1   **Genomic instability caused by Arp2/3 complex inactivation results in**

2   **micronucleus biogenesis and cellular senescence**

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4   Running Title: Arp2/3 complex inactivation and senescence

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Abstract

The Arp2/3 complex is a ubiquitous actin nucleator with well-characterized activities in cell organization and movement, but its roles in chromatin-associated and cell cycle-related processes are relatively understudied. We investigated how the Arp2/3 complex affects genomic integrity, mitosis, and cell proliferation using mouse fibroblasts containing an inducible knockout (iKO) of the ArpC2 subunit. We show that permanent Arp2/3 ablation results in DNA damage, the formation of cytosolic micronuclei, and cellular senescence. Upon Arp2/3 depletion, cells undergo an abrupt proliferation arrest that is accompanied by activation of the tumor suppressor p53, upregulation of its downstream cell cycle inhibitor *Cdkn1a*/p21, and recognition of micronuclei by the cytosolic DNA sensor cGAS. Micronuclei arise in ArpC2 iKO cells due to chromosome segregation defects during mitosis and premature mitotic exits. Such phenotypes are explained by the presence of damaged chromatin fragments that fail to attach to the mitotic spindle, abnormalities in actin assembly during metaphase, and asymmetric microtubule architecture during anaphase. These studies establish functional requirements for the mammalian Arp2/3 complex in genome stability and mitotic spindle organization. They further expand our understanding of the intracellular mechanisms that lead to senescence and suggest that cytoskeletal dysfunction is an underlying factor in biological aging.

Author Summary

The actin cytoskeleton consists of protein polymers that assemble and disassemble to control the organization, shape, and movement of cells. However, relatively little is understood about how the actin cytoskeleton affects genome maintenance, cell multiplication, and biological aging. In this study, we show that knocking out the Arp2/3 complex, a core component of the actin assembly machinery, causes DNA damage, genomic instability, defects in chromosome partitioning during mitosis, and a permanent cell proliferation arrest called senescence. Since senescent cells are major contributors to both age-associated diseases and tumor suppression, our findings open new avenues of investigation into how natural or experimental alterations of cytoskeletal proteins impact the process of aging and the regulation of cancer.

Introduction

44

45   The actin cytoskeleton consists of dynamic protein polymers that have well-known functions in

46   cell morphogenesis and motility. Globular (G-) actin monomers are present in the cytoplasm

47   and nucleus, and their polymerization into filamentous (F-) actin is driven by proteins called

48   nucleators [1]. These include actin monomer-oligomerizing proteins, Formin-family

49   nucleation/elongation proteins, and the Arp2/3 complex – a heteroheptameric actin assembly

50   factor that binds to the sides of existing filaments and nucleates new filaments to create

51   branched networks [2]. The Arp2/3 complex is highly conserved across almost all eukaryotes

52   [3,4] and is required for viability in such organisms; inactivation of genes encoding its subunits

53   prevents growth of *S.cerevisiae* [5,6] and *D.discoideum* [7] and is embryonic lethal in animals

54   including *D.melanogaster* [8,9], *C.elegans* [10,11], and *M.musculus* [12-14]. However, the

55   cellular basis underlying the essential nature of the Arp2/3 complex is not well understood.

56   Many processes that involve plasma membrane dynamics, especially cell adhesion and

57   motility, rely on actin networks assembled by the Arp2/3 complex [15]. In fact, conditional

58   knockouts in mice indicate that the complex is crucial for maintaining normal tissue architecture,

59   promoting changes in cell shape, and powering cell migration during development [14,16-20].

60   These *in vivo* results are consistent with molecular and cellular studies of Arp2/3-mediated actin

61   assembly using *in vitro* systems [21], dominant negative regulatory proteins [22], transient

62   RNAi-mediated knockdowns [23], and pharmacological inhibitors of the complex [24,25].

63   In contrast to the well-characterized roles of the Arp2/3 complex in protrusion and

64   motility, its functions in nuclear processes are only beginning to emerge. During interphase, all

65   3 classes of actin nucleators promote nuclear actin filament assembly in response to acute DNA

66   damaging agents [26-28]. In the nuclei of *Drosophila* and mammalian cells, Arp2/3-mediated

67   actin polymerization is crucial for repositioning damaged heterochromatin to the nuclear

68   periphery, which enables subsequent DNA repair activities [27,28]. As a result, depletion of the

69　Arp2/3 complex using RNAi in *Drosophila* larvae leads to chromosomal abnormalities and

70　genomic instability [27]. Depletion of the Arp2/3 complex in human cells additionally results in

71　defects in DNA damage-induced apoptosis [29].

72　　　　Apart from their functions in chromatin-associated processes during interphase, actin

73　and its nucleators, especially Formins and the Arp2/3 complex, are increasingly being found to

74　support proper chromosome movements during meiosis and mitosis. In starfish oocytes,

75　following nuclear envelope breakdown, several types of F-actin structures promote chromosome

76　transport and coordinate capture by microtubules [30,31]. Studies in mouse oocytes further

77　indicate that actin filaments permeate the meiotic microtubule spindles and facilitate proper

78　chromosome congression [32,33]. Chemical inhibition of actin dynamics or genetic inactivation

79　of Formin-2 prevents proper formation of kinetochore microtubules and leads to chromosome

80　alignment and segregation errors [33,34]. Similarly, during mitosis, several actin structures

81　have been shown to interact with and possibly guide microtubule spindle components. Actin

82　filaments that run between the microtubule spindle poles and F-actin fingers that project from

83　the cell cortex into the spindle have been identified in *Xenopus* epithelial cells [35].

84　Centrosomes, which serve as major microtubule nucleation and organizing centers, are also

85　sites of actin assembly [36]. The Arp2/3 complex localizes to centrosomes in multiple

86　mammalian cell types, and pharmacological inhibition of Arp2/3 results in decreased

87　centrosomal actin levels and impaired mitotic spindle formation [37-39]. Thus, disruption of

88　either actin or Arp2/3 function during meiosis or mitosis can lead to defects in chromosome

89　dynamics, highlighting the actin cytoskeleton as a key player in maintaining genomic integrity

90　during nuclear division.

91　　　　Although the effects of transient Arp2/3 depletion or inhibition on chromatin repair are

92　now evident, and several aberrations in chromosome movement have been characterized, the

93　impact of total and permanent Arp2/3 ablation on these processes has not been established.

94　The development of several cellular systems for studying long-term Arp2/3 depletion or deletion

95   has allowed more clear-cut assessments of the requirements for the Arp2/3 complex in a given

96   cellular process [14,40-43]. For example, these models have already provided fundamental

97   insights into the function of the Arp2/3 complex in cell migration. Studies using mouse

98   embryonic fibroblasts (MEFs) expressing shRNAs targeting the ArpC2 and Arp2 subunits [40],

99   embryonic stem cell-derived mouse fibroblasts lacking the ArpC3 subunit [14], and mouse

100   fibroblasts harboring a tamoxifen-inducible knockout of the Arp3 subunit [43], indicate that the

101   Arp2/3 complex is crucial for lamellipodia formation and directional migration. Additionally,

102   mouse fibroblasts containing a tamoxifen-inducible knockout of the ArpC2 subunit exhibit

103   impaired lamellipodia formation and reduced motility speeds [42].

104   To determine the outcome of Arp2/3 complex ablation on chromatin-associated

105   processes related to cell viability and multiplication, we employed the inducible ArpC2 knockout

106   cell model [42]. Our findings establish a key connection between Arp2/3 complex functions in

107   genomic integrity during interphase and mitosis in normal cells to the biogenesis of micronuclei

108   and induction of a cellular senescence pathway upon Arp2/3 inactivation.

6

Results

ArpC2 iKO cells undergo an abrupt proliferation arrest and morphological enlargement

Given that the Arp2/3 complex is required for viability in many eukaryotic organisms, we sought

to better understand its essential nature – apart from its well-recognized roles in adhesion and

motility – in mammalian cells. The Arp2/3 complex is composed of seven subunits: two Actin-

related proteins (Arp2 and Arp3) and five Complex subunits (ArpC1-C5), with ArpC2 and ArpC4

forming a structural core and multiple isoforms of Arp3, ArpC1, and ArpC5 providing peripheral

diversity [4,44]. Previous work indicates that MEFs subjected to RNAi-mediated depletion of the

ArpC2 and Arp2 subunits are viable and remain culturable when generated in a genetic

background lacking the *p16Ink4a/Arf* tumor suppressors [40]. More recently, to circumvent

problems with knockdown instability, a conditional knockout model was created using

p16Ink4a/Arf $^{-/-}$ mouse tail fibroblasts (MTFs) harboring a floxed *ArpC2* allele and engineered to

express the CreER recombinase upon treatment with 4-hydroxytamoxifen (4-OHT) [42]. Since

the latter system is inducible, causes a permanent loss of the critical ArpC2 subunit, and leads

to degradation of other members of the complex, we adopted this Arp2/3 complex null cellular

model for our studies. In all of our experiments, parental MTFs carrying the conditional *ArpC2*

allele were exposed to DMSO to maintain a control (Flox) cell population or to 4-OHT to

generate ArpC2 induced knockout (iKO) cells.

For initially assessing the kinetics of Arp2/3 complex depletion, DMSO- and 4-OHT-

treated cells were collected at various timepoints, lysed, and immunoblotted with antibodies to

the ArpC2 and Arp3 subunits plus antibodies to GAPDH and tubulin as loading controls (Fig

1A). After 1 day in 4-OHT, ArpC2 protein levels were reduced by nearly half compared to

DMSO-treated Flox cells (Fig 1A and 1B). By 2 days, ArpC2 expression was diminished by

approximately 80%, and a reduction in Arp3 levels became noticeable (Fig 1A and 1B). The

amounts of both subunits continued to steadily decline in the iKO cells over time until they were

134   absent following 5 days in 4-OHT (Fig 1A and 1B). DMSO and 4-OHT were removed from

135   culture media after 6 days, but ArpC2 and Arp3 remained undetectable in the iKO population

136   out to 13 days (Fig 1A and 1B). To independently confirm the loss of the Arp2/3 complex by

137   fluorescence microscopy, Flox and iKO cells were stained with an antibody to label Arp3 and

138   with fluorescent phalloidin to visualize F-actin. As expected, Flox cells exhibited prominent Arp3

139   staining within F-actin-rich peripheral membrane ruffles, whereas Arp3 staining and ruffles were

140   both missing from the iKO cells (Fig 1C). Collectively, these results demonstrate that in this

141   cellular context, the Arp2/3 complex knockout is rapid, complete, and stable over time.

142   To determine the impact of abolishing Arp2/3 complex expression on cell proliferation,

143   we next quantified cell titers on a daily basis following the addition of DMSO or 4-OHT. For the

144   first 3 days, Flox and iKO cells multiplied at identical rates, but by 4 days, the growth

145   characteristics of Flox and iKO cultures began to diverge, and at 5 days, the iKO samples were

146   proliferating at a clearly slower pace (Fig 1D). After approximately 10-12 days, virtually all iKO

147   cells stopped dividing (Fig 1D). To quantify the differences in cell multiplication rates, we

148   calculated the population doubling times in the 0-4 and 5-12 day time periods following DMSO

149   or 4-OHT exposure. While the doubling times were similar for Flox and iKO populations (18h

150   vs. 20h) during the first interval, the iKO doubling times quadrupled to >80h in the 5-12 day

151   range (Fig 1E). Moreover, cell counts in iKO samples remained unchanged for an additional

152   week (Fig 1D), except in instances where colonies of 4-OHT escapees or revertants re-

153   expressing the Arp2/3 complex emerged (not depicted). Consistent with a requirement for the

154   Arp2/3 complex in intrinsic apoptosis [29], apoptotic cell phenotypes were not observed in the

155   iKO cell population. Thus, following loss of the Arp2/3 complex, MTF cells undergo an abrupt

156   and stable proliferation arrest.

157   When examining the different growth characteristics of the Flox and iKO cultures, it also

158   became apparent that the two cell types had distinct morphologies and sizes. Fluorescent

159   phalloidin staining, in addition to revealing a lack of F-actin-rich ruffles (Fig 1C), demonstrated

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160   that iKO cells were flatter and larger than Flox cells (Fig 1F). Quantification of Flox and iKO cell

161   areas showed that the iKO cells were, on average, about twice as large as Flox cells (Fig 1G).

162   So in addition to losing their ability to multiply, Arp2/3-deficient cells display significant increases

163   in their size.

164

165   **ArpC2 iKO cells exhibit the canonical nuclear and cytoplasmic features of senescence**

166   A loss of proliferative capacity and an increase in adherent cell area are common characteristics

167   of senescent cells. Cellular senescence refers to a permanent state of replicative arrest [45,46],

168   and is reflected in several additional physiological changes, including increased production of

169   pro-inflammatory proteins, a response known as the senescence-associated secretory

170   phenotype, or SASP [47,48]. Notably, previous global gene expression profiling using the

171   ArpC2/Arp2-depleted MEF model revealed that several genes common to the SASP were up-

172   regulated [41], suggesting a link between the loss of Arp2/3 function and this aspect of cellular

173   senescence. To investigate whether the ArpC2 iKO cells also displayed this senescence

174   feature, we performed RT-PCR (Fig 2A) and RT-qPCR (Fig 2B) to compare transcript levels for

175   Interleukin-6 (Il-6), a pro-inflammatory cytokine consistently present in the SASPs derived from

176   senescent cells with diverse origins [49,50]. In agreement with findings from the ArpC2/Arp2

177   RNAi MEF studies, *Il-6* expression was greater in iKO cells than in Flox cells at 3, 6, and 9 days

178   after the onset of 4-OHT treatment (Fig 2A). RT-qPCR revealed that *Il-6* transcript levels were

179   nearly 4-fold higher in the iKO compared to Flox cells at 9 days (Fig 2B), indicating that a

180   permanent loss of the Arp2/3 complex induces the production of this key SASP component.

181   Senescence is also frequently associated with changes in nuclear structure, including

182   decreased levels of Lamin B1, a structural protein of the nuclear lamina [51]. To determine if

183   Lamin B1 abundance was altered by the deletion of ArpC2, we immunoblotted Flox and iKO

184   cells for Lamin B1 and found that Lamin B1 protein levels were indeed 3-fold lower in iKO cell

185   populations (Fig 2C and 2D). To confirm the reduction in Lamin B1 expression, Flox and iKO

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186   cells were also subjected to immunofluorescence microscopy. In Flox cells, nuclear Lamin B1

187   staining was consistently bright (Fig 2E and 2F). Contrastingly, in iKO cells, nuclear Lamin B1

188   levels were visibly lower (Fig 2E and 2F). In agreement with the immunoblotting data,

189   quantification of nuclear fluorescence intensities demonstrated that, on average, Lamin B1 was

190   nearly 3-fold less abundant in iKO cells (Fig 2F).

191   In addition to the production of a transcriptional SASP response and a decrease in their

192   nuclear Lamin B1 levels, senescent cells typically exhibit a cytoplasmic senescence-associated

193   β-galactosidase (SA-βgal) activity at pH 6 [52]. This is the most widely accepted biomarker of

194   senescence. SA-βgal staining in Flox and iKO cells from 0-22 days revealed that by 7 days,

195   and at later timepoints, the number of SA-βgal-positive cells was significantly higher in iKO than

196   Flox populations (Fig 3A and 3B). In many instances, SA-βgal activity is thought to reflect an

197   increase in lysosomal content [53,54]. So to assess lysosomal abundance, we treated Flox and

198   iKO cells with LysoTracker, a fluorescent probe that labels acidic intracellular structures, and

199   examined the cells microscopically. LysoTracker intensely stained discrete circular puncta

200   resembling lysosomes in Flox cells, but broadly stained large portions of the cytoplasm in iKO

201   cells (Fig 3C). Quantification of LysoTracker fluorescence at day 9 revealed that more than

202   80% of iKO cells versus 5% of Flox cells showed the more expansive staining pattern (Fig 3D).

203   Furthermore, >10% of the areas within iKO cells stained positive for LysoTracker compared to

204   <1% of the areas in Flox cells (Fig 3D). Thus, the arrest, morphological, and SASP

205   observations, when taken together with these Lamin B1, SA-βgal, and LysoTracker staining

206   results, establish that ArpC2 iKO cells undergo senescence.

207

208   **The formation of micronuclei and DNA damage clusters precedes iKO cell senescence**

209   Cellular senescence can be induced by a variety of stimuli, including DNA damage, oncogene

210   activation, telomere shortening, and mitochondrial dysfunction [55-57]. Micronuclei and

211   cytosolic chromatin fragments are common senescence-associated phenotypic traits and

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212   influence the senescent state [58-60]. During the course of our characterization of the

213   senescence parameters described above, we noticed that iKO cells frequently contained

214   micronuclei whereas Flox cells did not (Fig 1C; Fig 2E; Fig 3C). Following specific examinations

215   of samples containing DAPI-stained DNA, it became obvious that small cytosolic micronuclei

216   were often present in iKO cells (Fig 4A). Of iKO cells with micronuclei, most had one

217   micronucleus (Fig 4B; i, ii, iii), but some harbored 2 or 3 micronuclei (Fig 4B; iv, v, vi).

218   Micronuclei ranged in size and could be found completely detached (Fig 4B; i, ii, vi) or tethered

219   to the periphery (Fig 4B; iii, iv, v) of the main nucleus. We next quantified the timing and

220   frequency with which micronuclei formed in Flox and iKO cells. Surprisingly, after just one day

221   in 4-OHT, about 13% of iKO cells had micronuclei (Fig 4C). The percentage of iKO cells with

222   micronuclei plateaued at approximately 20% by 3 days and remained steady out to 13 days,

223   whereas the proportion of Flox cells with micronuclei stayed at around 5% throughout the entire

224   time course (Fig 4C). Therefore, a notably rapid formation of micronuclei precedes the

225   proliferation arrest and SA-βgal positivity that results from *ArpC2* inactivation.

226   Since micronuclei are often indicative of genomic instability, we next assessed the extent

227   of DNA damage in Flox and iKO cells. Staining with an antibody to the modified histone protein

228   H2AX (γH2AX), which is phosphorylated in response to double-stranded DNA breaks [61],

229   demonstrated that iKO cells contained prominent DNA damage clusters in their nuclei and

230   micronuclei (Fig 5A). Upon closer inspection, Flox cells typically exhibited no or diffuse γH2AX

231   staining in their nuclei (Fig 5B), whereas iKO cells frequently had 1-3 isolated γH2AX clusters

232   that localized to the nuclear periphery and/or within micronuclei (Fig 5B). Quantification of the

233   percentage of cells with γH2AX clusters following the addition of DMSO or 4-OHT revealed that

234   clusters began to increase in iKO cells by 2 days, and that from 3 days onward, clusters were

235   significantly more common in iKO cells than in Flox cells (Fig 5C). The fraction of iKO cells with

236   γH2AX clusters leveled out at approximately 25%, while the proportion of Flox cells containing

237   clusters was always 3-6% (Fig 5C). These results are consistent with previous observations in

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238 which *Drosophila* and mouse cells exposed to ionizing radiation and subjected to RNAi-

239 mediated Arp2/3 depletion were found to contain DNA damage and micronuclei [27]. However,

240 our results indicate that even without exposure to acute genotoxic agents, losing the Arp2/3

241 complex can cause an accumulation of damaged DNA elements that incorporate into

242 micronuclei, thereby illustrating that the Arp2/3 complex is a crucial player in maintaining

243 genomic integrity under relatively normal cell culture conditions.

244

245 **Activation of p53 and upregulation of *Cdkn1a*/p21 accompany the ArpC2 iKO cell arrest**

246 In light of the biogenesis of γH2AX clusters and micronuclei, we hypothesized that a DNA

247 damage response might be activated in the ArpC2 iKO cells. To explore this possibility, we

248 immunoblotted Flox and iKO cell extracts with antibodies to the crucial tumor suppressor protein

249 and transcription factor p53, which typically becomes stabilized, phosphorylated, and enriched

250 in the nucleus following DNA damage [62,63]. Consistent with this expectation, by 3 days after

251 4-OHT vs DMSO treatment, p53 levels were higher in iKO than in Flox cells, and p53 was

252 phosphorylated on Serine-15 (Fig 6A). Furthermore, immunofluorescence microscopy revealed

253 that nuclear p53 fluorescence was more intense in iKO cells than in Flox cells (Fig 6B). These

254 results imply that a p53-mediated DNA damage response is induced in Arp2/3-depleted cells.

255   Two of the major factors involved in the cell cycle arrest that leads to senescence are

256 the cyclin-dependent kinase inhibitors *Cdkn2a*/p16INK4A (p16) and *Cdkn1a*/p21CIP/WAF (p21).

257 Elevated levels of both of these anti-proliferative transcripts/proteins are frequently used as

258 indicators of the senescent state [55,64], although populations of cells expressing high levels of

259 p16 appear to be distinct from those expressing high levels of p21, at least in tissues from aged

260 mice [65]. Since p21 is a known transcriptional target of p53 [66,67], and the MTFs used in our

261 studies lack p16, we postulated that *Cdkn1a*/p21 was associated with the onset of senescence

262 in the iKO cells. To test this, we performed RT-PCR (Fig 6C) and RT-qPCR (Fig 6D) to

263 compare *Cdkn1a* transcript levels in Flox and iKO cells over a 9-day time period. Indeed,

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264   *Cdkn1a* expression appeared greater in the iKO cells than in the Flox cells at 6 and 9 days after

265   the initiation of 4-OHT treatment (Fig 6C). RT-qPCR revealed that *Cdkn1a* transcript levels

266   were doubled in the iKO cells relative to the Flox cells at 9 days (Fig 6D), showing that deletion

267   of the Arp2/3 complex leads to an upregulation of this key cell cycle regulator. To evaluate

268   whether the increase in *Cdkn1a* transcript corresponded to greater p21 protein levels, Flox and

269   iKO cells were treated with antibodies to p21 and subjected to immunofluorescence microscopy

270   (Fig 6E). Quantification of p21 nuclear intensity at the 6 day timepoint verified that p21 levels

271   were significantly higher in iKO than in Flox cells (Fig 6E and 6F). Together, the p53

272   enrichment and p21 upregulation in iKO cells support the idea that a DNA damage response

273   signaling pathway accompanies cell cycle arrest after the Arp2/3 complex is removed.

274

275   **Cytoplasmic cGAS and STING are recruited to micronuclei**

276   In addition to the above nuclear changes that took place upon Arp2/3 ablation, it seemed likely

277   that cytoplasmic changes arising from the presence of micronuclei in ArpC2 iKO cells would

278   also be linked to the onset of senescence. We hypothesized that a cytosolic DNA detection and

279   signaling pathway involving the cyclic GMP-AMP Synthase (cGAS) enzyme, which recognizes

280   extra-nuclear chromatin and relays a signal to the downstream effector molecule STING [60,

281   68,69], might also be activated in iKO cells. Tagged cGAS can be recruited to micronuclei, and

282   through its detection of cytosolic DNA and activation of STING, promotes pro-senescence and

283   pro-inflammatory gene expression [70-72]. To determine if tagged cGAS localizes to the

284   micronuclei in ArpC2 iKO cells, we transiently transfected MTFs with plasmids encoding

285   mCherry-cGAS or mCherry as a control (Fig 7A), and treated them with 4-OHT to induce *ArpC2*

286   deletion. mCherry was highly expressed in the iKO cells but was not recruited to micronuclei

287   (Fig 7B). In contrast, mCherry-cGAS showed intense localization to micronuclei (Fig 7B),

288   indicating that it can detect the cytosolic DNA in iKO cells.

289   We next wanted to determine the localization of endogenous cGAS and STING in the

290 MTFs. Immunoblotting indicated that cGAS and STING were expressed at similar levels in Flox

291 and iKO cells (Fig 7C), so to test whether cGAS and/or STING were recruited to the

292 micronuclei, we visualized these proteins via immunofluorescence microscopy after 3-4 days,

293 when micronuclei become more abundant in iKO cells and just before Flox and iKO

294 multiplication rates begin to diverge. Consistent with previous studies [73,74], endogenous

295 cGAS was present in the nuclei of both Flox and iKO cells (Fig 7D). It was also readily apparent

296 in nearly 40% of iKO cell micronuclei (Fig 7D). The rare micronuclei that formed in Flox cells

297 also recruited cGAS (Fig 7D magnification). For STING, an integral membrane protein that

298 localizes to organelles of the conventional secretory pathway, including the endoplasmic

299 reticulum (ER) and Golgi [75], immunofluorescence revealed a speckled ER-like localization in

300 both Flox and iKO cells, and enrichment around micronuclei (Fig 7D inset). Immunolabeling for

301 STING, the ER chaperone GRP94, and the *cis*-Golgi protein GM130 implied that ER and not

302 Golgi membranes are more likely to surround subsets of micronuclei in iKO cells (Fig 7E).

303 These findings are consistent with a function for cGAS in recognizing damaged DNA in the

304 cytosol and initiating a signaling pathway that locally activates STING near micronuclei in

305 Arp2/3-deficient cells.

306   To explore whether cGAS affects the cell proliferation arrest that takes place when the

307 Arp2/3 complex is lost, MTFs were exposed to 4-OHT for 3 days to induce the deletion of *ArpC2*

308 and then transferred into media containing the cGAS inhibitor RU.521 [76] or DMSO as a

309 control. Quantification of cell replication rates during days 4-6 following the onset of 4-OHT

310 treatment revealed that RU.521 caused a modest but statistically faster population doubling time

311 in the iKO cells (Fig 7F), implying that cGAS inhibition has the potential to oppose the initiation

312 of senescence in some of the cells in this experimental system. Collectively, the above

313 localization and inhibitor results support the idea that cGAS/STING signaling contributes to the

314 establishment of senescence in Arp2/3-deficient cells.

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14

Micronuclei form as a result of mitotic defects in ArpC2 iKO cells

Because ArpC2 iKO cells accumulate DNA damage, form micronuclei with high frequency, and undergo senescence in a p53/p21- and cGAS/STING-associated manner, we next wanted to determine how this critical process of micronucleus biogenesis takes place. Micronuclei can form as a result of chromosome missegregation during mitosis and from expulsion of damaged DNA from the nucleus during interphase [58,77-80]. To differentiate between the possibilities that micronuclei are a product of defects in mitosis versus aberrant nuclear remodeling in interphase, we expressed the GFP-tagged histone H2B in MTFs and visualized chromatin dynamics in live cells. These experiments were performed within the first 2 days of DMSO or 4-OHT treatment, when Arp2/3 complex levels were declining the fastest (Fig 1B), the cells were still dividing rapidly (Fig 1E), and the incidence of micronucleus formation was highest (Fig 4C).

Timelapse imaging of H2B-GFP-expressing Flox cells revealed that the majority of mitoses resulted in an equal partitioning of nuclear chromatin into two daughter cells (Fig 8A). However, iKO cell mitoses often yielded micronuclei due to errors in chromatin segregation (Fig 8A). This was repeatedly observed when chromatin fragments near the metaphase plate were not properly distributed to daughters during anaphase (Fig 8A). Another common phenotype of iKO cells that entered mitosis was premature mitotic exit (Fig 8B). Such cells underwent a prolonged prometaphase or metaphase, failed to enter anaphase, and ultimately returned to interphase with nuclei containing twice their normal chromatin content (Fig 8B). Micronuclei also formed in some of these cells that failed to complete mitosis (Fig 8B).

We next measured the frequencies with which the mitotic defects occurred. First, approximately 23% of completed mitoses in iKO cells yielded micronuclei compared to only about 6% of completed mitoses in Flox cells (Fig 8C). Second, after categorizing the stages of mitosis and determining the number of timelapse frames spent specifically in metaphase (judged by the presence of at least 95% of H2B-GFP fluorescence aligned compactly at the cell equator), we found that >80% of Flox cells that completed mitosis spent only one frame in

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342   metaphase, whereas only 40% of iKO cells proceeded through metaphase with this speed (Fig

343   8D). The other 60% of iKO cells that completed mitosis spent two or more frames in metaphase

344   (Fig 8D), suggesting that iKO cells experience an unusually prolonged metaphase period.

345   Third, when evaluating the incidence of premature mitotic exits (defined as cells that entered

346   prophase but did not complete anaphase), we discovered that nearly 30% of iKO cells that

347   entered prophase underwent premature mitotic exits compared to only 10% of Flox cells (Fig

348   8E). Approximately 14% of premature mitotic exits that took place in the iKO cells also gave

349   rise to micronuclei. Fourth, among the 330 iKO interphase nuclei that were observed during live

350   cell imaging, at most 2 (i.e., ≤0.6%) appeared to create micronuclei via budding or extrusion,

351   indicating that micronuclei form almost exclusively during mitosis. As a final point, to confirm

352   that the prevalent chromatin missegregation events in iKO cells were, at least in part,

353   attributable to the presence of broken DNA fragments, we fixed and stained mitotic cells for

354   γH2AX. In agreement with this possibility, during prometaphase and metaphase, iKO cells

355   contained γH2AX foci at the periphery of or completely detached from the main chromatin mass

356   (Fig 8F). Furthermore, in early anaphase, iKO cells displayed prominent DNA damage clusters

357   near their equators, and by late anaphase, γH2AX-positive lagging chromosomes became

358   apparent (Fig 8F). Overall, these live and fixed cell studies show that, during Arp2/3-depleted

359   conditions, damaged chromatin fragments persist and are incorporated into micronuclei as a

360   result of defects in mitotic chromosome segregation.

361

362   **Actin filament penetration into the central spindle is diminished in Arp2/3-depleted cells**

363   Upon discovering that knocking out the Arp2/3 complex leads to chromatin partitioning defects,

364   we wanted to also assess how the loss of this actin nucleator might alter the actin and

365   microtubule cytoskeletons during mitosis. Actin has been observed in various parts of meiotic

366   and mitotic spindles in diverse organisms [30-39], so to examine the organization and intensity

367   of actin filaments in relation to microtubule spindles in MTFs, we stained mitotic Flox and iKO

16

368 cells with phalloidin to label F-actin, an anti-tubulin antibody to visualize microtubules, and DAPI

369 to detect DNA. We focused on metaphase, when the chromosomes are either properly or

370 improperly aligned at the central spindle, corresponding to when Flox mitoses rapidly proceed or

371 iKO mitoses frequently stall. Imaging of metaphase Flox cells in multiple focal planes revealed

372 that several distinct F-actin structures were present. In the lower portions of cells, linear actin

373 filaments in the shape of a spindle and several bundles of microtubules were observed (Fig 9A).

374 In the middle planes of cells, multiple thick finger-like F-actin structures penetrated the

375 chromosomal region, and numerous microtubules comprising the main microtubule spindle were

376 apparent (Fig 9A). In the upper parts of cells, fewer F-actin and microtubule structures

377 intercalated the central spindle area (Fig 9A). Staining with anti-ArpC2 antibodies indicated that

378 the Arp2/3 complex was not enriched along the thin linear actin filaments at the lower part of the

379 cell, but was present near F-actin and microtubules in the middle and upper spindle structures

380 (Fig 9A). Together, these observations expand the catalog of F-actin and Arp2/3-associated

381 cytoskeletal structures that are found within dividing mammalian cells.

382   For more closely examining the spatial positioning of actin filaments, microtubules, and

383 the Arp2/3 complex at the metaphase plate, we performed fluorescence intensity line scan

384 analyses through the chromatin-containing region. Peaks of spindle microtubule intensity often

385 coincided with peaks of F-actin intensity as well as sites of ArpC2 enrichment (Fig 9B). In

386 contrast, DNA staining levels were highest where microtubules and F-actin were lowest (Fig

387 9B). These observations show a positive relationship between F-actin and microtubule

388 localization in the central metaphase spindle.

389   To determine whether loss of the Arp2/3 complex causes any abnormalities in F-actin

390 organization during metaphase, we compared F-actin staining in Flox and iKO cells. Actin

391 filament levels in the metaphase chromatin-containing region appeared less prominent in iKO

392 cells than in Flox cells (Fig 9C). Quantification of phalloidin fluorescence intensities in these

393 regions of the central spindle revealed that F-actin levels were significantly lower in iKO cells

394   compared to Flox cells (Fig 9D). This was not due to a general deficit in central spindle staining,

395   because the fluorescence intensity of microtubules in the same region was not significantly

396   different between iKO and Flox cells (Fig 9D). A reduction in the penetration of actin filaments,

397   but not microtubules, into the metaphase chromatin-containing region of iKO cells was further

398   reflected by fewer prominent F-actin peaks in fluorescence intensity plot profiles (Fig 9E).

399   Collectively, these results indicate that a functional Arp2/3 complex is required for the

400   polymerization of actin filaments in the vicinity of metaphase chromosomes.

401

402   **Arp2/3 depletion results in anaphase microtubule asymmetry**

403   Since metaphase spindle-associated F-actin overlapped with microtubules in Flox cells and was

404   less prominent in Arp2/3 knockout cells, we considered that the subsequent arrangement of

405   microtubules during chromosome segregation might be altered. To explore this possibility, we

406   evaluated microtubule organization and intensity during anaphase, when the chromosomes are

407   either correctly or incorrectly partitioned. Flox cells exhibited uniform distributions of

408   microtubules across the width of the separating spindle (Fig 10A), as evidenced by the relatively

409   evenly-spaced peaks in fluorescence intensity plots perpendicular to the presumed position of

410   the cytokinetic ring (Fig 10B). In contrast, iKO cells displayed unbalanced tubulin intensities in

411   which the microtubules appeared to be more heavily concentrated on one side of the spindle

412   (Fig 10A and 10B). These results suggest that in addition to the presence of misplaced

413   damaged chromatin and a decrease in actin filaments at the metaphase plate, chromosome

414   missegregations arising from alterations in anaphase microtubule organization may be a

415   contributing factor in the formation of micronuclei in Arp2/3-deficient cells. Thus, loss of the

416   Arp2/3 complex has multiple significant molecular consequences – beginning with unrepaired

417   DNA damage and including mitotic actin and microtubule cytoskeleton abnormalities – that lead

418   to a p53/p21 response, the biogenesis of micronuclei, cGAS/STING recruitment, and the onset

419   of senescence.

18

Discussion

The Arp2/3 complex is a key driver of many cellular processes that are mediated by actin assembly at the plasma membrane, namely adhesion, endocytosis, protrusion, and migration. While the complex is evolutionarily conserved among nearly all eukaryotes and is essential for viability in animals, the molecular basis underlying its indispensability is unclear. Roles for the Arp2/3 complex in enabling DNA repair during interphase, promoting chromosome partitioning during meiosis/mitosis, and controlling apoptosis following DNA damage have recently emerged and may help explain why Arp2/3 is essential. Such studies prompted us to determine the effects of permanent Arp2/3 complex ablation on viability, proliferation, and chromatin-associated processes in mammalian cells. Our results reveal important functions for the Arp2/3 complex in maintaining genomic integrity and supporting mitotic progression, while uncovering striking mechanistic connections between actin cytoskeleton defects and senescence.

Various endogenous stresses are known to induce cellular senescence, including replicative, telomeric, genotoxic, oncogenic, oxidative, and mitochondrial stress [81]. Our studies now add cytoskeletal dysfunction as a trigger of senescence. Arp2/3 complex knockout cells, in addition to a stable proliferation arrest and physical enlargement, display multiple phenotypic hallmarks of senescence. These include an increase in *Il-6* transcript reflective of SASP expression and a reduction of nuclear Lamin B1 levels. The iKO cells also harbor high cytoplasmic SA-βgal activity and increased acidic organelle content. Given that the Arp2/3 complex influences most cellular functions, from plasma membrane remodeling [2,15,82] to mitochondrial dynamics [83] to autophagy [84] to DNA repair [27,28], it seems likely that multiple intracellular defects derived from Arp2/3 deficiency can impact senescence induction. Potential changes in mitochondrial turnover, combined with altered lysosomal content could signify increases in mitochondrial, oxidative, and proteotoxic stress in iKO cells. The extent to which these stressors contribute to the initiation or maintenance of senescence will be an important

19

445   area of future investigation.

446   Despite uncertainties in the severity of cytoplasmic organelle dysfunction in iKO cells,

447   obvious defects in nuclear and chromatin-associated processes emerged as major factors in

448   promoting senescence in our studies. Kinetic analyses indicate that 1-3 days after exposure to

449   4-OHT, ArpC2 (and thus functional Arp2/3 complex) levels are 50-90% depleted (Fig 1A and

450   1B). This time period coincides with the steepest increases in detection of micronuclei via DAPI

451   staining (Fig 4C) and visualization of dsDNA breaks in both nuclei and micronuclei via γH2AX

452   staining (Fig 5C). A DNA damage response is activated, as evidenced by increased p53

453   expression and phosphorylation (Fig 6A and 6B). By 4-6 days, the p53-regulated cell cycle

454   inhibitor p21 accumulates in the nucleus (Fig 6C-F) and cell multiplication stops (Fig 1D). By 7

455   days, SA-βgal-positivity begins to increase (Fig 3A and 3B). At 7-10 days, the entire population

456   of iKO cells appears to be senescent, given the complete lack of proliferation (Fig 1D), as well

457   as consistent single cell phenotypes in enlargement (Fig 1G), nuclear Lamin B1 reduction (Fig

458   2F), and cytoplasmic acidity (Fig 3D). Beyond 10 days, the iKO cell population remains viable

459   but non-replicative while maintaining 20-25% positivity for micronuclei and SA-βgal activity.

460   While it is clear that all Arp2/3 knockout cells senesce, it is unclear why only a fraction of

461   the culture is SA-βgal positive. This could be a technical matter of assay sensitivity, or intense

462   SA-βgal-positivity may simply develop later in some cells. Additionally, the reasons why the

463   entire culture senesces when only a quarter of the iKO cells harbor detectable micronuclei

464   remain ambiguous. It is conceivable that aneuploidy as a result of receiving insufficient

465   chromatin following mitosis or from excess DNA content following premature mitotic exits

466   contributes to a p53-mediated arrest program in micronucleus-negative cells. Another

467   possibility is that paracrine signaling from mature senescent cells to others in the culture

468   supports the global proliferation block.

469   In response to damaging stimuli, the cyclin-dependent kinase inhibitors p16 and p21 are

470   frequently upregulated and act as key players in pathways that promote the replicative arrest

471   that defines senescence [55-57]. Earlier studies showed that inactivation of *p16Ink4a/Arf* is

472   necessary for establishing Arp2/3-depleted mouse fibroblast cultures, suggesting that the loss of

473   Arp2/3 can induce growth arrest or death in a p16-mediated manner [40,41]. More recent

474   experiments in human cells treated with a genotoxic agent indicate that depletion of the Arp2/3

475   complex or inactivation of two of its upstream regulators prevents the execution of apoptosis

476   [29]. Such cells undergo a p53- and p21-associated cell cycle arrest but fail to properly

477   complete an apoptosome-based caspase cleavage cascade [29]. These observations, together

478   with our data showing that ArpC2 iKO cells activate p53 and accumulate p21 in a p16-

479   independent manner, suggest that losing the Arp2/3 complex can trigger multiple cell cycle

480   arrest pathways. While p53 is known to cause upregulation of *Cdkn1a*/p21 to promote arrest

481   [85-87], deciphering the degree to which other anti-proliferative mechanisms cooperate with p21

482   in iKO cells requires more investigation. Interestingly, studies in aged mice show that cells

483   expressing high levels of p21 are mostly distinct from those expressing high levels of p16 [65],

484   further highlighting the possibility that loss of the Arp2/3 complex *in vivo* may instigate different

485   pathways to senescence depending on the physiological state of the cell and its tissue of origin.

486   In addition to the functions of p53 and p21 in the nucleus, it seems likely that recognition

487   of micronuclei in the cytoplasm also participates in the senescence response in Arp2/3-deficient

488   cells. We observed cGAS and STING localization at or around micronuclei in ArpC2 iKO cells,

489   and a chemical inhibitor of cGAS seemed to slightly increase multiplication of iKO cultures.

490   Recent work has also implicated cGAS/STING in regulating p21 expression, as depletion of

491   cGAS or STING results in reduced p21 levels and premature mitotic entry [88]. These results

492   support the notion that multiple nuclear and cytoplasmic signaling factors contribute to the

493   induction and maintenance of the proliferation arrest in ArpC2 iKO cells.

494   The main mechanism underlying micronucleus biogenesis in Arp2/3-deficient cells is a

495   lack of fidelity in chromatin partitioning during mitosis, as revealed by our live imaging studies.

496   Such segregation defects can be primarily explained by broken DNA fragments failing to

497   properly attach to the microtubule spindle, since most micronuclei formed from cells completing

498   inaccurate mitoses. However, cytoskeletal abnormalities may also contribute to chromosome

499   segregation errors, as changes in the organization of metaphase F-actin and anaphase

500   microtubules are also prevalent in the knockout cells. Our findings complement previous

501   experiments showing altered F-actin levels in meiotic/mitotic structures and defects in spindle

502   formation following chemical inhibition of Arp2/3 [37-39]. Interestingly, many mitoses in ArpC2

503   iKO cells feature a prolonged metaphase period. Delayed mitoses arising from failure to satisfy

504   spindle assembly checkpoints can cause chromosome segregation mistakes and premature

505   mitotic exits, both of which give rise to aneuploid cells [89], and both of which were observed at

506   high frequencies in iKO cells. Thus, Arp2/3 complex deficiency leads to multiple deleterious

507   consequences in M-phase of the cell cycle. More work is needed to determine how Arp2/3,

508   actin, and microtubules collaborate in controlling spindle positioning, chromosome alignment,

509   and DNA segregation.

510   Given that high mutation rates and aneuploidy are associated with organismal aging and

511   tumorigenesis [90], impairment of Arp2/3 function *in vivo* could be a contributor to the

512   development of age-related dysfunction and cancers. One central feature of the aging process

513   is an accumulation of senescent cells, which can drive several aging phenotypes [91,92]. Such

514   discoveries have led to the development of new "senolytic" classes of drugs to treat and slow

515   the progression of age-related pathologies [93]. Our findings suggest that impaired Arp2/3

516   complex function, and potentially other actin cytoskeletal misregulation, may contribute to

517   premature senescence and aging. Taken together with previous observations that F-actin

518   integrity affects aging and lifespan in *C.elegans* [94,95], preventing or correcting cytoskeletal

519   defects may promote cellular longevity and help reduce the senescent cell burden during

520   organismal aging.

521   In contrast to the negative impacts of cellular senescence on aging, the anti-proliferative

522 effects of senescence can serve as a positive form of tumor suppression [96]. Several

523 therapeutics that induce senescence in cancer cells have been developed to reduce and

524 prevent metastatic growth [97]. The human *CDKN2A* locus, which encodes p16^{INK4A}/Arf, is

525 frequently inactivated or epigenetically suppressed in various types of cancers [98]. Thus, the

526 *ArpC2* floxed *Cdkn2a* null cells used in our studies present a unique opportunity to study Arp2/3

527 complex function in a genetic background that is particularly relevant to preventing the growth of

528 cancer cells. Enhancing our understanding of the connection between the actin cytoskeleton

529 and cellular senescence will therefore provide insight into therapeutic strategies used to

530 regulate cell proliferation, arrest, and death in the context of both age-related diseases and

531 cancers.

Materials and Methods

Ethics statement

Research with biological materials was approved by the UConn Institutional Biosafety

Committee. This study did not include research with human subjects or live animals.

Mammalian cell culture

MTFs containing the floxed *ArpC2* allele (from James Bear, University of North Carolina) [42]

were cultured in DMEM (with 4.5g/L glucose, L-Glutamine, 110mg/L sodium pyruvate), 10%

fetal bovine serum (FBS), GlutaMAX, and antibiotic-antimycotic (Gibco). Cells were treated with

media containing 0.01% DMSO or 2µM 4-OHT (Sigma) to obtain Flox or iKO populations. For

treatments exceeding 3 days, culture supernatants were replaced with fresh media containing

DMSO or 4-OHT on day 4. Cultures were returned to normal media after day 6. All experiments

were performed using cells that had been in active culture for 2-12 trypsinized passages.

Cell proliferation assays

MTFs were cultured in 12-well plates and cell titers were routinely determined using a

hemocytometer. Cells were initially seeded at multiple concentrations ranging from $2x10^3$ to

$2x10^4$ cells per well. After 5 days, confluent Flox samples were subcultured daily into multiple

wells at concentrations of $1-2x10^4$ cells per well, while iKO samples were subcultured if/when

they reached 95% confluency. All cultures were expanded into 6-well plates and 6cm dishes

when necessary. Population doubling times were calculated based on initial and final cell titers

every 24-48h using the equation [time x log(2)] / [log(final) - log(initial)]. Due to their continuous

proliferation, the plotted values of cell numbers for Flox samples at days 8-12 were

extrapolations based on doubling rates at those time points. For cGAS inhibitor experiments,

MTF supernatants were replaced with fresh media containing 5µM RU.521 every 12h from days

4-6 after 4-OHT exposure and counted at 0, 24, and 48h of DMSO or RU.521 treatment.

Transfections and fluorescent probes

557 For mCherry-cGAS cloning, mouse cGAS was amplified via PCR from a pMSCVpuro-eGFP-

558 cGAS template (Addgene, 108675) using primers containing KpnI and NotI restriction sites (S1

559 Table), and inserted into the pKC-mCherryC1 vector [99]. For transfections, Flox cells were

560 grown in 12-well plates for 24h and then transfected with 350ng of mCherry-cGAS plasmid in

561 DMEM. After 3h, DMEM was replaced with MTF media, and 18h later cells were trypsinized and

562 transferred onto 12mm glass coverslips in 24-well plates. Media containing DMSO or 4-OHT

563 was added after 3h, and cells were subjected to additional 29h growth before fixation as

564 described below. For H2B-GFP transfections, Flox cells were grown in 24-well plates for 24h

565 and then transfected with 130ng of H2B-GFP plasmid (Addgene, 11680). After 5h, DMEM was

566 replaced with MTF media containing DMSO or 4-OHT. Cells were imaged live 15-40h later, as

567 described below. All plasmids were maintained in NEB5-alpha *E.coli* and purified using

568 Macherey-Nagel kits. For imaging acidic cytoplasmic organelles, cells were incubated for 30min

569 in media containing 100nM LysoTracker Red (Invitrogen) prior to fixation.

570 **RT-PCR and RT-qPCR**

571 RNA from Flox and iKO fibroblasts grown in 6-well plates was isolated using TRIzol reagent

572 (Ambion). Following chloroform extraction, isopropanol precipitation, and a 75% ethanol wash,

573 total RNA was resuspended in water. cDNA was reverse transcribed using the iScript cDNA

574 synthesis kit (Bio-Rad) and then PCR-amplified using Taq polymerase (New England Biolabs)

575 and primers listed in S1 Table. Primers were designed to amplify ~340-480bp with each cDNA

576 target. The resulting PCR products were visualized on ethidium-bromide stained agarose gels.

577 RT-qPCR was performed using SYBR-green (Bio-Rad) on a CFX96 Real-Time System (Bio-

578 Rad). 1µl of cDNA was used in each 10µl RT-qPCR reaction, and all samples were run in

579 duplicate. Primer dilution curves were analyzed to ensure primer specificity. Ct values were

580 normalized to GAPDH and/or actin. The iKO:Flox ratio (fold difference) at each timepoint was

581 calculated by the comparative ΔΔ cycle threshold method.

582 **Cell extracts**

583   For preparation of whole cell extracts, fibroblasts cultured in 6-well plates were collected in

584   phosphate buffered saline (PBS) containing 1mM EDTA, centrifuged at 2,900rpm for 5.5min at

585   4ºC, and lysed in 25mM HEPES (pH 7.4), 100mM NaCl, 1% Triton-X-100, 1mM EDTA, 1mM

586   Na_3VO_4, 1mM NaF, 1mM phenylmethylsulfonyl fluoride, and 10µg/ml each of aprotonin,

587   leupeptin, pepstatin, and chymostatin on ice. Lysates were mixed with Laemmli sample buffer,

588   boiled, and centrifuged prior to SDS-PAGE analyses.

589   **Immunoblotting**

590   Cell extract samples were separated on 12% SDS-PAGE gels before transfer to nitrocellulose

591   membranes (GE Healthcare). Membranes were blocked in PBS containing 5% milk before

592   probing with primary antibodies at concentrations listed in S2 Table. Following overnight

593   incubation at 4ºC, membranes were washed and treated with IRDye-680/800- (LI-COR) or

594   horseradish peroxidase-conjugated (GE Healthcare) secondary antibodies. Infrared and

595   chemiluminescent bands were visualized using a LI-COR Odyssey Fc Imaging System. Band

596   intensities were measured using LI-COR Image Studio software. Densitometries of proteins-of-

597   interest were normalized to GAPDH, tubulin, and/or actin loading controls.

598   **Immunostaining**

599   For immunofluorescence, fibroblasts cultured on glass coverslips in 24-well plates were washed

600   with PBS and fixed using PBS containing 2.5% paraformaldehyde for 30min. Following PBS

601   washes, cells were permeabilized using PBS containing 0.1% Triton X-100 for 2min, washed,

602   and stained with primary antibodies in PBS containing 1% bovine serum albumin, 1% FBS, and

603   0.02% azide for 45-60min as described in S2 Table. Cells were washed and treated with Alexa

604   Fluor 488-, 555-, or 647-conjugated goat anti-rabbit, anti-mouse, or anti-rat secondary

605   antibodies, 1µg/mL DAPI, and/or 0.2U/mL Alexa Fluor 488- or 647-labeled phalloidin

606   (Invitrogen) for 35-45min as detailed in S2 Table. Following washes, coverslips were mounted

607   on glass slides in Prolong Gold anti-fade (Invitrogen).

608   **Fluorescence microscopy**

26

609 All fixed and live cells were imaged using a Nikon Eclipse Ti microscope equipped with Plan

610 Apo 100X (1.45 NA), Plan Apo 60X (1.40 NA), or Plan Fluor 20X (0.5 NA) objectives, an Andor

611 Clara-E camera, and a computer running NIS Elements Software. Most images were taken as

612 single epifluorescence slices, whereas 60X images of mCherry-transfected cells (Fig 7) were

613 taken as z-stacks with a 0.3μm step size, and fixed mitotic cells were taken with a 0.3μm (Fig 9)

614 or 0.5μm (Fig 8, Fig 10) step size. Live cell imaging was performed in a 35ºC chamber (Okolab).

615 During live imaging, cells were cultured in fresh media containing 25mM HEPES (pH 7.4) and

616 DMSO or 4-OHT. Images were captured using the 20x objective at 12min intervals. All image

617 processing and analysis was conducted using ImageJ/FIJI software [100].

618 **Fluorescence quantification**

619 Microscopic quantifications were performed using ImageJ. For fibroblast area calculations, cell

620 perimeters were outlined based on phalloidin staining. For Lamin B1 abundance, the mean

621 fluorescence intensity (MFI) of Lamin B1 staining in the nuclear area of the cell was measured

622 and the background signal from outside the cell was subtracted from the MFI values. For

623 normalization purposes, all MFI values for individual Flox and iKO cells were divided by the

624 overall average MFI across 2 replicates for Flox cells. For acidic organelle analyses, the number

625 of cells with diffuse LysoTracker staining was scored as positive or negative and divided by the

626 total number of cells analyzed. To determine the LysoTracker area as a % of the total cell area,

627 cell perimeters were outlined based on phalloidin staining and the Threshold tool was used to

628 quantify the % of this area containing LysoTracker staining. For micronuclei and γH2AX

629 quantifications, the number of cells with DAPI-stained micronuclei and the number of cells with

630 prominent nuclear γH2AX clusters were quantified and divided by the total number of cells

631 analyzed. Clusters were defined as isolated and discrete regions of γH2AX staining. For

632 analyses of p21, the MFI of p21 staining in the nuclear area of the cell was measured and the

633 average p21 MFI across 3 replicates for Flox and iKO cells was calculated. The average MFI of

634   Flox cells was set to 1. For area-based assays of F-actin and microtubule intensities during

635   metaphase, the DAPI-stained DNA mass was outlined in ImageJ and the MFI of phalloidin and

636   anti-tubulin staining in this area was measured relative to DAPI. In linescan analyses of F-actin,

637   tubulin, ArpC2, and DNA staining intensities in metaphase and/or anaphase cells, the ImageJ

638   plot-profile tool was used. For metaphase, 14-16µm lines were drawn across the chromatin

639   mass. For anaphase, 10-15µm lines were drawn across the mitotic spindle halfway between

640   one set of chromosomes and the equator of the two forming daughter cells. In each plot, the

641   minimum pixel intensity recorded along the line was subtracted from all values along the line to

642   set the minimum to 0, and then all values were divided by the maximum to set the highest peak

643   to 1. The numbers of cells analyzed in each type of assay are listed in the Figure Legends.

644   **β-galactosidase assays**

645   SA-βgal activity was assessed using the Senescence β-Galactosidase Staining Kit (Cell

646   Signaling Technologies, 9860). Fibroblasts were cultured in 6-well plates, washed once with

647   PBS, fixed for 20min, and washed twice with PBS before incubation in SA-βgal staining solution

648   at 37ºC in the dark for 20-24h. Images were captured using an iPhone 7 on a bright-field

649   microscope equipped with a 10x objective. The % of SA-βgal-positive cells was quantified by

650   counting the number of intensely blue-colored cells divided by the total number of cells.

651   Samples were coded and scored in a blinded fashion.

652   **Reproducibility and Statistics**

653   All conclusions were based on observations made from at least 4 separate experiments, while

654   quantifications were based on data from 2-5 representative experiments. Statistical analyses

655   were performed using GraphPad Prism software as noted in the Figure Legends. P-values for

656   data sets including 2 conditions were determined using unpaired t-tests unless otherwise noted.

657   Analyses of data sets involving +/- scoring used Fisher's exact test. P-values <0.05 were

658   considered statistically significant.

659 **Acknowledgements**

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934 **Figure Legends**

935 **Fig 1. ArpC2 iKO cells undergo a proliferation arrest and enlargement. (A)** Mouse

936 fibroblasts were treated with DMSO (Flox) or 4-OHT (iKO) for 0-6d and collected at 0-13d.

937 Samples were lysed, subjected to SDS-PAGE, and immunoblotted with antibodies to ArpC2,

938 Arp3, and GAPDH. **(B)** ArpC2 and Arp3 band intensities were normalized to GAPDH or tubulin

939 band intensities and plotted as the iKO:Flox ratio. Each dot represents the mean ratio ±SD from

940 2-3 experiments. **(C)** Flox and iKO cells were fixed at 7d and stained with phalloidin (F-actin;

941 magenta), an Arp3 antibody (green), and DAPI (DNA; blue). The arrowhead highlights a

942 micronucleus. Scale bar, 10µm. **(D)** Flox and iKO cell titers were quantified at 0-18d. Each point

943 represents the mean ±SD from 2 Flox and 2-3 iKO experiments, except for the 14d and 18d

944 timepoints, which were from a representative iKO sample that did not exhibit outgrowth of

945 colonies expressing ArpC2. **(E)** Flox and iKO population doubling times were quantified daily

946 from 0-4d and 5-12d. For each time range, the bar represents the mean doubling time ±SD from

947 n=3 experiments. **(F)** Flox and iKO cells were fixed at 7d and stained with phalloidin and DAPI.

948 The arrowhead highlights a micronucleus. Scale bar, 25µm. **(G)** Flox and iKO cells were

949 outlined in ImageJ and their areas quantified. Each dot represents an individual cell and the

950 lines represent the mean area ±SD from analyses of 100 cells.

951

952 **Fig 2. Loss of the Arp2/3 complex results in elevated *Il-6* transcript and decreased**

953 **nuclear Lamin B1 levels. (A)** Mouse fibroblasts were treated with DMSO (Flox) or 4-OHT

954 (iKO) for 0-6d and collected at 0, 3, 6, and 9d. RNA was isolated and RT-PCR performed using

955 primers for *Il-6* and *Gapdh*. PCR products were visualized on ethidium bromide stained agarose

956 gels. **(B)** RT-qPCR was performed using primers for *Il-6* and *Gapdh* at 9d. *Il-6* product levels

957 were normalized to *Gapdh*. Each bar represents the mean transcript abundance ±SD from n=3

958 experiments. AU = Arbitrary Units. **(C)** Flox and iKO cells were collected at 10d and

41

959   immunoblotted with antibodies to Lamin B1, ArpC2, GAPDH, and actin. **(D)** Lamin B1 band

960   intensity was normalized to GAPDH and actin band intensities. Each bar represents the mean

961   intensity ±SD from n=3 experiments. **(E)** Flox and iKO cells were fixed at 9d and stained with a

962   Lamin B1 antibody (green) and DAPI (DNA; blue). Arrowheads point to micronuclei. Scale bar,

963   25µm. **(F)** Nuclear Lamin B1 levels were quantified by outlining the DAPI-stained nucleus of

964   each cell in ImageJ and measuring the mean Lamin B1 pixel intensity. Each dot represents an

965   individual cell and the line depicts the average Lamin B1 pixel intensity from analyses of 492-

966   556 cells pooled from n=2 experiments (denoted in black or green). RFU = Relative

967   Fluorescence Units.

968

969   **Fig 3. SA-βgal activity and lysosomal content are increased in ArpC2 iKO cells. (A)**

970   Mouse fibroblasts were treated with DMSO (Flox) or 4-OHT (iKO) for 0-6d and senescence-

971   associated beta-galactosidase (SA-βgal) staining was performed over a 22d period. **(B)** The %

972   of SA-βgal-positive cells was quantified by calculating the number of intensely blue-colored cells

973   divided by the total number of cells. Each point represents the mean % ±SD from a

974   representative 0d timepoint, n=2 experiments for the 3d and 22d timepoints, and

975   n=3 experiments for the 7d and 13d timepoints (727-2197 cells per point). **(C)** Flox and iKO

976   cells at 9d were treated with LysoTracker (magenta), fixed, and stained with DAPI (DNA; blue).

977   The magnifications illustrate punctate versus diffuse LysoTracker staining, and the arrowhead

978   indicates the position of a micronucleus. Scale bars, 25µm. **(D)** The % of cells exhibiting diffuse

979   LysoTracker staining was quantified by scoring the number of cells with broad instead of

980   punctate cytoplasmic fluorescence and dividing by the total number of cells. The relative area

981   within each cell occupied by LysoTracker was quantified using the threshold function in ImageJ.

982   Each bar represents the mean % ±SD from n=2 experiments (151-155 cells per bar).

983 **Fig 4. Arp2/3 complex depletion leads to the formation of cytoplasmic micronuclei. (A)**

984 Mouse fibroblasts were treated with DMSO (Flox) or 4-OHT (iKO) for 6d, fixed at 7d, and

985 stained with DAPI. Arrowheads point to micronuclei. Scale bar, 25μm. **(B)** Magnifications show

986 different micronucleus phenotypes in iKO cells. Scale bar, 5μm. **(C)** The % of cells with

987 micronuclei was quantified over a 13d period following DMSO or 4-OHT exposure. Each point

988 represents the mean % ±SD from n=3 experiments, except for the 2d and 4d timepoints, which

989 are from n=2 experiments (432-631 cells per point in each experiment).

990

991 **Fig 5. Prominent DNA damage clusters are found in the nuclei and micronuclei of ArpC2**

992 **iKO cells. (A)** Mouse fibroblasts were treated with DMSO (Flox) or 4-OHT (iKO) for 6d, fixed at

993 7d, and stained with a γH2AX antibody (green) and DAPI (DNA; blue). Arrowheads point to

994 γH2AX clusters in micronuclei and arrows indicate γH2AX clusters in nuclei. Scale bar, 25μm.

995 **(B)** Magnifications show no or diffuse γH2AX staining in Flox nuclei and γH2AX clusters in iKO

996 cell nuclei and micronuclei. Scale bar, 5μm. **(C)** The % of cells with γH2AX clusters was

997 quantified over a 13d period following DMSO or 4-OHT exposure. Each point represents the

998 mean % ±SD from n=3 experiments, except for 2d and 4d timepoints, which are from n=2

999 experiments (432-631 cells per point in each experiment).

1000

1001 **Fig 6. Activation of p53 and upregulation of *Cdkn1a*/p21 occur in ArpC2 iKO cells. (A)**

1002 Mouse fibroblasts were treated with DMSO (Flox) or 4-OHT (iKO) for 3d, collected, and

1003 immunoblotted with antibodies to p53, phospho-p53(Ser15), ArpC2, tubulin, actin, and GAPDH.

1004 Total and phosphorylated p53 intensities relative to loading controls are indicated beneath the

1005 respective bands. **(B)** Flox and iKO cells were fixed at 3d and stained with a p53 antibody

1006 (green) and DAPI (DNA; blue). Arrowheads point to micronuclei. Scale bars, 25μm. **(C)** Flox

1007 and iKO cells were collected at 0, 3, 6, and 9d. RNA was isolated and RT-PCR performed using

1008   primers for *Cdkn1a*, *Gapdh* and *β-actin*. PCR products were visualized on ethidium bromide

1009   stained agarose gels. **(D)** RT-qPCR was performed using primers for *Cdkn1a* and *Gapdh* at 0,

1010   3, 6, and 9d. *Cdkn1a* product levels were normalized to *Gapdh* and plotted as the iKO:Flox

1011   ratio. Each point represents the mean ratio ±SD from n=3 experiments. **(E)** Flox and iKO cells

1012   were fixed at 6d and stained with a p21 antibody (green) and DAPI. **(F)** Nuclear p21

1013   fluorescence was quantified by outlining the DAPI-stained nucleus of each cell in ImageJ and

1014   measuring the mean p21 pixel intensity. Each bar represents the mean intensity ±SD from n=3

1015   experiments (653-658 cells per bar). RFU = Relative Fluorescence Units.

1016

1017   **Fig 7. cGAS and STING are recruited to micronuclei. (A)** Mouse fibroblasts were treated

1018   with 4-OHT (iKO), transfected with plasmids encoding mCherry or mCherry-cGAS, collected,

1019   and immunoblotted with antibodies to mCherry and GAPDH. **(B)** iKO cells expressing mCherry

1020   or mCherry-cGAS (magenta) were fixed at 1-2d and stained with DAPI (DNA; blue). Arrowheads

1021   point to a cGAS-positive micronucleus. Scale bars, 20µm. **(C)** Cells were treated with DMSO

1022   (Flox) or 4-OHT (iKO) for 3-4d and immunoblotted with antibodies to cGAS, STING, ArpC2,

1023   tubulin, actin, and GAPDH. **(D)** Flox and iKO cells were fixed at 3d and stained with cGAS

1024   (magenta) or STING (green) antibodies and DAPI. Arrowheads point to cGAS- or STING-

1025   positive micronuclei. 38.7% of micronuclei-containing iKO cells were cGAS-positive (75 cells

1026   from n=2 experiments). Insets show STING localization around an iKO micronucleus. **(E)** iKO

1027   cells were fixed at 3d and stained with STING antibodies (green) and either a GRP94 or GM130

1028   antibody (yellow). **(F)** Cells were treated with DMSO or 4-OHT for 3d, switched to media

1029   containing DMSO or RU.521, and population doubling times were quantified daily from 4-6d.

1030   Each bar represents the mean doubling time ±SD from n=5 experiments.

1031

1032   **Fig 8. Micronuclei form due to chromatin segregation errors and premature mitotic exits**

1033   **in ArpC2 iKO cells. (A-B)** Live H2B-GFP-expressing mouse fibroblasts were imaged every

1034   12min from 1-2d following DMSO (Flox) or 4-OHT (iKO) exposure. Panel A shows completed

1035   mitoses, while panel B shows iKO cells exiting mitosis prematurely. Arrowheads in the final 3-6

1036   frames point to micronuclei biogenesis events. Scale bars, 5µm. **(C)** The % of completed

1037   mitoses yielding micronuclei was calculated by dividing the number of mitoses yielding at least

1038   one micronucleus by the total number of completed mitoses captured during live imaging (Flox

1039   n=63; iKO n=52; pooled from 13 Flox and 22 iKO experiments). **(D)** All completed mitoses of

1040   Flox and iKO cells were binned into categories based on the number of timepoints observed in

1041   metaphase (Flox n=63; iKO n=52). Black and grey bars represent Flox and iKO data,

1042   respectively. **(E)** The % of premature mitotic exits was calculated by dividing the number of

1043   cells that entered prophase and returned to interphase without completing anaphase by the total

1044   number of cells that entered prophase (Flox n=71; iKO n=70; pooled from 13 Flox and 22 iKO

1045   experiments). 10 of the 70 iKO cells (14.3%) formed micronuclei. **(F)** iKO cells were fixed at 7d

1046   and stained with a γH2AX antibody (green) and DAPI (DNA; blue). Movies for panels A and B

1047   appear in Supporting Information (S1-S7 Videos).

1048

1049   **Fig 9. Arp2/3 complex depletion reduces actin filament density in the chromatin-**

1050   **containing central spindle during metaphase. (A)** Mouse fibroblasts were treated with

1051   DMSO for 1-2d, fixed, and stained with phalloidin (F-actin; magenta), an anti-tubulin antibody

1052   (microtubules; green), anti-ArpC2 antibodies (yellow), and DAPI (DNA; blue). Images represent

1053   the lower, middle, and upper regions of metaphase cells. Scale bars, 5µm. **(B)** The DNA-

1054   containing region was isolated from the middle spindle in A, magnified, and subjected to line-

1055   scan fluorescence intensity analyses. Plot profiles depict the pixel intensity values for tubulin

1056   (green), F-actin (magenta), ArpC2 (yellow), and DNA (blue) along the dashed lines drawn

1057   across the representative metaphase region. Color-coded arrowheads highlight the overlapping

1058   peaks of tubulin, F-actin, and ArpC2 and the inverse intensity pattern for DNA. RFU = Relative

1059   Fluorescence Units. **(C)** Mouse fibroblasts were treated with DMSO (Flox) or 4-OHT (iKO) for

1060   1-2d, fixed, and stained with phalloidin, an anti-tubulin antibody, and DAPI. The chromatin mass

1061   at the central metaphase spindle was outlined in ImageJ (dashed shapes) or a plot-profile line

1062   was drawn through it (dashed line). The bottom DAPI-stained iKO cell panel was overexposed

1063   in order to draw attention to the presence of 2 chromatin fragments erroneously missing from

1064   the metaphase plate (arrowheads). **(D)** Fluorescence intensities of F-actin and microtubules

1065   were measured in chromatin areas outlined as in panel C. Each bar represents the mean

1066   intensity ±SD from n=12 metaphase chromatin regions compiled from 3 experiments. **(E)** Plot

1067   profiles performed as in panel B depict the pixel intensity values for tubulin, F-actin, and DNA

1068   along the dashed lines drawn across the metaphase region from the representative iKO cell in

1069   panel C.

1070

1071   **Fig 10. Anaphase microtubules are unbalanced in ArpC2 iKO cells. (A)** Mouse fibroblasts

1072   were treated with DMSO (Flox) or 4-OHT (iKO) for 1d, fixed, and stained with a tubulin antibody

1073   (green) and DAPI (DNA; blue). Scale bar, 5μm. **(B)** Line-scan plot profiles depict the pixel

1074   intensity of tubulin along the horizontal dashed lines drawn across the representative mitotic

1075   spindles from panel A. Arrowheads highlight sites of aberrant chromatin localization. RFU =

1076   Relative Fluorescence Units.

1077 **Supporting Information Captions**

1078

1079 **S1 Table. Primers.**

1080

1081 **S2 Table. Immunofluorescence and immunoblotting reagents.**

1082

1083 **S1 Video. Timelapse movie of a H2B-GFP-expressing Flox cell (see Fig 8A first row).**

1084

1085 **S2 Video. Timelapse movie of a H2B-GFP-expressing Flox cell (see Fig 8A second row).**

1086

1087 **S3 Video. Timelapse movie of a H2B-GFP-expressing iKO cell (see Fig 8A third row).**

1088

1089 **S4 Video. Timelapse movie of a H2B-GFP-expressing iKO cell (see Fig 8A fourth row).**

1090

1091 **S5 Video. Timelapse movie of a H2B-GFP-expressing iKO cell (see Fig 8B first row).**
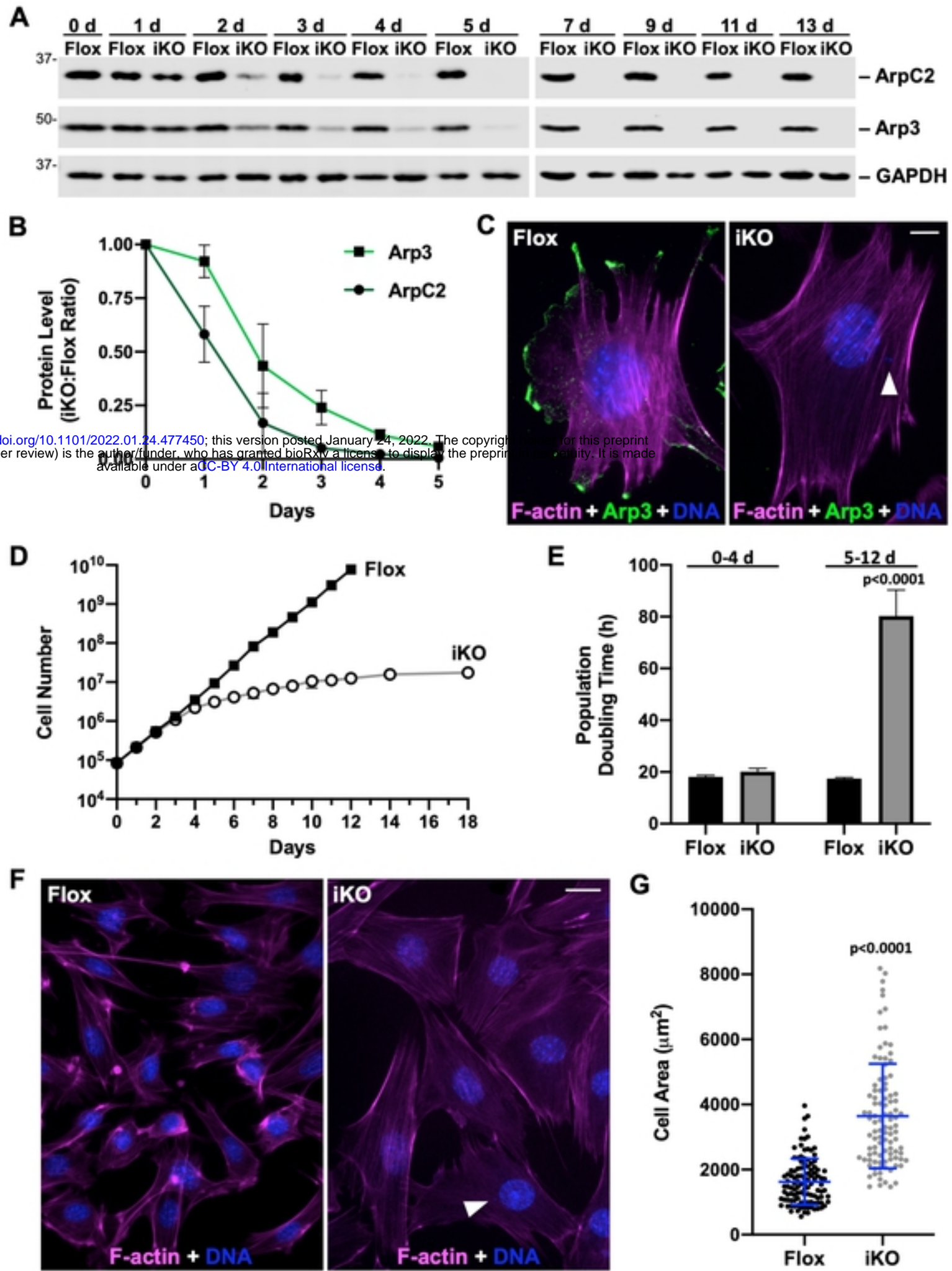
1092

1093 **S6 Video. Timelapse movie of a H2B-GFP-expressing iKO cell (see Fig 8B second row).**

1094

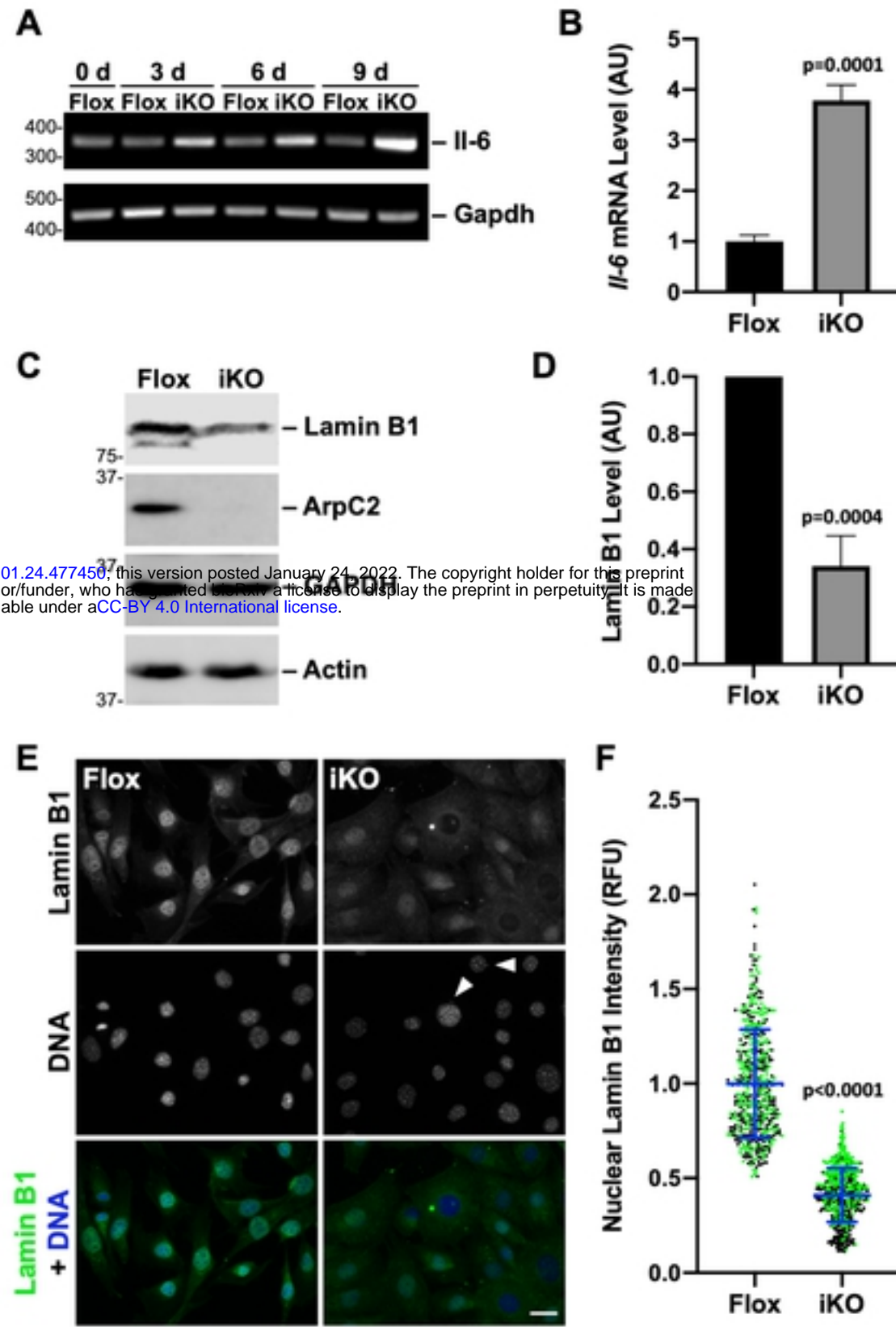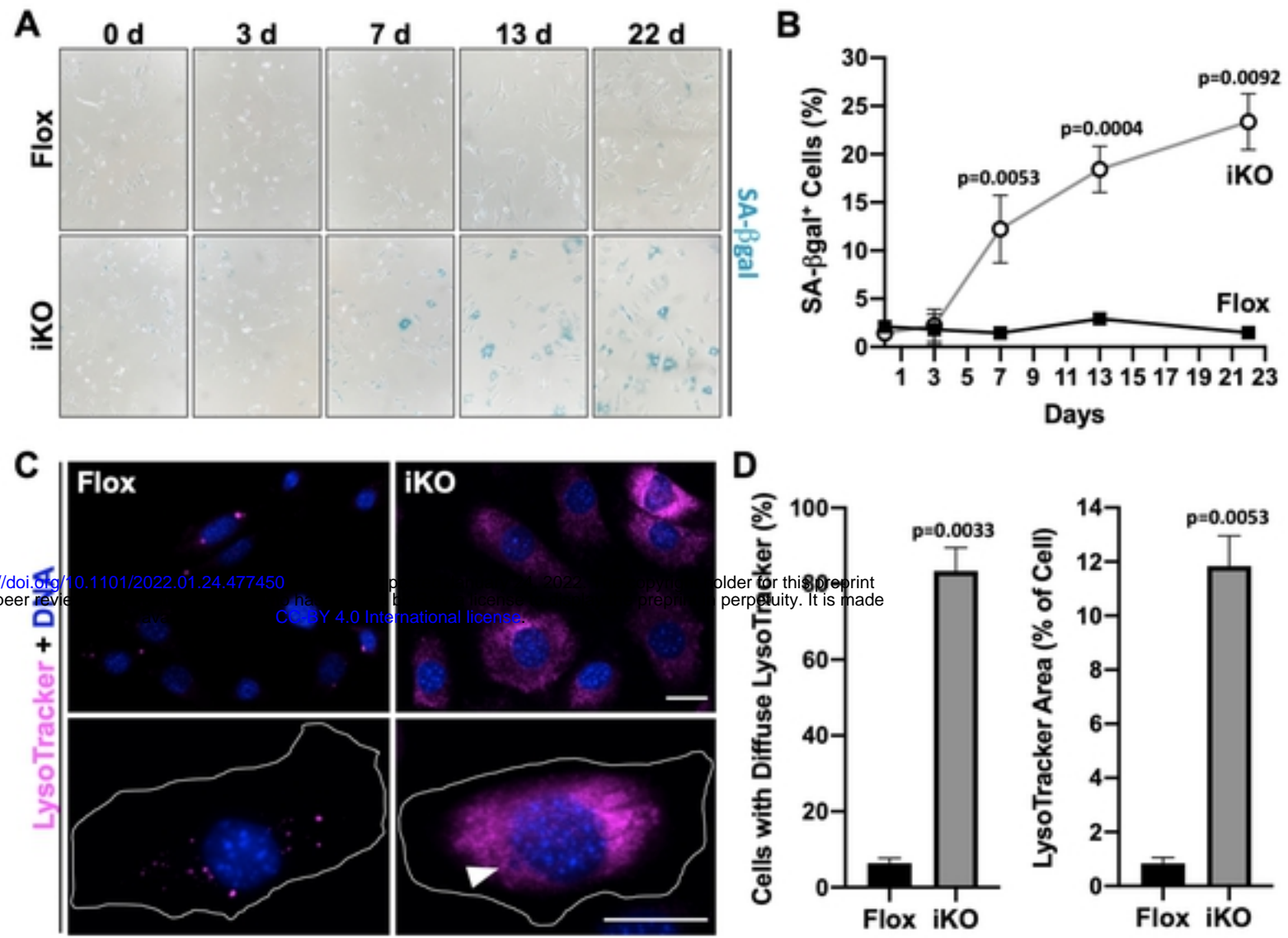1095 **S7 Video. Timelapse movie of a H2B-GFP-expressing iKO cell (see Fig 8B third row).**

A

	0 d	3 d		6 d		9 d	
	Flox	Flox	iKO	Flox	iKO	Flox	iKO

400-
300-   — Il-6

500-
400-   — Gapdh

B

p=0.0001

Il-6 mRNA Level (AU)

Flox   iKO

C

Flox   iKO

— Lamin B1

75-
37-   — ArpC2

37-   — (Gapdh)

37-   — Actin

D

p=0.0004

Lamin B1 Level (AU)

Flox   iKO

E

Flox   iKO

Lamin B1

DNA

Lamin B1 + DNA

F

p<0.0001

Nuclear Lamin B1 Intensity (RFU)

Flox   iKO

2

3

4

5

A

Flox iKO

50- — p53
1.0 2.0

50- — phospho-p53
1.0 10.7

37- — ArpC2
1.0 0.1

50- — Tubulin

— Actin
37-

37- — GAPDH

B

Flox iKO

p53

DNA

p53 + DNA

C

0 d   3 d   6 d   9 d
Flox Flox iKO Flox iKO Flox iKO

400- — Cdkn1a
300-

500- — Gapdh
400-

500- — β-actin
400-

D

$Cdkn1a$ mRNA Level (iKO:Flox Ratio)

p=0.0475

p<0.0001

Days

E

Flox iKO

p21

DNA

p21 + DNA

F

Nuclear p21 Intensity (RFU)

p=0.0168

Flox iKO

6

A

iKO

mCherry | mCh-cGAS

250-
150-
100-
75-

50-

37-

25-

anti-mCherry

37-

anti-GAPDH

B

iKO

| mCherry | DNA | mCherry DNA |
| mCherry-cGAS | DNA | mCh-cGAS DNA |

C

Flox iKO

50- — cGAS
37- — STING
37- — ArpC2
50- — Tubulin
— Actin
37-
37- — GAPDH

D

Flox | iKO

| cGAS | DNA | cGAS DNA | cGAS | DNA | cGAS DNA |
| STING | DNA | STING DNA | STING | DNA | STING DNA |

E

iKO

| STING | STING DNA | GRP94 DNA |
| STING | STING DNA | GM130 DNA |

F

Flox | iKO

Population Doubling Time (h)

p=0.060

p=0.002

DMSO RU.521 DMSO RU.521

A

| | 0 min | 12 min | 24 min | 36 min | 48 min | 60 min | 72 min | 84 min | 96 min |

Flox

| | 0 min | 12 min | 24 min | 36 min | 48 min | 60 min | 72 min | 84 min | 120 min |

iKO

| | | | | | | 108 min | 180 min | 228 min |

B

| | 0 min | 24 min | 36 min | 60 min | 84 min | 108 min | 120 min | 144 min | 168 min | 216 min |

iKO

C Complete Mitoses Yielding Micronuclei (%)
p=0.0139
Flox / iKO

D Complete Mitoses (%)
p<0.0001
p<0.0001
Frames in Metaphase: 1, 2+

E Premature Mitotic Exits (%)
p=0.0031
Flox / iKO

F iKO
Prometaphase   Metaphase
Early Anaphase   Late Anaphase
γH2AX + DNA

8