

1 **G-quadruplex DNA drives genomic instability and represents a targetable**  
2 **molecular abnormality in ATRX-deficient malignant glioma**

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25

26 **Abstract**

27           Mutational inactivation of *ATRX* ( $\alpha$ -thalassemia mental retardation X-linked)  
28 represents a defining molecular alteration in large subsets of malignant glioma. Yet the  
29 pathogenic consequences of *ATRX* deficiency remain unclear, as do tractable  
30 mechanisms for its therapeutic targeting. Here we report that *ATRX* loss in isogenic  
31 glioma model systems induces replication stress and DNA damage by way of G-  
32 quadruplex (G4) DNA secondary structure. Moreover, these effects are associated with  
33 the acquisition of disease-relevant copy number alterations over time. We then  
34 demonstrate, both *in vitro* and *in vivo*, that *ATRX* deficiency selectively enhances DNA  
35 damage and cell death following chemical G4 stabilization. Finally, we show that G4  
36 stabilization synergizes with other DNA-damaging therapies, including ionizing radiation,  
37 in the *ATRX*-deficient context. Our findings reveal novel pathogenic mechanisms driven  
38 by *ATRX* deficiency in glioma, while also pointing to tangible strategies for drug  
39 development.

40 Infiltrating gliomas are the most common primary brain tumors and, despite  
41 considerable molecular and clinical heterogeneity, remain uniformly deadly in the face of  
42 aggressive surgical and cytotoxic treatment regimens<sup>1</sup>. Recent large-scale genomic  
43 profiling has shown that inactivating mutations in *ATRX* ( $\alpha$ -thalassemia mental  
44 retardation X-linked) characterize large subclasses of both adult and pediatric glioma<sup>2-4</sup>.  
45 Despite these striking correlations, however, the precise mechanisms by which *ATRX*  
46 mutation promotes gliomagenesis remain unclear. Indeed, germline mutations in *ATRX*  
47 do not predispose affected individuals to cancer, causing instead a rare, congenital  
48 neurodevelopmental condition associated with intellectual disability (*ATR-X syndrome*)<sup>5</sup>.  
49 *ATRX* encodes a chromatin binding protein widely implicated in epigenetic regulation  
50 and remodeling<sup>6-12</sup>, suggesting that epigenomic dysfunction may, at least in part,  
51 underlie the oncogenic effects of *ATRX* deficiency. *ATRX* loss has also been implicated  
52 in alternative lengthening of telomeres (ALT), an abnormal telomerase-independent  
53 mechanism of telomere maintenance based on homologous recombination<sup>13,14</sup>. Finally,  
54 *ATRX* deficiency has been repeatedly linked to replication stress, DNA damage, copy  
55 number aberrations, and aneuploidy<sup>15-20</sup>. Whether and how such genomic instability  
56 contributes to the initiation or evolution of malignant glioma remains unclear.

57 *ATRX* binds widely across the genome at sites featuring tandem repeats and  
58 CpG islands<sup>21</sup>. Many such loci are GC-rich and susceptible to forming G-quadruplexes  
59 (G4s), abnormal secondary structures implicated in both transcriptional dysregulation  
60 and DNA damage. Accordingly, it has been hypothesized that, among its various  
61 functionalities, *ATRX* serves to resolve G4s genome-wide and mitigate their deleterious  
62 consequences<sup>21,22</sup>. The tendency of G4s to stall replication forks underlies their  
63 association with DNA damage<sup>23</sup>. Chemical stabilization of G4s induces replication  
64 stress at genomic loci prone to G4 formation<sup>24</sup>, and also promotes DNA damage and  
65 apoptosis in neural progenitor cells<sup>25</sup>. Moreover, recent work suggests that G4-induced

66 replication stress at telomeres may drive ALT in the ATRX-deficient setting through  
67 induction of homologous recombination<sup>13</sup>. Indeed, G4 stabilization hampers the ability of  
68 forced ATRX expression to abrogate the ALT phenotype *in vitro*. Taken together, these  
69 findings provide compelling links between ATRX, G4 biology, and genomic instability.  
70 Whether ATRX deficiency directly induces G4 formation and DNA damage, however,  
71 remains unestablished, as does the impact of G4s on the pathogenesis of ATRX-  
72 deficient neoplasia. Moreover, therapeutic strategies leveraging G4 biology in the  
73 selective targeting of ATRX-deficient cancers have not been extensively explored.

74 To characterize the role of G4-mediated genomic instability in glioma biology, we  
75 reversibly inactivated ATRX in an isogenic normal human astrocyte (NHA) model. We  
76 found that ATRX loss increased G4 formation, replication stress, and DNA damage  
77 genome-wide. Moreover, ATRX-deficient NHA's accumulated clinically relevant copy  
78 number aberrations (CNAs) at an accelerated rate relative to ATRX-intact counterparts.  
79 Chemical G4 stabilization was associated with enhanced DNA damage and cell death in  
80 ATRX-deficient context. Moreover, ATRX-mutant glioma stem cell (GSC) xenografts  
81 were selectively sensitive to G4 targeting *in vivo*. Finally, G4 stabilization in ATRX-  
82 deficient cells effectively synergized with other DNA-damaging treatment strategies,  
83 including ionizing radiation. These findings clarify distinct mechanisms by which G4s  
84 influence ATRX-deficient glioma pathogenesis and indicate that G4-stabilization may  
85 represent an attractive therapeutic strategy for the selective targeting of ATRX-mutant  
86 cancers.

87

## 88 **Results**

89 ***ATRX deficiency promotes G4 formation, DNA damage, and chromosome***  
90 ***breaks.*** To model the genomic consequences of ATRX deficiency in a glioma-relevant  
91 cellular context, we performed shRNA-mediated ATRX knockdown in TERT and E6/E7-

92 transformed normal human astrocytes (NHAs). Several studies have effectively  
93 employed immortalized NHAs to delineate key aspects of glioma biology<sup>26-30</sup>. In our  
94 investigations, we employed two distinct hairpin constructs to silence *ATRX*—shATRX1  
95 and shATRX2—the latter of which was driven by a doxycycline (dox)-inducible promoter  
96 (FIG. 1a). This framework allowed for the analysis of both immediate and long-term  
97 effects of *ATRX* deficiency as well as their reversibility. Using a monoclonal antibody  
98 known to recognize G4 structures *in situ* (1H6), we then demonstrated that *ATRX*  
99 deficiency increased nuclear G4s relative to levels seen in control shRNA-expressing  
100 parental NHAs (shCon), an effect that was reversible upon restored *ATRX* expression  
101 (FIG. 1b-1c). Increased G4s were also found in murine neuroepithelial progenitor cells  
102 featuring inactivated *Atrx* (Supplementary FIG. 1a). The specificity of 1H6 for DNA-  
103 based secondary structures was confirmed by DNAase treatment, which eradicated  
104 immunolabelling, and RNase treatment, which did not, in NHAs treated with the G4  
105 stabilizing agent CX-3543 (see below, Supplementary FIG. 1b). We also employed a  
106 synthetic single-chain antibody (hf2) to immunoprecipitate G4s in both *ATRX*-intact and  
107 *ATRX*-deficient contexts. hf2 specificity was validated by gel-shift assay showing  
108 specific capture of synthesized Kit2 nucleotides independently from random ssDNA and  
109 dsDNA (Supplementary FIG. 1c). We then performed pulldowns in our isogenic NHAs,  
110 finding that *ATRX* deficiency significantly increased the qPCR enrichment of known G4  
111 sites within the *MYC* and *ZNF618* loci, as well as in telomeric regions on chromosomes  
112 1, 2, and X (FIG. 1d)<sup>31-33</sup>. Consistent with the notion that *ATRX* resolves G4s as part of  
113 its normal functionality, we found a distinct absence of colocalization between *ATRX* and  
114 G4 immunofluorescence in *ATRX*-intact NHAs (FIG. 1e). Finally, functional studies  
115 demonstrated that *ATRX* knockdown failed to induce significant changes in apoptosis,  
116 BrdU incorporation, or cell cycle profile (Supplementary FIG. 1d-1f). Taken together,  
117 these findings confirm, in a true isogenic system, that *ATRX* deficiency promotes G4

118 formation. Moreover, they indicate that, at least in this glioma-relevant context,  
119 increased G4s as a consequence of ATRX deficiency are insufficient to drive apoptosis  
120 or impact cellular proliferation.

121 We then examined whether the G4s induced by ATRX deficiency promoted  
122 replication stress and DNA damage, as suggested by prior literature<sup>34</sup>. We found that  
123 ATRX knockdown significantly increased  $\gamma$ -H2AX-positive foci by immunofluorescence  
124 (FIG. 2a-2b), and did so in a pattern the extensively colocalized with nuclear G4  
125 distribution (FIG. 2c). Moreover, these changes were accompanied by engagement of  
126 the replication stress pathway, as evidenced by upregulated levels p-CHK1 and p-KAP1  
127 on Western blot (FIG. 2d). To determine whether increased levels of DNA damage in  
128 the ATRX-deficient setting might lead to structural abnormalities in chromosomes, we  
129 performed metaphase cytogenetic analysis coupled with telomere-fluorescence in situ  
130 hybridization (FISH) in Par-Con and ATRX-deficient NHAs uniformly aged to 15  
131 passages. Consistent with multiple prior reports, ATRX knockdown in this context was  
132 not associated with an obvious ALT phenotype (Supplementary Figure 2a-2b). However,  
133 we consistently observed increased numbers of chromosome breaks in ATRX-deficient  
134 NHAs relative to shCon counterparts (FIG. 2e and Supplementary FIG. 2b). These data  
135 establish pathogenic links between G4s arising with ATRX deficiency and the  
136 generalized genomic instability characteristic of ATRX-mutant tumors and cell lines.

137 ***ATRX deficiency drives clinically relevant CNA formation.*** Having confirmed  
138 that ATRX deficiency induces DNA damage and structural abnormalities in  
139 chromosomes, likely through G4-mediated mechanisms, we sought to assess whether  
140 these biological processes might promote acquisition of CNAs in ATRX-deficient tumors.  
141 *ATRX* mutations in adult glioma arise almost exclusively in the setting of concurrent  
142 mutations in *TP53* and either *IDH1* or *IDH2*. The glioma subclass defined by this  
143 combined genotype, termed “IDHmut-noncode1”<sup>2</sup>, features almost uniformly low-level

144 *ATRX* expression and exhibits a characteristic pattern of CNAs, distinct from that  
145 commonly seen in other adult disease subclasses (Supplementary FIG. 3). Moreover,  
146 multiple CNAs recurrently featured in *ATRX*-deficient glioma mobilize established  
147 oncogenic and/or tumor suppressive loci, including *MYC* and *CDKN2A*<sup>2</sup>, implying that  
148 such structural abnormalities may contribute to the malignant evolution of this inexorably  
149 progressive cancer.

150 To experimentally model CNA formation in the *ATRX*-deficient setting, we aged  
151 our isogenic NHAs in culture, monitoring DNA copy number by SNP array at 5 and 15  
152 passages—~1 month and ~3 months in culture, respectively (Supplementary FIG. 3).  
153 We found that while both sets of isogenics demonstrated increased CNAs over time,  
154 *ATRX*-deficient NHAs exhibited a distinct pattern of gains and losses that included larger  
155 (> 1 Mb) alterations not seen *ATRX*-intact counterparts (FIG. 3a). Analysis of TCGA  
156 SNP data revealed a similar subset of broad alterations included within the CNA profile  
157 of IDHmut-noncode gliomas (FIG. 3b). Moreover, two of the broad CNAs associated  
158 with *ATRX* deficiency *in vitro*, involving 12p gain and 14q loss, recapitulated events  
159 commonly seen in the IDHmut-noncode glioma subclass and associated with relatively  
160 unfavorable prognosis when present (FIG. 3c-3f). Taken together, these findings  
161 support the notion that G4-mediated DNA damage induces specific types and patterns of  
162 CNAs in *ATRX*-deficient glioma, which in turn influence the malignant evolution of the  
163 disease.

164 ***Chemical G4 stabilization selectively targets *ATRX*-deficient NHAs and***  
165 ***enhances their sensitivity to ionizing radiation.*** As indicated above, the pronounced  
166 effects of *ATRX* deficiency on G4 formation and replication stress in NHAs were not  
167 associated with increased cell death a baseline. Nevertheless, we reasoned that  
168 compensatory mechanisms to resolve G4s and otherwise maintain genomic integrity  
169 were likely under increased stress, and that chemical stabilization of G4s might,

170 therefore, selectively enhance DNA damage to an unsustainable degree in the ATRX-  
171 deficient context. To evaluate the therapeutic potential of this synthetic lethal approach,  
172 we treated our isogenic NHAs in culture with increasing concentrations of CX-3543  
173 (Quarfloxin), an established G4-stabilizing agent<sup>35,36</sup>. We found that ATRX knockdown,  
174 in both constitutive and inducible systems, was associated with increased sensitivity to  
175 CX-3543 (IC<sub>50</sub> = 42.449 nM (shATRX1) vs 328.835 nM (shCon) in constitutive NHAs  
176 and IC<sub>50</sub> = 357.424 nM (pre-induction) vs 53.415 nM (shATRX2) vs 247.700 nM (post-  
177 induction); FIG. 4a-4b). Clonogenicity studies revealed similarly enhanced vulnerability  
178 to CX-3543 in ATRX-deficient NHAs as well as normally ATRX-intact GSCs (TS 543)  
179 subjected to ATRX knockdown (FIG. 4c-4d). Restoring ATRX expression reverted  
180 NHAs to baseline levels of sensitivity (FIG. 4b).

181  $\gamma$ -H2AX immunofluorescence demonstrated dramatically increased levels of DNA  
182 damage in ATRX-deficient NHAs treated with CX-3543, accompanied by activation of  
183 the replication stress pathway as determined by western blot (FIG. 4e-4h). Moreover,  
184 53BP1-positive foci of DNA damage demonstrated extensive co-localization with G4s by  
185 immunofluorescence on confocal microscopy (Supplementary FIG. 4). Once again,  
186 these effects were reversed following ATRX re-expression (FIG. 4f, 4h). Annexin V flow  
187 cytometry confirmed that the heightened sensitivity of ATRX-deficient NHAs to CX-3543  
188 reflected increased apoptosis, and this enhanced cell death followed the kinetics of  
189 replication stress pathway activation in both constitutive and inducible isogenic contexts  
190 (FIG. 5a-5d). In total, these results indicate that chemical stabilization of G4 structures  
191 selectively promotes cell death in the ATRX-deficient context, likely by inducing toxic  
192 levels of DNA damage.

193 The experimental links, described above, between replication stress, DNA  
194 damage, and CX-3543 treatment prompted us to consider whether G4 stabilization might  
195 enhance the therapeutic efficacy of established DNA-damaging treatment strategies,



196 particularly in ATRX-deficient setting. To evaluate this possibility, we subjected vehicle  
197 and CX-3543-treated isogenic NHAs, cultured in soft agar, to increasing doses of either  
198 ionizing radiation (IR) or hydroxyurea (HU), assessing viable colonies at 21 days. We  
199 found that CX-3543 treatment potentiated the cytotoxicity of both IR and HU, and while  
200 these effects were significant for all cellular genotypes, they were particularly strong in  
201 ATRX-deficient NHAs (FIG. 5e-5f). Moreover, restoring ATRX expression markedly  
202 dampened the extent of cytotoxic synergy. These findings inform additional  
203 therapeutically relevant strategies combining chemical G4 stabilization with well-  
204 established treatment modalities in the targeting of ATRX-deficient cancer.

205 ***ATRX deficiency enhances sensitivity to chemical G4 stabilization in vivo.***

206 Having established the increased sensitivity of ATRX-deficient NHAs and GSCs to  
207 chemical G4 stabilization in cell culture, we sought to ascertain whether this approach  
208 could exhibit a similar degree of efficacy *in vivo*. To this end, we employed an *ATRX*-  
209 mutant, patient-derived GSC line (JHH-273) capable of forming tumors in murine hosts  
210 when embedded in the hind flank<sup>37</sup>. Following cellular implantation, we subjected  
211 xenografted mice to daily intravenous treatment with either CX-3543 or vehicle and  
212 monitored tumor growth over time. We found that CX-3543 dramatically slowed the  
213 growth of JHH-273 flank tumors (FIG. 6a-6b and Supplementary FIG. 5a) and  
214 significantly prolonged survival xenografted mice (FIG. 6c). Histopathological  
215 examination of CX-3543-treated xenografts revealed cellular depopulation, reduced  
216 proliferative activity by Ki-67 immunostaining, and increased  $\gamma$ -H2AX-positive DNA  
217 damage foci relative to untreated counterparts, recapitulating *in vitro* findings (FIG. 6d).  
218 Notably, telomere FISH failed to reveal changes in the level of ALT in residual viable  
219 tumor following CX-3543 treatment (FIG. 6d). To ascertain whether these effects were  
220 specific to the *ATRX*-mutant context, we performed analogous xenograft experiments  
221 using *ATRX*-wild type TS 543 cells. In these studies, we found that CX-3543 treatment

222 had no appreciable effect on either xenograft growth or mouse survival (FIG. 7a-7b and  
223 Supplementary FIG. 5b). However, when we subjected these same GSCs to ATRX  
224 knockdown, they were rendered sensitive to CX-3543 to an extent similar to that  
225 observed for JHH-273 cells (FIG. 7c-7d and Supplementary FIG. 5c). ATRX knockdown  
226 also recapitulated histopathological effects on cellular depopulation, proliferative activity,  
227 and  $\gamma$ -H2AX-positivity (FIG. 7e-7f). Consistent with prior reports<sup>16,19</sup>, ATRX knockdown  
228 was not associated with ALT in TS 543 cells (Supplementary FIG. 7e-7f). Taken  
229 together, these *in vivo* findings further support the therapeutic potential for chemical G4  
230 stabilization in the selective targeting of ATRX-deficient glioma.

231

## 232 Discussion

233 As indicated above, loss-of-function mutations in *ATRX* likely play central  
234 pathogenic roles in several distinct tumor variants, including multiple subtypes of  
235 incurable glioma. That *ATRX* itself encodes a chromatin regulatory protein suggests that  
236 epigenomic dysfunction underlies, at least in part, the oncogenic sequelae of its  
237 inactivation. To this point, we recently demonstrated that ATRX deficiency induces  
238 extensive chromatin remodeling and transcriptional shifts in putative glioma cells of  
239 origin, driving disease-relevant phenotypes that modulate both cellular motility and  
240 differentiation<sup>38</sup>. However, the full impact of ATRX deficiency on tumor initiation and  
241 evolution almost certainly includes other molecular mechanisms. The association of  
242 *ATRX* mutation and ALT<sup>14,19</sup>, for instance, is now extensively described and provides a  
243 vehicle to telomerase-independent immortalization in affected cancer cells. Moreover,  
244 recent work has linked ALT to the well-characterized genomic instability induced by  
245 ATRX deficiency<sup>13,39</sup>.

246 The pathogenic consequences of ATRX-dependent genomic instability in the  
247 context of cancer are unknown. Abundant prior work has demonstrated links between

248 ATRX deficiency, DNA damage, CNA development, and aneuploidy<sup>15-20</sup>. Indeed, p53-  
249 dependent apoptosis derived from genomic instability in the neuroepithelial progenitor  
250 compartment likely underlies the neuronal depopulation, microcephaly, and mental  
251 retardation associated with ATR-X syndrome<sup>40</sup>. Replication stress has been extensively  
252 implicated as a root cause of genomic instability in ATRX-deficient cells<sup>16,18,41</sup>. In  
253 addition to activating DNA damage pathway signaling, replication fork stalling and  
254 collapse can generate double-strand breaks and defective chromosome condensation  
255 during mitosis, both of which are known to drive CNAs and aneuploidy of the kind seen  
256 in *ATRX*-mutant glioma<sup>42-44</sup>. Recent work strongly supports the notion that the  
257 replication stress characterizing ATRX-deficient cells derives, at least in part, from G4  
258 DNA secondary structure<sup>13,23-25</sup>. ATRX binds widely at GC-rich genomic sites  
259 susceptible to forming G4s<sup>21</sup>, and restored ATRX expression in *ATRX*-mutant cell lines  
260 mitigates G4-associated phenotypes such as ALT<sup>13</sup>. Such data implies that ATRX may  
261 serve to protect the genome from unwanted G4 formation and the potentially deleterious  
262 consequences of ensuing genomic instability. Our findings support this conjecture by  
263 demonstrating, for the first time, that ATRX deficiency potently and reversibly induces  
264 G4 formation in an isogenic experimental context. As such, they confirm a novel  
265 functionality for a SWI/SNF epigenetic regulator already widely implicated in chromatin  
266 remodeling, structure, and organization.

267         That increased G4s were accompanied by replication stress signaling, DNA  
268 damage at spatially overlapping sites, and disease-relevant patterns of CNAs in our cell  
269 line models provides additional evidence that this pathobiological cascade features in  
270 *ATRX*-mutant neoplasia. Prior computational analysis across multiple tumor types  
271 established significant correlations between CNA breakpoints and genomic sites  
272 enriched in putative G4-forming sequences<sup>45</sup>, firmly implicating G4s in the process of  
273 cancer-associated CNA acquisition. In our NHA models, ATRX knockdown led to a

274 distinct CNA profile over time enriched in alterations over 1 Mb in size. While this  
275 pattern did not completely mirror the known CNA signature of *ATRX*-mutant gliomas<sup>2</sup>, it  
276 did recapitulate key elements involving larger, arm-level events. In particular, two CNAs  
277 (12p gain and 14q loss) were reminiscent of analogous alterations in human tumors  
278 associated with unfavorable prognosis. These data speak directly to the premise that  
279 CNA mobilization, driven at least in part by G4-mediated DNA damage, promotes  
280 malignant evolution in *ATRX*-deficient gliomas. As this tumor subtype characteristically  
281 progresses slowly over time<sup>46</sup>, such mechanistic insights are consistent with established  
282 clinical features.

283         Due to its sheer prevalence in glioma, *ATRX* deficiency represents a molecular  
284 target of intriguing therapeutic potential. That being said, effective strategies to drug an  
285 inactivated epigenetic regulator are not immediately obvious, as they might be in the  
286 setting of more conventional, kinase-predominant, oncogenic signaling networks. Given  
287 these challenges, leveraging specific vulnerabilities engendered by *ATRX* loss might  
288 offer alternative approaches. In particular, the longstanding association of *ATRX*  
289 deficiency with genomic instability, confirmed in this report, presents a tangible  
290 opportunity to explore a synthetic lethality paradigm, akin to that of Poly (ADP-ribose)  
291 polymerase (PARP) inhibitors in the treatment of *BRCA1*-inactivated breast cancer<sup>47</sup>.  
292 While the observed level of DNA damage in our *ATRX*-deficient cell line and tumor  
293 models was insufficient to induce apoptosis in isolation, due in part to coincident *TP53*  
294 inactivation, we hypothesized that its targeted enhancement would overwhelm  
295 compensatory mechanisms maintaining cell viability. Moreover, our identification of G4s  
296 as the likely source of *ATRX*-deficient genomic instability provided a viable approach to  
297 therapeutic selectivity.

298         CX-3543 is an established G4 stabilizing agent that, despite limited bioavailability,  
299 has advanced to Phase II clinical trials in at least one instance<sup>35</sup>. In our studies, *ATRX*-

300 deficient cell lines and tumors exhibited selective sensitivity to CX-3543 *in vitro* and *in*  
301 *vivo*. Moreover, cell death in these contexts was temporally associated with DNA  
302 damage and replication stress, speaking to likely mechanism of action. We cannot  
303 completely exclude the possibility that CX-3543 exerts some of its cytotoxic effects  
304 though the manipulation of ALT. As alluded to above, prior work has functionally linked  
305 increased G4s and DNA damage at telomeres with ALT induction in ATRX-deficient  
306 cells<sup>13</sup>. Nevertheless, ATRX knockdown was not associated with ALT in our NHA and  
307 TS 543 isogenics, consistent with multiple prior reports<sup>16,17,19</sup>, and CX-3543 failed to alter  
308 the pattern of telomere FISH in *ATRX*-mutant JHH-273 GSC xenografts. Taken together,  
309 these findings strongly suggest that the cytotoxicity of CX-3543 in the ATRX-deficient  
310 context is, at least in large part, mediated by increased DNA damage genome-wide, not  
311 limited to telomeric regions.

312 We also demonstrated that CX-3543 dramatically enhanced the effects of IR and  
313 HU in ATRX-deficient NHAs, highlighting possibilities for effective synergistic  
314 combinations in the clinical setting. Since its introduction almost 40 years ago, IR has  
315 remained one of the most important nonsurgical therapeutic modalities employed in the  
316 treatment of malignant glioma, with demonstrated efficacy across glioma subtypes<sup>48-50</sup>.  
317 Moreover, recent work has shown that ATRX-mutant gliomas in particular exhibit  
318 increased sensitivity to DNA-damaging combinations of IR and chemotherapy<sup>51,52</sup>. This  
319 vulnerability may derive in part from increased genomic instability at baseline. Defective  
320 non-homologous end joining (NHEJ), documented to arise with ATRX deficiency in  
321 preclinical models<sup>17</sup>, may also play a role. Moreover, IDH mutations, which almost  
322 invariable co-occur with ATRX deficiency in adult glioma, are known to disable  
323 homologous recombination (HR)-mediated DNA repair<sup>53-55</sup>. Regardless of the precise  
324 molecular mechanisms at work, therapeutically potentiating an already effective

325 treatment strategy for glioma represents an underexplored approach with the potential  
326 for considerable clinical impact.

327 Whether CX-3543 itself represents an optimal agent for clinical translation in  
328 glioma remains unclear. Phase I trials were reportedly terminated due to poor  
329 compound bioavailability<sup>36</sup>, and no formal blood-brain barrier penetration studies have  
330 been released. Nevertheless, our findings indicate that the targeted approach of G4-  
331 stabilization has considerable therapeutic potential in the treatment of ATRX-deficient  
332 glioma, along with other *ATRX*-mutant cancers. That the strategy is based on a tumor-  
333 specific vulnerability arising in association with an easily assessable biomarker should  
334 facilitate its clinical application, while also minimizing harmful side effects in treated  
335 patients. Moreover, alternative G4 stabilizing agents with more favorable  
336 pharmacokinetic profiles than CX-3543 are currently available for use both as tool  
337 compounds and starting points for chemical derivatization<sup>56,57</sup>.

338 In summary, we firmly implicate G4 secondary structure as a defining  
339 characteristic of *ATRX*-mutant glioma, one that drives disease-relevant genomic  
340 instability and presents opportunities for tangible therapeutic advancement. As such,  
341 our work has important implications for both the molecular pathogenesis of ATRX-  
342 deficient neoplasia as well as the development of more effective drugs specifically  
343 targeting a palette of deadly tumors.

344

## 345 **Methods**

346 **Study design.** The objective of this study was to determine the impact of ATRX  
347 deficiency on G4 formation, DNA damage, and genomic instability in glioma, and assess  
348 the potential of chemical G4 stabilization as a therapeutic strategy in ATRX-deficient  
349 tumors. This was a controlled, laboratory-based, experimental study using cell line  
350 models in culture and in xenografts. ATRX was inactivated by genetic approaches and,

351 in some cases, pharmaceutical agents and/or ionizing radiation were applied. Sample  
352 sizes were determined independently for each experiment without formal power  
353 calculation. No data was excluded from analysis. Unless otherwise specified,  
354 experiments employed three replicates per sample. Endpoints varied by experiment and  
355 are described below, in figure legends, or in the Results section. Histopathological and  
356 immunohistochemical review of xenografts was conducted by a Neuropathologist  
357 (J.T.H.) in a nonblinded fashion. Quantification of G4 and/or  $\gamma$ -H2AX immunostaining in  
358 NHAs was blinded.

359 **Cell culture and generation of ATRX-deficient cell lines.** The parental immortalized  
360 normal human astrocyte line was a gift from R.O. Peiper (UCSF)<sup>58</sup>. TS 543 is a patient-  
361 derived glioma tumorsphere line harboring *PDGFRA* amplification<sup>59</sup> and maintained in  
362 NeuroCult™ NS-A Proliferation media (#05751, Stemcell). ATRX knockdown was  
363 achieved by introducing either a modified FUGW vector (a gift from David Baltimore  
364 (Addgene plasmid # 14883)) carrying an shRNA expression cassette against *ATRX*  
365 (shATRX1) (see Supplementary Table 1 for shRNA sequences), a TRIPZ TET-inducible  
366 vector (Dharmacon) containing a distinct shRNA against ATRX (shATRX2), or a third  
367 shRNA against ATRX (sh590) from the TRC shRNA library (Sigma). shATRX1 and  
368 shATRX2 positive cells were FACS-sorted every 2 passages by fluorescent marker  
369 (RFP) for the top 5% of total population to ensure high shRNA expression. Sh590  
370 positive TS543 cells were subjected to puromycin-based selection.

371 **Proliferation, cell cycle and apoptosis analyses.** Flow-cytometry analyses of  
372 proliferation and cell cycle were performed using the BD Pharmingen BrdU Flow Kit (#  
373 559619). Apoptosis assays were performed using the Dead Cell Apoptosis Kit (#  
374 V13241, Thermo Fisher) with Propidium Iodide (PI) substituted by DAPI to avoid RFP  
375 interference.



376 **In Situ visualization of G-quadruplexes,  $\gamma$ -H2AX and 53BP1.** The 1H6 antibody was  
377 a gift from Dr. Peter M. Lansdorp<sup>60</sup>. For immunostaining, cells were grown in chamber  
378 slides (Nunc Lab-Tek II, cat no. 154526, Thermo Fisher) and synchronized to G0 phase  
379 by 24-hour serum starvation. The cells were digested with 10mg/ml proteinase K for 1h  
380 at 37°C, followed by fixation (4% paraformaldehyde in PBS for 10 min) and  
381 permeabilization (0.5% Tween, 0.2% Triton X-100 in PBS, 10 min). To eliminate RNA-  
382 structures, cells were treated with 20 ug/500 ul RNase A (Invitrogen). To confirm  
383 specificity towards DNA-G4, cells were incubated in 40mM Tris Cl (pH 8), 5mM CaCl<sub>2</sub>,  
384 2mM MgCl<sub>2</sub>, 100 ug/ml BSA alone or including 0.06 U/ul of DNase I (Promega) and 80  
385 gel units/ul of micrococcal nuclease (#M0247S, New England Biolabs) at 37°C for 2h.  
386 For staining, cells were blocked with goat serum (Sigma) for 4h at room temperature,  
387 then incubated with 0.5 ug/ml 1H6 antibody at 4°C overnight. Slides were then washed 5  
388 times with PBST, incubated with Alexa Fluor 488 or 568 goat anti-mouse IgG  
389 (Invitrogen) at room temperature for 2 h, washed 5 times with PBST and mounted with  
390 coverslips using ProLong Gold antifade reagent and DAPI counterstain (Invitrogen). For  
391  $\gamma$ -H2AX monostaining or 53BP1/G4 double staining, cells were treated with or without  
392 100 nM CX-3543 for 3 days prior to synchronization to G0, and stained with  $\gamma$ -H2AX  
393 antibody (# 05-636, Millipore), or 53BP antibody (cat# NB100-304, Novus Biologicals)  
394 together with the 1H6 G4 antibody.

395 **G4 pulldowns.** Plasmid expressing hf2 was a kind gift of Dr. Shankar  
396 Balasubramanian<sup>61</sup>. Hf2 antibodies were expressed in BI21 competent cells and purified  
397 with Protein A Sepharose (#P9424, Sigma) as previously described<sup>62</sup>. For G4 pulldowns,  
398 2  $\mu$ g of hf2 and 50  $\mu$ l of Protein A Dynabeads (#10001D, ThermoFisher) were mixed and  
399 incubated overnight rotating at 4°C. Beads were washed with PBS for 5 times. 10  $\mu$ g of  
400 genomic DNA from NHAs was sonicated and incubated with beads in 0.5% BSA  
401 overnight rotating at 4°C, followed by 6 washes with 10 mM Tris pH 7.4, 100 mM KCl,



402 0.1 % tween and one wash with 10 mM Tris pH 7.4, 100 mM KCl. Bound DNA was  
403 eluted in 50  $\mu$ L of 1% SDS, 0.1 M NaHCO<sub>3</sub> at 30 °C for one hour then purified by  
404 QIAquick PCR Purification Kit (Qiagen) to a final volume of 20  $\mu$ L. The recovered DNA  
405 was used to determine enrichment of telomeric sequence (Tel1, 2 and X) and the  
406 promoter regions of *MYC* and *ZNF618*, using the *ESR1* promoter as a negative control  
407 (See Supplementary Table 2 for primer sequences).

408 **TEL-FISH and metaphase cytogenetic analysis.** For cell lines, resuspended cells  
409 were incubated with Colcemid (0.1  $\mu$ g/ml) at 37 °C for 45 min, resuspended in 0.075 M  
410 KCl and incubated at 37 °C for 10 min, followed by fixation in methanol:acetic acid (3:1)  
411 solution. TEL-FISH was performed according to standard procedures using a CY3-  
412 conjugated, telomere-specific nucleic acid probe: 5'-TTAGGGTTAGGGTTAGGG-3'  
413 (Applied Biosystems). For xenograft tissues, tumors were removed and subjected to  
414 OCT embedding followed by 5  $\mu$ m sectioning. Frozen sections were fixed with 4%  
415 paraformaldehyde for 10 min. After denaturing at 85 °C for 5 minutes in 10 mM Tris-HCL  
416 pH 7.2, 70% formamide, 0.5% blocking solution reagent (Roche), hybridization was  
417 performed as described above.

418 **Cell viability and clonogenic assay.** For standard viability assays, cells (500  
419 cells/well) were incubated with a serial concentration of CX-3543 (10-300nM) for 7 days  
420 in 96-well plates. Cell viability was then assessed with the CellTiter-Glo Luminescent  
421 Assay (Promega) according to manufacturer-recommended procedures. To determine  
422 clonogenic ability, NHA or TS 543 cells were seeded at 5,000 cells/10-cm dish and  
423 incubated with vehicle or 50 nM CX-3543 for 14 days. Cells were fixed with 4%  
424 paraformaldehyde and stained with 0.005% crystal violet in PBS, followed by 3 washes  
425 in PBS and 2 washes in ddH<sub>2</sub>O. For soft agar colony formation assays, 50,000 cells  
426 were seeded in 6-well plates containing 1% bottom layer and 0.5% top layer soft agar.  
427 Cells were then cultured in growth media with or without 50 nM CX-3543. Radiation

428 dosing of 0, 1, 2 or 4 Gy was immediately applied after plating. The 1.5 ml growth media  
429 covering the agar cultures was replenished every week. At day 21, colonies were fixed  
430 with 4% paraformaldehyde for 30 min and stained with 0.005% crystal violet in PBS  
431 overnight. Stained colonies were then washed extensively in PBS and water, and  
432 quantified on a Gelcount colony counter (Oxford Optronix).

433 **SNP arrays.** Genomic DNA was isolated from ATRX-deficient NHAs at passages 5 and  
434 15. As controls, genomic DNA from ATRX-intact parental NHAs was derived at the start  
435 point (P0), P5 and P15. Extracted DNA was subjected to Affymetrix Genome-Wide  
436 Human SNP 6.0 array analysis(cat# 901182, ThermoFisher) according to the  
437 manufacturer's protocol. Preliminary copy number derivation and segmentation was  
438 conducted as previously described<sup>63,64</sup> to obtain CNV segment files with the following  
439 information: chromosome, start position, end position, probe number, and segment  
440 mean value. For analysis, we focused variations with absolute segment mean value >  
441 0.5 for LGG samples and > 0.1 for NHA lines. All variations associated with ChrX and  
442 ChrY were excluded. CNV length was calculated by using the end position minus the  
443 start position. Data were visualized using IGV and GISTIC2.0.

444 **Xenograft experiments.** All animal protocols and procedures were performed in the  
445 xenograft suite at Memorial Sloan-Kettering Cancer Center in accordance with  
446 Institutional Animal Care and Use Committee (IACUC) guidelines. JHH-273 samples  
447 were kind gifts from Dr. Gregory Riggins at the Johns Hopkins University. Tumor  
448 samples were mechanically dissociated and small pieces (0.2 mm<sup>3</sup>/flank) were  
449 embedded into the flanks of nude mice (Taconic Farms). In parallel, ATRX-intact and  
450 ATRX-deficient TS 543 cells at exponential growth phase were dissociated with  
451 Accutase (#07920, Stemcell), resuspended in Neurocult media, mixed with Matrigel  
452 (#356234, Corning) (1:1) and injected into nude mice flanks in a 50 ul mixture containing  
453 5x10<sup>6</sup> cells. Mice were randomized to vehicle or CX-3543 (12.5 mg/kg) treatment

454 groups. Drug delivery occurred via intravenous injection once per day, on a 5 day/week  
455 schedule until health-related defined end points. Tumor volumes were measured by  
456 calipers and calculated using the formula  $(l \times w^2)/2$ , where  $w$  is width and  $l$  is length in  
457 mm. For survival experiments, mice were treated until they reached health-related end  
458 points (2000 mm<sup>3</sup> tumor volume). For growth curve comparisons, all mice in a study  
459 cohort were sacrificed when the first mouse reached the 2000 mm<sup>3</sup> tumor size threshold.  
460 An independent cohort was used for Kaplan-Meier analysis. Xenografted tissues were  
461 removed, weighted and split into two parts. One part was snap frozen for TEL-FISH,  
462 while the other half was subjected to FFPE processing. 5 $\mu$ m FFPE sections were  
463 deparaffinized and subjected to antigen retrieval. Sections were blocked for non-specific  
464 binding with goat serum for 2h, followed by staining with Ki67 (5 $\mu$ g/ml, ab15580, Abcam)  
465 or  $\gamma$ -H2AX (1:1000, # 05-636, Millipore) antibodies at 4°C overnight. Sections were  
466 washed and incubated with secondary antibody. Ki67 staining were counterstained with  
467 Hematoxylin, and  $\gamma$ -H2AX staining were counterstained with DAPI.

468 **Statistics.** Unless otherwise stated, all results, representing at least three independent  
469 experiments, were plotted as mean  $\pm$  SEM. In general, data were statistically analyzed  
470 using unpaired Student's  $t$  tests. Log-rank (Mantel-Cox) test were used to determine the  
471 significance of differences in Kaplan-Meier analysis of LGG patients and of hind flank  
472 xenograft experiments. 2-way ANOVA was used to compare the growth curves of  
473 xenografts and the colony formation assays. P values are represented using \* for  $P <$   
474 0.05, \*\* for  $P < 0.01$ , \*\*\* for  $P < 0.001$ , and \*\*\*\* for  $P < 0.0001$ .

475 **Data and materials availability.** All data (raw and processed) and materials related to  
476 this manuscript will be made available upon request, utilizing material transfer  
477 agreements when appropriate.

478

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492 W.H.W., R.S., and C.D.; Analysis and interpretation of data, Y.W., K.K., T.A.C., and  
493 J.T.H.; Writing, review, and/or revision of the manuscript, Y.W., K.K., T.A.C., and J.T.H.;  
494 Administrative, technical, or material support, G.J.R., K.K., E.P.S., T.A.C., and J.T.H.;  
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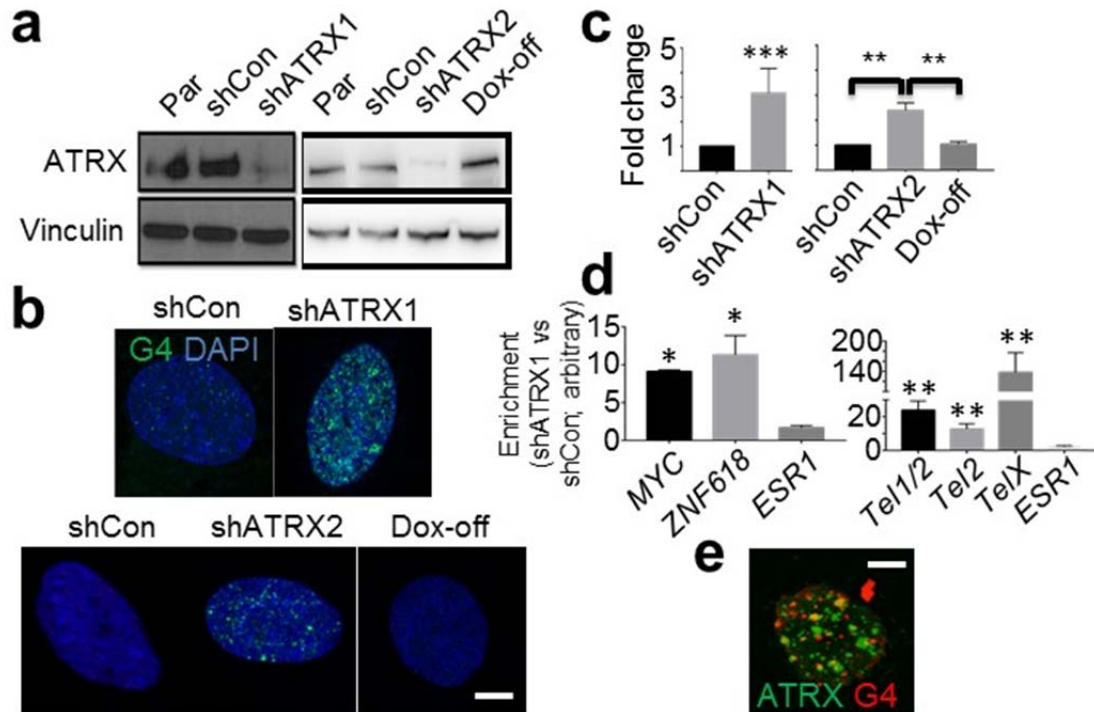
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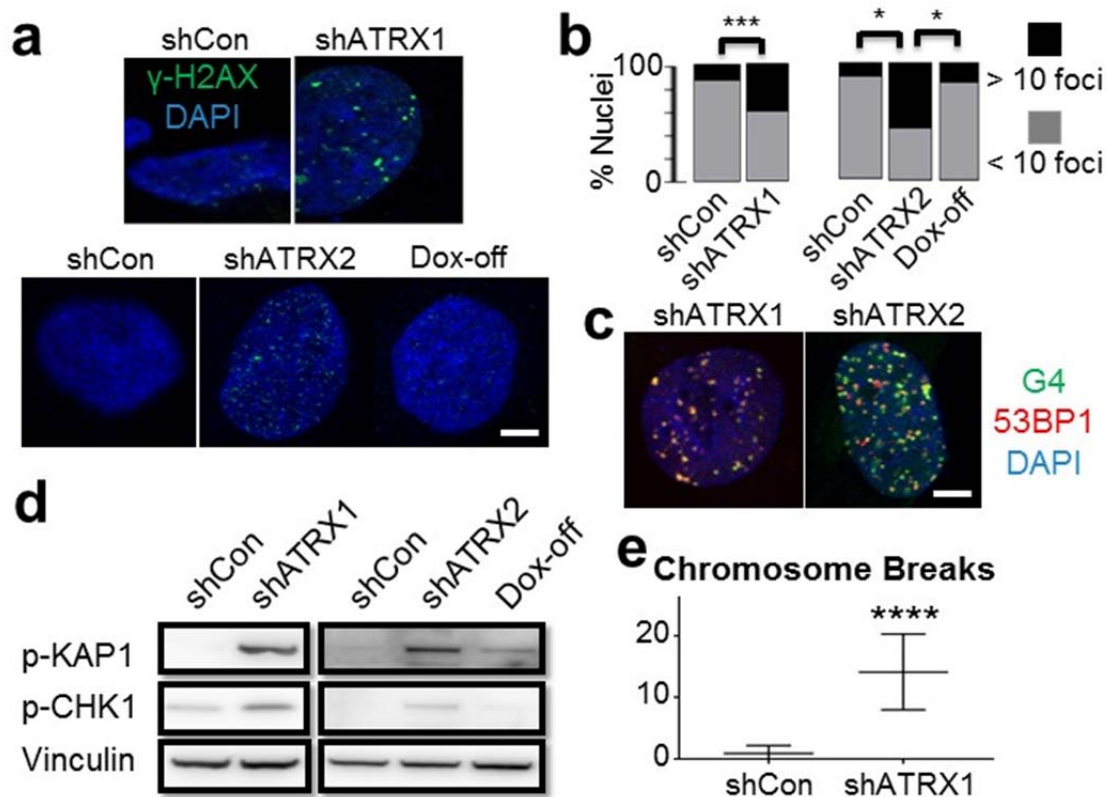
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657 **Figures and Legends:**

658

659 **FIG. 1: ATRX deficiency promotes G4 formation** **a:** Western blots for ATRX in  
 660 parental (Par), shControl (shCon) and shATR<sub>X</sub> NHA (Vinculin loading control). Left  
 661 panel shows stable NHA lines (shATR<sub>1</sub>) and right panel shows the inducible lines post  
 662 Doxycycline induction (shATR<sub>2</sub>) and withdrawal (Dox-off). **b:** Immunofluorescent  
 663 staining of G4 in the stable (upper panel) and inducible (lower panel) NHA lines (DAPI  
 664 counterstain). **c:** Quantified relative G4 signal intensity. **d:** G4-containing DNA fragments  
 665 from shCon and shATR<sub>1</sub> NHAs were pulled down from sheared genomic DNA with  
 666 purified hf2 antibodies. Recovered DNA was subjected to real-time PCR for telomeric  
 667 sequences or G4-rich promoter regions of *MYC* and *ZNF618* (*ESR1* used as negative  
 668 control). Graphs show enrichment over GAPDH scaled to shCon levels. **e:** ATRX and  
 669 G4 immunofluorescence in parental NHAs showing no significant colocalization.



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**FIG. 2: ATRX deficiency promotes replication stress, DNA damage, and**

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**chromosome breakage. a:**  $\gamma$ -H2AX immunofluorescence in stable (upper panel) and

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inducible (lower panel) NHAs (DAPI counterstain). **b:** Quantified percentage of cells with

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>10 immunopositive  $\gamma$ -H2AX foci. **c:** NHAs with ATRX knock down were double stained

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for G4 and 53BP1, showing extensive colocalization. **d:** Western blots of p-KAP1 and p-

676

CHK1 show activation of replication stress arising with ATRX knockdown. **e:** ATRX

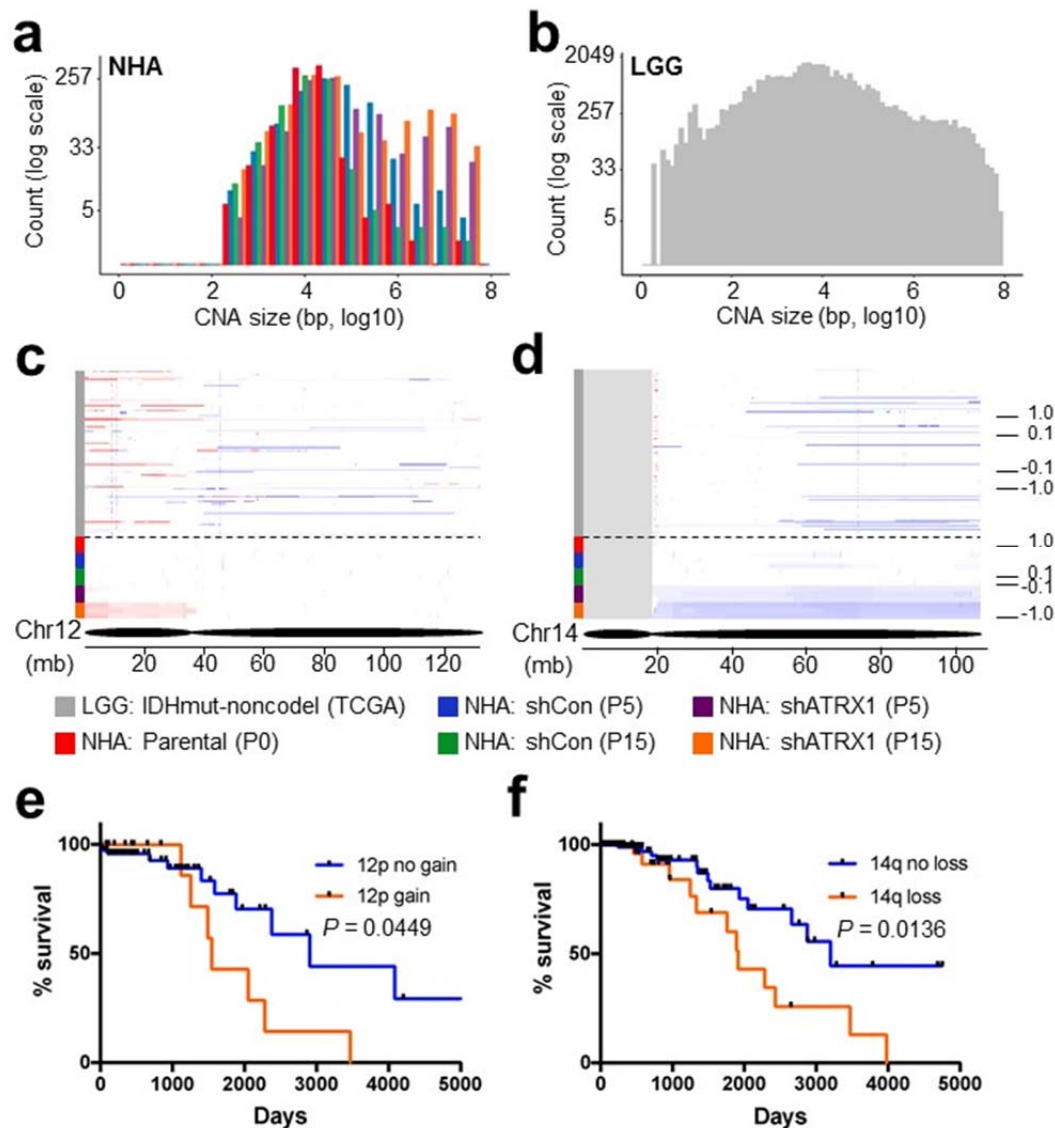
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deficient NHAs (shATR1, passage 15) showed significantly increased chromosome

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breaks by cytogenetic analysis.

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681 **FIG. 3: ATRX deficiency induces clinically relevant copy number alterations. a:**

682 Size distribution of CNAs in parental (P0), shCon (P5 and P15) and shATRX1 (P5 and

683 P15) NHAs. In ATRX knockdown cells, large CNAs (>1 Mb) arise over passage number.

684 **b:** Size distribution of CNAs in IDHmut-noncodel gliomas (TCGA)<sup>2</sup>. **c-d:** IGV plots for

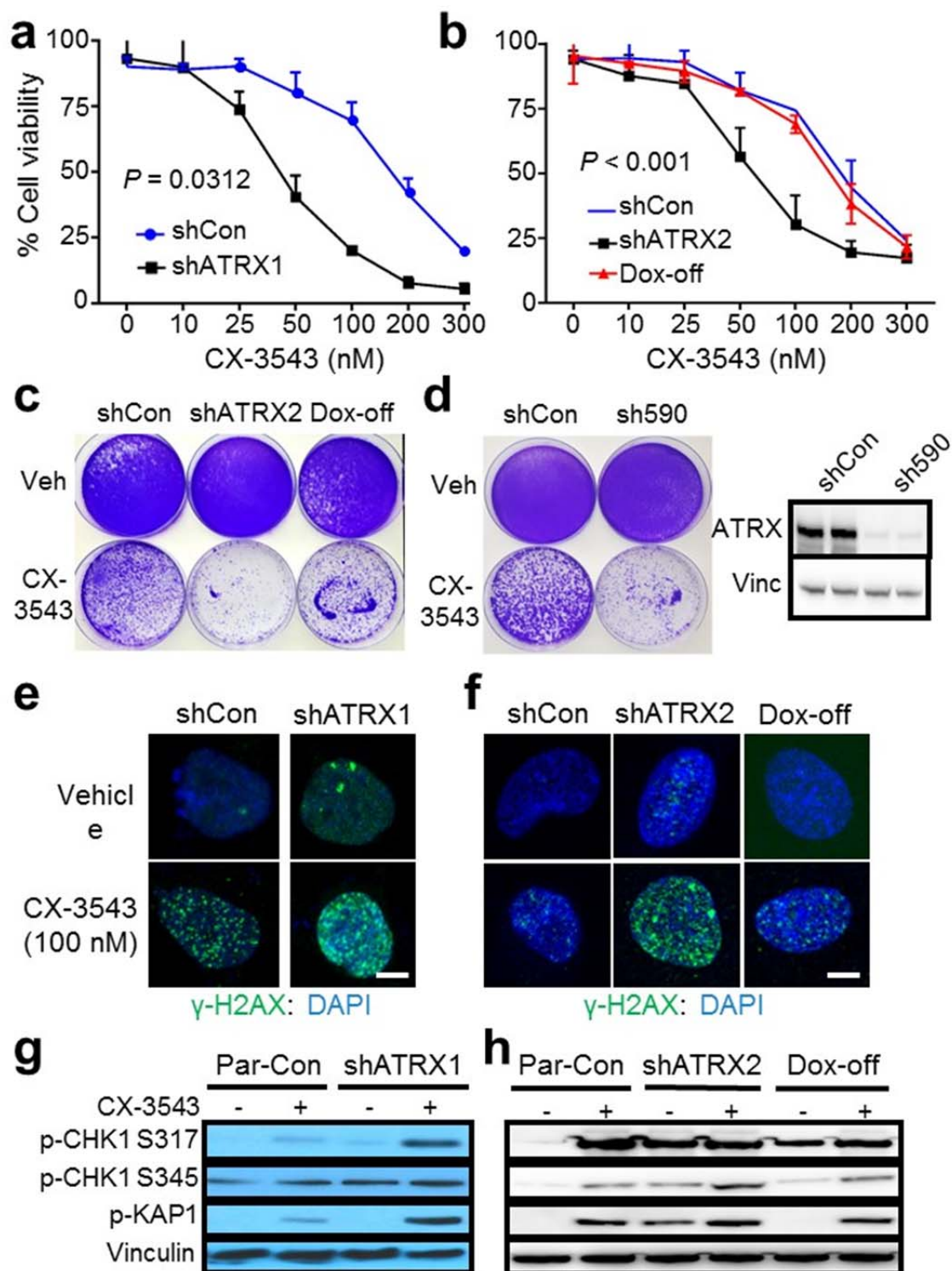
685 chromosome 12 (c) and 14 (d) comparing CNA regions in IDHmut-nocodel gliomas

686 (above dotted line) to NHAs (below dotted line). **e-f:** Kaplan Meier curves for IDHmut-

687 noncodel glioma patients with or without either 12p gain or 14q loss, showing significant

688 differences in overall survival.





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**FIG. 4: ATRX-deficient NHAs are selectively sensitive to CX-3543. a-b:** Cell viability

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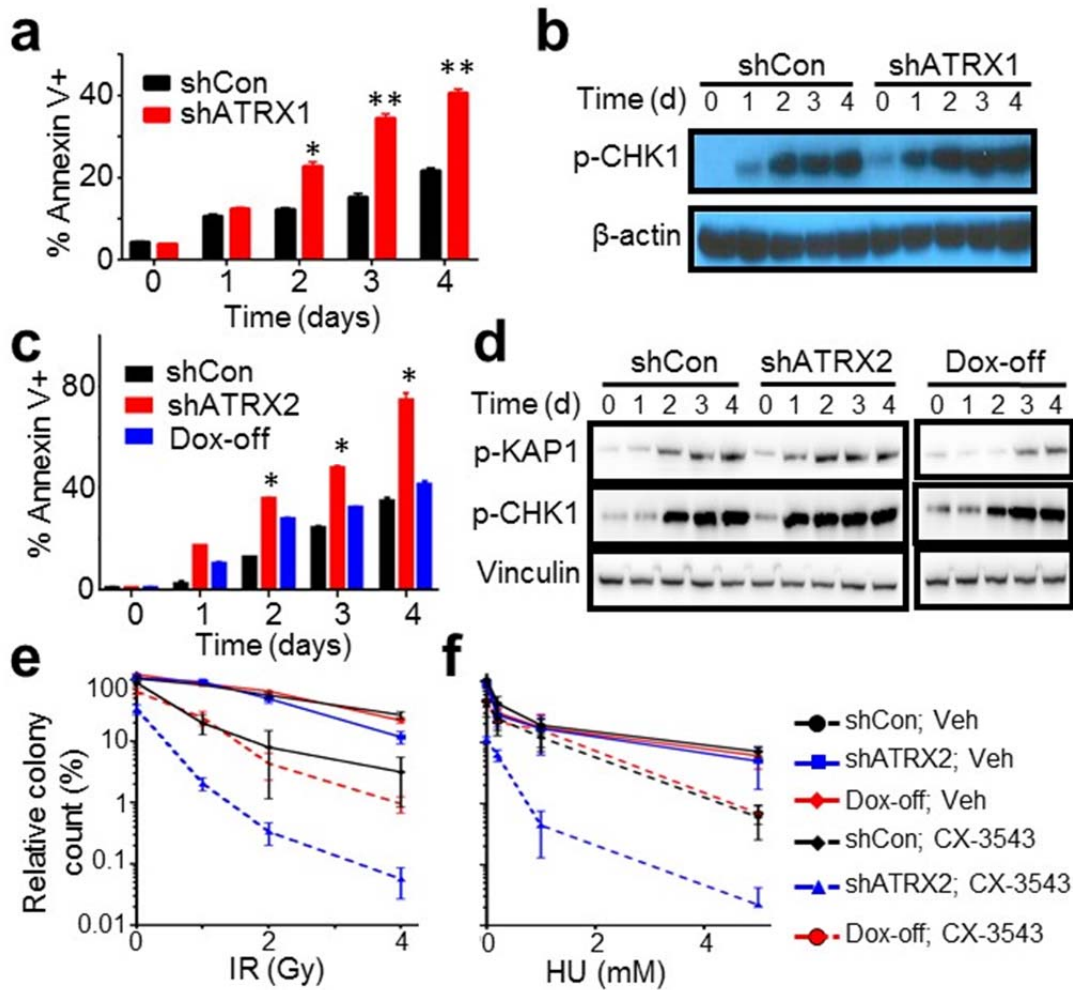
(CellTiter-Glo) of stable (a) and inducible (b) shCon and shATRX NHAs treated with CX-

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3543 from 0-300nM. **c:** Clonogenic assay of inducible shATRX2 NHAs demonstrates

693 enhanced and reversible sensitivity to CX-3543 (50 nM) with ATRX deficiency. **d:**  
694 Clonogenic assay of TS 543 with (sh590) and without (shCon) ATRX knockdown  
695 demonstrates enhanced sensitivity to CX-3543 (50 nM) with ATRX deficiency; western  
696 blot confirms robust ATRX knockdown (Vinculin control, duplicate loading). **e-f:**  $\gamma$ -H2AX  
697 immunofluorescence showing in stable (c) and inducible (d) shATRX NHAs showing  
698 increased DNA damage with CX-3543 treatment (100 nM), particularly in the setting of  
699 ATRX knockdown. **g-h:** Western blots showing increased phosphorylation of replication  
700 stress pathway constituents (CHK1 and KAP1) in stable (e) and inducible (f) shATRX  
701 NHAs following CX-3543 treatment (100 nM).  
702

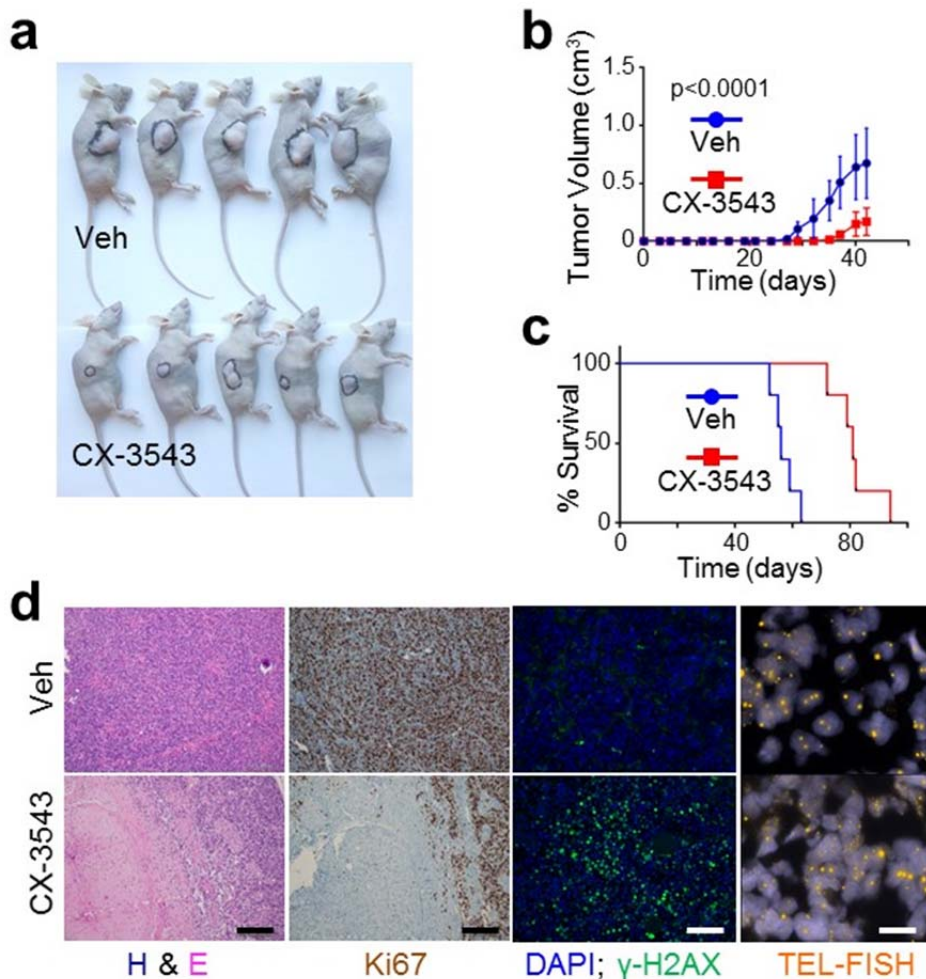




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**FIG. 5: CX-3543 selectively promotes apoptosis and synergizes with DNA-**

705 **damaging therapies in ATRX-deficient NHAs.** a-b: : Time course study in shCon and  
706 shATR1 NHAs treated with 100 nM CX-3543 showing parallel kinetics of apoptosis  
707 (Annexin V positivity; a) and p-CHK1/p-KAP1 levels by western blot (b). c-d: Analogous  
708 time course study in inducible shATR2 cells documenting p-CHK1 and p-KAP1 levels (d)  
709 by western blot and Annexin V positivity (c) over time. e-f: Soft agar colony counts for  
710 NHAs treated with either vehicle control (Veh) or 50 nM CX-3543 and either increasing  
711 doses of IR (a) or HU (b). Colony counts measured at 21 days scaled to that of shCon,  
712 Veh NHAs.



713

714 **FIG. 6: CX-3543 markedly slows the growth of ATRX-mutant glioma xenografts *in***

715 ***vivo*. a:** representative image of mice bearing JHH-273 xenografts following treatment

716 with either vehicle (veh) or 12.5 mg/kg CX-3543 for 42 days. **b:** Average xenograft

717 volume over time in 10 mice randomized (5 and 5) into 2 groups, receiving either 12.5

718 mg/kg CX-3543 or vehicle control (veh). **c:** Kaplan-Meier analysis of survival In a

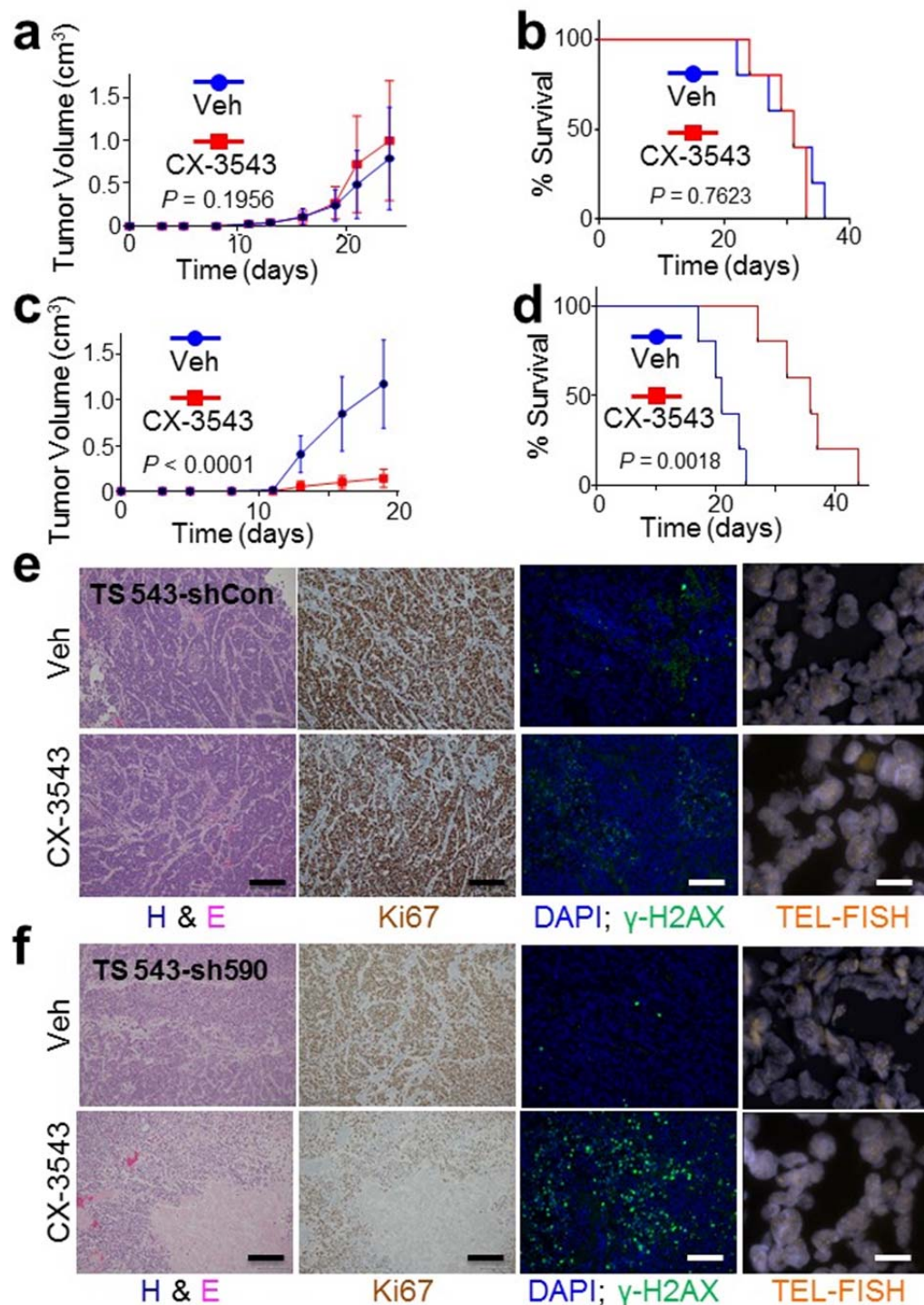
719 separate cohort of 10 mice randomized (5 and 5) into 2 groups, receiving either 12.5

720 mg/kg CX-3543 or vehicle control (Veh). **d:** Representative H&E-stained, immunostained

721 (Ki67 and  $\gamma$ -H2AX), and TEL-FISH stained sections of xenografts from vehicle (Veh) and

722 CX-3543-treated mice. CX-3543-treated tumors showed decreased cellularity,

723 decreased proliferative activity, and increased DNA damage.



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725

**FIG. 7: Efficacy of CX-3543 *in vivo* is dependent on ATRX deficiency. a:** TS 543

726 (ATRX intact) xenografts exhibited similar rates of growth when treated with either  
727 vehicle control (Veh, N=3) or 12.5 mg/kg CX-3543 (N=3) as reflected by tumor volume  
728 over time. **b:** Kaplan-Meier analysis of survival In a separate cohort of 10 mice

729 randomized (5 and 5) into 2 groups, receiving either 12.5 mg/kg CX-3543 or vehicle  
730 control (Veh). **c:** ATRX knockdown in TS 543 cells (sh590) restored the sensitivity of  
731 xenografts to CX-3543 treatment as shown by tumor volume over time in 10 mice  
732 randomized (5 and 5) into 2 groups, receiving either 12.5 mg/kg CX-3543 or vehicle  
733 control (Veh). **d:** Kaplan-Meier analysis of survival In a separate cohort of 10 mice  
734 randomized (5 and 5) into 2 groups, receiving either 12.5 mg/kg CX-3543 or vehicle  
735 control (Veh). **e-f:** Representative H&E-stained, immunostained (Ki67 and  $\gamma$ -H2AX), and  
736 TEL-FISH stained sections of xenografts from vehicle (Veh) and CX-3543-treated mice  
737 harboring either TS 543-shCon (a) or TS 543-sh590 (b) xenografts. CX-3543-associated  
738 histopathological effects were limited to TS 543-sh590 xenografts. Evidence of ALT was  
739 not seen in either GSC line.  
740