1 **CLASP2 lattice-binding near microtubule plus ends stabilizes**

2 **kinetochore attachments**

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Abstract

The fine regulation of kinetochore microtubule dynamics during mitosis ensures proper chromosome segregation by promoting error correction and spindle assembly checkpoint (SAC) satisfaction. CLASPs are widely conserved microtubule plus-end-tracking proteins that regulate microtubule dynamics throughout the cell cycle and independently localize to kinetochores during mitosis. Thus, CLASPs are ideally positioned to regulate kinetochore microtubule dynamics, but the underlying molecular mechanism remains unknown. Here we found that human CLASP2 can dimerize through its C-terminal kinetochore-targeting domain, but kinetochore localization was independent of dimerization. CLASP2 kinetochore localization, microtubule plus-end-tracking and microtubule lattice binding through TOG2 and TOG3 (but not TOG1) domains, independently sustained normal spindle length, timely SAC satisfaction, chromosome congression and faithful segregation. Measurements of kinetochore microtubule half-life in living cells expressing RNAi-resistant mutants revealed that CLASP2 kinetochore localization, microtubule plus-end-tracking and lattice binding cooperatively modulate kinetochore microtubule stability during mitosis. Thus, CLASP2 regulates kinetochore microtubule dynamics by integrating distinctive microtubule-binding properties at the kinetochore-microtubule interface to ensure chromosome segregation fidelity.

Keywords

Mitosis, kinetochore, microtubule dynamics, CLASP2, TOG, +TIPs

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Introduction

Microtubule dynamics are modulated by several Microtubule-Associated Proteins (MAPs) (Maiato et al., 2004). Some MAPs specifically accumulate at the growing plus ends of microtubules and are collectively known as microtubule plus-end-tracking proteins or +TIPs (Akhmanova and Steinmetz, 2008). CLIP-associated proteins (CLASPs) are widely conserved +TIPs that stabilize microtubule plus ends by suppressing catastrophes and promoting rescue (Aher et al., 2018; Al-Bassam et al., 2010; Lawrence et al., 2018; Majumdar et al., 2018). Humans have two CLASP paralogues, CLASP1 and CLASP2, which exist as multiple isoforms (Akhmanova et al., 2001; Inoue et al., 2000; Lemos et al., 2000). Structurally, CLASPs harbor three distinct functional domains: 1) a characteristic basic region, also found in other +TIPs, comprising two serine-x-isoleucine-proline (SxIP) motifs that enable microtubule plus-end-tracking via interaction with EB proteins (Honnappa et al., 2009; Mimori-Kiyosue et al., 2005); 2) the presence of two to three tumor overexpression gene (TOG) domains, which interact with the microtubule lattice by distinguishing the structural conformation of the αβ-tubulin dimer on microtubule protofilaments (Leano et al., 2013; Maki et al., 2015); and 3) a C-terminal domain required for kinetochore localization (Maia et al., 2012; Maiato et al., 2003a; Mimori-Kiyosue et al., 2006) and protein dimerization (Al-Bassam et al., 2010; Funk et al., 2014; Patel et al., 2012), as well as interaction with other kinetochore proteins, including CLIP170, CENP-E and Plk1 (Akhmanova et al., 2001; Dujardin et al., 1998; Maffini et al., 2009; Maia et al., 2012).

During mitosis, mammalian CLASPs play redundant roles in the organization of the mitotic spindle, and interference with their function results in

73 several mitotic abnormalities, including monopolar, short and multipolar spindles

74 (Logarinho et al., 2012; Maiato et al., 2003a; Maiato et al., 2003b; Mimori-Kiyosue

75 et al., 2006; Pereira et al., 2006). CLASPs localize at the fibrous corona region of

76 the kinetochore throughout mitosis, where they play a critical role in the regulation

77 of kinetochore microtubule dynamics required for microtubule poleward flux and

78 turnover, as well as the correct alignment and segregation of chromosomes

79 (Logarinho et al., 2012; Maffini et al., 2009; Maiato et al., 2003a; Maiato et al.,

80 2005; Maiato et al., 2003b; Pereira et al., 2006). Previous works revealed that,

81 during prometaphase, CLASP1 interacts with the kinesin-13 Kif2B to promote

82 kinetochore microtubule turnover, which is necessary for the correction of

83 erroneous attachments (Maffini et al., 2009; Manning et al., 2010). In metaphase,

84 CLASP1 interacts with Astrin, which promotes kinetochore microtubule

85 stabilization required for spindle assembly checkpoint (SAC) satisfaction

86 (Manning et al., 2010). This places CLASP1 as part of a regulatory switch that

87 enables the transition between labile-to-stable kinetochore microtubule

88 attachments by establishing temporally distinct interactions with different partners

89 at the kinetochore. CLASP2 is regulated by Cdk1 and Plk1 phosphorylation as

90 cells progressively reach metaphase, leading to a gradual stabilization of

91 kinetochore-microtubule attachments (Maia et al., 2012). While this broad picture

92 provides important information on the molecular context in which CLASPs

93 operate at the kinetochore-microtubule interface, we still lack a detailed

94 mechanistic view on how the intrinsic properties of CLASPs modulate

95 kinetochore microtubule dynamics.

96   Here we focused on human CLASP2 to investigate how its distinct

97 functional domains affect mitosis, with emphasis on the regulation of kinetochore

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98   microtubule dynamics. Our findings revealed that CLASP2 integrates multiple

99   independent features, including microtubule plus-end-tracking, microtubule

100   lattice binding through TOG domains and kinetochore localization, to modulate

101   kinetochore microtubule dynamics required for faithful chromosome segregation

102   during mitosis in human cells.

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Results and Discussion

CLASP2 can form dimers through its C-terminal domain

Previous studies have come to contradictory conclusions regarding the native structure of CLASPs. Based on the hydrodynamic profile of several kinetochore sub-complexes present in *Xenopus* egg extracts, it was proposed that the single *Xenopus* CLASP exists as an elongated monomer in solution (Emanuele et al., 2005). This was subsequently confirmed by Fluorescence Correlation Spectroscopy and Photon Counting for human GFP-CLASP2 (Drabek et al., 2006), as well as single molecule fluorescence intensity quantification of recombinant human GFP-CLASP2 (Aher et al., 2018). However, another set of studies with recombinant human CLASP1 and its counterparts in *S. pombe* and *S. cerevisiae* indicated that they can form homodimers in solution through their C-terminal domain (Al-Bassam et al., 2010; Funk et al., 2014; Patel et al., 2012). To shed light into this debate, we investigated the native state of CLASP2 in human cells. This was initially assessed by blue native polyacrylamide gel electrophoresis followed by western-blot analysis (Fig. 1A). We observed a fast migrating band, consistent with a monomeric form of CLASP2, as well as higher molecular weight species that may have arisen from formation of homodimers, heterodimers with CLASP1 and higher order multiprotein complexes. Interestingly, the migration profile of CLASP2 was similar in mitotic and asynchronous cell populations, indicating that the previously reported CLASP2 phosphorylation in mitosis (Maia et al., 2012) does not significantly alter any potential oligomerization or higher order state of CLASP2.

142   Next, we calculated the molecular weight of CLASP2 from mitotic cells

143   using the method of Siegel and Monty (Siegel and Monty, 1966), which combines

144   the Stokes' radius obtained from size exclusion chromatography (Fig. 1B) and

145   the sedimentation coefficients derived from density gradient centrifugation (Fig.

146   1C). Accordingly, we identified two pools of CLASP2 with apparent molecular

147   weights of 153 kDa and 278 kDa, respectively (Fig. 1D), which did not co-elute

148   with α-tubulin (Fig. 1C), suggesting that, unlike its yeast counterparts (Al-Bassam

149   et al., 2010; Funk et al., 2014; Majumdar et al., 2018), human CLASP2 is not

150   normally associated with free tubulin, in agreement with recent in vitro

151   reconstitution experiments (Aher et al., 2018; Maki et al., 2015). These results

152   are consistent with the existence of monomeric (theoretical value for the longest

153   CLASP2α isoform = 166 kDa) and dimeric forms of CLASP2 in human cells.

154   Further calculation of the frictional ratio of these two pools yielded a value of ∼2,

155   indicating that CLASP2 molecules are non-globular and considerably elongated.

156   To determine whether CLASP2 dimerization is mediated by the C-terminal

157   region, as previously reported for human CLASP1 and its counterparts in yeasts

158   (Al-Bassam et al., 2010; Funk et al., 2014; Patel et al., 2012), we investigated the

159   oligomerization of a C-terminal recombinant fragment of human CLASP2

160   (CLASP2-1213-1515). CLASP2 C-terminal eluted as a single peak after size

161   exclusion chromatography (Fig. 1E), in a volume corresponding to a Stokes'

162   radius of 35.1Å (Fig. 1F). Density gradient centrifugation analysis revealed a

163   sedimentation coefficient of 5.2 (Fig. 1G). Using the Siegel and Monty method,

164   the calculated molecular weight was 78.5 kDa, consistent with dimer formation

165   (theoretical value 75.5 kDa) (Fig. 1D). Analysis of CLASP2 C-terminal by

166   Dynamic Light Scattering (DLS), which measures Brownian motion and relates

167 this to the size of particles in solution, also revealed a single population with low

168 polydispersity values (10%) and a hydrodynamic radius of 36.1Å (Fig. 1H),

169 corresponding to an estimated molecular weight of 67.9 kDa. Taken together,

170 these results indicate that human CLASP2 can form dimers through its C-terminal

171 domain. The existence as monomer or dimer in solution or in cells might depend

172 on the physiological concentration of CLASP2 and its accumulation in particular

173 cellular structures (e.g. kinetochores).

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175 **CLASP2 C-terminal domain is required for kinetochore localization**

176 **independently of dimerization**

177 To investigate how CLASP2 impacts kinetochore microtubule dynamics during

178 mitosis, we used an RNAi-based rescue system with RNAi-resistant mRFP-

179 CLASP2γ-derived constructs harboring specific mutations that disrupt the

180 different CLASP2 functional domains (Maki et al., 2015), either alone or in

181 combination (Fig. 2A). In addition to the wild type (WT) mRFP-CLASP2γ

182 construct, we used a construct mutated in both SxIP motifs (IP12) (Honnappa et

183 al., 2009) that disrupts CLASP2-EB protein interaction and consequently

184 microtubule plus-end-tracking; a construct mutated in both TOG2 and TOG3

185 domains (2ea-3eeaa) (Al-Bassam et al., 2007; Leano et al., 2013) required for

186 microtubule lattice binding; as well as a construct combining mutations at both

187 SxIP motifs and in TOG3 (IP12-3eeaa). Additionally, we used a construct with a

188 truncated C-terminal domain (ΔC), which mediates CLASP2 dimerization (this

189 study) and kinetochore localization (Maia et al., 2012; Mimori-Kiyosue et al.,

190 2006). Finally, to dissect the requirement of CLASP2 dimerization for its

191 kinetochore localization, we generated a construct in which the human CLASP2

192   C-terminal was replaced by an artificial dimerization domain based on the Gcn4-

193   Leucine Zipper sequence (ΔC-Gcn4), as shown previously for the *S. cerevisiae*

194   CLASP orthologue Stu1 (Funk et al., 2014).

195   After stable transduction using a lentiviral system in a human U2OS cell

196   line stably expressing photoactivatable (PA) GFP-α-tubulin (Ganem et al., 2005),

197   all constructs expressed at comparable levels (Sup. Fig. 1). We then determined

198   the localization of the different constructs after RNAi-mediated depletion of both

199   CLASP1 and CLASP2 (Sup. Fig. 2) to avoid functional redundancy (Mimori-

200   Kiyosue et al., 2006; Pereira et al., 2006) and potential dimerization with the

201   endogenous proteins. As expected, the IP12 construct failed to localize at

202   microtubule plus ends, as determined by co-localization with EB1 in interphase

203   cells (Sup. Fig. 3A), whereas the 2ea-3eeaa construct failed to associate with

204   mitotic spindle microtubules (Sup. Fig. 3B). In agreement, the IP12-3eeaa

205   construct showed a compromised localization at both microtubule plus ends and

206   spindle microtubules (Sup. Fig. 3A, B). To investigate the localization of the

207   different CLASP2 constructs at unattached kinetochores we used mitotic cells

208   treated with nocodazole to depolymerize spindle microtubules. All constructs,

209   except the ΔC and ΔC-Gcn4, were able to localize at unattached kinetochores

210   (Fig. 2B). In order to substantiate these findings, we quantified the fluorescence

211   intensity of each CLASP2 construct relative to the signal from constitutive anti-

212   centromere antibodies from CREST patients. As expected, mutant CLASP2-ΔC

213   and CLASP2-ΔC-Gcn4 proteins were virtually undetectable at unattached

214   kinetochores (Fig. 2C), confirming that the C-terminal domain of CLASP2 is

215   necessary for kinetochore localization, while demonstrating that dimerization *per*

216   *se* is not sufficient to target CLASP2 to kinetochores. This reveals a critical

217 difference between human CLASP2 and its orthologue Stu1 in *S. cerevisiae*, in

218 which dimerization was shown to be sufficient for kinetochore targeting (Funk et

219 al., 2014). Surprisingly, although all other CLASP2 mutants that compromise

220 microtubule plus-end-tracking and association with the microtubule lattice were

221 detectable at unattached kinetochores, they all showed a statistically significant

222 decrease (∼20%) relative to WT CLASP2 (Fig. 2C). We attribute this reduction to

223 slight differences in the expression of the distinct constructs and/or possible

224 alterations in the tridimensional conformation/folding of CLASP2 that might

225 compromise its normal interaction with kinetochores.

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227 **Regulation of mitotic spindle length by CLASP2 depends on its kinetochore**

228 **localization and microtubule-binding properties**

229 CLASPs have been previously implicated in the control of mitotic spindle length

230 through their role in the incorporation of tubulin required for kinetochore

231 microtubule poleward flux (Logarinho et al., 2012; Maffini et al., 2009; Maiato et

232 al., 2003a; Maiato et al., 2005; Maiato et al., 2002; Mimori-Kiyosue et al., 2006).

233 However, it remains unknown how CLASPs mediate this process. One possibility

234 is that, similar to other TOG-domain containing proteins that localize to

235 kinetochores (Ayaz et al., 2012; Geyer et al., 2018; Miller et al., 2016;

236 Nithianantham et al., 2018), CLASPs act as microtubule polymerases by

237 interacting directly with free tubulin in solution. Another possibility is that CLASPs

238 regulate spindle microtubule flux by controlling the interaction between

239 kinetochores and the microtubule lattice, near polymerizing microtubule plus-

240 ends. To distinguish between these possibilities, we used fluorescence

241 microscopy in fixed cells and the same RNAi-rescue strategy to investigate how

242 the different CLASP2 functional domains contribute to ensure normal mitotic

243 spindle length. As expected, RNAi-mediated depletion of endogenous CLASPs

244 from U2OS cells resulted in shorter spindles (Maffini et al., 2009), and expression

245 of RNAi-resistant WT CLASP2γ completely rescued normal spindle length (Fig

246 3A, C). This further demonstrates CLASPs functional redundancy, while

247 revealing that the TOG1 domain that is lacking in the CLASP2γ isoform is

248 completely dispensable for the regulation of mitotic spindle length. In contrast,

249 expression of all the other mutant constructs failed to rescue normal spindle

250 length (Fig. 3A, C). Interestingly, disruption of the TOG2 and TOG3 domains

251 affected spindle length to a similar extent as disruption of the SxIP motifs,

252 whereas the combined disruption of both TOG3 domain and SxIP motifs

253 exacerbated this effect almost comparably with the disruption of the kinetochore-

254 targeting domain of CLASP2 or CLASPs depletion alone (Fig. 3A, C). Altogether,

255 these results indicate that microtubule plus-end-tracking and microtubule lattice-

256 binding through the TOG2 and TOG3 domains are independently required to

257 ensure normal spindle length. However, these properties per se are insufficient

258 to ensure the normal function of CLASP2, which requires targeting to

259 kinetochores, independently of dimerization. Finally, expression of all mutant

260 CLASP2 constructs resulted in a significant increase in anaphase and telophase

261 cells with chromosome missegregation events (Fig. 3B, D), suggesting a role for

262 CLASP2 in error correction.

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264 **Chromosome congression, timely SAC satisfaction and chromosome**

265 **segregation fidelity rely on both CLASP2 kinetochore- and microtubule-**

266 **binding properties**

267   To gain a deeper insight on how the distinct CLASP2 functional domains impact

268   mitosis we resorted to live-cell imaging analysis by spinning-disc confocal

269   microscopy. To monitor chromosome movements we transduced H2B-GFP in

270   each of the stable U2OS lines expressing the distinct CLASP2 constructs and

271   added SiR-tubulin at 20 nM to visualize microtubules (Lukinavicius et al., 2014).

272   As expected, CLASPs depletion from U2OS cells resulted in a significant delay

273   from nuclear envelope breakdown (NEB) to anaphase onset relative to controls

274   (Fig 4A, B), and this delay was fully rescued by expression of the WT CLASP2γ

275   construct, but not by any of the CLASP2 mutant constructs (Fig 4A, B). These

276   results suggest that CLASP2 at the kinetochore requires microtubule plus-end-

277   tracking and lattice binding capacities to establish a functional kinetochore-

278   microtubule interface necessary for timely SAC satisfaction.

279   Measurement of the duration from NEB to metaphase in the same

280   experimental setup revealed that the observed delay in anaphase onset after

281   CLASPs depletion, or inefficient rescue with the different CLASP2 mutant

282   constructs, was essentially due to a delay in completing chromosome

283   congression to the spindle equator (Fig. 4A, C), consistent with a role of CLASP2

284   in the regulation of kinetochore-microtubule attachments. Moreover, this delay

285   correlated with an increase in chromosome segregation errors during anaphase

286   and telophase (Fig. 4D), in agreement with our fixed cell analysis (Fig. 3D). Taken

287   together, these results strongly suggest that multiple domains of CLASP2 ensure

288   normal kinetochore microtubule dynamics required for chromosome congression,

289   timely SAC satisfaction and efficient error correction during mitosis.

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291   **Multimodal regulation of kinetochore-microtubule dynamics by CLASP2**

292   To directly test how the different CLASP2 domains impact kinetochore

293   microtubule dynamics we quantified kinetochore and non-kinetochore

294   microtubule half-life by Fluorescence Dissipation after Photoactivation (FDAPA)

295   (Ferreira et al., 2018). By fitting the fluorescence decay over time to a double

296   exponential curve, it is possible to discriminate two spindle microtubule

297   populations with fast and slow turnover that are thought to correspond to the less

298   stable non-kinetochore microtubules and more stable kinetochore microtubules,

299   respectively (Bakhoum et al., 2009a; Bakhoum et al., 2009b; Zhai et al., 1995).

300   Admittedly, while this approach is unable to distinguish multiple stable

301   microtubule populations that might exist in the spindle, it remains the golden

302   standard to detect subtle alterations in kinetochore microtubule dynamics that

303   cannot be disclosed by less sensitive methods, such as cold-shock or treatment

304   with microtubule poisons. As so, depletion of endogenous CLASPs by RNAi

305   resulted in a significant decrease in kinetochore microtubule half-life during

306   metaphase when compared to controls (Fig. 5A, B), without significantly affecting

307   the half-life of non-kinetochore microtubules (Fig. 5A, C). Interestingly, previous

308   measurements of kinetochore microtubule half-life after CLASPs depletion from

309   human U2OS cells suggested a role in kinetochore microtubule destabilization

310   (Maffini et al., 2009). However, this previous study did not distinguish between

311   late prometaphase and metaphase cells, reinforcing the idea that CLASPs are

312   part of a regulatory switch that controls the transition between labile-to-stable

313   kinetochore microtubule attachments (Maia et al., 2012; Manning et al., 2010).

314   Importantly, the WT CLASP2γ construct was able to completely rescue normal

315   kinetochore microtubule half-life (Fig. 5A, B), indicating full functionality and

316   dispensability of the TOG1 domain for the stabilization of kinetochore

microtubules during metaphase. In contrast, mutation of the TOG2 and TOG3 domains, or the SxIP motifs of CLASP2, resulted in less stable kinetochore microtubules and consequently incapacity to rescue the effect caused by CLASPs depletion, a tendency that was exacerbated by the combined mutation of the TOG3 and SxIP domains (Fig. 5A, B). These results suggest that the microtubule lattice binding properties of CLASP2 act synergistically with its microtubule plus-end-tracking ability to stabilize kinetochore-microtubule attachments during metaphase in human cells.

The incapacity to rescue normal kinetochore microtubule half-life after CLASPs depletion was maximal in both C-terminal deletion mutants (Fig. 5A, B). This indicates that the localization of CLASP2 at kinetochores is critical for normal kinetochore microtubule dynamics, independently of CLASP2 dimerization, association with the microtubule lattice and tracking of growing microtubule plus ends. Remarkably, none of the CLASP2 mutants significantly compromised non-kinetochore microtubule half-life in our assay. We conclude that the role of CLASPs in the regulation of spindle length, chromosome congression, error correction and timely SAC satisfaction relies on its capacity to integrate critical microtubule-binding properties at the kinetochore-microtubule interface that sustain microtubule growth and promote their stabilization. These results are consistent with the unique capacity of human CLASPs to bind to curved microtubule protofilaments (Leano et al., 2013; Maki et al., 2015) and recent electron microscopy work showing that both growing and shrinking microtubule plus ends, including those of kinetochore microtubules, are curved (McIntosh et al., 2018). They are also supported by recent in vitro reconstitution experiments that showed that CLASP2-coated spherical beads are able to mediate

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342   moderately long-lived attachments with polymerizing microtubule plus-ends

343   (Chakraborty et al., 2019). Unlike other TOG-domain containing proteins, such

344   as members of the XMAP215/ch-TOG family that work as microtubule

345   polymerases that bind to free α,β-tubulin heterodimers (Ayaz et al., 2012; Geyer

346   et al., 2018; Nithianantham et al., 2018), our data is most consistent with a model

347   in which microtubule lattice binding and EB protein-dependent microtubule plus-

348   end tracking of CLASP2 is required at the kinetochore to stabilize, or prevent

349   destabilization, of kinetochore-microtubule attachments as cells progress into

350   metaphase.

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Acknowledgements:

Author contributions:

H.G. performed and analyzed all the experiments, except Figure 1, under supervision by N.O. and H.M.. A.C.F and Z.G. performed the biochemical characterization of CLASP2, under supervision of S.M.R., J.A. and H.M.. I.A. provided tools. T.M.S. generated tools and preliminary data. H.M. conceived and coordinated the project, designed experiments and analyzed the data. H.G. and H.M. wrote the paper, with contributions from all the authors.

Declaration of Interests

The authors declare no competing interests.

386   **Material and Methods**

387   **Production of recombinant CLASP2 C-terminal protein**

388   Synthetic gene coding for the C-terminal comprising nucleotides 1213-1515 of

389   human CLASP2α with codon usage optimized for expression in *E. coli* was

390   obtained from GenScript. CLASP2 ORF was subcloned into the *EcoRI* and *NdeI*

391   restriction sites of the expression vector pPR-IBA2 (IBA LifeSciences) in fusion

392   with an N-terminal Strep-tag and a C-terminal His10-tag. *E.coli* BL21 Star (DE3)

393   cells (Life Technologies) transformed with pPR-IBA2-CLASP2 (1213-1515)

394   plasmid were grown at 37 °C in LB medium supplemented with 50 µg/ml ampicillin

395   to OD600 0.4, and expression was induced by addition of 0.4 mM IPTG. After

396   growing for 4 h at 37 °C, cells were harvested and lysed by sonication in 50 mM

397   Tris-HCl pH 8.0, 150 mM NaCl supplemented with protease inhibitors (Complete

398   EDTA-free, Roche). Clarified protein extracts were loaded onto a HisTrap HP

399   column (GE Healthcare) pre-equilibrated in 50 mM Tris-HCl pH 8.0, 500 mM

400   NaCl, 20 mM imidazole (buffer A) and eluted with 200 mM imidazole in buffer A.

401   CLASP2 C-term containing fractions were pooled, adjusted to 100 mM Tris-HCl

402   pH 8.0 and 1 mM EDTA and loaded onto a StrepTrap HP column (GE Healthcare)

403   pre-equilibrated in 100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA (buffer

404   B). Bound CLASP2 was eluted with 2.5 mM desthiobiotin in buffer B. Protein-

405   containing fractions were concentrated and further purified on a HiPrep 16/60

406   Sephacryl S-200 HR column (GE Healthcare) pre-equilibrated with 50 mM Tris-

407   HCl pH 8.0, 400 mM NaCl, 5% GlyOH, 1mM EDTA (buffer C). Pure protein was

408   concentrated using a 30 KDa molecular-weight cutoff ultracentrifugal

409   concentration device (Millipore).

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Size exclusion chromatography

412 Size exclusion chromatography experiments with HeLa cell extracts were

413 performed with a pre-packed Superose 6 10/300 GL column (GE Healthcare ©).

414 The column was equilibrated with buffer A (20 mM Hepes, 150 mM NaCl, 1mM

415 DTT, pH 7.9) and a 0.3 mL/min flow rate was used throughout the experiments.

416 Calibration was performed with protein standards (Stokes radius (a)):

417 Thyroglobulin (8.50 nm), Ferritin (6.10 nm), Aldolase (4.81 nm), BSA (3.55 nm),

418 Ovoalbumin (3.05 nm), Chymotrypsinogen (2.09 nm) and Ribonuclease (1.37

419 nm). 50 µg of standards and 30 µg of sample cell extract were used in each run.

420 Standard protein elution was monitored by measuring the absorbance at 280 nm.

421 In the sample run, 0.5 mL fractions were collected, TCA precipitated, subjected

422 to SDS-PAGE, blotted and immunodetected with a rat anti-CLASP2 antibody

423 (Maffini et al., 2009), and protein distribution was analyzed by densitometry.

424 Recombinant CLASP2 C-term (300 µg, injection volume of 100 µl) was analyzed

425 on a Superose 12 10/300 GL column (GE Healthcare) pre-equilibrated in buffer

426 C. The column was calibrated with protein standards of known molecular weight

427 and stokes' radius (Low and High Molecular Weight Kit, GE Healthcare)

428 according to manufacturer's protocol.

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Density gradient centrifugation

431 Continuous gradient of 5% and 30% sucrose was prepared in buffer A. 50 µg of

432 each standard protein were loaded on top of the gradient and centrifuged at

433 29.000 rpm in a SW41 rotor (Beckman) for 29 h at 4ºC. After centrifugation, 0.7

434 mL fractions were collected from the bottom of the tubes, subjected to SDS-

435 PAGE, Coomasie staining and densitometry analysis. Native protein standards

436 used were (Sedimentation coefficient, S) Ribonuclease (2S), Chymotrypsinogen

437 (2.6S), Ovoalbumin (3.5S), albumin (4.6S), immunoglobulins (7S) and catalase

438 (11.3S). 300 µg of sample cell extract were loaded on top of another gradient,

439 and processed as before. After centrifugation, 0.7 mL fractions were collected

440 from the bottom of the tube, TCA precipitated, subjected to SDS-PAGE, blotted

441 and immunodetected with a rat anti-CLASP2 or mouse anti-α-tubulin antibodies

442 (clone B-512, Sigma Aldrich). For analysis of recombinant CLASP2 C-term,

443 density gradients from 5 to 30% sucrose in 50 mM Tris- HCl pH 8.0, 400 mM

444 NaCl, 1mM EDTA were prepared using a gradient former. The gradients were

445 loaded with 50 µg of recombinant CLASP2 C-term or protein standards of known

446 sedimentation coefficient and centrifuged for 29 h at 29.000 rpm in a SW41 rotor

447 (Beckman). Fractions of 700 µl were collected starting from the bottom of the tube

448 using a peristaltic pump. Each fraction was TCA precipitated and analyzed by

449 SDS-PAGE followed by PageBlue Protein Staining (Fermentas).

450

451 **Estimation of molecular weight by size exclusion chromatography and**

452 **density gradient centrifugation**

453 The molecular weights of recombinant CLASP2 C-term and full length CLASP2

454 were calculated by the method of Siegel and Monty (Siegel and Monty, 1966)

455 using equation 1 and 2 and the respective Stokes radius and sedimentation

456 coefficient obtained from size exclusion chromatography and density gradient

457 centrifugations, respectively,

458   (1) $M = (s\, N_A\, f)/(1-v\, \rho)$

459   (2) $f = 6\, \pi\, \eta\, R_s$

460   where s is the sedimentation coefficient, NA is the Avogadro's constant (6.022 x

461   1023 mol- 1), f is the frictional coefficient, v is the partial specific volume of the

462   protein (0.7362 cm3 g- 1); ρ is the density of the medium (1g cm-3); η is the

463   viscosity of the medium (0.01 g cm-1 s-1) and RS is the stokes radius. The partial

464   specific volume was estimated based on the amino acid sequence of the protein

465   using the method of Cohn and Edsall and the program SEDNTERP v1.08

466   (http://www.jphilo.mailway.com/default.htm).

467

468   **Estimation of molecular weight by dynamic light scattering**

469   Molecular size measurements of CLASP2 C-term (5 mg/ml in buffer C) were

470   performed using a glass cuvette ZEN0023 and a Zetasizer Nano ZS DLS system

471   (Malvern Instruments). Three independent measurements were obtained at 20

472   °C and data were analyzed using Zetasizer software v7.03. The hydrodynamic

473   radius calculated from the diffusion coefficient using the Stokes-Einstein Equation

474   was determined from the volume distributions and used to estimate the molecular

475   weight.

476

477   **Constructs and lentiviral transduction**

478   The mRFP-CLASP2γ constructs WT, IP12 (I496A, P497A; I519A, P520A), 2ea-

479   3eeaa (W106E, K191A; W667E, K833E, K838A, R839A), IP12-3eeaa (I496A,

480   P497A; I519A, P520A; W667E, K833E, K838A, R839A) and ΔC (CLASP2γ

481 without C-Terminal domain 1017-1294), inserted in a CSII-CMV-MCS vector,

482 were reported previously (Maki et al., 2015). The ΔC-Gcn4 construct was

483 generated by inserting the Gcn4 sequence after the mRFP-CLASP2γ-ΔC

484 sequence, using Gibson assembly (Gibson et al., 2009). The entire Gcn4

485 sequence was contained in the two primers used in the reaction mixed with the

486 vector containing the mRFP-CLASP2γ-ΔC construct cut with *NotI*. Lentiviral

487 supernatants were produced in HEK293T cells using pmd2.G, Pax2 and the CSII-

488 CMV-MLS plasmids, with the presence of the Lipofectamine 2000 (Invitrogen)

489 and Opti-MEM (Gibco). A U2OS cell line stably expressing Photoactivatable (PA)

490 GFP-α-tubulin (Ganem et al., 2005) was transduced with the viral supernatants,

491 in the presence of 6 μg/mL Polybrene (Sigma-Aldrich). The same procedure was

492 used to produce the double expressing H2B-GFP and mRFP-CLASP2γ cell lines.

493

494 **Cell line maintenance**

495 All cell lines used in this work were cultured in DMEM with 10% FBS,

496 supplemented with 10 μg/μL of antibiotic-antimycotic mixture (Gibco) and

497 selected with Zeocine 1 μg/μL. Cell lines were maintained at 37°C in a 5% CO_2,

498 humidified atmosphere. To synchronize HeLa cell cultures in mitosis 5 μM of S-

499 Trytil-L-Cysteine were added to the medium 16 h before harvesting by mitotic

500 shake off.

501

502 **Cell sorting**

503 Cells expressing the different mRFP-CLASP2γ constructs were enriched by cell

504 sorting using a FACS Aria II cell sorter (Becton Dickinson). Cells were re-

505 suspended in Basic Sorting Buffer [Phosphate Buffered Saline (PBS) 1X, Ca^{2+}

506 and Mg^{2+} free; 5 mM EDTA; 25 mM HEPES; 2% FBS] and cells expressing red

507 fluorescence signal were sorted to a tube containing DMEM with 10% FBS and

508 then transferred to an appropriate growth flask. The same procedure was used

509 to enrich the double expressing H2B-GFP and mRFP-CLASP2γ cell lines,

510 selecting in cell with double fluorescence signal (GFP and mRFP).

511

512 **siRNA**

513 The siRNA experiments were performed in 0.2×10^6 cells cultured in 1.5 mL

514 DMEM with 5% FBS. A solution containing 2 µL of Lipofectamine RNAiMax

515 (Invitrogen) diluted in 250 µL Opti-MEM was mixed with other containing 100

516 pmol of siRNA (Sigma Aldrich) diluted in 250 µL Opti-MEM. The solution mix was

517 incubated for 30 minutes at room temperature and then added dropwise to the

518 cells. The siRNA oligonucleotides used were: Scrambled siRNA

519 CUUCCUCUCUUUCUCUCCCUUGUGA, CLASP1#A siRNA

520 GCCAUUAUGCCAACUAUCU, CLASP2#A siRNA

521 GUUCAGAAAGCCCUUGAUG, CLASP1#B siRNA

522 GGAUGAUUUACAAGACUGG and CLASP2#B siRNA

523 GACAUACAUGGGUCUUAGA (Mimori-Kiyosue et al., 2005). After 6 h incubation

524 at 37°C, the medium was removed and replaced by 2 mL of DMEM with 10%

525 FBS.

526

22

Western blotting

Protein extracts were obtained from cells by addition of Lysis Buffer (20 mM HEPES/KOH, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 2 mM DTT at -20°C and Protease inhibitor 4C+PMSF 0.1 mM at -20°C 1:100, pH 7.9) and frozen with liquid Nitrogen (N_2). The suspension was centrifuged 5 minutes at 14,000 rpm at 4°C, collecting the supernatant. Total protein levels were quantified using Bradford Reagent (Thermoscientific), using Bovine Serum Albumin (BSA; Thermofisher) solutions as standards. 15 μg of total protein per sample were mixed with Sample Buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue) and denatured at 95°C for 5 minutes. Samples were loaded in a 6.5% Acrylamide Gel mounted in a Mini-PROTEAN vertical electrophoresis apparatus (Bio-Rad), using NZYColour Protein Marker II (NZYTech). Blotting was performed with an iBlot Gel Transfer System (Invitrogen). Membranes were blocked with 5% powder milk in PBS Tween 0.1% for 45 minutes. For primary antibodies we used hybridoma supernatant of monoclonal antibody 6E3 Rat anti-CLASP2 (Maffini et al., 2009) at 1:150 and Mouse anti-α-tubulin clone B-512 (Sigma Aldrich) at 1:10000, diluted in 5% powder milk in PBS Tween 0.1%, and incubated overnight (4°C) with agitation. Anti-Rat HRP (Horseradish Peroxidase) and anti-Mouse HRP secondary antibodies (Santa Cruz Biotechnology) were used at 1:2000 proportions in 5% powder milk in PBS Tween 0.1% and incubated for 1 h. Signal was developed with Clarity Western ECL Blotting Substrate (Bio-Rad) and detected in a Bio-Rad Chemidoc XRS system, with Image Lab software.

Immunofluorescence

Cells were fixed using paraformaldehyde at 4% in Cytoskeleton Buffer (CB; 137 mM NaCl, 5mM KCl, 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO4, 2mM EGTA, 2mM $MgCl_2$, 5 mM PIPES, 5 mM Glucose), or in alternative, Methanol at -20°C, for 10 minutes (for Nocodazole arrested cell experiments, Nocodazole was added at final concentration of 3.3 µM to the culture, 2-3 h prior to fixation). Extraction was accomplished using CB-Triton X-100 0.5% for 10 minutes. Primary antibodies used in this work were: mouse anti-α-tubulin clone B-512 at 1:1500 (Sigma Aldrich), mouse anti-EB1 at 1:500 (BD Biosciences), human anti-CREST 1:1000 (Abyntek), rat anti-RFP (ChromoTek) 1:1000, diluted in PBS-Triton X-100 0.1% with 10% FBS. Secondary antibodies Alexa Fluor anti-Mouse 488, anti-rat 568 and anti-Human 647 (Invitrogen) were diluted 1:1000 in PBS-Triton X-100 0.1% with 10% FBS. DNA staining was achieved by addition of 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Coverslips were mounted using mounting medium (20 mM Tris pH8, 0.5 mM N-propyl gallate, 90% glycerol). Imaging was performed in a Zeiss Axio Observer Widefield Microscope, using a 63x 1.46 N.A. plan-apochromatic objective. 3D-deconvolution was performed using AutoQuant X Image Deconvolution Software (Media Cybernetics). Image processing and quantifications were made using ImageJ Software. Statistical analysis was performed using GraphPad Prism 8 Software, using Student t-test or Mann-Whitney test, according to the normality of the distribution.

Live-cell imaging

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574   The mRFP-CLASP2γ U2OS cells expressing H2B-GFP were cultured in glass

575   coverslips using DMEM without phenol red and supplemented with 25 mM of 4-

576   (2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco) and 10% FBS.

577   SiR-Tubulin (Spirochrome) was added to a final concentration of 20 nM, 30-60

578   minutes prior to observation. Time-lapse imaging was performed in a heated

579   chamber (37°C) using a 60x oil-immersion 1.4 N.A. plan-apochromatic objective

580   mounted on an inverted microscope (Nikon TE2000U) equipped with a CSU-X1

581   spinning-disk confocal head (Yokogawa Corporation of America) and with three

582   laser lines (488 nm, 561 nm and 647 nm). Images were detected with an

583   iXonEM+ EM-CCD camera (Andor Technology). Eleven z-planes separated by 1

584   µm were collected every 2 minutes. Image processing and quantifications was

585   performed using ImageJ software and NIS Viewer from Nikon. Statistical analysis

586   was performed using GraphPad Prism 8 Software, using Student t-test or Mann-

587   Whitney test, according to the normality of the distribution.

588

589   **Photoactivation experiments**

590   Microtubule turnover rates were measured in the mRFP-CLASP2γ U2OS cells

591   stably expressing the PA-GFP-α-tubulin. Cells were grown in glass coverslips

592   with DMEM without phenol red and with 25 mM of HEPES (Gibco), supplemented

593   with 10% FBS. Time-lapse imaging was performed in a heated chamber (37°C)

594   using a 100x 1.4 N.A. plan-apochromatic differential interference contrast (DIC)

595   objective mounted on an inverted microscope (Nikon TE2000U) equipped with a

596   CSU-X1 spinning-disk confocal head (Yokogawa Corporation of America) and

597   with two laser lines (488 nm and 561 nm). Images were detected with an

598   iXonEM+ EM-CCD camera (Andor Technology). Photoactivation was performed

599   with a Mosaic DMD-based patterning system (Andor) equipped with a 405 nm

600   diode laser. Cells in metaphase were chosen using DIC acquisition. A thin stripe

601   of spindle microtubules was locally photoactivated in one half-spindle by pulsed

602   near-UV-irradiation (405 nm laser; 500 microseconds exposure time) and

603   fluorescence images (seven 1 μm-separated z-planes centered at the middle of

604   the mitotic spindle) were captured every 15 seconds for 4.5 minutes with a 100x

605   oil-immersion 1.4 N.A. plan-apochromatic objective. To quantify fluorescence

606   dissipation after photoactivation (FDAPA), spindle poles were aligned

607   horizontally and whole-spindle, sum-projected kymographs were generated (sum

608   projections using ImageJ and kymographs generated as previously described

609   (Pereira and Maiato, 2010). Fluorescence intensities were quantified for each

610   time point (custom-written routine in Matlab, "LAPSO" software) and normalized

611   to the first time point after photoactivation for each cell following background

612   subtraction and correction for photobleaching. Correction for photobleaching was

613   performed by normalizing to the values of fluorescence loss obtained from whole-

614   cell (including the cytoplasm), sum projected images for each individual cell. This

615   method allows for the precise measurement of photobleaching for each cell at the

616   individual level and avoids any potential unspecific Taxol-associated phenotypes.

617   Under these conditions, the photoactivated region dissipates, yet the

618   photoactivated molecules are retained within the cellular boundaries defined by

619   the cytoplasm. To calculate microtubule turnover, the sum intensity at each time

620   point was fit to a double exponential curve $A1*exp(-k_1*t) + A2*exp(-k_2*t)$ using

621   Matlab (Mathworks), in which t is time, *A1* represents the less stable (non-KT-

622   MT) population and *A2* the more stable (KT-MT) population with decay rates of

26

623   k_1 and k_2, respectively. When running the routine to fit the data points to the model

624   curve, the rate constants are obtained as well as the percentage of microtubules

625   for the fast (typically interpreted as the fraction corresponding non-kinetochore

626   microtubules) and the slow (typically interpreted as the fraction corresponding to

627   kinetochore microtubules) process are obtained. The half-life for each process

628   was calculated as $\ln 2/k$ for each population of microtubules. All experiments were

629   performed in the presence of MG132 (5 µM) added 30 min prior to imaging to

630   ensure that cells were in metaphase and prevent mitotic exit. Statistical analysis

631   for photoactivation experiments was performed in the GraphPad Prism8

632   software, using the Mann-Whitney test.

633

634   **Statistical Analysis**

635   Statistical analysis was performed with Graphpad Prism, version 8. Quantification

636   of kinetochore co-localization, spindle length, segregation errors, NEB to

637   Anaphase and NEB to Metaphase durations were performed using the

638   parametric unpaired Student's t-test. Quantification of both kinetochore

639   microtubule and non-kinetochore microtubule half-lives was performed using the

640   non-parametric Mann-Whitney U-Test.

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811   **Figure Legends**

812   **Figure 1. CLASP2 can form dimers through its C-terminal kinetochore-targeting**

813   **domain. (A)** Extracts from asynchronous (A) and mitotic (M) HeLa cells resolved by BN-

814   PAGE and analyzed by western blot for CLASP2 (left) or α-tubulin (right). Indicated

815   apparent molecular weights were estimated by comparison with the gel mobility behavior

816   of protein standards of known molecular weights, as indicated. Dimerization of α-tubulin

817   (130 kDa) was used as a control. Single or double asterisks indicate the position of

818   putative monomers and dimers, respectively. **(B)** Mitotic enriched HeLa cell extracts

819   were separated by size exclusion chromatography. Collected fractions were TCA-

820   precipitated and analyzed by western blot with anti-CLASP2 antibodies. **(C)** Mitotic

821   enriched HeLa cell extracts were loaded on a 5%-30% sucrose gradient. Fractions

822   collected after centrifugation were TCA-precipitated and analyzed by western blot with

823   the indicated antibodies. Note that CLASP2 and free tubulin are collected in distinct

824   fractions. **(D)** Summary of Stokes radius (nm), sedimentation coefficients (S) and

825   estimated molecular weights (KDa) calculated for the different CLASP2 forms**. (E)** Size

826   exclusion chromatogram of human recombinant CLASP2 C-terminal, eluting in a single

827   peak. **(F)** Size exclusion chromatography calibration curve used to determine the Stoke's

828   radius of CLASP2 C-terminal. **(G)** Density gradient centrifugation curve used to

829   determine the sedimentation coefficient of recombinant CLASP-2 C-terminal. **(H)**

830   Dynamic light scattering analysis of the CLASP2 C-terminal showing an hydrodynamic

831   radius of 36.1 Å, corresponding to an estimated molecular weight of 67.9 KDa.

832

833   **Figure 2. CLASP2 C-terminal domain is required for kinetochore localization**

834   **independently of dimerization. (A)** Schematic representation of the RNAi-resistant

835   mRFP-CLASP2γ constructs used in this project: Wild Type (WT) CLASP2γ; the mutant

836   constructs IP12, 2ea-3eeaa, IP12-3eeaa; the truncated version at C-terminal ΔC; and

837   the hybrid construct ΔC-Gcn4. Mutations in TOG2 and TOG3 domains and SxIP motifs

35

838   are indicated. **(B)** Immunofluorescence analysis of the U2OS PA-GFP-α-tubulin and

839   mRFP-CLASP2γ transduced cell lines, arrested in mitosis by the addition of nocodazole

840   to depolymerize microtubules, showing the localization of the different CLASP2

841   constructs at kinetochores, except the ΔC and ΔC-Gcn4 constructs. Cells were

842   immunostained with anti-centromere antibodies (ACA) from CREST patients (green) and

843   the mRFP signal of the different CLASP2 constructs (red). Detailed images of mRFP and

844   ACA co-localization are magnified 3X. Scale bar is 5 μm. **(C)** Quantification of

845   kinetochore fluorescence intensity (F.I.) of mRFP/ACA signals for each cell line after

846   background subtraction. Each data point represents an individual kinetochore. Bars

847   represent mean and standard deviation. Quantifications from 3 independent

848   experiments: WT = 26 cells, 260 kinetochores; 2ea-3eeaa = 27 cells, 267 kinetochores;

849   IP12 = 27 cells, 269 kinetochores, IP123eeaa = 26 cells, 253 kinetochores ΔC = 26 cells,

850   253 kinetochores ΔC-Gcn4 = 18 cells, 196 kinetochores. All mutant constructs showed

851   a decreased intensity of the signal at the kinetochores compared with WT. **** =

852    $p < 0.0001$.

853

854   **Figure 3. Regulation of mitotic spindle length by CLASP2 depends on its**

855   **kinetochore localization and microtubule-binding properties. (A)**

856   Immunofluorescence analysis of the U2OS PA-GFP-α-tubulin cell lines in metaphase.

857   Parental (without exogenous mRFF-CLASP2γ constructs) and mutant cell lines depleted

858   from endogenous CLASPs with siRNA #12A, showed shorter spindles relative to cells

859   expressing WT mRFF-CLASP2γ. Scale bar is 5 μm. **(B)** Immunofluorescence analysis

860   of the U2OS PA-GFP-α-tubulin cell lines in anaphase. Segregation errors are indicated

861   with red arrows in the DAPI channel. Parental and CLASP2 mutant cell lines depleted of

862   endogenous CLASPs by siRNA #12A showed increased chromosome segregation

863   errors relative to WT. Scale bar is 5 μm. **(C)** Quantification of mitotic spindle length in

864   each cell line: the first column represents parental U2OS PA-GFP-α-tubulin cell line

865   without RNAi treatment, the remaining columns represent treatment with siRNA #12A.

866   Each data point represents an individual cell. Bars represent mean and standard

867   deviation. Quantifications from 3 independent experiments: Parental without RNAi = 88

868   cells; Parental with RNAi = 74 cells; WT = 108 cells; 2ea-3eeaa = 96 cells; IP12 = 105

869   cells; IP12-3eeaa = 88 cells; ΔC = 112 cells; ΔC-Gcn4 = 142 cells. Parental and mutant

870   cell lines depleted of endogenous CLASPs show a statistical significant decrease in

871   spindle length. ns = non-significant, *** = p<0.001, **** = p<0.0001. **(D)** Quantification of

872   chromosome segregation errors in anaphase and telophase cells: each column

873   represents the percentage of anaphase and telophase cells with at least one segregation

874   error; the first column represents parental U2OS PA-GFP-α-tubulin cell line without RNAi

875   treatment, the remaining columns represent treatment with the siRNA #12A.

876   Quantifications from 3 independent experiments: Parental without RNAi = 92 cells;

877   Parental with RNAi = 117 cells; WT = 153 cells; 2ea-3eeaa = 126 cells; IP12 = 135 cells;

878   IP12-3eeaa = 121 cells; ΔC = 120 cells; ΔC-Gcn4= 106 cells. CLASPs-depleted cells

879   and mutant cell lines showed higher percentage of cells with segregation errors. ns =

880   non-significant, * = p<0.05; ** = p< 0.01; *** = p<0.001.

881

882   **Figure 4. Chromosome congression, timely SAC satisfaction and chromosome**

883   **segregation fidelity relies on both CLASP2 kinetochore- and microtubule-binding**

884   **properties. (A)** Live-cell imaging of the parental U2OS PA-GFP-α-tubulin cells and

885   respective cell lines expressing the mRFP-CLASP2γ constructs, after endogenous

886   depletion of CLASPs with RNAi. mRFP-CLASPγ (red), DNA (blue) and SiR-tubulin

887   (magenta) are depicted. Timing 00:00 starts at Nuclear Envelope Breakdown (NEB). The

888   anaphase panel additionally shows the H2B-GFP signal to highlight the segregation

889   errors. Time is in hours:minutes. Scale bar is 5 μm. **(B)** Mitotic timing quantification

890   between NEB and Anaphase (Ana) onset. First column represents parental U2OS PA-

891   GFP-α-tubulin cells. The remaining columns represent CLASPs RNAi after treatment

892   with siRNA #12A. Each data point represents an individual cell. Bars represent mean

893   and standard deviation. Parental without RNAi = 11 cells, 3 independent experiments;

894   Parental with RNAi = 12 cells, 3 independent experiments; WT = 28 cells, 6 independent

895   experiments; 2ea-3eeaa = 32 cells, 14 independent experiments; IP12 = 33 cells, 13

896   independent experiments; IP12-3eeaa = 19 cells, 7 independent experiments; ΔC = 23

897   cells, 9 independent experiments; ΔC-Gcn4 = 15 cells, 5 independent experiments.

898   Parental and mutant cells depleted of endogenous CLASPs showed a significant

899   increase in mitotic duration. ns = non-significant, ** = $p<0.01$, *** = $p<0.001$, **** =

900    $p<0.0001$. **(C)** Quantification of the time between NEB and Metaphase (Meta) for the

901   same data set as in B. First column represents parental U2OS PA-GFP-α-tubulin cells.

902   The remaining columns represent CLASPs RNAi after treatment with siRNA #12A. A

903   significant delay in chromosome congression is observed in parental and mutant cell

904   lines after CLASPs depletion. ns = non-significant, ** = $p<0.01$, *** = $p<0.001$, **** =

905    $p<0.0001$. **(D)** Quantification of segregation errors during live cell imaging for the same

906   data set as in B and C; each column represents the percentage of cells with at least one

907   segregation error detected in the total cells imaged for each cell line. Parental and mutant

908   cells depleted of endogenous CLASPs showed a higher frequency of cells with

909   segregation errors.

910

911   **Figure 5. Multimodal regulation of kinetochore microtubule dynamics by CLASP2.**

912   **(A)** Photoactivation experiments in live U2OS parental PA-GFP-α-tubulin cells and

913   expressing the different mRFP-CLASP2γ constructs, after RNAi against endogenous

914   CLASPs; the expression of PA-GFP-α-tubulin enables the activation of a stripe near the

915   metaphase plate by a near-UV laser. Panels represent DIC, the mRFP-CLASP2γ signal

916   (red) and the PA-GFP-α-tubulin signal before photoactivation (Pre-PA), immediately after

917   photoactivation (PA) and at 2 and 4.5 minutes after photoactivation. The PA-GFP-α-

918   tubulin signal was inverted for better visualization. Scale bar is 5 µm. **(B)** Quantitation of

919 kinetochore microtubule (KT-MT) half-life for all cell lines. The first column corresponds

920 to the parental cell line, and the remaining columns correspond to experiments

921 performed after CLASPs depletion with siRNA #12A. Each data point represents an

922 individual cell. The boxes represent median and interquartile interval, the bars represent

923 minimum and maximum values. Parental without RNAi = 43 cells, 5 independent

924 experiments; Parental with RNAi = 20 cells, 6 independent experiments; WT = 67 cells,

925 7 independent experiments; 2ea-3eeaa = 105 cells, 8 independent experiments; IP12 =

926 108 cells, 9 independent experiments; IP12-3eeaa = 55 cells, 4 independent

927 experiments; ΔC = 60 cells, 4 independent experiments; ΔC-Gcn4 = 34 cells, 8

928 independent experiments. Mutant constructs in the TOG domains and in the SxIP motifs,

929 as well the kinetochore-lacking constructs ΔC and ΔC-Gcn4 showed less stable

930 kinetochore microtubules in the absence of endogenous CLASPs. ns = non-significant,

931 * = $p < 0.05$, ** = $p < 0.01$. **(C)** Quantitation of non-KT-MT half-life for all cell lines for the

932 same data set as in B. All cell lines showed non-KT-MT stability that was

933 indistinguishable from controls. ns = non-significant.

934

935 **Supplementary Figure 1.** Western blot with anti-CLASP2 antibody, revealing the total

936 (endogenous and exogenous) CLASP2 levels for each mRFP-CLASP2γ construct cell

937 line. Endogenous CLASP2α and the mRFP-CLASP2γ constructs WT, 2ea-3eeaa, IP12

938 and IP12-3eeaa could not be distinguished due to similar molecular weight (165.7 kDa

939 for endogenous CLASP2α and 167.9 kDa for mRFP-CLASP2γ constructs), while in the

940 ΔC (120.8 KDa) and ΔC-Gcn4 (125.3 KDa) it was possible to visualize both bands for

941 endogenous CLASP2α and exogenous mRFP-CLASP2γ. All cell lines expressed

942 comparable levels of mRFP-CLASP2γ, slightly lower in 2ea-3eeaa and slightly higher in

943 ΔC. α-tubulin was used as loading control.

944 **Supplementary Figure 2.** Western blot analysis, using an anti-CLASP2 antibody,

945 highlighting the depletion of endogenous CLASPs by siRNA #12A and siRNA #12B.

946 Since endogenous CLASP2α and the mRFP-CLASP2γ constructs WT, IP12, 2ea-3eeaa

947 and IP12-3eeaa displayed similar molecular weights (MW) (165.7 kDa for endogenous

948 CLASP2α and 167.9 kDa for the mRFP-CLASP2γ constructs), depletion efficiency of

949 endogenous CLASPs were obtained by comparing the treatments with Scramble siRNA,

950 siRNA #12A that deplete endogenous CLASPs and siRNA #12B that deplete both

951 endogenous and exogenous CLASPs. However, for ΔC and ΔC-Gcn4 cases, because

952 they have different MW from endogenous CLASP2α, it was possible to discriminate both

953 bands. α-tubulin was used as loading control.

954

955 **Supplementary Figure 3. (A)** Immunofluorescence analysis showing the co-localization

956 of the mRFP-CLASP2γ constructs with EB1 at the microtubule plus ends in interphase.

957 Cells were stained with anti-EB1 antibody (green) and anti-RFP antibody (red). The anti-

958 RFP antibody was used in order to increase detection of the mRFP-CLASP2γ constructs

959 at the plus tips after methanol fixation. IP12 and IP12-3eeaa showed a decreased co-

960 localization with EB1 at the microtubule plus ends. Scale bar is 5 µm. **(B)**

961 Immunofluorescence analysis showing localization of the mRFP-CLASP2γ constructs

962 with mitotic spindle microtubules. Cells were immunostained with an anti-α-tubulin

963 antibody to visualize the spindle (green). The 2ea-3eeaa and IP12-3eeaa constructs

964 showed a decreased localization on spindle microtubules. Scale bar is 5 µm.

965

966

967

968

Movie Legends

Movie S1 – Human parental U2OS cell stably expressing PA-GFP-α-tubulin (not shown) and H2B-GFP (green in the merged image; shown alone in white in the right panel) after CLASP1 and CLASP2 RNAi, with microtubules (magenta) visualized with 20 nM SiR-tubulin. Note the presence of a lagging chromosome during anaphase. Time is in h:min.

Movie S2 – Human parental U2OS cell stably expressing PA-GFP-α-tubulin and wild type (WT) mRFP-CLASP2γ (red) and H2B-GFP (green in the merged image; shown alone in white in the right panel) after CLASP1 and CLASP2 RNAi, with microtubules (magenta) visualized with 20 nM SiR-tubulin. Time is in h:min.

Movie S3 – Human parental U2OS cell stably expressing PA-GFP-α-tubulin and mRFP-CLASP2γ 2ea-3eeaa mutant (red) and H2B-GFP (green in the merged image; shown alone in white in the right panel) after CLASP1 and CLASP2 RNAi, with microtubules (magenta) visualized with 20 nM SiR-tubulin. Note the presence of 2 lagging chromosomes during anaphase, one of which resolves. Time is in h:min.

Movie S4 – Human parental U2OS cell stably expressing PA-GFP-α-tubulin and mRFP-CLASP2γ IP12 mutant (red) and H2B-GFP (green in the merged image; shown alone in white in the right panel) after CLASP1 and CLASP2 RNAi, with

41

991   microtubules (magenta) visualized with 20 nM SiR-tubulin. Note the presence of

992   a chromosome bridge in anaphase and telophase. Time is in h:min.

993

994   **Movie S5** – Human parental U2OS cell stably expressing PA-GFP-α-tubulin and

995   mRFP-CLASP2γ IP12-3eeaa mutant (red) and H2B-GFP (green in the merged

996   image; shown alone in white in the right panel) after CLASP1 and CLASP2 RNAi,

997   with microtubules (magenta) visualized with 20 nM SiR-tubulin. Note the

998   presence of a transient lagging chromosome during anaphase. Time is in h:min.

999

1000   **Movie S6** – Human parental U2OS cell stably expressing PA-GFP-α-tubulin and

1001   mRFP-CLASP2γ ΔC mutant (red) and H2B-GFP (green in the merged image;

1002   shown alone in white in the right panel) after CLASP1 and CLASP2 RNAi, with

1003   microtubules (magenta) visualized with 20 nM SiR-tubulin. Note the presence of

1004   transient lagging chromosomes in the beginning of anaphase. Time is in h:min.

1005

1006   **Movie S7** – Human parental U2OS cell stably expressing PA-GFP-α-tubulin and

1007   mRFP-CLASP2γ ΔC-Gcn4 mutant (red) and H2B-GFP (green in the merged

1008   image; shown alone in white in the right panel) after CLASP1 and CLASP2 RNAi,

1009   with microtubules (magenta) visualized with 20 nM SiR-tubulin. Note the

1010   presence of persistent lagging chromosomes in anaphase. Time is in h:min.

1011

A

C 30% Sucrose gradient 5%

B Size exclusion chromatography

D

Pool	Stokes Radius	Sedimentation Coefficient	Estimated Molec. Weight
1 (dimer)	9.9 nm	6.6S	278 KDa
2 (monomer)	7.6 nm	4.7S	153 KDa
CLASP2 C-term	3.5 nm	5.2S	78.5 KDa

E

F

$y = 0.007x + 0.439$
$R^2 = 0.995$

G

$y = -1.568x + 27.916$
$R^2 = 0.996$

H

A mRFP-CLASP2γ / α-tubulin / DAPI

CLASPs RNAi

Columns: Parental | Parental | Wild Type | IP12 | 2ea-3eeaa | IP12-3eeaa | ΔC | ΔC-Gcn4

Rows: Merged (α-tubulin / DAPI); α-tubulin

B mRFP-CLASP2γ / α-tubulin / DAPI

CLASPs RNAi

Columns: Parental | Parental | Wild Type | IP12 | 2ea-3eeaa | IP12-3eeaa | ΔC | ΔC-Gcn4

Rows: Merged; DAPI

C Pole to pole distance (μm) — categories: Parental, Parental, WT, 2ea-3eeaa, IP12, IP12-3eeaa, ΔC, ΔC-Gcn4 (CLASPs RNAi)

D % Ana/Telo Cells w/ Segregation Errors — categories: Parental, Parental, WT, 2ea-3eeaa, IP12, IP12-3eeaa, ΔC, ΔC-Gcn4 (CLASPs RNAi)

Figure A: Rows labeled Parental, Wild Type, 2ea-3eeaa, IP12, IP12-3eeaa, ΔC, ΔC-Gcn4. Columns labeled DIC, mRFP-CLASP2, Pre-PA, PA, 2 mins, 4.5 mins.

B. KT-MTs — KT-MT half-life (min). Categories: Parental, Parental, WT, 2ea-3eeaa, IP12, IP12-3eeaa, ΔC, ΔC-Gcn4. Significance: **, ns, *, *, *, **, **. CLASPs RNAi.

C. non-KT-MTs — non KT-MT half-life (min). Categories: Parental, Parental, WT, 2ea-3eeaa, IP12, IP12-3eeaa, ΔC, ΔC-Gcn4. Significance: ns, ns, ns, ns, ns, ns, ns. CLASPs RNAi.