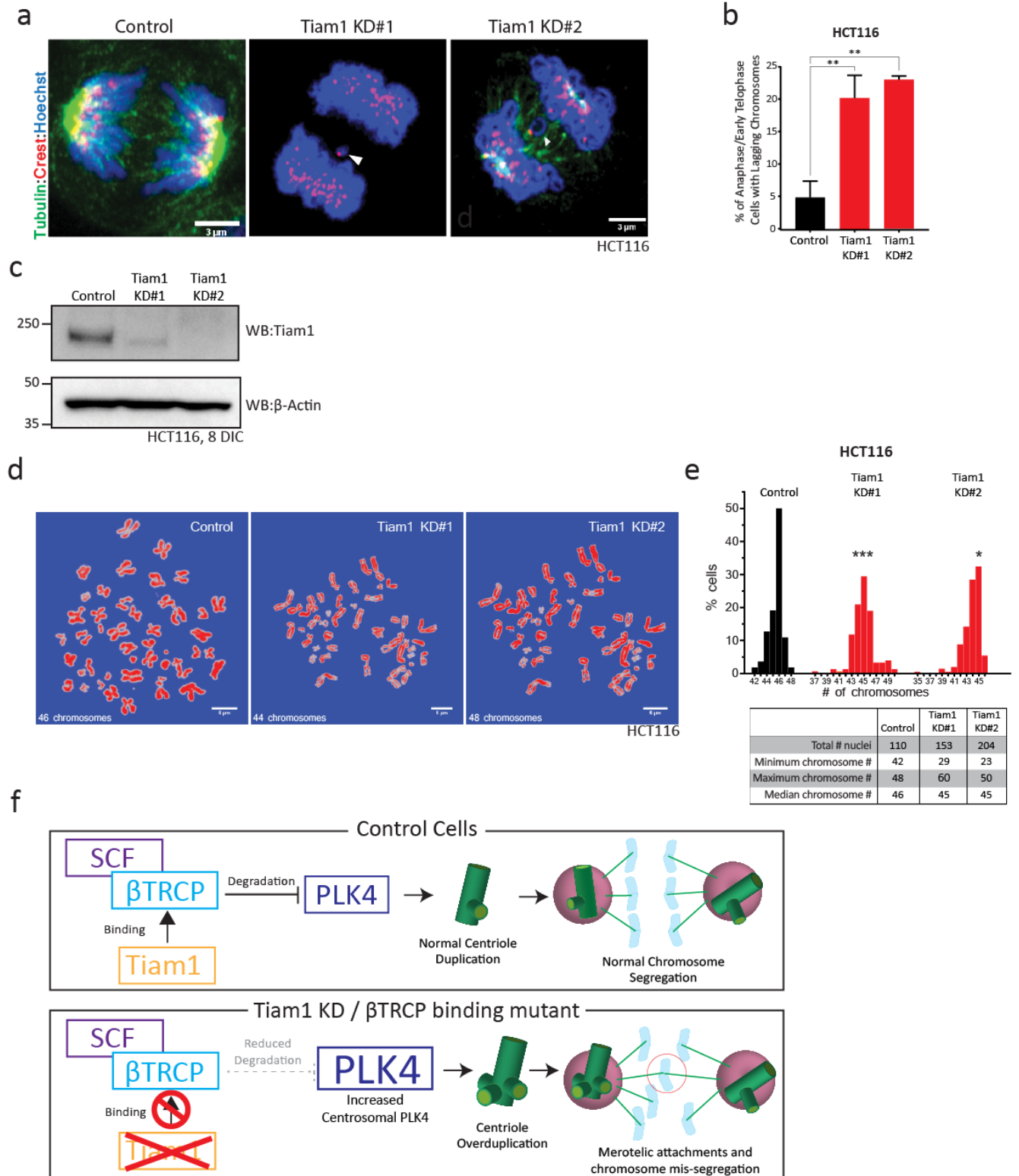
Figure 3



318 **Figure 3 – Tiam1 requires β TRCP binding to maintain normal centriole number**

319 a) Schematic showing the domain structure of wild-type (WT) Tiam1, and the mutant
320 versions. b) Western blots from HCT116-Tiam1-GEF* and HCT116-C1199 cells showing
321 depletion of endogenous Tiam1 by two independent siRNAs and, following treatment with
322 doxycycline (Dox), restoration of near-endogenous levels of either a GEF-dead (GEF*)
323 Tiam1 or an N-terminally truncated mutant (C1199, marked with an arrowhead), both of
324 which are resistant to siRNA treatment. c) Maximal z-projection confocal images of HCT116
325 control, Tiam1 knockdown and Tiam1-rescue cells. Cells exhibit abnormal centriole number
326 following Tiam1 knockdown; this is rescued by expression of RNAi-resistant GEF* Tiam1,
327 but not by expression of RNAi-resistant C1199. d) Quantification of HCT116 cells with
328 excess centrioles from rescue experiments as in (c), from 3 independent experiments; more
329 than 50 cells counted per condition per experiment (one-way ANOVA, corrected for multiple
330 comparisons). e) Western blot from HCT116 cells showing immunoprecipitation of wild-type
331 Tiam1, C1199 (marked with an arrowhead) and Tiam1-AA (all constructs HA-tagged); only
332 wild-type Tiam1 is able to co-immunoprecipitate endogenous β TRCP. f) Western blot from
333 HCT116-Tiam1-AA cells showing depletion of endogenous Tiam1 by two independent
334 siRNAs and restoration of near-endogenous levels of Tiam1 following treatment with
335 doxycycline (Dox) to express the RNAi-resistant, non- β TRCP binding mutant Tiam1-AA. g)
336 Maximal z-projection confocal images of control and Tiam1-knockdown HCT116 cells,
337 showing excess centriole number following Tiam1 knockdown, which is not rescued by
338 doxycycline-induced expression of siRNA-resistant Tiam1-AA. h) Quantification of cells with
339 excess centrioles from rescue experiments as in (g) from 4 independent experiments; more
340 than 50 cells counted per condition per experiment (one-way ANOVA, corrected for multiple
341 comparisons; t-test for comparing +/- dox cells treated with the same siRNA). For all
342 quantification: *** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$; error bars show S.E.M. Scale bars show 3 μ m.
343 β -Actin was used as a loading control in all blots.

Figure 4



344

345 **Figure 4 – Depletion of Tiam1 leads to lagging chromosomes at anaphase and early**
346 **telophase and aneuploidy**

347 a) Maximal z-projection confocal images of anaphase HCT116 cells; chromosomes stained
348 with Hoechst and marked with CREST as a centromere marker, showing lagging
349 chromosomes following Tiam1 knockdown with two independent siRNAs. Scale bars are
350 3 μ m. b) Quantification of percentage of HCT116 cells in anaphase or early telophase with
351 lagging chromosomes following transfection with control or Tiam1 knockdown siRNAs, as in
352 (a). Data quantified from three independent experiments, at least 50 cells quantified per
353 condition per experiment (one-way ANOVA, corrected for multiple comparisons). c) Western
354 blot showing Tiam1 levels in HCT116 cells following 8 days growth in culture with repeated
355 siRNA transfection. β -Actin was used as a loading control. d) Representative images of
356 metaphase spreads from HCT116 cells treated with either control or Tiam1 siRNAs. Scale
357 bars are 5 μ m. e) Histogram of chromosome number from control and Tiam1 knockdown
358 HCT116 cells; data collected from three independent experiments. Table shows additional
359 statistics. Kruskal-Wallis test adjusted for multiple comparisons. * $p < 0.05$ *** $p < 0.001$. f)
360 Model of how the Tiam1- β TRCP interaction affects PLK4 protein levels, centriole duplication
361 and chromosome segregation.

362 **Materials and Methods**

363 **Cell Culture**

364 All cell lines were cultured at 37°C in a humidified incubator (5% CO₂ atmosphere). U2OS
365 and HCT116 cells were cultured in DMEM High Glucose (Gibco) supplemented with 10%
366 FBS (Gibco). MCF7 cells were cultured in DMEM High Glucose (Sigma) supplemented with
367 10% FBS, 1% L-Glutamine (Gibco). Cell lines were routinely tested to exclude Mycoplasma
368 contamination and for cell line authentication (via in-house facilities).

369 **Generation of cell lines**

370 Plasmids were introduced into cells either by transfection using TransIT-LT1 (Mirus)
371 according to the manufacturer's instructions or by retroviral transduction as previously
372 described [20]. For inducible overexpression, HCT116 were retrovirally transduced with
373 pRetro-Tet-ON followed by selection with G418 (1mg/ml, Sigma-Aldrich). pRetro-XT-based
374 constructs were then retrovirally transduced and cells selected with puromycin (2 µg/ml,
375 Sigma-Aldrich).

376 **Plasmids**

377 The doxycycline-inducible PLK4 plasmid was generated by PCR of the wild-type PLK4
378 cDNA from Addgene plasmid #41165 by PfuUltra II Fusion HS DNA polymerase (Agilent
379 #600670.) pcDNA Plk4(Sak) wt (Nigg HR9) was a gift from Erich Nigg[38]. The sequence
380 was confirmed by PCR, and digested with NotI and MluI enzymes (restriction sites
381 introduced via the PCR primer sequences) for introduction into similarly-digested pRetro-X-
382 Tight (Takara Bio). The final insertion was confirmed by sequencing.

383 The doxycycline-inducible Tiam1-AA (non-βTRCP-binding) mutant was generated by
384 Quikchange II (Agilent) site-directed mutagenesis of WT-Tiam1 mouse cDNA at S329 and
385 S334, and successful mutagenesis confirmed by PCR. A portion of the cDNA containing the
386 mutated region was digested with HpaI and NotI, and inserted into the existing pRetro-X-
387 Tight-Tiam1-WT plasmid. Insertion was confirmed by PCR.

388 **siRNA transfection**

389 For transfection of cells for immunofluorescence staining, cells were plated at a density of
390 2x10⁵ onto glass coverslips (6 well dish), and reverse transfected using RNAiMax
391 (Invitrogen) following the manufacturer's instructions. Cells were grown for 72 hours before
392 being fixed in MeOH. For Tiam1, four siRNA sequences were used - Tiam1 KD#1 5'-
393 GAGGTTGCAGATCTGAGCA-3'; Tiam1 KD#2 5'-GAGGUUGCAGAUCUGAGCA-3'; Tiam1

394 KD#3 5'-AGAGCGCACCUACGUGAAA-3'; Tiam1 KD#4 5'GGTTCTGTCTGCCCAATAA3' -
395 and synthesized by Eurofins MWG. In all of the reported assays, a negative control siRNA
396 was used typically siLuc (control) 5'-CGUACGCGGAAUACUUCGA-3', or Dharmacon
397 siGENOME Non-Targeting siRNA #4. 4 siRNA sequences were used to deplete PLK4 to
398 test the specificity of the anti-PLK4 antibody. These were: PLK4 siRNA#1: 5'-
399 GAAUGAACAGGUAUCUAA-3'; PLK4 siRNA#2: 5'-GAAACAUCUUCUAUCUUG-3';
400 PLK4 siRNA#3: 5'-GUGGAAGACUCAAUUGAUA-3'; and PLK4 siRNA#4: 5'-
401 GGACCUUAUUCACCAGUUA-3' (sequences derived from [39]).

402 **Centrinone Treatment**

403 Following siRNA transfection and plating, cells were treated with 20nM Centrinone (Tocris
404 Bioscience) or vehicle control for 3 days before fixation.

405 **Depolymerisation of the cytoplasmic microtubule network**

406 Tiam1 was transiently depleted in U2OS cells by reverse transfection of siRNA on glass
407 coverslips. 72 hours post transfection cells were transferred to pre-cooled DMEM (4°C) and
408 incubated on ice for 30 minutes to depolymerise cytoplasmic microtubules.

409 Cells were fixed in ice-cold methanol immediately following 30 minute incubation on ice.
410 Centrioles were visualised by IF using Centrin and centrosomes using Pericentrin. Diffuse
411 alpha-Tubulin staining around the centrioles revealed depolymerisation of the microtubule
412 network. Centriole number was quantified using the Deltavision Core microscope.

413 **Antibodies**

414 Antibodies against the following were used for western blotting (WB), immunofluorescence
415 (IF) and immunoprecipitation (IP):

- 416 • α -tubulin (DM1A; Sigma-Aldrich, T6199 mouse; 1:2500 IF, methanol; 1:5000 WB)
- 417 • β -actin (Sigma-Aldrich, clone AC-15 mouse; 1:10,000 WB)
- 418 • Centrin (Millipore, (20H5) Mouse; 1:5000 IF, methanol)
- 419 • CREST (Europa Bioproducts, FZ90C-CS1058, Human, 1:2000, methanol)
- 420 • HA tag (Roche Diagnostics, 3F10 rat; 1:200 IF, formaldehyde; 1:1000 WB)
- 421 • PCNA (Abcam, ab18197, rabbit, 1:2000 IF, methanol)
- 422 • Pericentrin (Covance, PRB-432C; rabbit, 1:2000 IF, methanol)
- 423 • PLK4 (Sigma-Aldrich, clone 6H5 mouse, 1:1000 IF, methanol; 1:500 WB)
- 424 • Tiam1 (Bethyl Laboratories, rabbit, A300-099A, 1:1000 WB)
- 425 • Tiam1 (R&D Systems, AF5038, sheep 1:200, methanol)

426 Secondary antibodies and stains used were: IgG peroxidase-conjugated anti-mouse IgG
427 from donkey (GE Healthcare, NA931), anti-rabbit IgG from donkey (GE Healthcare, NA934);
428 Alexa Fluor 488 chicken anti-mouse IgG (H+L) (Molecular Probes, A21200), Alexa Fluor 488
429 donkey anti-rat IgG (H+L) (Molecular Probes, A21208), Alexa Fluor 568 donkey anti-mouse
430 IgG (H+L) (Molecular Probes, A10037), Alexa Fluor 647 chicken anti-rabbit IgG (H+L)
431 (Molecular Probes, A21208) (1:500 IF); Alexa Fluor 647 goat anti-human IgG (H+L)
432 (Molecular Probes, A21445) (1:500 IF); Hoechst 3342 (Life Technologies, H3570).

433 **Protein analysis**

434 Cells were lysed in an appropriate volume of IP lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM
435 NaCl, 1% Triton-x-100 (v/v), 10% glycerol (v/v), 2 mM EDTA, 25 mM NaF and 2 mM
436 NaH₂PO₄ containing 1% protease inhibitor cocktail (P8340, Sigma-Aldrich) and 1%
437 phosphatase inhibitor cocktails 2 and 3 (P5726 and P0044, Sigma-Aldrich) added fresh] or
438 RIPA buffer [25 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS (v/v), 0.5% sodium deoxycholate
439 (v/v), 1% Triton-x-100 containing 1 EDTA-free protease inhibitor tablet (Roche) and 1%
440 phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich) added fresh] for 10 min on ice and
441 proteins were resolved by SDS-PAGE for western blotting.

442 For immunoprecipitation of myc-tagged PLK4, lysates were incubated with 50 µL of Myc-tag
443 beads (A7470, Sigma-Aldrich, blocked with 5% BSA for one hour at room temperature), for
444 2 hours at 4 °C with rotation. Beads were subsequently washed with lysis buffer, and eluted
445 with 2× SDS-PAGE sample buffer (Nupage, Invitrogen).

446 **Immunofluorescence**

447 For immunofluorescence, cells were grown on coverslips and fixed with 100% ice-cold
448 methanol for 5 min at -20 °C. Cells were washed and then blocked in 1% BSA in PBS (v/v)
449 for 1 hour, before successive incubation with primary antibodies (overnight at 4°C) and then
450 secondary antibodies (1 hour at room temperature). Coverslips were mounted onto glass
451 slides using Fluoromount-G (Southern Biotech) along with Hoechst 33342 (1:5000) for
452 nuclear staining, or droplet of ProLong® Gold anti-fade reagent containing the DNA stain
453 DAPI. Staining of endogenous Tiam1 was performed with the same protocol as in [21]
454 which was shown to specifically detect centrosomal Tiam1.

455 **Metaphase spreads**

456 HCT116 cells were grown for 8 days, with an initial siRNA transfection followed by two
457 subsequent trypsinisation, replating and transfections (total of three rounds of siRNA
458 transfection). Cells were treated with 150nM Nocodazole (M1402, Sigma-Aldrich) for four

459 hours to arrest cells in mitosis. Mitotic cells were collected by a mitotic shake off and
460 harvested, followed by resuspension in 3ml pre-warmed hypotonic buffer (40% RPMI
461 (R8758, Sigma-Aldrich), 60% ddH₂O) for 20 minutes. Cells were fixed by repeated addition
462 of Carnoy's fixative (3:1 v/v solution of Methanol:Glacial Acetic Acid), centrifugation and
463 aspiration before final collection in 100% glacial acetic acid. Cells were dropped onto
464 precooled (4°C) wet slides from a height of 40-50cm, left to dry and stained with Hoechst.
465 Images were taken using the Zeiss AiryScan confocal and chromosome number determined
466 by manual counting using ImageJ.

467 **Microscopy**

468 Centrosome and centriole images were acquired using a Zeiss Observer equipped with a
469 Zeiss LSM 880 scan head with the AiryScan detector, with Argon laser 458, 488, 514nm
470 (Lasos, Jena, Germany), Diode 405-30 (Lasos), DPSS 561-10 (Lasos) and HeNe 633nm
471 (Lasos) were utilised for illumination and a Plan-Apochromat 40x/1.4 Oil (Zeiss) objective
472 lens. Centriole, lagging chromosome and metaphase spread images were acquired utilising
473 the 'Fast' AiryScan mode. All equipment control, acquisition and processing of AiryScan
474 images was performed in Zen Black (Zeiss). All images were processed in ImageJ. Images
475 in the same figure panel stained with the same antibody are all set to the same minimum
476 and maximum brightness for comparison of localisation and intensity.

477 PLK4 images were captured using a Zeiss Axiovert 200 M microscope (Solent Scientific).
478 The system uses an Andor iXon 888 camera and a 300 W Xenon light source is used for
479 fluorescence illumination with a variety of ET-Sedat filters (406, 488, 568, 647 nm). The
480 system utilises the Metamorph software to capture and process images. Images were taken
481 using the 100 × oil lens.

482 Immunofluorescence images of centrosomes and centrioles in MCF7 cells and mitotic U2OS
483 cells, as well as in cold-treated U2OS cells, were captured using the Deltavision Core
484 system (based on an Olympus IX71 microscope; fluorescence is achieved using a 300 W
485 Xenon light source with a variety of Sedat filter sets (406, 488, 568, 647 nm) and the
486 attached Roper Cascade 512B camera; images were taken using 100 × / 60 × oil lens.) The
487 Deltavision core system utilises softWorx to capture and process images.

488 **Centriole counts**

489 Widefield or confocal z-stacks of cells stained for Centrin and Pericentrin were used to
490 determine centriole number. Planes were taken at the minimum distance recommended by
491 the system (typically either 200nm or 140nm, depending on the system). Centriole counts

492 were conducted manually using ImageJ, with any cell containing at least one centrosome
493 (marked with Pericentrin) which was associated with three or more puncta of Centrin
494 counted as having excess centriole number. Also included were cells with more than two
495 distinct centrosomes, as these were considered to have arisen from earlier centriole
496 overduplication events (although these typically accounted for only 2-4% of abnormal cells).

497 **Quantification of centrosomal PLK4 intensity**

498 Maximal projections of widefield microscopy image stacks were generated using ImageJ.
499 Two square regions of interest were drawn of fixed size: the first encompassing the
500 centrosome area as marked with Pericentrin (35px^2) (a) and a second larger region of
501 interest, centred on this first box (55px^2) (b). The formula $a - ((b-a)(55^2/35^2))$ was used to
502 calculate the intensity of the PLK4 signal at the centrosome, adjusted for background
503 intensity surrounding the centrosome.

504 **Statistical analysis**

505 Appropriate statistical tests were chosen to minimise type I error associated with significance
506 values. Statistical differences between data were analysed in Prism (GraphPad Software)
507 with appropriate post hoc multiple comparisons test. Tests are specified in figure legends.

508

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517

518 **Competing interests**

519 The authors declare no competing or financial interests.

520

521 **Author contributions**

522 Conceptualization: A.P.P, E.L.O, H.J.W, A.M; Methodology: A.P.P, E.L.O, H.J.W; Validation:
523 A.P.P., G.R.M.W.; Formal analysis: A.P.P, E.L.O; Investigation: A.P.P, G.R.M.W., E.L.O.;
524 Resources: A.P.P, G.R.M.W., H.J.W; Writing - original draft preparation: A.P.P, A.M.;
525 Visualization: A.P.P; Supervision: A.M.; Project administration: A.P.P, A.M.; Funding
526 acquisition: A.P.P., A.M.

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531

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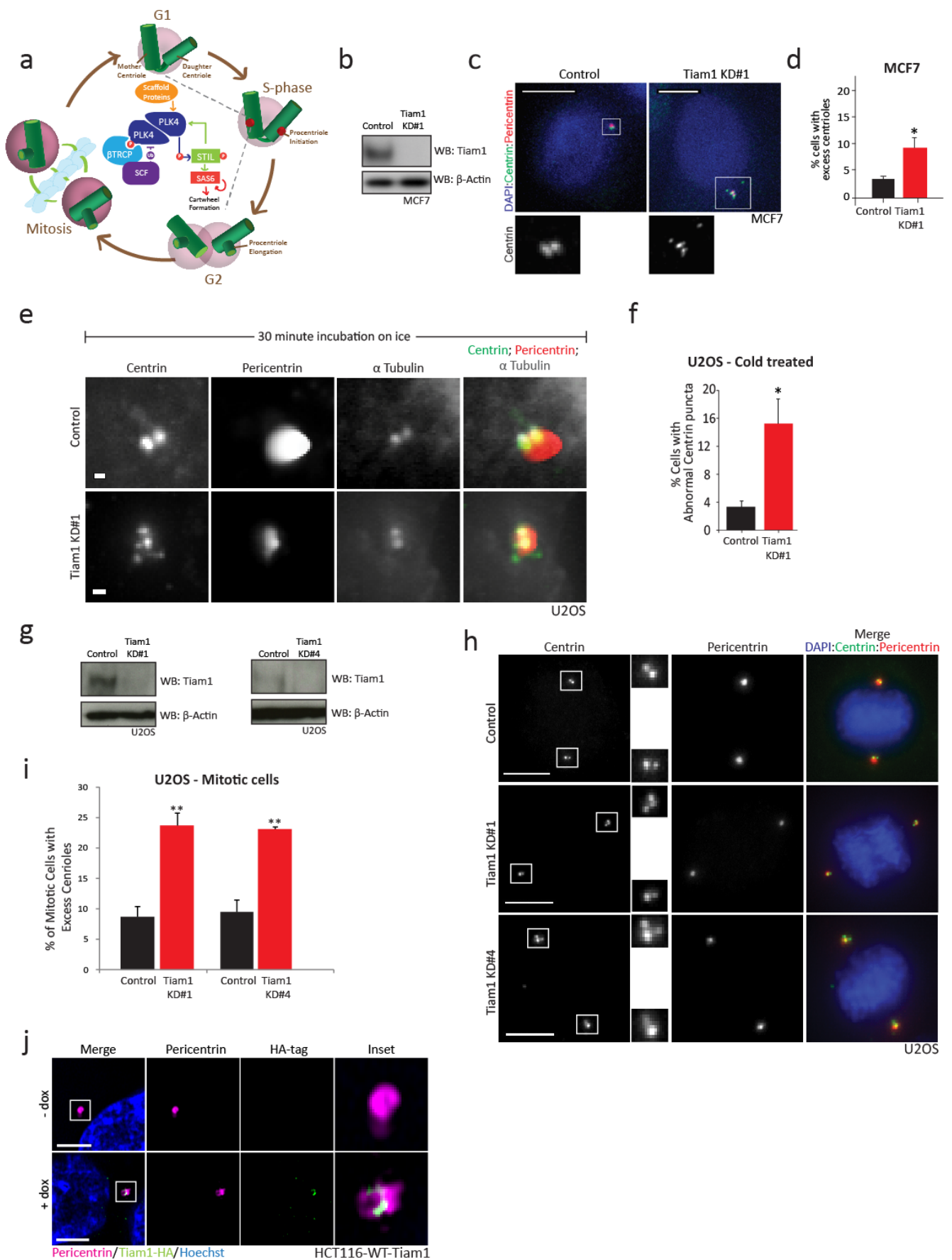
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Supplementary Materials

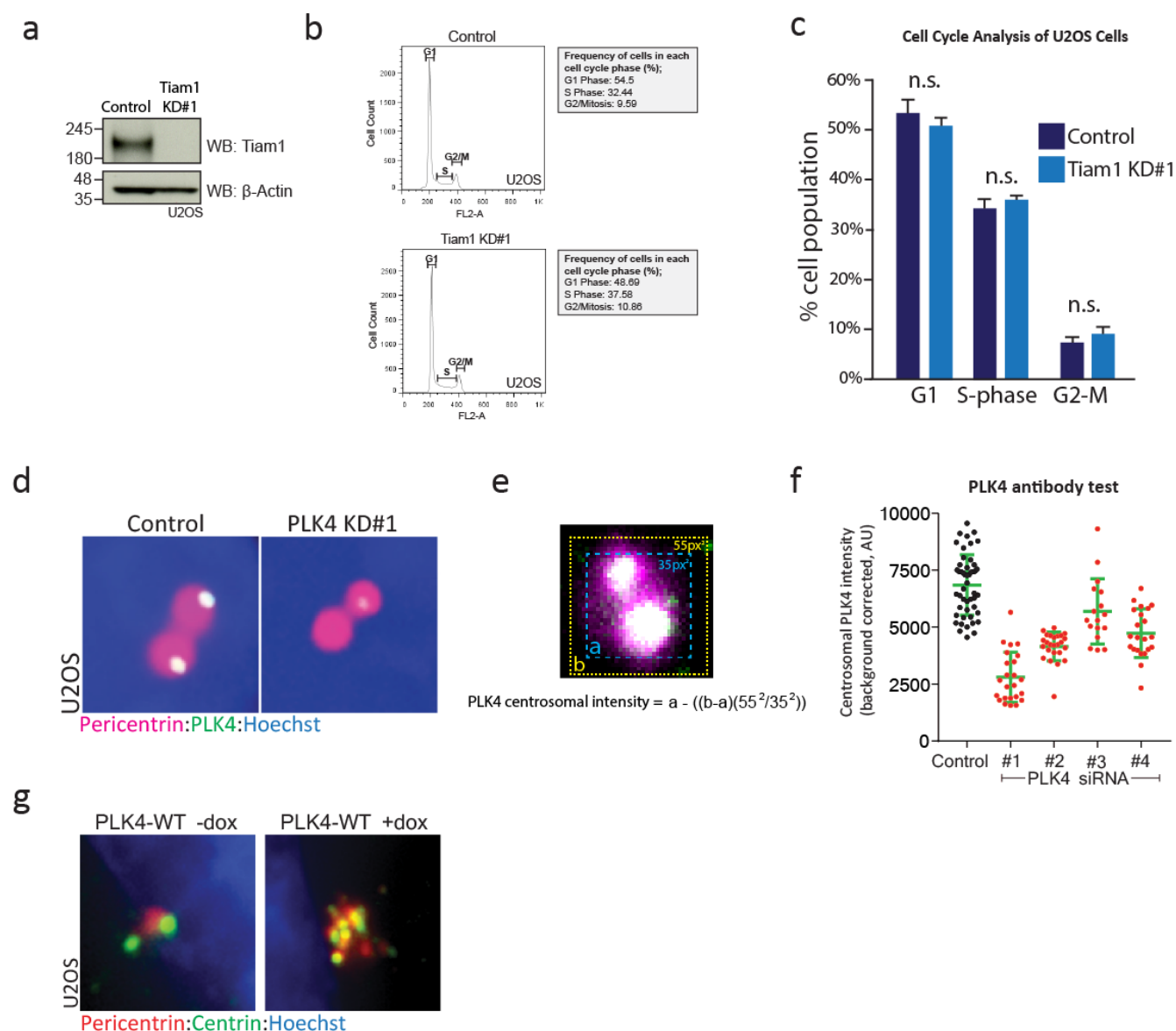
Supplementary Figure 1



641 **Supplementary Figure 1**

642 a) Schematic of the centriole duplication cycle. b) Western blot showing knockdown of
643 endogenous Tiam1 in MCF7 cells. c) Deltavision maximal z-projection images of MCF7
644 cells showing an increase in centriole number following Tiam1 knockdown. Scale bar shows
645 10 μm . d) Quantification of centriole number in MCF7 cells as in (c), from three independent
646 experiments, >145 cells quantified per experimental replicate. e) Deltavision images of
647 centrosomes in U2OS cells (marked with Pericentrin), showing stable centriole staining (as
648 marked with Centrin) in both control and Tiam1 knockdown cells, following cold treatment
649 (see Methods). Scale bars show 1 μm . f) Quantification of centriole number in control and
650 Tiam1 knockdown cells following cold treatment as in (e), from three independent
651 experiments. ≥ 70 cells were quantified per experimental replicate. g) Western blots
652 showing knockdown of Tiam1 in U2OS cells following treatment with either Tiam1 KD#1 or
653 Tiam1 KD#4 siRNAs. h) Deltavision maximal z-projection images of mitotic U2OS cells
654 treated with either control or Tiam1 KD#1 or KD#4 siRNA, showing an increase in centriole
655 number in Tiam1 knockdown cells. Scale bars show 10 μm . i) Quantification of cells with
656 excess centrioles from images as in (h) from three independent experiments (Tiam1 KD#1;
657 100 mitotic cells quantified per experimental replicate; Tiam1 KD#4; ≥ 45 mitotic cells
658 quantified per experimental replicate; N=3). * = $p < 0.05$ ** = $p < 0.01$. j) Single confocal z-
659 planes of HCT116-Tiam1-WT cells showing centrosomal localisation of exogenous Tiam1
660 (as detected with an antibody against the HA tag on WT-Tiam1) following doxycycline (+dox)
661 treatment. Scale bars show 3 μm . All bars show mean \pm SEM. β -Actin was used as a
662 loading control in all blots.

Supplementary Figure 2



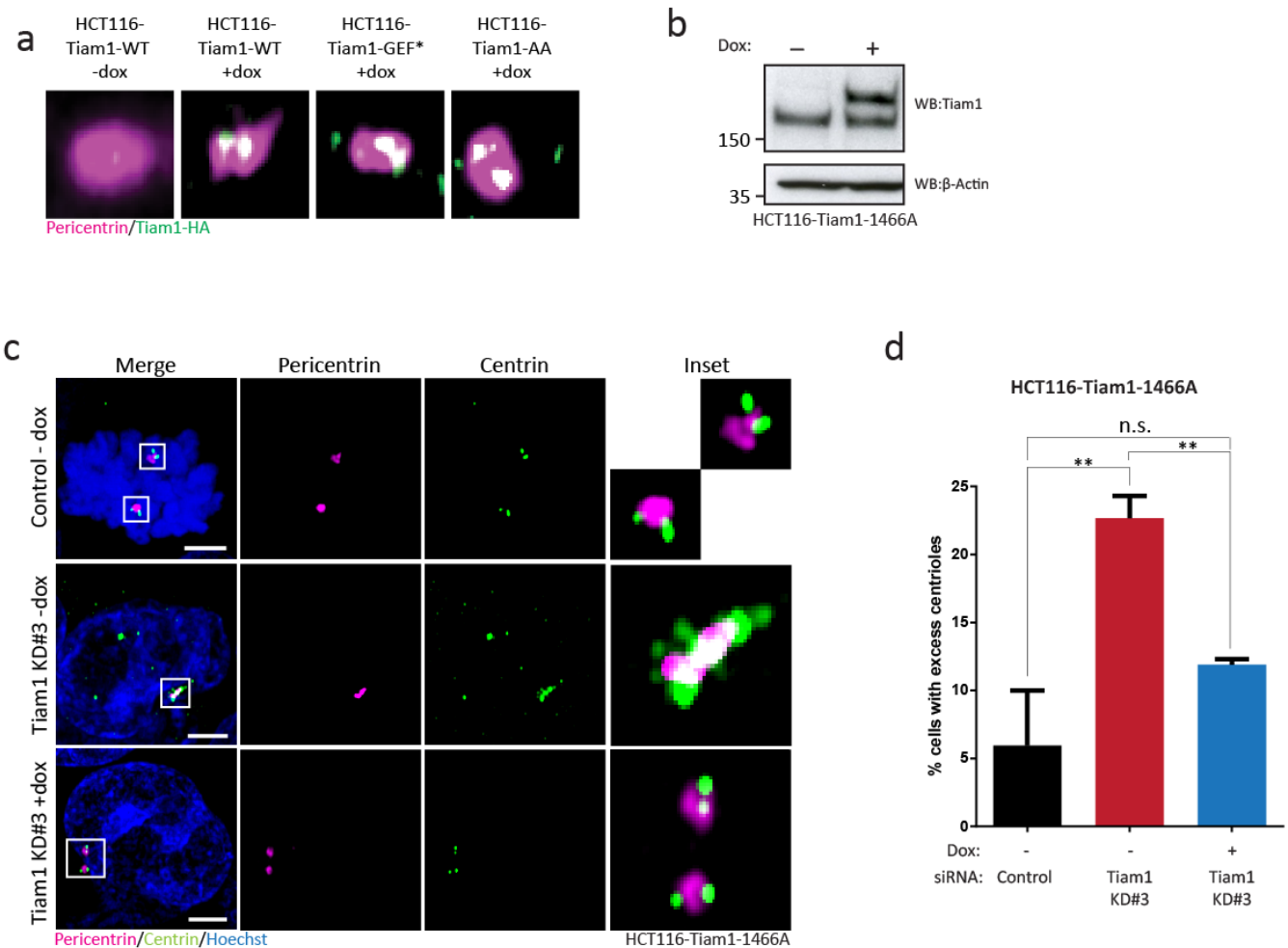
663

664 **Supplementary Figure 2**

665 a) Western blot showing Tiam1 knockdown in U2OS cells. β -Actin was used as a loading
 666 control. b) Representative FACS plots from control and Tiam1 KD#1 U2OS cells. c)
 667 Summary of FACS analysis of cell cycle data from U2OS cells showing no change in cell
 668 cycle progression following Tiam1 knockdown, compared with control cells. Taken from
 669 three independent FACS experiments. d) Representative images of U2OS centrosomes
 670 (marked with Pericentrin), showing PLK4 staining at centrosomes in control cells, and
 671 decrease in staining in PLK4-knockdown cells. e) Schematic of centrosomal PLK4 intensity
 672 quantification. f) Quantification of centrosomal PLK4 intensity from U2OS cells treated with

673 a panel of siRNAs targeting PLK4. g) Images of U2OS cells stained with Pericentrin (red,
674 marking whole centrosomes) and Centrin (green, marking centrioles) showing an increase in
675 centrosome and centriole number after treatment with doxycycline (+dox) to induce
676 expression of WT-PLK4. t-tests comparing control and Tiam1 KD#1 for each cell cycle
677 phase. n.s. = not significant

Supplementary Figure 3

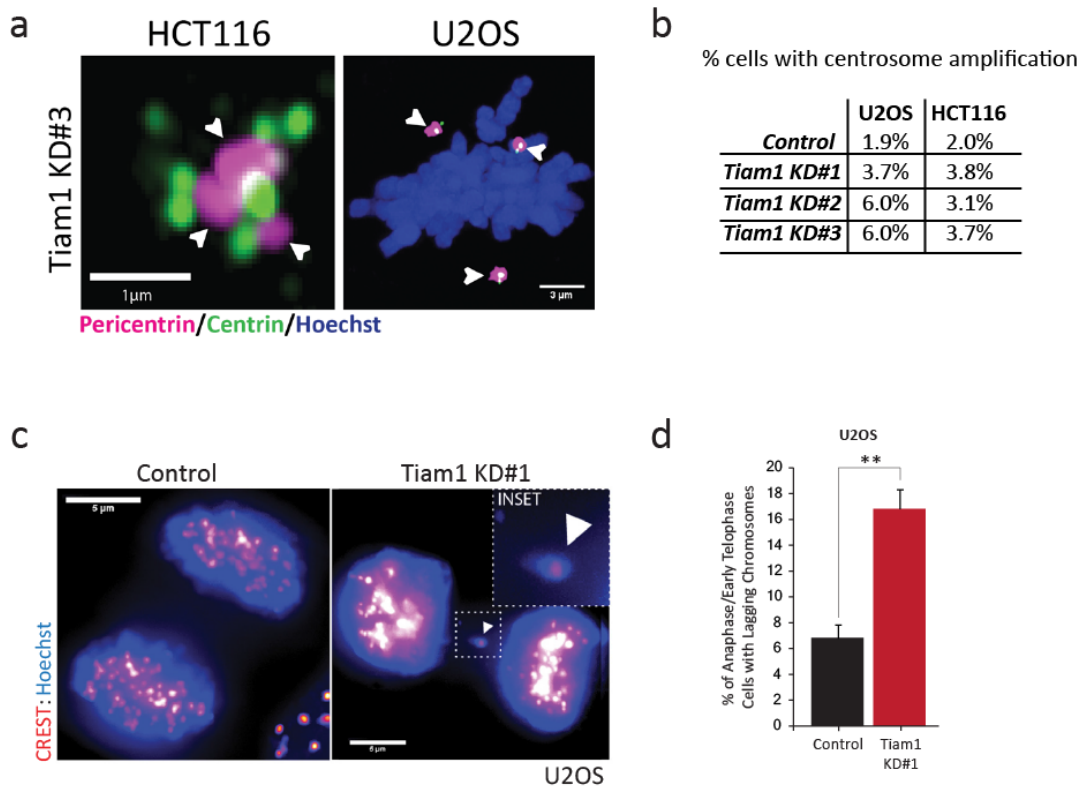


678

679 Supplementary Figure 3

680 a) Confocal images of centrosomes (marked with Pericentrin, magenta) in HCT116 cells,
 681 showing localization of exogenous WT, GEF* and AA-mutant Tiam1 to the centrosome
 682 (marked with an antibody against the HA-tag, green.) b) Western blot showing expression of
 683 Tiam1-1466A in HCT116 following treatment with doxycycline (dox). β -Actin was used as a
 684 loading control. c) Maximal z-projection confocal images of HCT116 cells treated with either
 685 control or Tiam1 KD#3 siRNA, showing an increase in centriole number following Tiam1
 686 knockdown, which is rescued by expression of Tiam1-1466A following doxycycline (Dox)
 687 treatment. d) Quantification of HCT116 cells with excess centrioles from cells as in (c) from
 688 3 independent experiments; more than 50 cells counted per condition per experiment (one-
 689 way ANOVA, corrected for multiple comparisons). ** $p < 0.01$ n.s. = not significant; error bars
 690 show S.E.M. Scale bars show 3 μ m.

Supplementary Figure 4



691

692 **Supplementary Figure 4**

693 a) Representative confocal images showing HCT116 and U2OS cells with centrosome
 694 amplification (marked with arrowheads) following Tiam1 knockdown. Centrosomes are
 695 marked with Pericentrin (magenta) and Centrin (green). Scale bars are 1μm (HCT116) and
 696 3μm (U2OS). b) Table showing increase in percentage of U2OS and HCT116 cells with
 697 excess centrosomes following three days of transfection with either control or Tiam1
 698 knockdown siRNAs. c) Images of U2OS cells (stained with Hoechst and CREST as a
 699 centromere marker), with a lagging chromosome at telophase in the cell treated with Tiam1
 700 KD#1 siRNA (marked with an arrowhead). Scale bars are 5μm. d) Quantification of control
 701 and Tiam1 knockdown U2OS cells with lagging chromosomes at anaphase and early
 702 telophase from three independent experiments. ** $p < 0.001$ (t-test); error bars show S.E.M.