1 Lysosomal degradation ensures accurate chromosomal

2 segregation to prevent genomic instability

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ABSTRACT

Lysosomes, as primary degradative organelles, are the end-point of different converging pathways including macroautophagy. To date, lysosome function has mainly focused on interphase cells, while their role during mitosis remains controversial. Mitosis dictates the faithful transmission of genetic material among generations, and perturbations of mitotic division lead to chromosomal instability, a hallmark of cancer. Heretofore, correct mitotic progression relies on the orchestrated degradation of mitotic factors, which was mainly attributed to ubiquitin-triggered proteasome-dependent degradation. Here, we show that mitotic transition does not only rely on proteasome-dependent degradation, as impairment of lysosomes increases mitotic timing and leads to mitotic errors, thus promoting chromosomal instability. Furthermore, we identified several putative lysosomal targets in mitotic cells. Among them, WAPL, a cohesin regulatory protein, emerged as a novel p62-interacting protein for targeted lysosomal degradation. Finally, we characterized an atypical nuclear phenotype, the toroidal nucleus, as a novel biomarker for genotoxic screenings. Our results establish lysosome-dependent degradation as an essential event to prevent genomic instability.

KEYWORDS: Lysosome, Mitosis, Chromosomes segregation, Selective autophagy, Chromosomal instability, Toroidal nucleus.

Abbreviations: **3D**: three Dimensional; **APC/C**: anaphase-promoting complex; **Arl8b**: ADP-ribosylation factor-like protein 8b; **Atg**: autophagy-related proteins; **BORC**: BLOC-one-related complex; **CDK**: cyclin-dependent kinases; **CENP-E**: centromere-associated protein E; **CIN**: chromosomal instability; **ConcA**: concanamycin A; **CQ**: chloroquine; **DAPI**: 4,6-diamidino-2-penylinole; **FTI**: farnesylation inhibitors; **GFP**: green fluorescent protein; **GO**: gene ontology; **H2B**: histone 2B; **KIF**: kinesin-like protein; **LAMP1/2**: lysosome associated membrane protein 1/2; **LC3**: microtubule-associated protein 1A/1B-light chain 3; **MCAK**: mitotic centromere-associated kinesin; **MEF**: mouse embryonic fibroblast; **NPC**: nuclear pore complex; **PDS5B**: cohesin associated factor B; **SAC**: spindle assembly checkpoint; **SKIP**: pleckstrin homology domain-containing family M member 2; **TEM**: transmission electron microscopy; **ULK1**: unc-51 like autophagy activating kinase 1; **UPS**: ubiquitin-proteasome system; **v-ATPase**: vacuolar-ATPases; **WAPL**: wings apart-like protein.

INTRODUCTION

Chromosomal instability (CIN) is defined as an abnormal loss or rearrangement of chromosomes during cell division[1] and positively correlates with poor cancer patient prognosis[2]. The mechanisms underlying CIN remain poorly characterized but reflect abnormalities in kinetochore-microtubule attachment, sister chromatids cohesion, centrosome duplication, telomeres or the spindle assembly checkpoint (SAC)[3].

To maintain genome integrity, the cell cycle must be tightly coordinated to ensure the faithful transmission of hereditary information between generations. Cells spend more than 90% of their time in interphase and interphase length correlates with total length of the cell cycle[4]. In contrast, mitosis is extremely short, and the time spent in mitosis is remarkably constant and uncoupled from variability in other phases. Mitosis is the process by which a cell properly divides its genetic material and consists of five active phases from prophase to telophase. The major mitotic checkpoint comprises the metaphase-to-anaphase transition, separating mitotic entry and exit. Coordination of the mitotic regulatory network relies on hierarchal phosphorylation cascades driven by cyclin-dependent kinases (CDK)[5,6] and the ubiquitin-proteasome system (UPS) under the control of the anaphase-promoting complex (APC/C)[7,8]. During mitosis, known degradative functions are mainly restricted to UPS, specialized in ubiquitin-triggered protein degradation and presumably faster than lysosome-dependent degradation.

Lysosomes are acidic cytosolic vesicles responsible to enzymatically degrade all types of biological material. During macroautophagy, double-membrane vesicles (autophagosomes) engulf cytosolic material, converge and fuse with lysosomes. The lysosomal proton pump v-ATPase drives lumen acidification while the BORC-associated protein complex, including KIF5B motor protein, is the main driver of anterograde lysosomal transport and also contributes to autophagosome-lysosome fusion[9–11]. Autophagy-dependent degradation of cargos can either be

67   non-selective (bulk) or targeted (selective). Adaptor proteins, such as p62 (also known as

68   Sequestosome-1) regulate selective autophagic degradation[12,13]. Being cytosolic vesicles,

69   research on autophagy and lysosomes has mainly focused on interphase cells, omitting their

70   implication on mitosis. As cells undergo mitosis, dramatic structural rearrangements of organelles

71   occur[14–16]. However, several recent studies show controversial observations about the function of

72   lysosomes and autophagy in mitosis. Some studies claim that autophagy signaling is shut-down

73   at mitotic entry, that autophagic structures are barely detected in dividing cells and that

74   proteasome-dependent degradation of WIPI2 upon mitotic induction suppress autophagic flux[17–

75   19]. In contrast, lysosomal-dependent degradation is implicated in Cyclin A2 proteolysis during

76   mitosis[20] and mitophagy is active in prophase[21,22]. ULK1, an autophagy initiator protein, was

77   shown to drive SAC recruitment to kinetochores through Mad1 phosphorylation[23]. Furthermore,

78   selective autophagic degradation of centriolar satellites was recently demonstrated to support

79   correct karyokinesis[24,25]. Loss of Beclin1, another key autophagic protein, induced gene

80   amplification and consequent aneuploidy[26]. Better understanding of the regulatory mechanisms

81   driving mitotic transitions is crucial for preventing CIN and developing novel cancer treatment

82   strategies.

83   Here, we show, for the first time, that correct mitotic progression does not rely only on UPS-

84   dependent degradation. Dissecting the mitotic transition, we define a novel function of selective

85   autophagy and lysosome-dependent degradation specifically in dividing cells and identify new

86   mitotic lysosomal substrates. Furthermore, impairment of lysosome function during cell division

87   triggers chromosome mis-segregation and induces a striking nuclear phenotype, the toroidal

88   nucleus, which provides a new tool for genotoxicity tests.

89

90 **RESULTS**

91 **Lysosomes and autophagic vesicles are present and active during cell division**

92 To investigate the presence of lysosomes in mitotic subphases, LAMP2-positive vesicles and

93 DAPI-stained DNA were analyzed by immunofluorescence. Here we showed that lysosomes are

94 present and dynamic in all mitotic subphases (**Fig.1A; Video 1**). Morphological analysis of mitotic

95 lysosomes indicated that lysosomes decreased in number while increased in size from prophase

96 to late telophase, when they started recovering their interphase-like morphology (**Fig.1A-C; Video**

97 **1**). In addition, lysosome distribution is modified once cells proceeded into cell division. Mitotic

98 lysosomes surrounded the chromosomes in prophase and moved to the edges of chromosomes

99 during anaphase/telophase until chromosomes decondensed (**Fig.1D**). To analyze whether

100 mitotic lysosomes maintain their degradation capacity during cell division, we stained live U2OS

101 cells stably expressing Histone 2B-GFP (H2B-GFP U2OS) with Lysosensor for acidic organelle

102 detection and MagicRed for lysosomal cathepsin B activation. Colocalization between Lysosensor

103 and MagicRed supported the presence of functional lysosomes in dividing cells (**Fig.1E**).

104 As we found that lysosomes are present and active in mitosis, we analyzed the autophagic flux

105 during cell division. To this end, the expression of p62 and the lipidated form of LC3 (LC3-II) were

106 analyzed in mitosis after inhibition of lysosomal acidification with ConcA. Established

107 synchronization protocols such as serum starvation or double-thymidine block were insufficient to

108 efficiently synchronize U2OS cells in G2. Therefore, based on the ability of CDK1-specific inhibitor

109 RO3306 to reversibly block cells in G2, we established a synchronization-release protocol to

110 obtain enriched mitotic population (**Fig.1F**). Cell cycle analysis validated the efficiency of the

111 protocol in control cells or upon lysosome inhibition (**Fig.1G**). Upon ConcA treatment,

112 synchronized cells showed specific accumulation of LC3-II and of the autophagic adaptor protein

113 p62 without major changes in the global cell cycle profile (**Fig.1G-H**). Time-course assay after cell

114   synchronization and release demonstrated that both autophagic proteins LC3-II and p62 gradually

115   accumulated during mitosis transition upon acidification blockade (**Fig.S1A-B**). In parallel,

116   immunofluorescence analysis of both endogenous LC3 and p62 corroborated the presence of

117   autophagic vesicles in dividing cells (**Fig.1I**). Indeed, Pearson's correlation coefficient (Rr)

118   between LC3 and p62 significantly increased during mitotic subphases compared to interphase

119   cells (**Fig. 1I**). Finally, double-membrane vesicles (autophagosomes), as well as dense single-

120   membrane vesicles (autolysosomes/lysosomes), were detected by Transmission Electron

121   Microscopy (TEM) in mitotic cells (**Fig.1J**).

122   Collectively, our results demonstrate that both autophagic vesicles and functional lysosomes are

123   present and active in mitotic cells.

124   **Lysosome acidification capacity and trafficking maintain correct mitotic progression**

125   To investigate the role of lysosomes in cell division, we studied mitotic cells with impaired

126   lysosomes either by inhibiting their degradative capacity or their intracellular trafficking.

127   Impairment of lysosome acidification by the v-ATPase inhibitor Concanamycin A (ConcA) led to

128   a reduced number of mitotic lysosomes while increasing lysosome size, according to their

129   defective degradation capacity (**Fig.2A, C-D**). Parallel to ConcA treatment, we assessed the effect

130   of KIF5B depletion on lysosome morphology, number and distribution in mitotic cells. While in

131   interphase, KIF5B depletion induced a dramatic clustering of lysosomes in the perinuclear region

132   and a reduction in the lysosomal number. However, these effects were negligible in mitotic cells.

133   Interestingly, KIF5B depletion did not affect either lysosome number or size in dividing cells

134   (**Fig.2B-E and Fig.S2A**). When we analyzed the lysosomal distribution in the different mitotic

135   subphases, we observed that the hierarchical distribution of lysosomes observed in control cells

136   was almost completely absent in KIF5B-depleted cells (**Fig.2E**). Interestingly, in this setting,

137   KIF5B did not induce a clustering of lysosomes in a central zone as in interphase but impeded

138   the spatial organization of lysosomes during mitosis suggesting the implication of this kinesin on

139   the heterogeneous distribution of lysosomes during mitosis (**Fig.2E**).

140   Mitotic timing is strictly regulated and remarkably constant among different cell types and

141   species[4], taking approximately one hour in normal mitosis. Thus, after identifying active

142   lysosomes in mitotic cells the direct implication of lysosomes in mitotic progression was tested.

143   To this end, we used live-imaging to analyze H2B-GFP U2OS cells treated with ConcA or KIF5B-

144   depleted and recorded mitotic timing from prophase (chromosome condensation) to telophase

145   (chromosome decondensation). Mitosis was 22% slower in ConcA-treated cells compared to

146   control, and KIF5B depletion delayed mitotic progression to the same extent (**Fig.2F-G; Videos**

147   **2-4**). Combination of ConcA-treatment with KIF5B knockdown increased the average mitotic

148   duration by 45% verse control (**Fig.2G**).

149   In all, integrity of both lysosome transport and functionality is key for preserving mitotic schedule.

150   To precisely characterize the involvement of lysosomes in mitotic progression, we separately

151   analyzed the timing of mitotic entry (prophase to metaphase) and exit (metaphase to telophase).

152   Interestingly, both episodes were delayed by treatments that impaired lysosomes (**Fig.S2B**).

153   Consistently, the additive effect was reflected in both mitotic entry and exit (**Fig.S2B**).

154   One of the main causes of mitotic delay is the accumulation of mitotic errors such as misaligned

155   chromosomes at metaphase plate or chromosome bridges appearing during chromosome

156   segregation in anaphase[27] (**Fig.2H**). Thus, we next quantified the acquirement of mitotic errors in

157   synchronized cells treated or not with ConcA. Our results demonstrated that lysosome

158   acidification blockade significantly induces the accumulation of misaligned chromosomes and

159   chromosome bridges (**Fig.2I**). In parallel, we tested whether, beyond acidification, the protective

160   role of lysosomes against mitotic errors also relies on trafficking. Consistent with the observed

161   mitotic delay (**Fig.2F-G**), KIF5B-depleted synchronized cells showed a higher frequency of both

162 types of mitotic errors (**Fig.2J**). Thus, active lysosomes are important organelles to ensure correct

163 mitosis progression and limit CIN signature.

Mitotic errors induced by lysosome impairment correlate with an abnormal nuclear

phenotype: the toroidal nucleus

166 Defective mitotic progression generated a robust and striking nuclear phenotype in interphase

167 cells. Precisely, we observed a DAPI-stained nucleus with a hole devoid of chromatin (**Fig.3A**).

168 To understand how this structure was originated, we followed the change on nuclear structure

169 during mitosis by live-imaging using H2B-GFP U2OS cells. Our results showed that this

170 unconventional nuclear phenotype forms upon mitosis, being detectable in at least one of the

171 daughter cells once chromosomes decondense (**Fig.3B; Video 5**). To assure that this

172 unconventional structure does not correspond to an enlarged nucleolus[28], immunofluorescence

173 analysis was performed and results showed that none of the tested nucleolar markers (nucleolin,

174 fibrillarin and UBF) co-stained with the DAPI-free area (**Fig.3C** and **S3A**). To resolve whether this

175 section is nuclear or cytosolic, we imaged the nuclear envelope by immunofluorescence using

176 Lamin B1 and Nuclear Pore Complex (NPC) antibodies (**Fig.3D** and **S3B**). Interestingly, the

177 nuclear envelope was correctly formed around DNA. Further immunofluorescence analysis

178 showed that cytoskeleton filaments such as Phalloidin-stained actin fibers and microtubules, as

179 well as LAMP2-positive lysosomes or LC3-p62-positive autophagosomes were present within e

180 void at the center of the nuclei (**Fig.3E** and **S3C-D**). To discard a nuclear invagination and/or an

181 artifact, 3D reconstruction of high-quality images using IMARIS software resulted in the

182 characterization of a donut-like shaped structure that we refer to as a "toroidal nucleus" (**Fig.3F**

183 and **Video 6**). Ultrastructure analysis of these toroidal nuclei by TEM confirmed that cytosolic

184 material, including autophagic vesicles and lysosomes were present within the hole (**Fig.3G**).

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185   This phenotype was once reported as donut-shaped nucleus[29]. The authors proposed that protein

186   farnesylation inhibitors (FTI) induce the formation of toroidal nuclei by causing a pericentrin-

187   related centrosome separation defect in HeLa cells. Thus, we tested whether FTI induced the

188   formation of toroidal nuclei in our model. After confirming the production of toroidal nuclei following

189   FTI treatment in HeLa cells (**Fig.S3E, left**), we demonstrated that U2OS cells did not respond to

190   FTI. This is contrast to our results with impairment of lysosome acidification by ConcA, which

191   significantly increased the percentage of toroidal nuclei in both cell types (**Fig.S3E, right**). To

192   verify that FTI does not stimulate toroidal nuclei formation with a different kinetics in U2OS, we

193   treated and analyzed cells for 24h, 48h and 72h with FTI or ConcA. While ConcA gradually

194   induced a robust increase of toroidal nuclei with time; FTI did not (**Fig.S3F**). Next, we analyzed

195   whether inhibition of lysosome acidification by ConcA or Chloroquine (CQ) induced the inhibition

196   of farnesylation of Lamin A/C. Neither ConcA nor CQ do not impede Lamin A/C farnesylation like

197   FTI (**Fig.S3G**). Our results suggest that the formation of toroidal nuclei is not due to the inhibition

198   of farnesylation *per se* but to a defect in mitotic progression regardless its inducer.

199   Mitotic stresses have been linked to prolonged mitosis and accumulation of mitotic errors, both of

200   which we detected after lysosomal impairment. As toroidal nuclei were formed following

201   karyokinesis, we examined by live imaging a putative link between the occurrence of mitotic errors

202   and the formation of toroidal nuclei. The results showed a linear correlation between detectable

203   mitotic errors and toroidal nucleus generation with a Pearson's correlation coefficient of 0.85

204   (**Fig.3H**). Indeed, single-cell analysis demonstrated that more than 80% of toroidal nuclei resulted

205   from cells undergoing apparent defective mitosis (**Fig.3I**). Based on this, we next investigated the

206   role of mitotic stresses on generating toroidal nuclei. To this end, we depleted cells of KIF11, a

207   kinesin required for bipolar spindle establishment[30], and analyzed the formation of toroidal nuclei.

208   Disruption of KIF11 by small interfering RNA (siKIF11) or by the KIF11 specific inhibitor Monastrol

209   induced a significant increase in toroidal nucleus frequency (**Fig.3J**) without dramatically affecting

210   lysosome morphology and localization (**Fig.S3H**). Depletion of other kinesins implicated in

211   chromosome segregation such as mitotic centromere-associated kinesin (siMCAK/KIF2C)[31] or

212   Centromere-associated protein E (siCENP-E)[32] produced a 3.3- and 3.8-fold increase of toroidal

213   nucleus population, respectively (**Fig.S3I**). In all tested conditions, lysosomes morphology or

214   distribution were not apparently perturbed (**Fig.S3J**). Furthermore, cells treated with Nocodazole,

215   a microtubule de-polymerizing agent, showed a 4.5-fold increased prevalence of toroidal nuclei

216   accompanied by the expected lysosomal collapse (**Fig.S3I-J**). Thus, the formation of toroidal

217   nucleus seems to be attributable to impairment of karyokinesis.

218   We next investigated whether this phenotype is a consequence of impaired nuclear envelope

219   reformation. Live imaging of H2B-GFP U2OS cells stably expressing mCherry-Lamin A/C,

220   indicated that the reformation of the nuclear envelope preceded the formation of toroidal nuclei

221   (**Fig.S3K; Video 7**). This nuclear phenotype was versatile in terms of size, nuclear localization

222   and morphology (**Fig.S3L1-4**). Furthermore, the nucleus could contain more than one void

223   (**Fig.S3L5-8**) and be accompanied by micronucleus (**Fig.S3L6**). After mitosis, one or both

224   daughter cells could harbor this phenotype (**Fig.S3L7-8**).

225   We investigated whether toroidal nucleus was a common feature or specific to U2OS cells. To

226   this end, we screened through a panel of cell lines. Toroidal nuclei were not detected under the

227   tested conditions in the colon carcinoma cell lines RKO, LoVo or HCT116, hepatocarcinoma cells

228   Huh7 or MC-PED17 glioma-derived cell line[33–36] (**Fig.3K**). However, detection of toroidal nuclei

229   was successful in various cells, among which non-transformed human skin fibroblast (HFF) and

230   embryonic mouse fibroblasts (MEF), as well as cells from lung carcinoma (A549), cervix

231   carcinoma (HeLa) or diffuse intrinsic pontine glioma (SU-DIPG-XVII, SF-8628 and SF-7761)[33,34,37]

232   (**Fig.3K-L**). All those cells, except SF-8628, responded to ConcA treatment by presenting a

233   significant increase of toroidal nuclei (**Fig.3K**).

234   Thus, toroidal nuclei can be scored as a read-out for mitotic errors in interphase cells.

235   **Lysosome disruption induces the formation of toroidal nuclei**

236   The presence of toroidal nuclei in interphase cells facilitates the analysis of mitotic impairment in

237   whole cell populations, favoring the toroidal nucleus as a powerful tool for quantitative analysis of

238   chromosomal instability. Here we aimed to screen for lysosome-specific stresses using toroidal

239   nucleus as a biomarker for chromosomal instability. To this end, cells were treated with ConcA or

240   depleted for KIF5B and toroidal nuclei frequency was quantified. Consistently, v-ATPase inhibition

241   as well as blockage of anterograde transport led to a robust increase of toroidal nuclei population

242   (**Fig.4A-B**).

243   To discard ConcA-side effects not related to v-ATPase inhibition, we genetically or chemically

244   inhibited lysosome acidification. Thus, cells were treated with Chloroquine or depleted of the V0c

245   v-ATPase subunit (siATP6V0c). In agreement, inhibition of lysosome acidification increased the

246   formation of toroidal nucleus by 2.5-3-fold (**Fig.4C**). To validate treatment efficiency, lysosome

247   positioning and size were assessed by immunofluorescence, confirming the expected increase in

248   lysosomal volume[38] (**Fig.4D**). Both ConcA and Chloroquine increased toroidal nucleus formation

249   but their kinetics differed (**Fig.S4A**). Indeed, ConcA treatment acted faster and produced a peak

250   effect at 24 hours, while Chloroquine produced a similar effect after 48 hours, inducing the

251   maximum impact at 72 hours (**Fig.S4A**). To corroborate the specificity of mitosis impairment to

252   lysosome-related stresses, we tested compounds targeting other cellular processes or

253   organelles. In contrast to lysosomal inhibition, treatment with the mitochondrial complex I inhibitor

254   Phenformin or the proteasome inhibitor MG132 had no significant effect on the frequency of

255   toroidal nuclei in unsynchronized cells (**Fig.S4B**). We further asked whether defects in BORC-

256   dependent lysosome trafficking would also induce formation of toroidal nuclei as we determined

257   in cells depleted for KIF5B (**Fig.4E**). Cells genetically depleted of BORC-associated proteins Arl8b

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258   or SKIP also showed a significant increase in the population of toroidal nuclei (2.3- and 4.1-fold

259   respectively) confirming the importance of lysosomal trafficking for correct mitotic progression

260   (**Fig.4F**). Depletion efficacy was corroborated by the perinuclear clustering of lysosomes (**Fig.4F**).

261   Additionally, we examined the specificity of the motor function of KIF5B in the formation of toroidal

262   nucleus by complementing control or KIF5B-depleted cells with neuronal KIF5A, which resulted

263   in a rescue of normal toroidal nucleus frequency (**Fig.4G**). Interestingly, depletion of other motor

264   proteins involved in lysosomal trafficking such as KIF3A, KIF2A or KIF1A also led to a significant

265   increase in toroidal nuclei (**Fig.S4C**). Parallel to a delay in mitotic timing (**Fig.2F-G**), we found that

266   simultaneous disruption of lysosome acidification (ConcA) and trafficking (siKIF5B or siSKIP) had

267   an additive effect on toroidal nucleus formation compared to single treatments (**Fig.4H**). In all, our

268   results confirm that both v-ATPase-dependent lysosome acidification and BORC-associated

269   lysosomal trafficking are required to preserve mitotic integrity.

270   Impairment of lysosome functionality does not prevent mitotic cells to progress into G1 phase but

271   leads to CIN. In cells synchronized and released specifically in mitosis, ConcA and Chloroquine

272   similarly led to toroidal nuclei formation (2.6- and 2.7-fold increase, respectively) (**Fig.S4D**),

273   indicating that the different kinetics observed might be due to effect of the drugs in other phases

274   of the cell cycle. Interestingly, neither MG132 nor Monastrol induced toroidal nuclei in these

275   conditions (**Fig.S4D**), but it is noteworthy that these drugs imped cells from properly concluding

276   cell division as previously demonstrated[39,40], thus limiting the generation of subsequent daughter

277   cells harboring toroidal nucleus (**Fig.S4E**). These results further suggest that lysosomes are not

278   required to undergo mitosis, but genomic instability is triggered when lysosomes are impaired.

279   **Macroautophagy is a key player to maintain mitosis fidelity**

280   As we have detected the presence of autophagic vesicles in mitosis (**Fig.1**), we next analyzed

281   the involvement of macroautophagy for faithful mitotic progression by scoring for toroidal nucleus

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282   frequency after macroautophagy inhibition. Genetic depletion of Atg5 (siAtg5) induced a

283   significant increase in the proportion of toroidal nuclei (1.6-fold increase) (**Fig.5A**). Notably,

284   ConcA did not significantly affect the formation of toroidal nucleus in Atg5-depleted cells (**Fig.5A**).

285   In agreement with the correlation between mitotic errors occurrence and formation of toroidal

286   nuclei, the inhibition of autophagy by Atg5 depletion provoked a significant increase in misaligned

287   chromosomes and chromosome bridges (**Fig.5B-C**). Protein depletion efficiency was monitored

288   by Western Blot (**Fig.S5A**). Chemical inhibition of autophagy by 3MA, a PI3K-class III inhibitor,

289   also resulted in an increase in toroidal nuclei frequency together with an enhanced detection of

290   chromosomal misalignment (**Fig.S5B-C**). As U2OS is a cancer cell line already susceptible to

291   genomic instability and chromosome alterations[41], we used mouse embryonic fibroblasts (MEFs)

292   to analyze the impact of defective autophagy on mitotic progression in a non-tumoral model. First,

293   we validated ConcA efficiency and autophagic flux impairment by analyzing Atg5 and LC3 protein

294   levels. As expected, Atg5-/- MEFs harbored a defective autophagic flux (**Fig.5D**). Then, we

295   quantified the frequency of toroidal nuclei in Atg5 wt (Atg5+/+) and Atg5 KO (Atg5-/-) MEFs.

296   ConcA treatment in Atg5+/+ MEFs significantly increased toroidal nuclei population,

297   demonstrating that lysosomal inhibition impacts mitotic progression in various cell lines (**Fig.5E-**

298   **F and Fig.3K-L**). Like the effect of the depletion of Atg5 in U2OS cells, Atg5-/- MEFs presented

299   a significantly increased percentage of toroidal nuclei under normal growing conditions,

300   supporting that basal autophagy prevents cells from mitotic errors. ConcA-induced toroidal

301   nucleus formation was not further increased in Atg5 KO MEFs (**Fig.5E**). Atg5-deficient MEFs were

302   significantly more susceptible to chromosomal misalignment and chromosomal bridges compared

303   to parental MEFs (2.36- and 1.48-fold increase respectively) (**Fig.5G-H**). Inhibition of v-ATPase-

304   dependent lysosomal acidification by ConcA clearly increased chromosome misalignment and

305   chromosomal bridges in control Atg5+/+ MEFs (**Fig.5G-H**). Cells lacking Atg5 showed a milder

306   response to inhibition of lysosomal acidification regarding both phenotypes (**Fig.5G-H**).

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307　Altogether, our results establish the involvement of macroautophagy machinery in correct mitotic

308　progression.

309　Cohesin proteins WAPL and PDS5B are novel lysosome substrates during cell division

310　Based on the characterization of a remarkable role for lysosomes and autophagic vesicles in

311　mitotic progression, we next aimed to identify novel substrates of the autophagic-lysosomal

312　pathway specifically in mitosis by mass spectrometry analysis. Following synchronization of cells

313　at G2/M transition, cells were released in normal media or in media containing ConcA for 7 hours.

314　G1-enriched cell fractions were processed for mass spectrometry analysis. Drug efficacy was

315　validated by LC3 and p62 accumulation in the three experimental replicates after ConcA treatment

316　(**Fig.S6A**). Proteomic data analysis identified a total of 1749 peptides present in both ConcA and

317　control fractions, while only 13 and 141 were uniquely detected in control and ConcA fractions,

318　respectively (**Fig.6A**). Enrichment analysis validated 853 peptides being differently expressed (q-

319　value<0.05) that were selected based on ± 1.2-fold change cut-off (**Fig.6B**). In concordance with

320　its role in inhibiting lysosome-dependent degradation, ConcA significantly induces protein

321　accumulation. The obtained list of candidates was clustered into cellular and organism functions

322　using Reactome free database[42,43]. Reactome analysis highlighted that proteins accumulated

323　after lysosome inhibition specifically during mitosis were mainly involved in cell cycle, vesicle-

324　mediated transport or DNA repair (**Fig.S6B**). In agreement with previous data (**Fig.1I, S1B** and

325　**S6A**), p62 was significantly increased by 2.73-fold in the ConcA fraction compared to control

326　(**Fig.6B**). Next, we selected the 56 protein candidates that were clustered in functional

327　annotations related to mitosis and cell cycle regulation and we performed a GO enrichment

328　analysis (**Fig.S6C**). A list of 20 proteins involved in mitotic cell cycle progress (GO:1903047) were

329　further studied to define the involvement of lysosomes in chromosome segregation (**Fig.6C**).

330　Interestingly, among the principal enriched proteins, two cofactors of the cohesin complex such

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331   as Cohesin-associated factor B (PDS5B) and Wings apart-like protein (WAPL) were significantly

332   increased by 4.78- and 1.64-fold, respectively (**Fig.6B-C**). To validate proteomic data, we isolated

333   mitotic cells by shake-off after synchronization-release and evaluated protein levels in control and

334   ConcA-treated cells. As expected, autophagic proteins p62 and LC3-II accumulated in ConcA-

335   treated mitotic fraction (**Fig.6D and S6D**). Significant increase of both WAPL and PDS5B protein

336   levels occurred in mitotic cells upon acute impairment of lysosomal acidification capacity (**Fig.6D-**

337   **E**), indicating that both PDS5B and WAPL are novel putative lysosomal substrates specifically

338   during mitotic progression. To validate our findings in another cell line, we obtained shake-off

339   fractions from non-transformed MEFs. Mitotic fractions of non-synchronized MEFs corroborated

340   that PDS5B and WAPL significantly accumulated more in Atg5 -/- MEFs cells compared to

341   parental MEFs (**Fig.6F-G and S6E**). WAPL and PDS5B accumulation upon lysosome inhibition

342   could potentially be due to the observed mitotic delay independent of the autophagy machinery.

343   To address this point, U2OS cells were transfected with exogenous WAPL-GFP protein and

344   treated or not with ConcA for 3 hours after synchronization. Mitosis-enriched fractions were then

345   subjected to GFP-immunoprecipitation. First, we validated the binding between WAPL and its

346   well-described partner PDS5B[44] (**Fig.6H**). WAPL-GFP interacts with PDS5B to the same extent

347   in control or ConcA-treated cells, suggesting that their interaction is maintained upon lysosomal

348   inhibition. Interestingly, WAPL-GFP interaction with autophagic adaptor protein p62 significantly

349   increased in ConcA-treated cells (**Fig.6H**). These results support that WAPL is recognized by

350   p62-dependent selective autophagy machinery and emerges as a novel lysosome substrate

351   during mitosis.

352   In all, our results shed light into a novel regulatory mechanism for accurate mitotic progression,

353   depicting lysosomes as key players for correct chromosomal congression and faithful genetic

354   transmission (**Fig.7**). In addition, we identified the toroidal nuclei as a read-out of mitotic defects

355   in interphase cells, and such, are a novel biomarker of genotoxicity for cancer research.

356 **DISCUSSION**

357 **Faithful mitotic progression relies on autophagy and lysosomes**

358 During mitosis, dramatic cellular rearrangement occurs to support proper chromosome

359 segregation and productive partitioning of intracellular organelles. Many membranous

360 compartments undergo massive spatiotemporal disruption[16] and scheduled protein degradation

361 is required. While the role of UPS has been extensively studied, the implication of lysosomes and

362 autophagy in cell division still remains elusive[17,18,20–23,45]. Although lysosome studies are mainly

363 focused on interphase cells, endocytic vesicles (including lysosomes) were proposed to serve as

364 a membrane source for plasma membrane extension and retraction during mitotic transition[14,15].

365 Recently, PCM1-driven selective degradation of centriolar satellites was shown to maintain

366 centrosome function for correct mitotic progression[24]. Here, our results agree with the persistence

367 of a robust autophagic flux and lysosomal-dependent degradation during mitosis. Lysosome

368 enlargement suggests that fusion events are occurring in early mitosis and that, in contrast to

369 other organelles, lysosomes play an active role during cell division. The finding that under basal

370 conditions Atg5 KO MEFs harbor a higher population of toroidal nuclei corroborates that

371 autophagy is active and necessary for mitosis even in non-transformed cells. Among the identified

372 lysosomal substrates in mitosis, the accumulation of p62, an autophagic adaptor protein for

373 ubiquitinated cargos, validates the persistence of autophagic flux and suggests a potential

374 alternative route for the degradation of mitotic factors[46,47]. To note, PCM1 was not detected in our

375 proteomic analysis of mitotic cells, supporting that various selective autophagy processes might

376 probably coexist and should be timely regulated during mitotic progression. Recently, p62

377 emerged as a potential pivotal player in the crosstalk between UPS and selective

378 autophagosome-lysosome degradation[48,49]. Based on p62 bifunctionality and ubiquitin-lysine

379 linkages diversity, we speculate a potential interconnection between the two main degradative

16

380 pathways to correctly distribute genetic material. Control of sister chromatid cohesion during cell

381 division is crucial for equal distribution of genetic material. The canonical evolutionary conserved

382 cohesin complex is one of the main molecular entities involved in this process. The cohesin

383 complex consists of four subunits (SMC1, SMC3, SCC1 and STAG1/3)[50]. Currently, most

384 research focuses on cohesin loading processes during replication and cohesin-dependent

385 extrusion of chromatin loops for DNA-damage repair[51]. In mitosis, cohesin supports both mitotic

386 entry and metaphase-to-anaphase transition. In prophase, cohesin complexes dissociate to allow

387 chromatid condensation and separation. At that precise moment, cohesin complex disruption is

388 triggered by WAPL and PDS5B along the chromatids arms[52,53]. WAPL-dependent dissociation at

389 the centromeric region is inhibited by Shugoshin to prevent premature chromatin separation[54,55].

390 During metaphase-to-anaphase transition, chromosome dissociation at the centromeric region is

391 regulated by APC/C-dependent securin ubiquitination, leading to separase activation that cleaves

392 SCC1 to culminate chromatids separation[56]. Since lysosomes can degrade most biological

393 material and, taking in account that in this study we identified more than 100 potential lysosomal

394 protein substrates in mitotic cells, we favor the idea that the implication of lysosomes in cell

395 division is a multifactorial process. Our finding that WAPL and PDS5B accumulate upon blockade

396 of lysosomal acidification, identifies them as potential lysosomal substrates specifically during

397 mitosis. Our study reports for the first time the involvement of autophagy- and lysosomal-

398 dependent degradation of cohesin cofactors. The identification of WAPL as a novel p62 interactor

399 supports the model of selective degradation of WAPL by lysosome during mitotic transition.

400 However, additional studies would be needed to better understand the interconnection between

401 lysosome-dependent degradation and the regulation of chromatid cohesion during mitotic

402 progression. Variations in PDS5B and WAPL expression correlate cohesin and chromosomal

403 segregation defects with aneuploidies and cancer progression[52,57–60].

404   While the implications of lysosome biology in cancer are still controversial, efficacy of

405   lysosomotropic agents has been proven in several malignancies either alone or in combination,

406   and clinical trials are ongoing[61,62]. CIN is considered a hallmark of cancer understood either as a

407   strength, due to mutation induction facilitating tumor diversification, or as a tumor vulnerability by

408   eventually triggering apoptotic cell death[2,63]. Defective autophagy was recently linked to increased

409   CIN[64]. Here we show that lysosomal acidification and trafficking as well as selective autophagy

410   are necessary to prevent CIN. Our data are consistent with the metaphor of autophagy and

411   lysosome-dependent degradation as a double-edged sword in cancer progression[65]. In all,

412   depending on cell type and tumor stage, a combination of lysosomotropic drugs with conventional

413   therapies might be beneficial, as autophagy inhibition can be detrimental for cancer cells and

414   increased CIN can lead to cell death. Therefore, better understanding of the mechanisms by which

415   selective autophagy functions in cell division will provide novel routes to improve cancer treatment

416   strategies.

417   **Toroidal nucleus, a new tool for genotoxicity screenings**

418   Study of mitosis is challenging due to the velocity and architectural changes accompanying cell

419   division. To escape these obstacles, most mitosis-focused studies take advantage of

420   synchronization protocols. However, drugs used for cell synchronization, such as Nocodazole, do

421   not specifically target mitosis but rather essential cellular components like microtubules, thus

422   altering basic cellular functions. Currently, the only marker of defective mitosis detectable in non-

423   mitotic cells is the micronucleus. Although micronuclei formation is a consequence of

424   chromosome missegregation[66], they can be reabsorbed during subsequent cell divisions or

425   destroyed by autophagy[67,68]. Therefore, complementary read-outs in interphase cells are needed

426   for the analysis of mitosis impairment. Here we show that toroidal nuclei form upon inexact mitosis

427   and linearly correlates with the occurrence of mitotic errors. Inhibition of farnesylation was

428 previously proposed to drive the formation of donut-shaped nuclei by affecting centrosome

429 function[29]. Here we demonstrate that toroidal nuclei do not specifically and uniquely form upon

430 inhibition of protein farnesylation. Indeed, FTI did not increase toroidal nucleus population in

431 U2OS, while mitotic stresses, such as inhibition of lysosomal function described here, significantly

432 and robustly increased the frequency of toroidal nuclei in various cell lines. In addition, our results

433 reveal that toroidal nuclei form prior to reformation of the nuclear envelope, as it was also

434 described for micronucleus[69]. The fate of micronuclei is still uncertain. Four possibilities are under

435 examination: degradation, reincorporation into the nucleus, extrusion or persistence in the

436 cytoplasm leading to apoptosis or chromothripsis[70]. We expect similarities for toroidal nuclei, but

437 further analyses are needed to decipher how cells can cope with toroidal nuclei. We detected

438 toroidal nuclei in transformed and non-transformed cells, designating presence of this structure

439 as a robust and conserved phenotype within cell lines. However, the occurrence of toroidal nuclei

440 might vary dependent on cell type specificities and/or genetic background. Variability in toroidal

441 nucleus frequency might also correlate with basal autophagy levels, which are known to be cell

442 type specific[71]. Noteworthy, detection of toroidal nuclei is a new convenient tool for impaired

443 mitosis in genotoxicity screenings.

444 Until now, orchestration of mitotic factors degradation has been exclusively attributed to the UPS.

445 Our data reveals an additional route in which active autophagy and lysosomes promote faithful

446 genetic transmission. Given the promising results of lysosomotropic drugs for cancer treatment,

447 our study opens an alternative line of research focusing on lysosomes modulation to exploit CIN

448 for cancer therapy.

19

449   **MATERIALS AND METHODS**

450   **Cell Culture**

451   U2OS, HeLa, A549, HCT116, RKO, Huh7, MCF7 and HEK293-T cell lines were obtained from

452   American Type Culture Collection (ATCC). Atg5 wt and Atg5 KO MEF were kindly provided by

453   Dr. Zorzano (IRB Barcelona). HFF cells were kindly provided by the ICO CellBank. U2OS H2B-

454   GFP cell line was kindly provided by Dr. Agell (UB). SF-7761 and SF-8628 were purchased from

455   Millipore Sigma (Burlington, MA). MC-PED17 cells were a gift from Dave Daniels (Mayo Clinic;

456   Rochester, MN). SU-DIPG-XVII cells were a gift from Michelle Monje (Stanford University; Palo

457   Alto, CA). U2OS mCherry-LaminA/C and U2OS H2B-GFP LAMP1-RFP were generated in our

458   laboratory by transient transfection of plasmids purchased in Addgene repository, LAMP1-GFP

459   (#34831), LAMP1-mRFP-Flag (#34611) and mCherry-LaminA/C (#55068). WAPL-GFP plasmid

460   was kindly shared by Dr. Fangwei Wang. Briefly, DNA transfection was performed following

461   manufacturer's instructions using Lipofectamine 2000 (Life Technologies) in 1:5 opti-MEM:

462   DMEM medium. Cells were selected using a fluorescence-activated cell sorter (MoFlo Atrios

463   SORTER). Cells were grown in DMEM high glucose (Gibco) [4mM L-Glutamine, 4.5 g/L glucose,

464   1 mM Pyruvate] supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma

465   Aldrich). Cells were incubated at 37ºC, 5% CO_2 and 90-95% of relative humidity. Specific

466   experimental conditions are indicated in figure legends. All chemicals used in this study are

467   reported in **Table 1**.

468   **Cell synchronization at G2**

469   Cells were seeded the day before starting the synchronization in DMEM medium supplemented

470   with FBS (complete DMEM). Thymidine was added at a final concentration of 2 μM for 24 hours

471   to synchronize cells at late G1. After PBS washing, cells were released to S phase by adding

20

472   complete DMEM for 2 hours. Finally, RO3306 (CDK1 inhibitor) was added to cell culture medium

473   at a final concentration of 9 μM for 12 hours to arrest cells at late G2 (**Fig.1F**). Experiments with

474   synchronized cells were performed by releasing cells in complete DMEM with or without the

475   corresponding drugs for the indicated times. For the obtention of shake-off fractions, synchronized

476   cells were subjected to consecutive strokes to the dishes to detach specifically the mitotic cells

477   while maintaining the integrity of the cell monolayer.

478   **Silencing RNA transfections**

479   siRNA transfections were performed following manufacturer's instructions in Opti-MEM medium

480   (Life Technologies) using Lipofectamine RNA-iMAX (Life Technologies). Unless otherwise

481   indicated, transfections were performed for 48 hours. siRNA sequences and concentrations used

482   in these studies are listed in **Table 2**.

483   **Cell Cycle analysis**

484   Cells were trypsinized, counted and placed on ice. 500,000 cells were centrifuged at 1200 rpm at

485   4ºC for 5 minutes and cell pellet was washed with ice-cold PBS, fixed with 70% ethanol and

486   placed at -20ºC for at least 24 hours. Cells were then washed with ice-cold FACS buffer [BSA

487   0.1%, EDTA 5 mM in PBS] and centrifuged for 5 minutes at 1000 rpm at 4ºC. Supernatant was

488   discarded and the cell pellet was resuspended in propidium iodide (PI) staining solution [PBS,

489   0.1% NP40, 20 μg/mL RNAse A (Invitrogen), 40 μg/mL PI (Sigma)]. Cell suspension was

490   transferred to a 5 mL tube with cell strainer cap (Corning) and maintained at room temperature

491   protected from light for 15 minutes. Samples were acquired using FACS Canto System (BD

492   Biosciences, USA) and analyzed with ModFit LT software (Verity Software House).

493   **Protein extraction and Western Blot**

494   Cells were washed twice with ice-cold PBS, scraped and lysed on Lysis Buffer [20 mM Tris-HCl

495   pH 8, 10 mM EDTA, 150 mM NaCl, 1% Triton-X100] supplemented with protease inhibitor cocktail

496   (1:100 Sigma-Aldrich) and phosphatase inhibitors cocktail 2 and 3 (1:100 Sigma-Aldrich). Cell

497   lysates were centrifuged at 13000 rpm for 10 minutes at 4⁰C. Protein concentration was analyzed

498   using Pierce BCA Protein Assay kit (ThermoFisher Scientific) following manufacturer's

499   instructions. Equal amounts of protein lysates were resuspended in Laemnli SDS-sample buffer

500   and incubated at 95⁰C for 5 minutes. Proteins were separated on SDS-PAGE and transferred to

501   PVDF membranes (Millipore). Membranes were blocked with 5% non-fat dry milk (BioRad) in

502   Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 hour at room temperature.

503   Incubation of primary antibodies was performed overnight at 4⁰C in 5% non-fat dry milk or 3.5%

504   BSA (Sigma-Aldrich). After three washes in TBS-T, membranes were incubated for 1 hour at room

505   temperature with secondary antibodies (1:5000) diluted in 5% non-fat milk. Upon incubation,

506   membranes were washed three times with TBS-T and protein detection was performed by using

507   enhanced chemiluminescence kit (GE Healthcare). Blots were scanned with iBright detection

508   system (Thermo Fisher). All the antibodies used in this study are reported with the corresponding

509   working concentrations in **Table 3**.

510   **Immunoprecipitation**

511   U2OS cells transfected with WAPL-GFP expression vector or GFP-empty vector as negative

512   control were washed twice with ice-cold PBS and cellular pellets were kept at -80⁰C before lysis.

513   Cellular pellet was lysed in ice-cold IP-RIPA buffer [100 mM Tris-HCl pH7.5, 300 mM KCl, 10 mM

514   MgCl$_2$, 2 mM EGTA, 20% Glycerol and 1.6 % NP40] supplemented with protease inhibitors (2X),

515   phosphatase inhibitors cocktails (1X), PMSF 1mM and DTT 1mM. After 5 minutes incubation on

516   ice, collected samples were mechanically lysed with cold syringe and centrifuged at 13,000 rpm

517   for 5 minutes at 4 ⁰C. Soluble fraction was subjected to BCA Pierce protein quantification. 1,500

518   µg of protein per sample were separated for immunoprecipitation at 1 µg/µl concentration. For the

519   immunoprecipitation, 30 µl of GFP-Trap beads (ChromoTek) were added to each lysate and

520   incubated by rotation overnight at 4 ⁰C. Immunoprecipitates were washed three times with IP-

521   RIPA buffer. Immunoprecipitated proteins were denatured by the addition of 2X sample buffer

522   followed by boiling for 10 minutes, resolved by 4 %-20 % Criterion TGX Gel (BIO-RAD)

523   electrophoresis and analyzed by immunoblotting. 20 µg of the total lysate were loaded as input

524   to control.

525   **Immunofluorescence**

526   Cells were grown as monolayers on coverslips and subjected to the indicated conditions. Cells

527   were fixed with 4% paraformaldehyde for 10 minutes at room temperature. After 5 minutes

528   washing with PBS, cells were permeabilized using 0.1% Triton X-100 in PBS for 10 minutes at

529   room temperature. Next, cells were blocked with 1% BSA/0.01% Triton X-100 in PBS plus 10 mM

530   Glycine for 30 minutes at room temperature. Cells were incubated with primary antibodies for one

531   hour at room temperature or overnight at 4⁰C. Following a series of PBS washes, cells were

532   incubated with secondary antibodies for 45 minutes at room temperature. After two washes of 5

533   minutes with PBS, coverslips were mounted using Vectashield Mounting Solution containing DAPI

534   (Vector Laboratories). All the antibodies used in this study are reported with the corresponding

535   working concentrations in **Table 2**.

536   **Vesicle acidification and detection assays in live cells**

537   U2OS cells were seeded onto glass coverslips and subjected to the indicated conditions. Live

538   cells were washed once with PBS and Magic Red (Immunochemistry Technologies) and

539   Lysosensor (Invitrogen) were added to cover the cell layer following manufacturer's instructions.

540   Cells were incubated for 10 minutes at room temperature before image acquisition.

541 **Image acquisition and analysis**

542 For detailed analysis, image acquisition was performed in Leica spectral confocal microscope

543 TCS SP5 using a 63x N.A 1.4 objective and LAS AF software or Carl Zeiss LSM880 confocal

544 microscope and ZEN software. Fluorophores were excited with Argon 488, DPSS 561, Diode 640

545 and Diode 405 lasers. Image analysis was performed using FIJI Image J software (NIH USA).

546 For toroidal nucleus and mitotic errors quantification, images were acquired using Nikon

547 Epifluorescence microscope using a 40x dry objective. Image analysis was performed using Cell

548 counter plugin from FIJI Image J software (NIH USA). Pearson's correlation coefficient was

549 calculated based on confocal images using FIJI ImageJ plugin Intensity Correlation Analysis.

550 Mitotic lysosomes size and number was analyzed with FIJI ImageJ fixing particles size from 0.3

551 μm^2 to infinity. The distribution of mitotic lysosomes was calculated with FIJI ImageJ plugin Radial

552 Profile Angle as previously described[72].

553 **3D reconstitution**

554 Stacks of images acquired at optimal settings with Carl Zeiss LSM880 confocal microscope were

555 processed with IMARIS Software (Bitplane) for 3D image reconstruction and to generate in silico

556 animations and images.

557 **Live-cell time-lapse videos**

558 For mitotic delay analysis, U2OS H2B-GFP cells were grown onto glass bottom 8-well slides

559 (IBIDI). After indicated treatments, medium was replaced by FluoroBright medium (Gibco) and

560 live-cell imaging was performed on the Leica spectral confocal microscope TCS SP5. Images

561 were taken every 6 minutes and 33 seconds for a total time of 24 hours using the 63x glycerol

562 objective.

24

563   For lysosome trafficking analysis, U2OS cells stably expressing LAMP1-RFP and H2B-GFP were

564   grown onto glass bottom 8-well slides (IBIDI). After indicated treatments, medium was replaced

565   by FluoroBrite medium (Gibco) and live-cell imaging was performed on Carl Zeiss LSM880

566   confocal microscope. Images were taken every 5 minutes for a total time of 24 hours using the

567   63x glycerol objective.

568   For toroidal nucleus and nuclear lamina reformation assays, U2OS cells stably expressing H2B-

569   GFP and mCherry-Lamin A/C were grown onto bottom-glass 8 chambers slides (IBIDI). After

570   addition of FluoroBrite with indicated treatments, mitotic cell division was analyzed on a Carl Zeiss

571   LSM880 confocal microscope. For toroidal nucleus formation, images were taken every 93

572   seconds for 16 hours using the 63x glycerol objective. For nuclear envelope reformation

573   experiment, images were taken every 5 minutes for 16 hours using the 63x glycerol objective.

574   **Transmission Electron Microscopy (TEM)**

575   U2OS cells with or without ConcA (10 nM) treatment for 24 hours were fixed with Glutaraldehyde

576   2.5% in Sodium Cacodylate Trihydrate 0.1 M pH 7.2 for 2 hours at 4ºC. After three washes with

577   Sodium Cacodylate Trihydrate 0.1 M of 15 minutes each, samples were incubated for 2 hours

578   with Osmium tetroxide 1% in Sodium Cacodylate Trihydrate 0.1 M at room temperature. Samples

579   were washed three times for 15 minutes with Sodium Cacodylate Trihydrate 0.1 M. Then, samples

580   were processed through dehydration with Ethanol 30% to 100% gradually. Samples were then

581   embedded into Resin EPOXY. After sample orientation, polymerization occurred at 60ºC for 48

582   hours. Samples were cut using ultramicrotome EM UC6 Leica, first in semithin of 250 nm and

583   then ultrathin of 70 nm. Image acquisition was performed with TEM microscope JEOL JEM-1011.

584   Images were analyzed and processed with FIJI ImageJ software (NIH).

585 **Mass Spectrometry**

586 Three replicates of treated samples were processed for protein extraction in RIPA lysis buffer

587 [Tris-HCl pH8 50mM, NaCl 150mM, NP-40 1%, Sodium deoxycholate 0.5%, SDS 0.1%]. Protein

588 concentration was determined and 50 μg of each sample was digested using a FASP (Filter-Aided

589 Sample Preparation) approach. Briefly, proteins were reduced with dithiothreitol 10 mM (60

590 minutes, 32ºC) and alkylated with iodoacetamide 20 mM (30 minutes at 25ºC in the dark).

591 Samples were loaded onto an Amicon Ultra filter 10 KDa, 0.5 mL (Millipore) to remove interfering

592 agents with 2 rounds of centrifugations/washes with 100 mM ammonium bicarbonate buffer

593 (13,600 g; 25 minutes at room temperature). Digestion was carried out in two steps: first, samples

594 were digested (1:50 w sample/w enzyme) with Lys-C (Wako) in 6 M urea buffer for 3 hours at

595 35ºC, second, the samples were diluted 10-fold with 100 mM ammonium bicarbonate buffer and

596 digested with modified porcine trypsin (Promega-Gold) (1:25 w sample/w enzyme) for 16 hours

597 at 37ºC. The resulting peptide mixture was recovered by centrifuging the filter. Then, the filter was

598 washed twice with 300 μL of 50 mM ammonium bicarbonate and once with 200 μL of 20%

599 acetonitrile/50 mM ammonium bicarbonate (13,600g for 25 min at room temperature). All the

600 fractions were pooled, and the final peptide mixture was acidified with formic acid. Finally, the

601 final volume of the acidified peptide solution was reduced on a SpeedVac vacuum system

602 (Thermo Fisher Scientific), and the peptide solution was desalinated with a C18 spin column

603 (Thermo Fisher Scientific) following supplier's indications.

604 Samples were analyzed in a Proxeon 1,000 liquid chromatographer coupled to an Orbitrap Fusion

605 Lumos (Thermo Fisher Scientific) mass spectrometer. Samples were re-suspended in 0.5%

606 formic acid in water, and 2 μL (1 μg/ μL) were injected for LC-MSMS analysis. Peptides were

607 trapped on an NTCC-360/75-3-123 LC column and separated using a C18 reverse phase LC

608 column-Easy Spray (Thermo Fisher Scientific). The gradient used for the elution of the peptides

26

609 was 1% to 35% in 90 minutes followed by a gradient from 35% to 85% in 10 minutes with 250

610 nL/min flow rate. Eluted peptides were subjected to electrospray ionization in an emitter needle

611 (PicoTipTM, New Objective, Scientific Instrument Services) with an applied voltage of 2,000 V.

612 Peptide masses (m/z 300-1,700) were analyzed in data-dependent mode where a full scan MS

613 was acquired on the Orbitrap with a resolution of 60,000 FWHM at 400 m/z. Up to the 10 most

614 abundant peptides (minimum intensity of 500 counts) were selected from each MS scan and then

615 fragmented using CID (collision-induced dissociation) in the linear ion trap using helium as

616 collision gas with 38% normalized collision energy. The scan time settings were full MS at 250

617 ms and MSn at 120 ms. Generated raw data files were collected with Thermo Xcalibur (v.2.2)

618 (Thermo Fisher Scientific). MaxQuant 1.6.1.0 Software (Department for Proteomics and Signal

619 Transduction, Max-Planck Institute for Biochemistry) was used to search the raw data obtained

620 in the MS analyses against a SwissProt/Uniprot human database with Andromeda Search engine

621 (1.5.6.0). A target and decoy database were used to assess the false discovery rate (FDR).

622 Trypsin was chosen as enzyme and a maximum of two misscleavages were allowed.

623 Carbamidomethylation (C) was set as a fixed modification, whereas oxidation (M) and acetylation

624 (N-terminal) were used as variable modifications. Searches were performed using a peptide

625 tolerance of 7 ppm and a product ion tolerance of 0.5 Da. Resulting data files were filtered for

626 FDR <1%. Statistical analysis was performed in Perseus 1.6.2.1 (Department for Proteomics and

627 Signal Transduction, Max-Planck Institute for Biochemistry).

628 **Statistical Analysis**

629 Data was analyzed by Excel or GraphPad Prism4 software. Results are presented as Mean ±

630 S.D., for the indicated n independent experiments. Experimental data sets were compared by: (i)

631 Two-sampled, two-tailed Student's t-test for two experimental conditions sharing normal

632 distribution and variance (ii) One-way ANOVA test for more than 2 conditions sharing normal

27

633   distribution and variance. Multiple comparisons corrected using Bonferroni test (iii) Kruskal-Wallis

634   test for more than 2 conditions and data without assuming normal distribution. Multiple

635   comparisons corrected using Dunn's test.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

AUTHORS CONTRIBUTIONS

E.A. and C.M. conceived, designed and performed the experiments. C.D. performed experiments for detection of toroidal nuclei in glioma cells. E.A., C.M., S.A. and A.T. analyzed and discussed the data. E.A. and C.M. wrote the manuscript. A.T. and C.M. coordinated the study. All authors intellectually contributed and commented on the manuscript.

REFERENCES

1. McClelland SE. Role of chromosomal instability in cancer progression. Endocr Relat Cancer 2017; 24:T23–31.

2. Bakhoum SF, Cantley LC. The Multifaceted Role of Chromosomal Instability in Cancer and Its Microenvironment. Cell 2018; 174:1347–60.

3. Thompson SL, Bakhoum SF, Compton DA. Mechanisms of chromosomal instability. Curr Biol CB 2010; 20:R285-295.

4. Araujo AR, Gelens L, Sheriff RSM, Santos SDM. Positive Feedback Keeps Duration of Mitosis Temporally Insulated from Upstream Cell-Cycle Events. Mol Cell 2016; 64:362–75.

5. Sullivan M, Morgan DO. Finishing mitosis, one step at a time. Nat Rev Mol Cell Biol 2007; 8:894–903.

6. Bloom J, Cross FR. Multiple levels of cyclin specificity in cell-cycle control. Nat Rev Mol Cell Biol 2007; 8:149–60.

7. Peters J-M. The anaphase promoting complex/cyclosome: a machine designed to destroy. Nat Rev Mol Cell Biol 2006; 7:644–56.

8. Pines J. Cubism and the cell cycle: the many faces of the APC/C. Nat Rev Mol Cell Biol 2011; 12:427–38.

9. Jia R, Guardia CM, Pu J, Chen Y, Bonifacino JS. BORC coordinates encounter and fusion of lysosomes with autophagosomes. Autophagy 2017; 13:1648–63.

683 10. Guardia CM, Farías GG, Jia R, Pu J, Bonifacino JS. BORC Functions Upstream of Kinesins 1 and 3 to
684   Coordinate Regional Movement of Lysosomes along Different Microtubule Tracks. Cell Rep 2016;
685   17:1950–61.

686 11. Pu J, Schindler C, Jia R, Jarnik M, Backlund P, Bonifacino JS. BORC, a multisubunit complex that
687   regulates lysosome positioning. Dev Cell 2015; 33:176–88.

688 12. Mancias JD, Kimmelman AC. Mechanisms of Selective Autophagy in Normal Physiology and Cancer.
689   J Mol Biol 2016; 428:1659–80.

690 13. Lamark T, Svenning S, Johansen T. Regulation of selective autophagy: the p62/SQSTM1 paradigm |
691   Essays in Biochemistry [Internet]. [cited 2019 Sep 18]; Available from:
692   http://essays.biochemistry.org/content/61/6/609.full-text.pdf

693 14. Albertson R, Riggs B, Sullivan W. Membrane traffic: a driving force in cytokinesis. Trends Cell Biol
694   2005; 15:92–101.

695 15. Boucrot E, Kirchhausen T. Endosomal recycling controls plasma membrane area during mitosis.
696   Proc Natl Acad Sci 2007; 104:7939–44.

697 16. Jongsma MLM, Berlin I, Neefjes J. On the move: organelle dynamics during mitosis. Trends Cell Biol
698   2015; 25:112–24.

699 17. Eskelinen E-L, Prescott AR, Cooper J, Brachmann SM, Wang L, Tang X, Backer JM, Lucocq JM.
700   Inhibition of autophagy in mitotic animal cells. Traffic Cph Den 2002; 3:878–93.

701 18. Furuya T, Kim M, Lipinski M, Li J, Kim D, Lu T, Shen Y, Rameh L, Yankner B, Tsai L-H, et al. Negative
702   regulation of Vps34 by Cdk mediated phosphorylation. Mol Cell 2010; 38:500–11.

703 19. Lu G, Yi J, Gubas A, Wang Y-T, Wu Y, Ren Y, Wu M, Shi Y, Ouyang C, Tan HWS, et al. Suppression of
704   autophagy during mitosis via CUL4-RING ubiquitin ligases-mediated WIPI2 polyubiquitination and
705   proteasomal degradation. Autophagy 2019; :1–18.

706 20. Loukil A, Zonca M, Rebouissou C, Baldin V, Coux O, Biard-Piechaczyk M, Blanchard J-M, Peter M.
707   High-resolution live-cell imaging reveals novel cyclin A2 degradation foci involving autophagy. J Cell
708   Sci 2014; 127:2145–50.

709 21. Li Z, Zhang X. Autophagy in mitotic animal cells. Sci Bull 2016; 61:105–7.

710 22. Liu L, Xie R, Nguyen S, Ye M, McKeehan WL. Robust autophagy/mitophagy persists during mitosis.
711   Cell Cycle Georget Tex 2009; 8:1616–20.

712 23. Yuan F, Jin X, Li D, Song Y, Zhang N, Yang X, Wang L, Zhu W-G, Tian C, Zhao Y. ULK1 phosphorylates
713   Mad1 to regulate spindle assembly checkpoint. Nucleic Acids Res 2019;

714 24. Holdgaard SG, Cianfanelli V, Pupo E, Lambrughi M, Lubas M, Nielsen JC, Eibes S, Maiani E, Harder
715   LM, Wesch N, et al. Selective autophagy maintains centrosome integrity and accurate mitosis by
716   turnover of centriolar satellites. Nat Commun 2019; 10:4176.

717 25. Joachim J, Razi M, Judith D, Wirth M, Calamita E, Encheva V, Dynlacht BD, Snijders AP, O'Reilly N,
718   Jefferies HBJ, et al. Centriolar Satellites Control GABARAP Ubiquitination and GABARAP-Mediated
719   Autophagy. Curr Biol 2017; 27:2123-2136.e7.

720 26. Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, Chen G, Jin S, White E.
721   Autophagy suppresses tumor progression by limiting chromosomal instability. Genes Dev 2007;
722   21:1367–81.

723 27. Rieder CL, Maiato H. Stuck in Division or Passing through: What Happens When Cells Cannot
724   Satisfy the Spindle Assembly Checkpoint. Dev Cell 2004; 7:637–51.

725 28. Abella N, Brun S, Calvo M, Tapia O, Weber JD, Berciano MT, Lafarga M, Bachs O, Agell N. Nucleolar
726   Disruption Ensures Nuclear Accumulation of p21 upon DNA Damage. Traffic 2010; 11:743–55.

727 29. Verstraeten VLRM, Peckham LA, Olive M, Capell BC, Collins FS, Nabel EG, Young SG, Fong LG,
728   Lammerding J. Protein farnesylation inhibitors cause donut-shaped cell nuclei attributable to a
729   centrosome separation defect. Proc Natl Acad Sci U S A 2011; 108:4997–5002.

730 30. Liu Y, Zhang Z, Liang H, Zhao X, Liang L, Wang G, Yang J, Jin Y, McNutt MA, Yin Y. Protein
731   Phosphatase 2A (PP2A) Regulates EG5 to Control Mitotic Progression. Sci Rep [Internet] 2017
732   [cited 2019 Mar 8]; 7. Available from: http://www.nature.com/articles/s41598-017-01915-w

733 31. Ishikawa K, Kamohara Y, Tanaka F, Haraguchi N, Mimori K, Inoue H, Mori M. Mitotic centromere-
734   associated kinesin is a novel marker for prognosis and lymph node metastasis in colorectal cancer.
735   Br J Cancer 2008; 98:1824–9.

736 32. Yao X, Abrieu A, Zheng Y, Sullivan KF, Cleveland DW. CENP-E forms a link between attachment of
737   spindle microtubules to kinetochores and the mitotic checkpoint. Nat Cell Biol 2000; 2:484–91.

738 33. Hashizume R, Smirnov I, Liu S, Phillips JJ, Hyer J, McKnight TR, Wendland M, Prados M, Banerjee A,
739   Nicolaides T, et al. Characterization of a diffuse intrinsic pontine glioma cell line: implications for
740   future investigations and treatment. J Neurooncol 2012; 110:305–13.

741 34. Nagaraja S, Vitanza NA, Woo P, Taylor KR, Liu F, Zhang L, Li M, Meng W, Ponnuswami A, Sun W, et
742   al. Transcriptional Dependencies in Diffuse Intrinsic Pontine Glioma. Cancer Cell 2017; 31:635-
743   652.e6.

744 35. Zhang L, Peterson TE, Lu VM, Parney IF, Daniels DJ. Antitumor activity of novel pyrazole-based
745   small molecular inhibitors of the STAT3 pathway in patient derived high grade glioma cells. PLoS
746   ONE [Internet] 2019 [cited 2019 Sep 23]; 14. Available from:
747   https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6667205/

748 36. Chan K-M, Fang D, Gan H, Hashizume R, Yu C, Schroeder M, Gupta N, Mueller S, James CD, Jenkins
749   R, et al. The histone H3.3K27M mutation in pediatric glioma reprograms H3K27 methylation and
750   gene expression. Genes Dev 2013; 27:985–90.

751 37. Mueller S, Hashizume R, Yang X, Kolkowitz I, Olow AK, Phillips J, Smirnov I, Tom MW, Prados MD,
752   James CD, et al. Targeting Wee1 for the treatment of pediatric high-grade gliomas. Neuro-Oncol
753   2014; 16:352–60.

754   38.   Mauvezin C, Nagy P, Juhász G, Neufeld TP. Autophagosome–lysosome fusion is independent of V-
755   ATPase-mediated acidification. Nat Commun 2015; 6:7007.

756   39.   Kim OH, Lim JH, Woo KJ, Kim Y-H, Jin I-N, Han ST, Park J-W, Kwon TK. Influence of p53 and
757   p21Waf1 expression on G2/M phase arrest of colorectal carcinoma HCT116 cells to proteasome
758   inhibitors. Int J Oncol 2004; 24:935–41.

759   40.   Huszar D, Theoclitou M-E, Skolnik J, Herbst R. Kinesin motor proteins as targets for cancer therapy.
760   Cancer Metastasis Rev 2009; 28:197–208.

761   41.   Pontén J, Saksela E. Two established in vitro cell lines from human mesenchymal tumours. Int J
762   Cancer 1967; 2:434–47.

763   42.   Fabregat A, Jupe S, Matthews L, Sidiropoulos K, Gillespie M, Garapati P, Haw R, Jassal B, Korninger
764   F, May B, et al. The Reactome Pathway Knowledgebase. Nucleic Acids Res 2018; 46:D649–55.

765   43.   Stein LD. Using the Reactome Database. Curr Protoc Bioinforma 2004; 7:8.7.1-8.7.16.

766   44.   Shintomi K, Hirano T. Releasing cohesin from chromosome arms in early mitosis: opposing actions
767   of Wapl-Pds5 and Sgo1. Genes Dev 2009; 23:2224–36.

768   45.   Pohl C, Jentsch S. Midbody ring disposal by autophagy is a post-abscission event of cytokinesis. Nat
769   Cell Biol 2009; 11:65–70.

770   46.   Akutsu M, Dikic I, Bremm A. Ubiquitin chain diversity at a glance. J Cell Sci 2016; 129:875–80.

771   47.   Rogov V, Dötsch V, Johansen T, Kirkin V. Interactions between Autophagy Receptors and Ubiquitin-
772   like Proteins Form the Molecular Basis for Selective Autophagy. Mol Cell 2014; 53:167–78.

773   48.   Hewitt G, Carroll B, Sarallah R, Correia-Melo C, Ogrodnik M, Nelson G, Otten EG, Manni D,
774   Antrobus R, Morgan BA, et al. SQSTM1/p62 mediates crosstalk between autophagy and the UPS in
775   DNA repair. Autophagy 2016; 12:1917–30.

776   49.   Nam T, Han JH, Devkota S, Lee H-W. Emerging Paradigm of Crosstalk between Autophagy and the
777   Ubiquitin-Proteasome System. Mol Cells 2017; 40:897–905.

778   50.   Peters J-M, Nishiyama T. Sister Chromatid Cohesion. Cold Spring Harb Perspect Biol 2012;
779   4:a011130.

780   51.   Hassler M, Shaltiel IA, Haering CH. Towards a Unified Model of SMC Complex Function. Curr Biol
781   2018; 28:R1266–81.

782   52.   Haarhuis JHI, Elbatsh AMO, van den Broek B, Camps D, Erkan H, Jalink K, Medema RH, Rowland BD.
783   WAPL-mediated removal of cohesin protects against segregation errors and aneuploidy. Curr Biol
784   CB 2013; 23:2071–7.

785   53.   Losada A, Hirano M, Hirano T. Cohesin release is required for sister chromatid resolution, but not
786   for condensin-mediated compaction, at the onset of mitosis. Genes Dev 2002; 16:3004–16.

787   54.   Hara K, Zheng G, Qu Q, Liu H, Ouyang Z, Chen Z, Tomchick DR, Yu H. Structure of cohesin
788   subcomplex pinpoints direct shugoshin–Wapl antagonism in centromeric cohesion. Nat Struct Mol
789   Biol 2014; 21:864–70.

790   55.   Nishiyama T, Ladurner R, Schmitz J, Kreidl E, Schleiffer A, Bhaskara V, Bando M, Shirahige K, Hyman
791   AA, Mechtler K, et al. Sororin Mediates Sister Chromatid Cohesion by Antagonizing Wapl. Cell
792   2010; 143:737–49.

793   56.   Nakajima M, Kumada K, Hatakeyama K, Noda T, Peters J-M, Hirota T. The complete removal of
794   cohesin from chromosome arms depends on separase. J Cell Sci 2007; 120:4188–96.

795   57.   Misulovin Z, Pherson M, Gause M, Dorsett D. Brca2, Pds5 and Wapl differentially control cohesin
796   chromosome association and function. PLoS Genet 2018; 14:e1007225.

797   58.   Oikawa K, Ohbayashi T, Kiyono T, Nishi H, Isaka K, Umezawa A, Kuroda M, Mukai K. Expression of a
798   novel human gene, human wings apart-like (hWAPL), is associated with cervical carcinogenesis and
799   tumor progression. Cancer Res 2004; 64:3545–9.

800   59.   Losada A. Functional contribution of Pds5 to cohesin-mediated cohesion in human cells and
801   Xenopus egg extracts. J Cell Sci 2005; 118:2133–41.

802   60.   Ohbayashi T, Oikawa K, Yamada K, Nishida-Umehara C, Matsuda Y, Satoh H, Mukai H, Mukai K,
803   Kuroda M. Unscheduled overexpression of human WAPL promotes chromosomal instability.
804   Biochem Biophys Res Commun 2007; 356:699–704.

805   61.   Chude CI, Amaravadi RK. Targeting Autophagy in Cancer: Update on Clinical Trials and Novel
806   Inhibitors. Int J Mol Sci [Internet] 2017 [cited 2018 Sep 16]; 18. Available from:
807   https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5486101/

808   62.   Jiang P, Mizushima N. Autophagy and human diseases. Cell Res 2014; 24:69–79.

809   63.   Simonetti G, Bruno S, Padella A, Tenti E, Martinelli G. Aneuploidy: Cancer strength or vulnerability?
810   Int J Cancer 2018;

811   64.   Vessoni AT, Filippi-Chiela EC, Menck CF, Lenz G. Autophagy and genomic integrity. Cell Death Differ
812   2013; 20:1444–54.

813   65.   White E, DiPaola RS. The Double-edged Sword of Autophagy Modulation in Cancer. Clin Cancer Res
814   Off J Am Assoc Cancer Res 2009; 15:5308–16.

815   66.   Crasta K, Ganem NJ, Dagher R, Lantermann AB, Ivanova EV, Pan Y, Nezi L, Protopopov A,
816   Chowdhury D, Pellman D. DNA breaks and chromosome pulverization from errors in mitosis.
817   Nature 2012; 482:53–8.

818   67.   Rello-Varona S, Lissa D, Shen S, Niso-Santano M, Senovilla L, Mariño G, Vitale I, Jemaá M, Harper F,
819   Pierron G, et al. Autophagic removal of micronuclei. Cell Cycle Georget Tex 2012; 11:170–6.

820   68.   Bartsch K, Knittler K, Borowski C, Rudnik S, Damme M, Aden K, Spehlmann ME, Frey N, Saftig P,
821   Chalaris A, et al. Absence of RNase H2 triggers generation of immunogenic micronuclei removed by
822   autophagy. Hum Mol Genet 2017; 26:3960–72.

823   69.   Hatch EM, Fischer AH, Deerinck TJ, Hetzer MW. Catastrophic Nuclear Envelope Collapse in Cancer
824   Cell Micronuclei. Cell 2013; 154:47–60.

825   70.   Hintzsche H, Hemmann U, Poth A, Utesch D, Lott J, Stopper H. Fate of micronuclei and
826   micronucleated cells. Mutat Res Mutat Res 2017; 771:85–98.

827   71.   Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, Adachi H, Adams
828   CM, Adams PD, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring
829   autophagy (3rd edition). Autophagy 2016; 12:1–222.

830   72.   Chung JY-M, Steen JA, Schwarz TL. Phosphorylation-Induced Motor Shedding is Required at Mitosis
831   for Proper Distribution and Passive Inheritance of Mitochondria. Cell Rep 2016; 16:2142–55.

832   **FIGURE LEGENDS**

833   **Figure 1**. **Autophagic flux and lysosome-dependent degradation are active in dividing cells**.

834   (**A**) Representative single focal plan (1Z) confocal images of U2OS cells undergoing mitosis

835   labelled with lysosomal marker LAMP2 (green). Interphase cell and distinct mitotic subphases are

836   detectable with DNA staining with DAPI (blue). Scale bar, 10 µm. (**B**) Quantification of lysosome

837   number per cell in interphase compared to each mitotic subphases. Error bars represent S.D. of

838   ≥ 10 images. (**C**) Quantification of lysosome average size per cell in interphase and for each

839   mitotic subphases. Error bars represent S.D. of ≥ 10 images. (**D**) Upper panel: Analysis of the

840   distribution of lysosomes (green) and DNA (blue) in interphase and for each mitotic subphases

841   using Radial Profile Angle ImageJ plugin. A Radius of 20 µm was maintained constant for the

842   analysis. Lower panel: representative image of a cell in each analyzed phase. (**E**) Representative

843   single focal plan confocal images of U2OS H2B-GFP stable cells in interphase or undergoing

844   mitosis were stained with Lysotracker (green – arbitrary color) for lysosomes detection, Magic

845   Red (red) for active cathepsin B, H2B-GFP (blue – arbitrary color) for DNA staining. Scale bar, 10

846   µm. (**F**) Schematic representation of the synchronization protocol established for U2OS cells. (**G**)

847   Cell cycle analysis of cells at T0 (before release of the reversible CDK1 inhibitor – RO3306) and

848 of cells after release from RO3306 incubated either with normal growing media (CTRL) or with 10

849 nM ConcA-containing growing media. Percentage of cells in G1, S and G2/M cell cycle phases

850 are represented under the experimental conditions. (**H**) Analysis of autophagic flux by Western

851 Blot detection with specific antibodies of autophagic proteins p62 and LC3 in cell fractions of panel

852 G synchronized as in panel F. β-actin protein level was used as loading control. (**I**) Representative

853 maximal projection (z ≥ 15) of confocal images of U2OS cells in interphase or cells undergoing

854 mitosis. Endogenous autophagic proteins LC3 (red) and p62 (green) were detected by

855 immunofluorescence and DNA was marked with DAPI (blue). Pearson's correlation coefficient (r)

856 was calculated for each analyzed condition (n ≥ 10). Scale bar, 10 µm. (**J**) TEM images show

857 mitotic cells undergoing mitotic exit. Arrowheads indicate autophagic vesicles and lysosomes and

858 yellow asterisks point out mitochondria. Scale bars, as indicated. **Panels B** and **C**, statistical

859 significance between interphase and mitotic subphases is represented as: * $p < 0.05$, ** $p < 0.005$,

860 *** $p < 0.001$

861 **Figure 2**. **Lysosome impairment delays mitotic progression and leads to mitotic errors and**

862 **an atypical nuclear phenotype.** (**A**-**B**) U2OS cells were either treated with ConcA 10 nM for 24

863 hours (**A**) or transiently transfected with silencing RNA against KIF5B (siKIF5B) (**B**). Single plan

864 confocal images show LAMP2-positive lysosomes (green) detected by immunofluorescence in

865 cells undergoing mitosis. DNA is labelled with DAPI (blue). Scale bar, 10 µm. (**C**-**D**) Comparison

866 of the number of lysosomes per cell (**C**) or the average lysosome size (**D**) in interphase and each

867 mitotic subphases in control, ConcA-treated or KIF5B-depleted cells. Line represents the mean

868 in each condition. Total images analyzed ≥ 10 per condition. (**E**) Intracellular distribution of

869 lysosomes in interphase and in each mitotic subphases in control and KIF5B-depleted cells. Total

870 images analyzed ≥ 10 per condition. (**F**) H2B-GFP U2OS cells were subjected to time-lapse

871 imaging for 24 hours every 6 minutes 33 seconds. Single focal plan of representative images of

872 control cells, cells treated with ConcA 10 nM or depleted for KIF5B (siKIF5B) for 48 hours

873   undergoing mitosis are shown. H2B-GFP is depicted in grayscale (arbitrary color). Scale bars, 5

874   µm. (**G**) Quantification of mitotic timing of the indicated experimental conditions was performed

875   from prophase (chromosomes condensation) to telophase (chromosomes decondensation). Error

876   bars represent 5-95 percentiles of mitosis ≥ 90. (**H**) Representative images of misaligned

877   metaphase plate and chromosome bridges. Scale bar, 5 µm. (**I-J**) U2OS cells were synchronized

878   at late G2 phase and released with or without ConcA for 1 hour (**I**) or 3 hours (**J**). DAPI staining

879   was used for DNA detection. Chromosome misalignment (**I**) and chromosomal bridges (**J**) were

880   quantified compared to total number of metaphases and anaphases, respectively. Error bars

881   represent S.D. of n=3 independent experiments (>200 cells). (**K-L**) U2OS cells depleted for KIF5B

882   (siKIF5B) or transfected with siRNA control (siNT) were synchronized and released for 1 hour (**K**)

883   or 3 hours (**L**) to determine the percentage of chromosome misalignment or chromosome bridges

884   relative to the total number of cells undergoing metaphase or anaphase, respectively. Error bars

885   represent S.D. of n=3 experiments (>150 cells). In **Panels G** and **I-L**, statistical significance is

886   represented as: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

887   **Figure 3. Toroidal nucleus is a novel biomarker for chromosomal instability in interphase**

888   **cells.** (**A**) Representative image of toroidal nucleus. U2OS cells were fixed and stained with DAPI

889   for DNA detection. Scale bar, 5 µm. (**B**) Live imaging of H2B-GFP U2OS cells undergoing cell

890   cycle for 16 hours every 93 seconds. Mosaic of single focal plan images indicate aberrant nucleus

891   formation upon cell division. Scale bar, 10 µm. (**C-E**) U2OS cells were fixed and

892   immunofluorescence was performed using nucleolin antibody for nucleolus detection (**C**), Lamin

893   B1 antibody for nuclear envelope staining (**D**) or LAMP2 antibody to mark lysosomes (**E**) and

894   DAPI for DNA labelling. Scale bar, 5 µm. (**F**) 3D reconstruction using IMARIS software was

895   performed from confocal images stack of toroidal nucleus (z every 0.1 µm for 11 µm). Blue

896   corresponds to DAPI-stained nucleus and yellow dots mark LAMP2-positive lysosomes. (**G**)

897   U2OS cells were treated with ConcA for 24 hours, fixed and prepared for TEM. Images show

898   toroidal nucleus surrounded by the nuclear envelope. Abbreviations used: NE: nuclear envelop,

899   N: nucleus, Cyt: cytosol, AV: autophagic vesicles. Scale bars, as indicated. (**H**) Analysis of live

900   imaging experiment by following cell division of H2B-GFP U2OS cells for 24 hours every 9

901   minutes. Mitotic cells were detected and visualized to quantify the number of those with detectable

902   mitotic errors (misaligned chromosomes or chromosomal bridges) and those generating toroidal

903   nucleus. Linear regression was analyzed and the corresponding Pearson coefficient (r) was

904   calculated. (**I**) Analysis of the percentage of toroidal nucleus formed upon non-apparent mitotic

905   error or as a consequence of mitotic errors. Error bars represent S.D. of 295 mitosis. (**J**) U2OS

906   cells were depleted for KIF11 or MCAK for 48 hours using silencing RNA or treated with Monastrol

907   100 μM or Nocodazole 1 μM for 24 hours. Quantification of toroidal nuclei formed under these

908   conditions was performed by detection of DAPI-stained nuclei. Error bars represent S.D. of n > 3

909   experiments (10 fields / experiment). (**K**) Table summarizing a screen for toroidal nuclei through

910   various cell lines. Fold-increase was calculated upon ConcA treatment (10 nm for 24 h). (**L**)

911   Representative images of toroidal nuclei in screened cell lines from panel K. Panels **I** and **J**

912   statistical significance is represented as: * p < 0.05, ** p < 0.005, ***p < 0.001.

913   **Figure 4**. **Toroidal nucleus frequency increases upon impairment of lysosome trafficking**

914   **and acidification capacity**. (**A**) U2OS cells were treated with ConcA (10 nM) for 24 hours or

915   depleted for KIF5B for 48 hours using silencing RNA. Error bars represent S.D. of n > 5

916   experiments. (**B**) Representative confocal single plans showing morphology and distribution of

917   endogenous lysosomes (LAMP2 in green) under experimental conditions of panel A. Nuclei were

918   labelled with DAPi in blue. Scale bar, 10 μm. (**C**) U2OS cells were treated with Chloroquine CQ

919   (10 μM) or depleted for the V0c subunit of the v-ATPase (siATP6V0c) for 48 hours. Error bars

920   represent S.D. of n > 3 experiments. (**D**) Representative single plan confocal images of U2OS

921   cells treated as in panel **C**. Lysosomes were detected with LAMP2 antibody and nuclei were

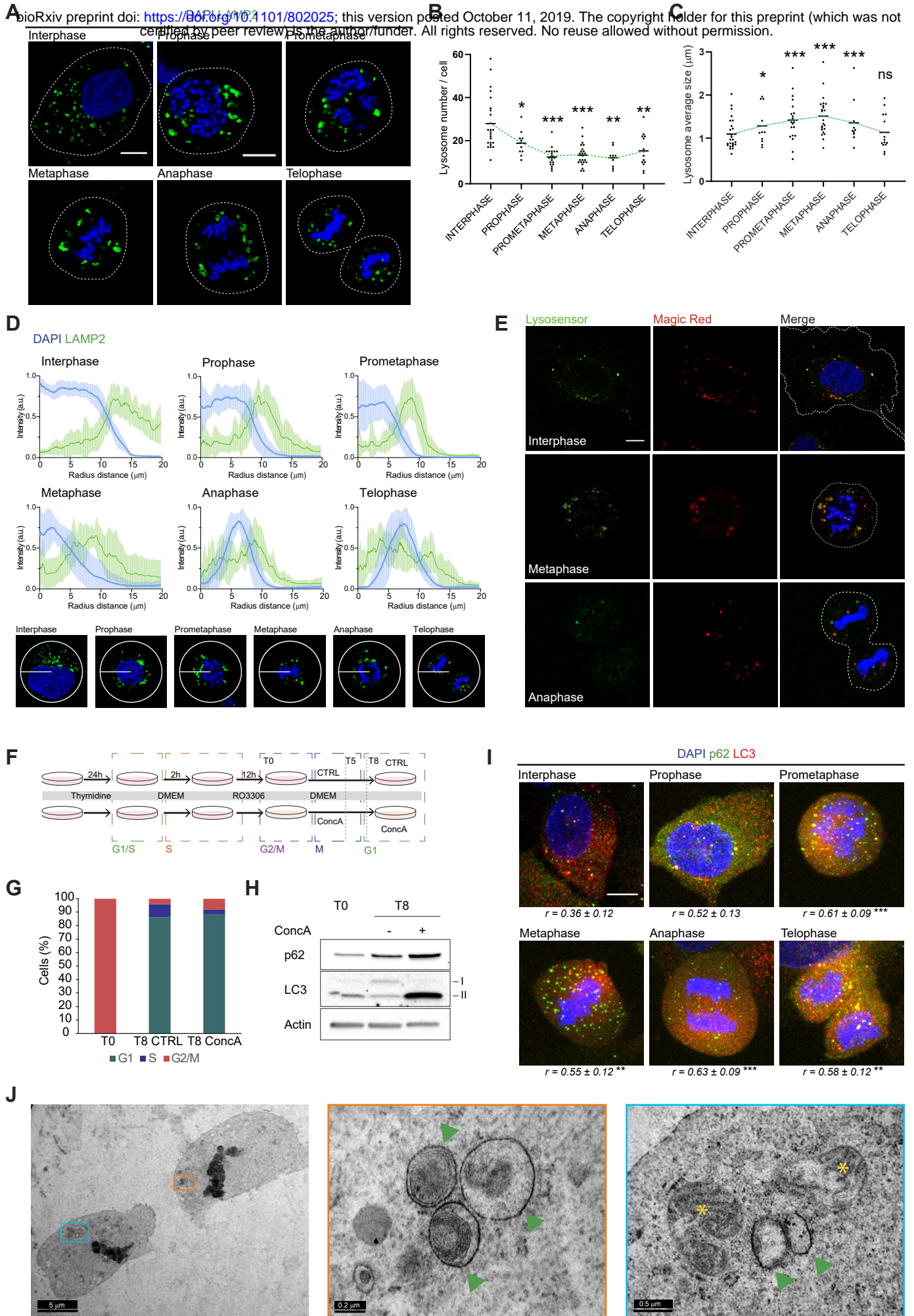922   stained with DAPI. Scale bar, 10 μm. (**E**) U2OS cells were depleted for BORC-associated proteins

923   SKIP or Arl8b using silencing RNA for 48 hours. Error bars represent S.D. of n > 3 experiments.

924   (**F**) Representative images of LAMP2-positive lysosomes (green) under the indicated conditions

925   (panel E). Nuclei are stained with DAPI (blue). Scale bar, 10 µm. (**G**) U2OS cells were transfected

926   with KIF5B silencing RNA and/or with KIF5A-GFP overexpression plasmid. Error bars represent

927   S.D. of n > 3 experiments. (**H**) U2OS cells were depleted for KIF5B or SKIP and the next day

928   treated or not with ConcA for 24 hours. Error bars represent S.D of n > 3 experiments. In panels

929   **A**, **C, E** and **G-H** nuclei were stained with DAPI for detection of toroidal nuclei compared to total

930   number of cells. Statistical significance is represented as: * p < 0.05, ** p < 0.005, ***p < 0.001,

931   ns: p > 0.05.

932   **Figure 5**. **Alterations of macroautophagy increase mitotic errors and toroidal nucleus**

933   **formation**. (**A**) U2OS cells were depleted for Atg5 for 48 hours and treated or not with ConcA for

934   24 hours. Error bars represent S.D. of n > 3 experiments. (**B-C**) Quantification of mitotic errors

935   (chromosome misalignment and chromosome bridges, respectively) in U2OS cells control (siNT)

936   or depleted for Atg5 (siAtg5) for 48 hours under normal growing condition (CTRL) or treated with

937   ConcA (10 nM for 24 h). Error bars represent S.D. of n=4 experiments (>300 cells). (**D**) Validation

938   of Atg5 depletion and ConcA efficacy in MEFs Atg5 +/+and -/- by Western Blot. β-actin was used

939   as loading control. (**E**) MEF Atg5 +/+ and Atg5 -/- were subjected or not to ConcA treatment for

940   24 hours. DNA was labelled with DAPI and frequency of toroidal nuclei was quantified. Error bars

941   represent S.D. of n=3 experiments. (**F**) Representative image of toroidal nucleus in MEF Atg5 KO

942   cells. Scale bar, 5 µm. (**G-H**) Quantification of mitotic errors (chromosome misalignment and

943   chromosome bridges, respectively) in MEF Atg5 +/+ and Atg5 -/-. Error bars represent S.D. of

944   n=5 experiments (>100 cells). Panels **A**-**C**, **E** and **G**-**H**, * represents the statistical significance of

945   ConcA inhibitory effect compared to control, while # represents the effects of Atg5 genetic

946   depletion. * p < 0.05, ** p < 0.005, *** p < 0.001, ns: p > 0.05, # p < 0.05 and ## p < 0.005.

38

947 **Figure 6**. **Identification of novel mitotic lysosomal substrates**. (**A**) Venn diagram representing

948 proteins detected by mass spectrometry of synchronized U2OS cells released in growing media

949 (CTRL) or ConcA-containing media. (**B**) Volcano plot depicting significant fold changes (FC) ($\log_2$)

950 in protein abundances in cells treated with 10 nM ConcA. Three independent experiments were

951 analyzed per condition. Dots denote the 1749 unique proteins detected in pre-filtered samples.

952 Threshold at q-value < 0.05 depicts peptides significantly modified under experimental conditions.

953 Blue dots represent peptides with FC < 0.8. Orange dots correspond to peptides presenting a FC

954 > 1.2. Within these candidates, red dots indicate target proteins WAPL, p62 and PDS5B. (**C**)

955 Table summarizing the 20 candidates identified by mass spectrometry that are clustered in the

956 Gene Ontology (GO) group GO:1903047 of mitotic cell cycle progress. (**D**) Western Blot of

957 synchronized U2OS mitotic cell fractions after mitotic cell enrichment by shake-off. Protein levels

958 of WAPL, PDS5B, p62 and LC3 were detected. β actin was used as loading control. (**E**)

959 Quantification of WAPL, PDS5B, p62 and LC3-II protein levels in experimental conditions as in

960 panel D and normalized to β actin protein level. Error bars represent S.D. of n=3 experiments. (**F**)

961 MEFs Atg5 +/+ and -/- under normal growing conditions were subjected to shake-Off. Mitotic

962 enriched cell fractions were then analyzed by Western-Blot and accumulation of WAPL and

963 PDS5B was detected in MEF Atg5 -/-. β-actin was used as loading control. (**G**) Quantification of

964 WAPL and PDS5B protein levels from panel F normalized to β-actin loading control. Error bars

965 represent S.D. of n=3 experiments. (**H**) Immunoprecipitation of exogenous WAPL-GFP in U2OS

966 cells treated or not with 10 nM ConcA for 24 hours. Direct interaction between WAPL and p62

967 was detected by Western-Blot. Cells expressing exogenous GFP tag were used as negative

968 control. Statistical significance is represented as: * $p < 0.05$, ** $p < 0.005$, ***$p < 0.001$, ns: p >

969 0.05.

970 **Figure 7. Impairment of lysosomes in mitotic cells leads to chromosomal instability**.

971 Lysosomal function is required for the maintenance of correct cell division and faithful

39

972   transmission of genetic material in the two daughter cells. Functional lysosomes degrade

973   essential mitotic factors necessary for appropriate chromosome segregation. On the contrary,

974   when lysosomes are dysfunctional either by impairment of their trafficking or acidification capacity,

975   mitotic cells are prompt to accumulate mitotic errors, to generate toroidal nucleus and mitosis is

976   delayed. These alterations are key features of chromosomal instability.

A DAPI LAMP2

Interphase, Prophase, Prometaphase, Metaphase, Anaphase, Telophase

B Lysosome number / cell — INTERPHASE, PROPHASE *, PROMETAPHASE ***, METAPHASE ***, ANAPHASE **, TELOPHASE **

C Lysosome average size (µm) — INTERPHASE, PROPHASE *, PROMETAPHASE ***, METAPHASE ***, ANAPHASE ***, TELOPHASE ns

D DAPI LAMP2

Interphase, Prophase, Prometaphase, Metaphase, Anaphase, Telophase
(Intensity (a.u.) vs Radius distance (µm))

E Lysosensor / Magic Red / Merge — Interphase, Metaphase, Anaphase

F Thymidine → DMEM → RO3306 → DMEM → T0, T5, T8, CTRL / ConcA
G1/S, S, G2/M, M, G1

G Cells (%) — T0, T8 CTRL, T8 ConcA — G1, S, G2/M

H T0, T8 — ConcA − +
p62, LC3 (−I, −II), Actin

I DAPI p62 LC3
Interphase r = 0.36 ± 0.12
Prophase r = 0.52 ± 0.13
Prometaphase r = 0.61 ± 0.09 ***
Metaphase r = 0.55 ± 0.12 **
Anaphase r = 0.63 ± 0.09 ***
Telophase r = 0.58 ± 0.12 **

J (scale bars 5 µm, 0.2 µm, 0.5 µm)

A

B INTERPHASE PROPHASE PROMETAPHASE METAPHASE

00:00 12:00 24:00 36:00 48:00

ANAPHASE TELOPHASE CYTOKINESIS

60:00 72.00 84.00 96.00 108.00

C DAPI Nucleolin Merge

D DAPI Lamin B1 Merge

E DAPI LAMP2 Merge

F 3D reconstruction

G (N, Cyt, NE labels; scale bars 2 μm, 0.5 μm, 0.2 μm; AV labels)

H r = 0.855 (Toroidal nucleus formation vs Mitotic error detection)

I % cells — Toroid from mitotic error, Toroid from normal mitosis (***)

J Toroidal nucleus (%) — siNT, siKIF11 (***), CTRL, Monastrol (***)

K

Source	Cell Type	Name	Toroidal Nuclei	Fold-increase upon ConcA
Human	Osteosarcoma	U2OS	YES	3.16
Human	Cervix epitheloid carcinoma	HeLa	YES	3.57
Human	Lung carcinoma	A549	YES	8.38
Human	Colorectal carcinoma	HCT116	NO	
Human	Hepato cellular carcinoma	HuH7	NO	
Human	Breast adenocarcinoma	MCF7	NO	
Human	Colon carcinoma	RKO	NO	
Human	Colorectal adenocarcinoma	LOVO	NO	
Human	Pediatric high-grade glioma	MC-PED17	NO	
Human	Pediatric diffuse intrinsic pontine glioma	SF-7761	YES	2.32
Human	Pediatric diffuse intrinsic pontine glioma	SF-8628	YES	ND
Human	Pediatric diffuse intrinsic pontine glioma	SU-DIPG-XVII	YES	1.52
Human	Skin fibroblast	HFF-1	YES	2.19
Human	Normal Kidney epithelium	HEK293-T	NO	
Mouse	Embryonic fibroblasts	MEF	YES	5.11

L U2OS HeLa A549 SF-7762

SF-8628 SUDIPG17 HFF MEF

A

CTRL 13 / 1749 / ConcA 141

B

Volcano plot: -log(p-value) vs -log2 (FC ConcA/CTRL); labeled points WAPL, p62, PDS5B; q value = 0.05; FC = 0.8; FC = 1.2

C GO:1903047

IDs	Protein names	Gene names	Fold-increase
Q00534	Cyclin-dependent kinase 6	CDK6	7.857
Q9NTI5	Sister chromatid cohesion protein PDS5 homolog B	PDS5B	4.780
Q15018	BRISC complex subunit Abro1	FAM175B	2.090
O00233	26S proteasome non-ATPase regulatory subunit 9	PSMD9	1.911
P28070	Proteasome subunit beta type-4	PSMB4	1.831
Q7Z5K2	Wings apart-like protein homolog	WAPAL	1.645
P50570	Dynamin-2	DNM2	1.644
P19784	Casein kinase II subunit alpha	CSNK2A2	1.547
P78406	mRNA export factor	RAE1	1.457
P62979	Ubiquitin-40S ribosomal protein S27a;Ubiquitin;40S ribosomal protein S27	RPS27A	1.450
O43823	A-kinase anchor protein 8	AKAP8	1.408
Q08209	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	PPP3CA	1.372
P18858	DNA ligase 1	LIG1	1.333
P83731	60S ribosomal protein L24	RPL24	1.321
Q9Y2Z0	Suppressor of G2 allele of SKP1 homolog	SUGT1	1.307
Q8NFH3	Nucleoporin Nup43	NUP43	1.297
P06730	Eukaryotic translation initiation factor 4E	EIF4E	1.264
Q99661	Kinesin-like protein KIF2C	KIF2C	1.233
Q13409	Cytoplasmic dynein 1 intermediate chain 2	DYNC1I2	1.225
O14818	Proteasome subunit alpha type-7	PSMA7	1.224

D U2OS Mitotic cells — ConcA (-/+): PDS5B, WAPL, p62, LC3 (I, II), Actin

E Relative protein level; ConcA (-/+): WAPL ***, PDS5B *; p62 *, LC3-II **

F MEFs Mitotic cells — Atg5 +/+ and -/-: PDS5B, WAPL, Actin

G Relative protein levels; Atg5 +/+ and Atg5 -/-: WAPL *, PDS5B *

H INPUTS / IP; GFP, WAPL-GFP, ConcA; PDS5B, WAPL-GFP, p62, GFP

FUNCTIONAL LYSOSOMES

IMPAIRED LYSOSOMES

MITOSIS

INTERPHASE

DELAY

Lysosomal substrates

Mitotic factors
PDS5B
WAPL

Degradation

MITOTIC ERRORS

Lagging chromosome

Chromosomal bridge

TOROIDAL NUCLEUS

FAITHFUL GENETIC TRANSMISSION

CHROMOSOMAL INSTABILITY

Functional lysosome
Autophagic vesicle
Impaired lysosome
MTOC / Centrosome
Chromosomes