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1 **Germline *RBBP8* variants associated with early-onset breast cancer**
2 **compromise replication fork stability**

3
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21
22 The authors have declared that no conflict of interest exists.

23 **Abstract**

24 Haploinsufficiency of factors governing genome stability underlies hereditary breast and
25 ovarian cancer. Homologous recombination (HR) repair is a major pathway disabled in these
26 cancers. With the aim of identifying new candidate genes, we examined early-onset breast
27 cancer patients negative for *BRCA1* and *BRCA2* pathogenic variants. Here, we focused on
28 CtIP (*RBBP8* gene) that mediates HR repair through the end-resection of DNA double-strand
29 breaks (DSB). Notably, the patients exhibited a number of rare germline *RBBP8* variants, and
30 functional analysis revealed that these variants did not affect DNA DSB end-resection
31 efficiency. However, expression of a subset of variants led to deleterious nucleolytic
32 degradation of stalled DNA replication forks in a manner similar to cells lacking *BRCA1* or
33 *BRCA2*. In contrast to *BRCA1* and *BRCA2*, CtIP deficiency promoted the helicase-driven
34 destabilization of RAD51 nucleofilaments at damaged DNA replication forks. Taken together,
35 our work identifies CtIP as a critical regulator of DNA replication fork integrity, which when
36 compromised, may predispose to the development of early-onset breast cancer.

37 **Introduction**

38 Hereditary breast and ovarian cancer (HBOC) is causally linked with
39 germline pathogenic variants in proteins implicated in homologous
40 recombination repair (HRR), the protection of stalled DNA replication forks and
41 cell cycle checkpoint control (1-6). *BRCA1* and *BRCA2* are the most commonly
42 mutated genes in HBOC, accounting for approximately 15% of cases (7).
43 However, a number of less frequent genetic alterations that predispose to
44 breast cancer have been uncovered in other genes e.g. *RECQL1*, *PALB2* and
45 *BRIP1* (3, 8-10). For the majority of emerging HBOC genes, it is currently not
46 possible to provide accurate risk estimates because they are rare. This poses
47 challenges to cancer risk management and counseling of women who carry
48 variants in these genes as well as burdens their families. Consequently, it has
49 been proposed that functional analyses should be employed in the
50 classification of novel genetic variants (1).

51 Notably, genetic and functional analysis of breast cancer associated
52 variants have uncovered substantial locus heterogeneity. Several HRR factors,
53 other than *BRCA1* and *BRCA2*, increase the risk of breast cancer including
54 *PALB2* and *RAD51C* (1, 8, 11). CtIP, encoded by the *RBBP8* gene, is a major
55 HRR factor that has thus far not been functionally linked with HBOC. CtIP is a
56 key regulator of double-strand break (DSB) resection operating within the
57 *BRCA1/BRCA2* pathway, and generates the single-stranded DNA segment
58 needed for *RAD51*-mediated recombination. Here, we examined a high-risk
59 population of early-onset *BRCA1* and *BRCA2* mutation-negative breast cancer
60 patients for germline variants in *RBBP8*. Compared to a Danish control cohort,
61 these patients were enriched for a subset of rare *RBBP8* variants. Functional

62 analysis revealed that whilst these CtIP variants did not affect DSB resection
63 efficiency, their expression led to deleterious nucleolytic degradation of stalled
64 replication forks in a manner similar to cells lacking BRCA1/BRCA2. Notably,
65 CtIP deficiency promoted the helicase-driven destabilization of RAD51
66 nucleofilaments at damaged replication forks. Taken together, our work
67 identifies CtIP as a critical regulator of replication fork integrity that when
68 mutated may predispose to the development of early-onset breast cancer.
69

70 **Results**

71 **Identification of *RBBP8* germline variants.** We screened a group of 129
72 Danish high-risk *BRCA1* and *BRCA2* pathogenic variant-negative breast
73 cancer patients for germline variants in *RBBP8* encoding CtIP (Patient group I,
74 outlined in Supplementary Figure 1). Fifty percent of women had a 1st or 2nd
75 degree relative with breast or ovarian cancer and included women below 35
76 years of age at the time of diagnosis, male breast cancer patients and six
77 women with early-onset ovarian cancer (Supplementary Table 1). This initial
78 screening identified five different non-synonymous, heterozygous *RBBP8*
79 variants (Table 1 and Figure 1A-B). Three patients were carriers of an in-frame
80 3-bp deletion in exon 18 (c.2410_2412del; p.E804del), which was detected at
81 an allele frequency of 1.16%. The p.E804del variant is significantly
82 overrepresented in our cohort with respect to 2,000 Danes (12). In addition, two
83 patients were carriers of different missense *RBBP8* variants (c.693T>A,
84 p.S231R in exon 9 and c.1928A>C, p.Q643P in exon 13, respectively), and one
85 patient carried two different missense variants, (c.298C>T, p.R100W in exon 6
86 and c.2131G>A, p.E711K in exon 15). Only the p.R100W variant was detected
87 in 2,000 Danes, whilst the p.Q643P and the p.E711K variant had not been
88 reported previously (12).

89 We subsequently sequenced *RBBP8* in a larger series of 1,092 patients
90 negative for *BRCA1* and *BRCA2* pathogenic variants with breast cancer and/or
91 ovarian cancer or other related cancer types, as well as unaffected individuals
92 of families with HBOC (Patient group II, outlined in Supplementary Figure 1).
93 Nine different heterozygous missense variants in *RBBP8* (Table 1 and Figure
94 1A-B) were identified in 14 females from this cohort. Three patients carried a

95 p.R110Q variant, two a p.H456R variant, and three carried the p.Q643P variant
96 previously identified among patient group I. A further six variants, p.R502L,
97 p.T675I, p.R805G, p.R839Q, p.P874A and p.E894D, were identified in
98 individual patients. In total, we identified 13 *RBBP8* variants in 21 patients
99 (Table 1), nine of which were observed once. Finally, we explored an
100 international cohort of 1054 breast cancer patients without pathogenic variants
101 in *BRCA1* or *BRCA2* for rare variants in *RBBP8*. Here, we identified 17 different
102 rare variants in 22 patients of which the clinically annotated (n=7) had a median
103 age of 38 at the time of diagnosis (Supplementary Table 2). These *RBBP8*-
104 variants also included the p.Q643P variant and two loss-of-function variants
105 (Supplementary Table 2).

106

107 ***RBBP8*/CtIP variants display a genome maintenance defect.** Since our
108 genetic screening indicated that *RBBP8* variants could be associated with
109 early-onset breast cancer, we investigated whether they affect known CtIP
110 function(s). Hence, we examined DNA DSB end resection as well as genome
111 stability after exposure to irradiation (IR) or replication stress induced with
112 aphidicolin (APH) or hydroxyurea (HU). To create an isogenic system for our
113 assays, we first depleted endogenous *RBBP8*/CtIP from breast cancer MCF7
114 cells with siRNA, and complemented cells with re-expression of siRNA-
115 resistant CtIP variants (Figure 2A-B). All variants were well expressed;
116 surprisingly, however, none of these variants affected the ability of cells to
117 perform DNA DSB end resection after irradiation (Figure 2C; Supplementary
118 Figure 2A). The phosphorylation of RPA32 on the S4/S8 residues was used as
119 a readout for the proficiency of DNA DSB end resection after IR in these assays

120 (13). Next, we monitored genome stability after exposure to IR, APH or HU,
121 using the accumulation of extranuclear micronuclei as a readout
122 (Supplementary Figure 2B). In a manner similar to Wt-CtIP, and in keeping with
123 our findings above, all the tested variants were able to complement the IR-
124 induced genome instability caused by the loss of CtIP (Table 2). Together,
125 these data indicate that the identified germline *RBBP8* variants do not give rise
126 to a detectable impairment of DNA DSB repair. However, expression of several
127 variants (Q643P, E804del, and R805G) as well as the C-terminal truncated
128 CtIP (Δ C) mutant, failed to complement the genome instability induced by APH
129 and HU following depletion of endogenous CtIP (Table 2). This suggests that
130 these variants perturb a function of CtIP specifically associated with the
131 replication stress response. In addition to the Danish breast cancer cohort, we
132 also investigated the *RBBP8* variants present in the international COMPLEXO
133 cohort for genome stability after exposure to APH or HU using variant
134 complementation in CtIP depleted cells. The CtIP-Q643P variant, as well as the
135 truncating variants CtIP-R185* and CtIP-L372* all displayed increased genome
136 instability after replication stress (Supplementary table 3).

137

138 **CtIP-E804del is proficient in HR repair**

139 To further examine the potential HR repair status of CtIP variants in the Danish
140 breast cancer cohort, we focussed on the CtIP-E804del variant as it was
141 significantly enriched in the cohort. We used the tractable U-2-OS cell line,
142 which is commonly used to evaluate CtIP function (14, 15), and generated an
143 inducible complementation system expressing siRNA resistant GFP-tagged
144 full-length CtIP or the CtIP-E804del variant (Supplementary Figure 2C-H).

145 Consistent with our previous results in MCF7 cells (Figure 2C), expression of
146 the CtIP-E804del variant in the U-2-OS cells could rescue the DSB resection
147 deficiency resulting from CtIP depletion (Supplementary Figure 2C-D). We then
148 set out to assess HRR efficiency in CtIP-E804del U-2-OS cells, since CtIP-
149 dependent DSB end resection is crucial for efficient HRR. As expected,
150 expression of CtIP-E804del variant could rescue the HRR deficiency caused
151 by CtIP depletion (Supplementary Figure 2E-G). Since HRR deficiency can be
152 therapeutically exploited through the use of PARP inhibitors (PARPi), we also
153 investigated whether CtIP-E804del variant expression promotes PARPi
154 sensitivity. As shown in Supplementary Figure 2H using variant
155 complementation of siRNA depleted cells, CtIP-E804del variant did not display
156 any increase in PARPi sensitivity over and above Wt-CtIP complemented cells.
157 Taken together, these results indicate that the CtIP-E804del variant displays
158 proficient DSB end resection and HRR. Furthermore, this suggests that CtIP
159 variants deficient in responding to replication stress may promote
160 tumorigenesis independently of HRR.

161

162 **CtIP promotes RAD51 function during replication stress.**

163 In order to functionally characterize a subset of variants in greater detail, the
164 CtIP-Q643P and CtIP-E804del variants were chosen because they were
165 significantly enriched in our Danish breast cancer cohort and were associated
166 with increased genome instability upon HU and APH treatment (Table 2).
167 Additionally, the CtIP-R805G variant was also chosen due to its close amino
168 acid sequence proximity to the CtIP-E804del variant and its defective response
169 to replication stress (Table 2).

170 Notably, Q643P, E804del and R805G CtIP variants could not be linked with
171 deficiency in DNA end resection. As an alternative explanation underlying their
172 functional contribution, we hypothesized that these variants may instead be
173 deficient in replication fork degradation which is a recently emerging role for
174 CtIP (16). To test this hypothesis, we first analyzed the prevalence of RPA foci.
175 This is a robust marker of ssDNA accumulating at replication forks after HU
176 treatment, with both increases and decreases in the number of RPA foci per
177 cell being indicative of replication stress response perturbations. Consistent
178 with previous reports (17), CtIP depletion led to an increase in HU-induced RPA
179 foci formation, which could be rescued by expressing exogenous Wt-CtIP-GFP
180 (Figure 3A-B). Interestingly, this was not the case for both the CtIP-E804del or
181 CtIP-R805G variants (Figure 3A-B). Serving as negative control, variants that
182 did not display genomic instability after replication stress could also rescue the
183 elevated level RPA foci formation resulting from CtIP knockdown
184 (Supplementary Figure 3A). Intriguingly, the CtIP-Q643P variant suppressed
185 RPA in a manner comparable to Wt-CtIP (Figure 3A-B).

186 To obtain insight into the underlying mechanisms, we further examined how
187 CtIP-depleted cells responded to replication stress. Since RAD51
188 nucleofilaments protect stalled replication forks from uncontrolled nucleolytic
189 degradation (5, 18), we addressed whether CtIP affects RAD51 localisation at
190 damaged forks. As shown in Figure 3C-D, HU-induced RAD51 foci formation
191 was reduced in MCF7 cells depleted of CtIP. Notably, neither expression of the
192 E804del nor R805G CtIP variants could complement the loss of HU-induced
193 RAD51 foci formation caused by CtIP depletion (Figure 3C-D and
194 Supplementary Figure 3B), whilst this could be restored by transient expression

195 of CtIP-Wt and several other CtIP potentially non-pathogenic variants. These
196 data therefore suggest that the Sae2-like domain of CtIP might play a role in
197 recruiting/stabilizing RAD51 after replication stress. Intriguingly, the HU-
198 induced RAD51 response was comparable in cells expressing the CtIP-Q643P
199 variant as to compared to cells expressing Wt-CtIP (Figure 3C-D), which
200 suggests that this variant promotes replication stress-induced genome
201 instability via another mode of action.

202

203 In order to directly visualize RAD51 recruitment to the stalled forks after HU
204 treatment, we turned to isolation of proteins on nascent DNA (iPOND), using
205 CLiCK chemistry to conjugate biotin to a nucleoside analog (EdU) incorporated
206 into newly synthesized DNA (19). Our analyses primarily focused on
207 comparing Wt-CtIP with the CtIP-E804del variant, since this variant was the
208 most significantly enriched variant from the Danish cohort that exhibited a
209 defective response to replication stress. In agreement with our previous data
210 (Figure 3C-D), using iPOND, the recruitment of RAD51 to nascent DNA
211 damaged with HU was reduced in the absence of CtIP. Moreover, this
212 deficiency was restored by the complementation with Wt- CtIP. Importantly,
213 however, this was not the case after complementation with the CtIP-E804del
214 mutant (Supplementary Figure 3C). To understand if CtIP is recruited directly
215 to stalled forks after HU treatment, we employed a proximity ligation assay
216 (PLA)-based approach that measures the association of proteins on nascent
217 DNA (20, 21). Following the depletion of CtIP from U-2-OS cells, the expression
218 of Wt-CtIP or CtIP-E804del was induced in cells with doxycycline. Cells were
219 then labeled with EdU for 10 min prior to treatment with 4 mM HU for 5 h. Click

220 chemistry was then used to conjugate Biotin to EdU and PLA was conducted
221 to detect protein binding to biotin-labeled nascent DNA. Using this approach,
222 our data revealed that Wt-CtIP is present at nascent DNA after replication
223 stress, while CtIP-E804del was absent under the same conditions
224 (Supplementary Figure 3D-E).

225 Together, these data suggest that CtIP prevents the accumulation of ssDNA at
226 damaged replication forks by recruiting/stabilizing RAD51 and that the cancer-
227 associated CtIP variant E804del compromises this function.

228

229 **CtIP antagonizes excessive degradation of stalled replication forks**
230 **through FBH1**. Since RAD51 is known to protect stalled replication forks from
231 degradation and loss of CtIP is causing a decrease in RAD51 foci formation,
232 we sought to measure replication fork degradation directly, using the single
233 molecule DNA fibre-based assay (18, 22). CtIP was depleted from U-2-OS cells
234 and expression of Wt-CtIP, the E804del or ΔC variants were induced. These
235 cells were then sequentially pulse-labelled with CldU and IdU to label nascent
236 DNA before prolonged fork stalling with HU (Figure 4A). In keeping with
237 previous reports (23), these analyses showed that loss of CtIP results in
238 increased degradation of nascent DNA at stalled replication forks (Figure 4B).
239 Moreover, this was abolished upon the expression of Wt-CtIP, but not by
240 expression of the ΔC mutant (Figure 4C). Importantly, the E804del variant was
241 partially deficient in replication fork protection after HU (Figure 4C). Thus, we
242 surmise that the role of CtIP in preventing nascent DNA degradation at stalled
243 forks involves its C-terminal Sae2-like domain.

244

245 Finally, we asked whether CtIP plays a role in recruiting RAD51 to stalled forks
246 in a manner similar to BRCA1/2 or stabilizing RAD51 at these structures like
247 BOD1L and WRNIP1. Unlike BRCA1/2, BOD1L and WRNIP1 protect damaged
248 forks by suppressing the anti-recombinase activity of proteins such as FBH1
249 and BLM (15, 20). Moreover, it has been shown that loss of the anti-
250 recombinase FBH1 increases RAD51 foci formation at stalled replication forks
251 (24). Therefore, we hypothesized that FBH1 might be involved in evicting
252 RAD51 from stalled forks in the absence of CtIP. In keeping with this prediction,
253 concomitant depletion of FBH1 and CtIP rescued RAD51 accumulation in
254 HU-treated conditions to control levels (Figure 4D-E). To further explore the link
255 between CtIP and FBH1, we performed fork degradation assays in HU-treated
256 cells depleted of CtIP, FBH1 or CtIP/FBH1 together (Figure 4F). These
257 experiments revealed that loss of FBH1 restored nascent DNA stability in the
258 absence of CtIP (Figure 4F), suggesting that CtIP stabilises RAD51
259 nucleofilaments to suppress fork degradation. Depletion of FBH1 in cells
260 expressing the CtIP-E804del variant also restored nascent strand stability, and
261 re-stabilized RAD51 at stalled replication forks (Figure 4G-H, Supplementary
262 Figure 4A-B). These data therefore suggest that CtIP regulates replication fork
263 stability by suppressing FBH1-mediated eviction of RAD51 from stalled forks,
264 and that cancer-associated mutations in the C-terminus of CtIP perturb this vital
265 function (Figure 4H).

266

267 **Discussion**

268 Our study demonstrates a role for rare *RBBP8* variants in the control of DNA
269 replication fork integrity. Altogether we identified 13 *RBBP8* germline variants
270 in 21 patients, of which the C-terminal E804del variant was observed in three
271 patients. Importantly, we identified 3 *RBBP8* variants that displayed increased
272 genome instability. These variants were located in the C-terminus (E804del and
273 R805G) and LMO4-interacting (Q643P) regions of CtIP. The C-terminus region
274 is crucial for CtIP functions in genome maintenance and consistent with this,
275 localization of RAD51 and RPA to sites of damage was impaired by E804del
276 and R805G variants. Regarding the variant in the LMO4-interacting region
277 (Q643P), although the functional role for this domain is unclear, it is conceivable
278 that the breast cancer risk associated with this variant may relate to the
279 dysregulation of LMO4. However, we were unable to detect a variant-
280 dependent interaction between CtIP and LMO4 (Supplementary Figure 4C).
281 Additional studies of this variant may identify additional roles for CtIP in
282 maintaining genome stability and suppressing cancer susceptibility.

283

284 Surprisingly, the subset of CtIP variants promoting genome instability were
285 functionally wildtype for DNA DSB end resection and HRR. Instead, we
286 demonstrate that CtIP protects stalled replication forks against enhanced fork
287 degradation by promoting RAD51 nucleofilament stability, and it is this function
288 that is perturbed by variants associated with early-onset breast cancer. Thus,
289 these results suggest that CtIP insufficiency may predispose to breast cancer
290 by allowing deleterious replication fork degradation (Figure 4I). Interestingly,
291 loss of fork protection is a potential target for cancer therapy, since the ability

292 of BRCA1/2-deficient cells to acquire drug resistance is intimately linked to fork
293 protection (6).

294

295 A pathway protecting stalling DNA forks from degradation was first uncovered
296 in cells with BRCA2 insufficiency, and more recently has been reported in cells
297 lacking critical tumor suppressors known to be involved in regulating HRR,
298 including BRCA1, PALB2, and FANCD2 (6, 18, 21, 25). Our research now links
299 CtIP with these factors that allow stable RAD51 accumulation when forks are
300 challenged. However, unlike BRCA1, BRCA2 and PALB2, we suggest that CtIP
301 belongs to a family of replication fork protection factors, including BOD1L and
302 WRNIP1, that regulate the FBH1 helicase, a RAD51-evicting factor. Thus, in
303 the absence of RAD51-stabilising factors, FBH1 reduces the presence of
304 RAD51 at stalled forks, allowing uncontrolled fork degradation that can trigger
305 genome instability. This is an emerging biological response to fork stalling, and
306 the links with tumorigenesis are only now starting to be dissected. Notably, our
307 functional findings on CtIP are in agreement with recently published data that
308 also identify a role for CtIP in suppressing degradation of stalled replication
309 forks (16). The authors focused on the role of the N-terminal region of CtIP that
310 helps to minimize nucleolytic degradation by the DNA2 nuclease. Thus far, we
311 have not identified cancer-associated disabling variants in this CtIP region.

312

313 The roles of CtIP in breast cancer predisposition and progression are not well
314 understood, though studies have indicated that a lack or low levels of CtIP
315 expression in tumor cells is associated with a reduced survival rate (23, 26).
316 Furthermore, tumors lacking CtIP display an impaired ability to repair DSB,

317 which leads to increased sensitivity to PARP inhibitors (26, 27). Thus,
318 determining the impact of identified variants in CtIP on its function should be
319 considered when trying to personalise a therapeutic approach for treating a
320 specific patient. Intriguingly, analysis of a cohort of 129 *BRCA1* and *BRCA2*
321 mutation-negative Australian breast cancer patients failed to demonstrate an
322 enrichment of coding variants in *RBBP8* (28). In fact, no coding *RBBP8* variants
323 were identified except for a polymorphism in intron 6. In contrast, a recent
324 Spanish study identified two truncating *RBBP8* variants in two early-onset
325 *BRCA1/2* mutation negative BC patients (29). Furthermore, we identified two
326 functionally damaging truncating variants in the COMPLEXO cohort in addition
327 to the Q643P variant also described here. The differences between studies may
328 reflect population differences, cohort sizes as well as age of BC onset in the
329 cohorts.

330

331 Murine studies have indicated that CtIP haploinsufficiency is tumor promoting,
332 whereas a complete loss of CtIP is detrimental leading to inviability of mice (30).
333 In contrast, murine tissue-specific conditional CtIP ablation systems indicated
334 that a complete loss of CtIP suppresses tumorigenesis (31). However, it is likely
335 that these observations are due to a deleterious decrease in cellular fitness
336 linked to a complete loss of HRR. Importantly, we have shown that a subset of
337 *RBBP8* variants identified in this study are hypomorphic in a manner where
338 they impair some functions of CtIP but not all. Based on this, we propose that
339 hypomorphic but not loss-of-function mutations in *RBBP8* predispose to early-
340 onset breast cancer. We cannot exclude the possibility that these variants may
341 represent rare variants with little association with cancer development.

342 However, it is noteworthy that we identified *RBBP8* germline variants in early-
343 onset breast cancer patients at a frequency similar to that previously reported
344 for HBOC-associated mutations in *BRIP1*, *RECQL1* and *PALB2* (3, 8-10).
345 Thus, since our data indicates that *RBBP8* variants are more frequent in early-
346 onset breast cancer cases than in unaffected population-matched controls, this
347 warrants consideration of *RBBP8* being included in the gene panel when
348 carrying out breast cancer predisposing sequencing studies. Finally, our study
349 shows the usefulness of combining genetic screening in a high-risk phenotype
350 with comprehensive variant-centered functional analysis to identify and classify
351 new variants implicated in hereditary cancer syndromes.

352

353 **Experimental procedures**

354 **Patients**

355 All patient samples were consecutively recieved for HBOC diagnostics over a
356 period of 20 years, according to the contempary national HBOC guidelines
357 (Danish Breast Cancer Cooperative Group (DBCG)). Clinical and
358 histopathological data were retrieved from the Danish Pathology Registry and
359 the DBCG registry. Patients were diagnosed between year 1978 to 2016.

360

361 Patient group I included 129 breast and/or ovarian cancer patients, previously
362 identified as *BRCA1/2* pathogenic-variant-negative as part of their diagnostic
363 work-up (124 females and 5 males). Female patients diagnosed with ovarian
364 or with breast cancer at the age of 35 years or younger, while male breast
365 cancer patients were included regardless of age at time of diagnosis. Among
366 the female patients, 116 had breast cancer only, six had ovarian cancer only,
367 one had breast and ovarian cancer, and one had breast and cervical cancer.
368 Four of the men had breast cancer only, whereas 1 had both breast and
369 prostate cancer. Mean age at time of diagnosis of the female patients was 30
370 years and for male 59 years.

371

372 Patient group II included 1,092 *BRCA1/2* - negative samples from unselected
373 and consecutive patients undergoing genetic screening for HBOC according to
374 clinical guidelines as described above.

375

376 **Sequencing of *RBBP8* in patient group I**

377 Genomic DNA was purified from peripheral blood samples and library
378 preparation was performed using SeqCap EZ Human Exome Library v3 (Roche
379 NimbleGen, Madison, WI, USA) or SureSelect All Exon kit v5 (Agilent
380 Technologies, Santa Clara, CA, USA) following manufactures' instructions.
381 Sequencing was conducted using the HiSeq2500 or NextSeq500 platforms
382 from Illumina (San Diego, Ca, USA). The average coverage of all exomes was
383 65x.

384

385 **Data processing**

386 Fastq files were processed using CLC Biomedical Genomics Workbench v3
387 (Qiagen, Hilden, Germany). Reads were mapped to the human reference
388 genome hg19/GRCh37 and variant calling was performed by a Maximum
389 Likelihood approach on a Bayesian model. Variants were called with a minimum
390 of 10 reads, 3 counts and a frequency of >25 %. Called variants were filtered
391 using Ingenuity Variant Analysis (<http://ingenuity.com>). First, variants with call
392 quality <20 and read depth <10; were disregarded. Second, variants with an
393 allele frequency >1% of the public variant database including 1000 genomes
394 project (www.1000genomes.org), ExAC (<http://exac.broadinstitute.org>) or
395 gnomAD (<http://gnomad.broadinstitute.org>), or unless established as a
396 pathogenic common variant, were excluded. Third, only coding non-
397 synonymous variants and splice-site variants (+/-2bp) were kept. Finally, output
398 was filtered to include the *RBBP8* gene. Samples (n=1054) from the
399 COMPLEXO consortium were initially processed from raw fastq reads and
400 aligned to the human genome reference (hg19) using bwa (v0.5.9) on a per
401 lane basis. Alignment file pre-processing and germline variant calling was

402 performed by The Genome Analysis Toolkit (GATK) v3.1 (v3.1-144).
403 HaplotypeCaller algorithm was used to generate variant files (.vcf) which were
404 filtered to include only rare variants in the *RBBP8* gene (<1% in ExAC).

405

406 **Sequencing of *RBBP8* in Patient group II**

407 Screening of the larger group for *RBBP8* variants was performed using a gene
408 panel. The library was designed to capture all exons as well as the first and last
409 50 bp of the intronic regions. Samples were pooled into groups of four and
410 deeply sequenced (average coverage of 5.500x). Mapping and variant calling
411 was done as described for patient group I, however as samples were pooled
412 into groups of four, variants were called with a minimum of 100 reads, 10 counts
413 and frequency of 6.25 % (corresponding to a variant detection rate of 25 % pr.
414 sample). Variant filtering was performed using Ingenuity Variant Analysis.

415

416 **Sanger sequencing**

417 All non-polymorphic *RBBP8* variants identified by sequencing of the two patient
418 groups were verified by PCR and Sanger sequencing (for primer sequences
419 see Supplemental table 4).

420

421 **Statistical Analysis of allelic association with Breast Cancer**

422 Fisher's exact test was employed to determine if identified *RBBP8* variants
423 were enriched in the examined breast cancer patient cohorts compared to the
424 2,000 Danes were used as controls in the statistical analysis.

425

426 **Cell culture**

427 The human cancer cell lines were cultured for 5 days at 37°C and 5% CO₂ as
428 follows: The human breast cancer cells (MCF7) were cultured in RPMI (GIBCO,
429 Life Technologies), supplemented with 10% FBS (Sigma Aldrich), and 1%
430 penicillin/streptomycin (GIBCO, Life Technologies). The human osteosarcoma
431 cell line (U-2-OS), harboring inducible GFP-tagged siRNA resistant CtIP were
432 grown in Dulbecco's modified Eagle's medium with 10% tetracycline-free FBS
433 (Clontech) 1% penicillin/streptomycin (GIBCO, Life Technologies), 100 ug/ml
434 Zeocin (Invitrogen) and 5 ug/ml Blasticidin (Invitrogen). The human embryonic
435 kidney 293FT cells were grown in Dulbecco's modified Eagle's medium with
436 10% FBS (Sigma Aldrich) and 1% penicillin/streptomycin (GIBCO, Life
437 Technologies).

438

439 **Lentiviral infection**

440 The doxycycline inducible stable U-2-OS cell lines expressing the pcDNA4/TO
441 tagged siRNA-resistant versions of wild-type and mutant CtIP were established
442 by cloning CtIP cDNA into pcDNA4/TO-GFP vector (Invitrogen). The Δ C
443 truncation of CtIP is lacking amino acids 790–897.

444 The GFP-CtIP plasmids were sub-cloned into pLVX-TetOne Vector (Clontech)
445 and were co-transfected with Pax8 (Clontech) and VSVG (Clontech) into
446 HEK293 FT cells using FugeneHD (Promega). The generated CtIP lentivirus
447 were then transduced into U-2-OS using polybrene according to the
448 manufacturer's protocol resulting in cell lines expressing GFP- tagged siRNA-
449 resistant CtIP Wt, E804del, or Δ C in a Tet-on system. To induce expression of
450 siRNA resistant CtIP, doxycycline (1 ng/ml) was added to the medium for
451 approximately 24 h.

452

453 **Site directed mutagenesis**

454 The mutant CtIP plasmids were generated by site-directed mutagenesis of the
455 siRNA-resistant Wt-CtIP. The following primers were used:

456 CtIP R100W: Fw: 5'-ACTGAAGAACATATGTGGAAAAACAGCAAG

457 CtIP R100W: Re: 5'-CTTGCTGTTTTTCCACATATGTTCTTCAGT

458 CtIP R110Q: Fw: 5'-GAGTTTGAAAATATCCAGCAGCAGAATCTTAAA

459 CtIP R110Q: Re: 5'-TTTAAGATTCTGCTGCTGGATATTTTCAAATC

460 CtIP R185*: Fw: 5'-AGAACCCCATGTCTGATACATAGAACAAA

461 CtIP R185*: Re: 5'-TTTGTCTATGTATCAGACATGGGGGTTCT

462 CtIP V198M: Fw: 5'-AAATTGGAGCACTCTATGTGTGCAAATGAAAT

463 CtIP V198M: Re: 5'-ATTCATTTGCACACATAGAGTGCTCCAATTT

464 CtIP S231R: Fw: 5'-CACTTATGACCAAAGACAATCTCCAATGGCC

465 CtIP S231R: Rev: 5'-GGCCATTGGAGATTGTCTTTGGTCATAAGTG

466 CtIP E267G: Fw: 5'-ACTTGGTGTTC AAGGAGAATCTGAAACTC

467 CtIP E267G: Re: 5'-GAGTTTCAGATTCTCCTTGAACACCAAGT

468 CtIP Q272E: Fw: 5'-AAGAATCTGAAACTGAAGGTCCCATGAG

469 CtIP Q272E: Re: 5'-CTCATGGGACCTTCAGTTTCAGATTCTT

470 CtIP G331A: Fw: 5'-ATCTCCTGTATTTGCAGCTACCTCTAGTA

471 CtIP G331A: Re: 5'-TACTAGAGGTAGCTGCAAATACAGGAGAT

472 CtIP Q352P: Fw: 5'-CCTTCTCTTTTACCGCCTGGGAAAAAAA

473 CtIP Q352P: Re: 5'-TTTTTTTCCCAGGCGGTAAAAGAGAAGG

474 CtIP I369V: Fw: 5'-CTTTTAGCAACACTTGTGTATCTAGATTAGAAAA

475 CtIP I369V: Re: 5'-TTTTCTAATCTAGATACACAAGTGTTGCTAAAAG

476 CtIP L372*: Fw: 5'-CACTTGTATATCTAGATGAGAAAAAACTAGATCA

477 CtIP L372*: Re: 5'-TGATCTAGTTTTTTCTCATCTAGATATACAAGTG
478 CtIP E414D: Fw: 5'-AATAAAAATATAAGTGATTCCCTAGGTGAACAGA
479 CtIP E414D: Re: 5'-TCTGTTACCTAGGGAATCACTTATATTTTTATT
480 CtIP H456R: Fw: 5'-GAGGAAGAAAGTGAACGTGAAGTAAGCTGC
481 CtIP H456R: Re: 5'-GCAGCTTACTTCACGTTCACCTTCCTC
482 CtIP R502L: Fw: 5'-TTTTCAGCTATTCAGCTTCAAGAGAAAAGCCAA
483 CtIP R502L: Re: 5'-TTGGCTTTTCTCTTGAAGCTGAATAGCTGAAAA
484 CtIP E552D: Fw: 5'-ATTCCCCAGGGGATCCCTGTTTACA
485 CtIP E552D: Re: 5'-TGTGAACAGGGATCCCCTGGGGAAT
486 CtIP R589H: Fw: 5'-TTTAAAATTCCTCTACATCCACGTGAAAGTTTG
487 CtIP R589H: Re: 5'-CAAACCTTTCACGTGGATGTAGAGGAATTTTAAA
488 CtIP Q643P: Fw: 5'-AAAATAAAGTCTCTACCAAACAACCAAGATGTA
489 CtIP Q643P: Re: 5'-TACATCTTGGTTGTTTGGTAGAGACTTTATTTT
490 CtIP E711K : Fw: 5'-CAAGAGCAGAAGGGAAAAAAAAGTTCAAATG
491 CtIP E711K : Re: 5'-CATTTGAACTTTTTTTTTCCCTTCTGCTCTTG
492 CtIP E716K: Fw: 5'-GGGAGAAAAAAGTTCAAATAAAGAAAGAAAAATGAA
493 TG
494 CtIP E716K: Re: 5'-CATTCATTTTTCTTTCTTTATTTGAACTTTTTTCTCCC
495 CtIP E804del: Fw: 5'-GTGGTTCGGAAAAAAGAGAGAAGA...CAG
496 CtIP E804del : Re: 5'-GTGTGCCCAAGCAGTTTTTCTTCTC...CAC
497 CtIP R805: Fw: 5'-GTTTCGGAAAAAAGAGGAGGGAAGAAAAGCTGCTTGGGC
498 CtIP R805G : Re: 5'-GCCCAAGCAGTTTTTCTTCCCTCTTTTTTCCGAA
499 CtIP R839G : Fw: 5'-GGAATGTAGCGGAATCCGTGTCTTGAGCAGGAA
500 CtIP R839G : Re: 5'-TTCCTGCTCAAGACACGGATTCCGCTACATTCC
501 CtIP P847A : Fw: 5'- AAGGAAGATCTTGATGCTTGTCTCGTCCAA

502 CtIP P847A : Re: 5'- TTGGACGAGGACAAGCATCAAGATCTTCCTT
503 CtIP R877H: Fw: 5'-TTGATCCTTGTCTCATCCAAAAGACGT
504 CtIP R877H: Re: 5'-ACGTCTTTTTGGATGAGGACAAGGATCAA
505 CtIP E894D : Fw: 5'- TCCAAAAGGCAAGGACCAGAAGACATAGACG
506 CtIP E894D : Re: 5'- CGTCTATGTCTTCTGGTCCTTGCCTTTTGA
507 CtIP ΔC: Fw: 5'-GAAAGAGAGACTAGCTAGCAAAATTTTCCTCAT
508 CtIP ΔC: Re: 5'-ATGAGGAAAATTTTGCTAGCTAGTCTCTCTTTC

509 The PFU ultra-high-fidelity polymerase (Agilent) was used according to the
510 manufacturer's protocol.

511

512 **Oligonucleotides and transfection**

513 For siRNA transfections (48 h), Lipofectamine RNAiMAX (Invitrogen) was used
514 according to the manufacturer's protocol. MISSION® siRNA universal negative
515 control (UNC, Sigma) was used as a negative control, and the oligonucleotide
516 sequences used for knockdown of CtIP was 5'-GCUAAAACAGGAACGAAU
517 which was obtained from Microsynth (Balgach,Switzerland), for depleting
518 FBH1, a mix of two sequences 5'-GGGAUGUUCUUUUGAUAAA and 5'-
519 CCAUCCAACUUACACAUGA was used.

520

521 **Reagents**

522 Hydroxyurea (Sigma aldrich) was used at a final concentration of 4 mM for the
523 indicated time. Aphidicolin (Sigma aldrich) was used at a final concentration of
524 0.3 μM for the indicated time. Furthermore, Cytochalasin B (Sigma aldrich) was
525 used at a concentration of 1 μg/ml.

526

527 **Western blotting and antibodies**

528 Cells were lysed on ice in EBC buffer (50 mM Tris, pH 7.4, 120 mM NaCl, 0.5%
529 NP-40, and 1 mM EDTA) containing protease inhibitors (1% vol/vol aprotinin, 5
530 µg/ml leupeptin, 1 mM PMSF), phosphatase inhibitors (1 mM NaF, 10 mM β-
531 glycerophosphate), and 1 mM DTT. The lysates were sonicated, using a digital
532 sonifier (102C CE Converter; Branson), followed by centrifugation at 20,000xg
533 for 15 min. Proteins were resolved by SDS-PAGE and transferred to
534 nitrocellulose membrane. The membrane were incubated with primary antibody
535 followed by incubation with secondary antibody (HRP-conjugated anti-mouse
536 or -rabbit IgG; Vector Laboratories). Immunoblots were performed using the
537 following antibodies: CtIP (#A300488A, Bethyl Laboratories), RAD51 (#8349,
538 Santa Cruz), RAD51 (#63801, Abcam), PCNA (#18197, Abcam), FBH1
539 (FBXO18, #81563, Santa Cruz), GFP (#1181446000, Roche), Actin (#AB1501;
540 Millipore), RPA2 S4/8 (#A300245A, Biosite), RPA (#NA29L, Millipore), Vinculin
541 (#V9131, Sigma), BRCA2 (#OP95, Calbiochem), H3 (#1791, Abcam), HA
542 (#MMS-101P-500, Covance).

543

544 **Immunofluorescence**

545 The cells were grown on coverslips and treated as indicated and then prepared
546 for immunofluorescence staining. Primary antibodies used were RAD51
547 (1:1,000, 70-001, BioAcademia Jpn), GFP (1:1,000, #1181446000, Roche),
548 RPA (#NA29L, Millipore), RPA2 S4/8 (#A300245A, Biosite). Anti-rabbit Alexa
549 Fluor 647, anti-mouse Alexa Fluor 488 (1:2,000, A21245, A11017, Life
550 Technologies) were used as secondary antibodies. For RAD51,
551 immunofluorescence cells were pre-extracted twice for 3 min in CSK buffer

552 (0.5% Triton X-100, 20 mM Hepes pH 7.4, 100 mM NaCl, 3 mM MgCl₂, and
553 300 mM sucrose) followed by fixation in 4% paraformaldehyde (PFA) (VWR).
554 Cells were permeabilized in 0.5% Triton X-100 followed by incubation in
555 blocking buffer (1% BSA, 0.15% glycine, 0.1% Triton X-100 in PBS wash buffer
556 (1x PBS, 0.1% Tween-20, 1 mM CaCl₂, 0.5 mM MgCl₂). Primary antibody was
557 incubated for 1 h at room temperature in blocking buffer, followed by three
558 washes with PBS wash buffer. Secondary antibody was incubated for an
559 additional hour, washed 3x with PBS wash buffer, and mounted in mounting
560 vectashield with diamidino-2-phenylindole (DAPI) (vector Laboratories). EdU
561 staining was done per manufacturer's instructions (Life Technologies). Z-stack
562 images were acquired on a confocal Zeiss LSM 510 meta microscope
563 workstation, and images were processed and foci enumerated using Fiji
564 (ImageJ).

565

566 **Micronuclei assay**

567 Cells were cultured on coverslips post-transfection and were incubated for
568 another 24 h before starting the treatment of the cells. Cells were treated with
569 Aphidicolin (Sigma aldrich) for 16 h or Hydroxyurea (Sigma Aldrich) for 5 h. In
570 addition, cells were treated with Cytochalasin B (Sigma Aldrich) for 36 h
571 (MCF7), to inhibit cytokinesis, and then fixed in 4% paraformaldehyde (PFA)
572 (VWR). Next, the cells were permeabilized with 0.25% Triton X-100 solution,
573 washed twice with 1xPBS and mounted in Vectashield with diamidino-2-
574 phenylindole (DAPI) (vector Laboratories), binucleated cells with a micronuclei
575 was counted manually using a confocal Zeiss LSM 510 meta microscope and
576 a Scan^R workstation (Olympus).

577

578 **HR assay**

579 U-2-OS cells transfected with CtIP siRNA followed by transfection of gRNAs
580 targeting the LMNA locus and the Ruby Donor plasmid as described in
581 reference (32) together with empty vector or siRNA-resistant Wt, E804del or
582 Δ C CtIP. After 48 h, Lamin A (LMNA) genes were monitored by microscopy.

583

584 **iPond**

585 DOX-inducible U-2-OS cells were transfected with both UNC (negative control)
586 or CtIP siRNA and 24 h later, cells were induced with DOX for 24 h. Cells were
587 incubated with 10 μ M EdU for 15 min, washed in media, then incubated with
588 media containing 4 mM HU for 5 h, cross-linked with 1% formaldehyde,
589 harvested and permeabilised. Biotin azide was covalently attached to EdU
590 within newly replicated DNA using a Click reaction, and EdU containing DNA
591 was precipitated using Streptavidin agarose beads. Eluted proteins were then
592 analysed by SDS-PAGE and WB.

593

594 **DNA fibres**

595 DNA fibres were carried out as described previously (22). Twenty-four hours
596 post siRNA transfection cells were treated with doxycycline to induce CtIP
597 expression, and left for a further 24 h. Cells were then pulse-labelled with CldU
598 and IdU for 20 min each before a 5 h exposure to 4 mM HU. At least 200
599 replication forks were analysed per condition. Tract lengths were measured
600 using Fiji, and ratios calculated.

601

602 **Proximity ligation assay on nascent DNA**

603 Twenty-four hours post siRNA transfection cells were treated with doxycycline
604 to induce CtIP expression, and left for a further 24 h. Cells were then pulse-
605 labelled with 10 mM EdU for 10 min followed by 4 mM HU for 5 hr. After the
606 indicated treatment, cells were pre-extracted for 5 min in buffer (0.5% Triton X-
607 100, 10 mM PIPES pH 6.8, 20 mM NaCl, 3 mM MgCl₂, and 300 mM sucrose)
608 followed by fixation in 4% paraformaldehyde (PFA) (VWR). Cells were
609 incubated in blocking buffer (3% BSA, in PBS with 0.1% Na Azide for 1hr room
610 temperature or O/N in the cold room). After blocking, cells were subjected to
611 Click reaction with biotin-azide for 30 min and incubated overnight with the two
612 relevant primary antibodies at 4°C. The primary antibodies were diluted in PBS
613 with 3% FCS. The primary antibodies used were rabbit polyclonal anti-biotin
614 (1:500, #A150-109A, Bethyl), mouse monoclonal anti-biotin (1:500, #200-002-
615 211, Jackson immunoresearch), rabbit polyclonal anti-CtIP (1:500, # A300-
616 266A, Bethyl). The PLA reaction (Duolink, Sigma Aldrich) to detect anti-biotin
617 antibodies used were performed according to manufacturer instructions.

618

619 **Immunoprecipitation**

620 Extracts for immunoprecipitation were prepared using immunoprecipitation
621 buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10%
622 glycerol, 0.1% Tween) with protease inhibitors. Following preclearing with IgG-
623 coupled protein G beads (GE Healthcare), the lysates were incubated with
624 monoclonal anti-HA (Covance), and complexes were captured using Protein G
625 Sepharose beads (GE Healthcare) for at 4°C on a rotator. The beads were
626 washed five times followed by elution of bound proteins in Laemmli sample

627 buffer.

628

629 **PARPi sensitivity Assay**

630 DOX-inducible U-2-OS cells were seeded on to the CellCarrier-384 Ultra
631 Microplates (PerkinElmer, Massachusetts, United States) and reverse
632 transfection was performed using Lipofectamine RNAiMAX as per the
633 manufacturer's recommendation. After 24 h, DMSO and different
634 concentrations of Talazoparib (BMN 673, Axon Medchem, the Netherlands)
635 were added to the respective wells. On day 3, DMSO and PARPi containing
636 media were replenished. At day 5, CellTiter-Glo (Promega, Wisconsin, United
637 States) was used to quantify the number of viable cells as per the
638 manufacturer's recommendation. Surviving fractions were calculated relative to
639 DMSO-exposed cells for each PARPi concentration.

640

641 **Statistics**

642 Normal distribution was assessed for all experiment. Micronuclei data was
643 normally distributed and subsequently analyzed using a One-way ANOVA and
644 Dunnett's multiple comparison testing, comparing all variants to Wt-CtIP-GFP.
645 The PARP inhibitor data (Supplementary Figure 2i) were Johnson transformed
646 and the obtained, normally distributed data were fitted with a linear mixed
647 model, with replicates as random effect. Multiple comparisons were performed
648 with the lsmeans/diffmeans and the contrast function of the lmer package in
649 R. Significant codes shown are comparing siCtIP and siBRCA2 to the negative
650 control (siUNC). Foci counts, immunofluorescence intensities as well as HRR
651 data were not normally distributed. Therefore, ranks were assigned to all data

652 from three biologically independent replicates, based on the number of foci/
653 immunofluorescence intensity. The obtained ranks were used to fit a linear
654 mixed model. *P* values were adjusted using the holm method if more than two
655 comparisons were made. Biologically relevant *p* values are reported with the
656 following significant codes: $p < 0.0001$ ‘***’; $p < 0.001$ ‘**’; $p < 0.05$ ‘*’. All graphs
657 represent the mean (red line) \pm SEM (black).

658

659 **Study approval**

660 The study was approved by The Capital Region of Denmark (H-4-2010-050)
661 and The Danish data Protection Agency (RH-2016-353, I-Suite no.: 05097)
662 and DBCG (jr. no.: DBCG-2013-15).

663

664

665

666

667 **Author Contributions**

668 R.Z. designed and performed the cell biology experiments and iPOND
669 experiment. M.R.H. designed and performed DNA fibre assay. K.V. performed
670 Micronuclei and HRR assays. B.E. diagnosed and enrolled the breast cancer
671 patients. M.R., B.B. and F.C.N performed sequencing and data analysis. A.N.K.
672 generated an inducible complementation system in U-2-OS cells. H.R.
673 designed the cell biology experiments. M.B. performed the PARPi experiments.
674 R.Z, B.B., M.R.H., M.R., F.C.N., G.S.S. and C.S.S wrote the manuscript. The
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676

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689

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691

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- 785

849 **Tables**

850 Table 1. Identified *RBBP8* variants and allele frequencies. AF = Allele
 851 Frequency; Fisher Exact Test for AF in Group I and Group I + II compared to
 852 AF in controls (2.000 Danish individuals (Lohmueller et al., 2013)). Non-Finnish
 853 European (NFE) in gnomAD.

Nucleotide (HGVS)	Protein (HGVS)	Exon	Group I	Group II	AF Group I (%)	AF Group I + II (%)	AF controls (%)	AF NFE (%)	p-value AF (Group I vs controls)	p-value AF (Group I + II vs controls)
c.298C>T	p.R100W	6	1		0.388	0.041	0.025	0.008	ns	ns
c.329G>A	p.R110Q	6		3	-	0.123	0.153	0.070	-	ns
c.693T>A	p.S231R	9	1		0.388	0.041	-	-	ns	ns
c.1367A>G	p.H456R	12		2	-	0.082	0.127	0.220	-	ns
c.1505G>T	p.R502L	12		1	-	0.041	0.025	0.003	-	ns
c.1928A>C	p.Q643P	13	1	3	0.388	0.164	-	0.014	ns	0.02
c.2024C>T	p.T675I	14		1	-	0.041	-	0.011	-	ns
c.2131G>A	p.E711K	15	1		0.388	0.041	-	-	ns	ns
c.2410_2412del	p.E804del	18	3		1.163	0.123	-	0.015	0.0002	ns
c.2413A>G	p.R805G	18		1	-	0.041	-	0.003	-	ns
c.2516G>A	p.R839Q	19		1	-	0.041	-	0.088	-	ns
c.2620C>G	p.P874A	20		1	-	0.041	-	0.008	-	ns
c.2682G>C	p.E894D	20		1	-	0.041	-	-	-	ns

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866 Table 2. CtIP suppresses genomic instability at perturbed replication forks.
867 MCF7 cells transfected with the indicated siRNA followed by transfection of Wt
868 or its mutated CtIP variants. Further, cells were treated with IR or the indicated
869 dose of APH for 16 h or 4mM HU for 5h and Cytochalasin B for 36 h. DAPI stain
870 was used to visualize nuclei. Cells were imaged with a 20x objective on a
871 Scan^R workstation (Olympus). At least 100 green cells were counted for each
872 genotype per experiment. One-Way ANOVA with Dunnett's multiple
873 comparison test was performed on three independent replicates. All variants
874 were compared to Wt-CtIP-GFP.

CtIP variants	Cytochalasin B		IR		APH		HU	
	% of binuclei with micronuclei	p value	% of binuclei with micronuclei	p value	% of binuclei with micronuclei	p value	% of binuclei with micronuclei	p value
Vector (GFP)	49.95		67.30		66.67		67.14	
Wt	47.37	-	46.46	-	47.96	-	48.36	-
R1100W	45.13	Ns	48.38	ns	47.53	Ns	47.39	ns
R110Q	46.46	Ns	47.99	ns	49.31	Ns	50.18	ns
S231R	47.09	Ns	48.29	ns	49.29	Ns	47.36	ns
H456R	47.76	Ns	49.76	ns	47.99	Ns	45.34	ns
R502L	48.77	Ns	49.43	ns	50.12	Ns	50.14	ns
R589H	48.99	Ns	48.04	ns	48.77	Ns	47.70	ns
Q643P	49.33	Ns	48.45	ns	60.74	**** (0.0001)	57.83	** (0.0030)
E711K	46.35	Ns	48.03	ns	45.47	Ns	48.20	ns
E804del	49.34	Ns	47.99	ns	65.32	**** (0.0001)	69.32	**** (0.0001)
R805G	51.62	Ns	48.94	ns	66.67	**** (0.0001)	68.15	**** (0.0001)
R839Q	49.79	Ns	47.60	ns	49.12	Ns	48.28	ns
P874A	48.72	Ns	48.89	ns	50.20	Ns	48.53	ns
E894D	47.46	Ns	47.39	ns	49.09	Ns	49.17	ns
delta C	50.37	Ns	61.26	**** (0.0001)	59.38	*** (0.0002)	58.78	*** (0.0010)

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Figure 1 Identification of *RBBP8* germline variants

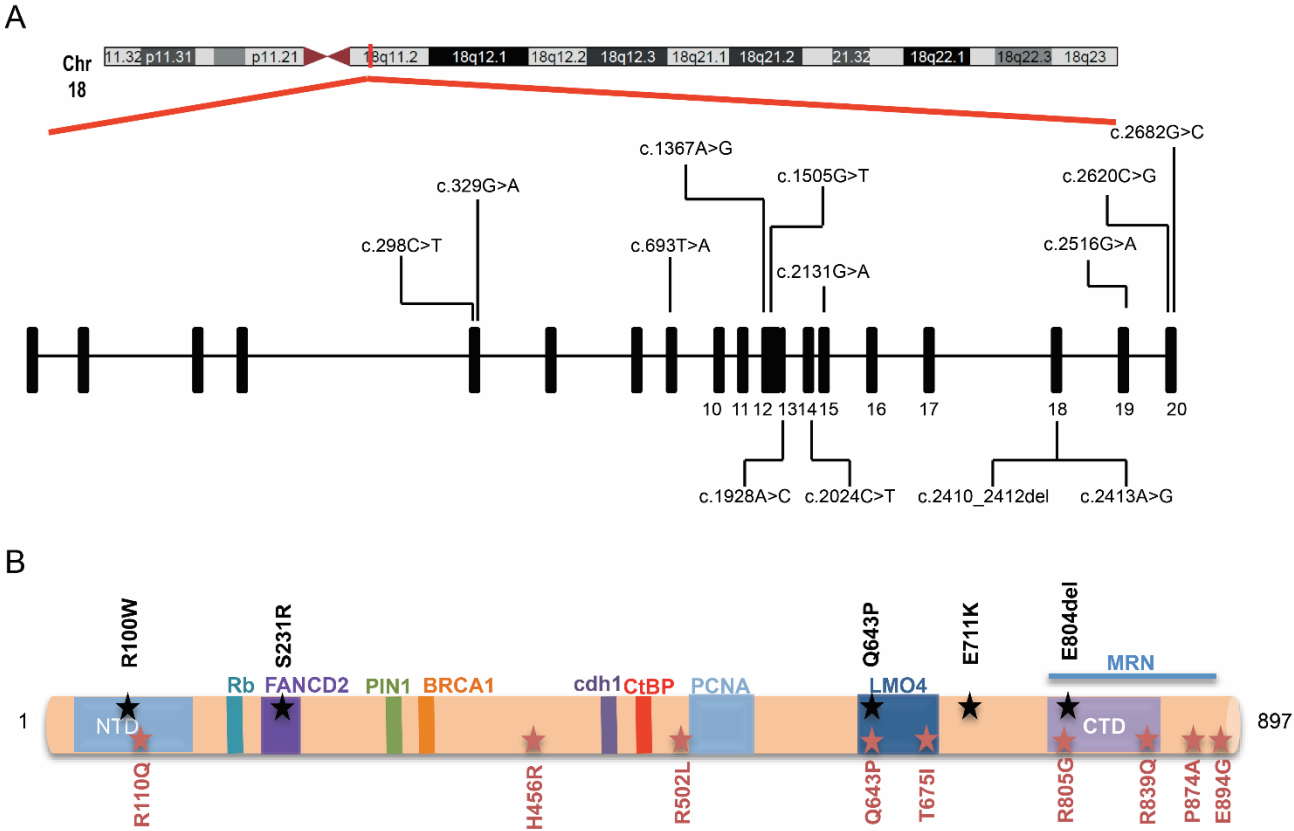


Figure 1. Identification of *RBBP8* germline variants. (A) Schematic representation of the identified variants at gene level indicated according to exon location. (B) Schematic representation of the identified variants at protein level indicated according to known functional domains. The multimerization domain (aa 45-165), the Sae2-like domain (aa 790-897) and the BRCA1 binding site are indicated. All variants further investigated in the functional studies are indicated in bold.

Figure 2. Subset of *RBBP8*/CtIP variants display a genome maintenance defect

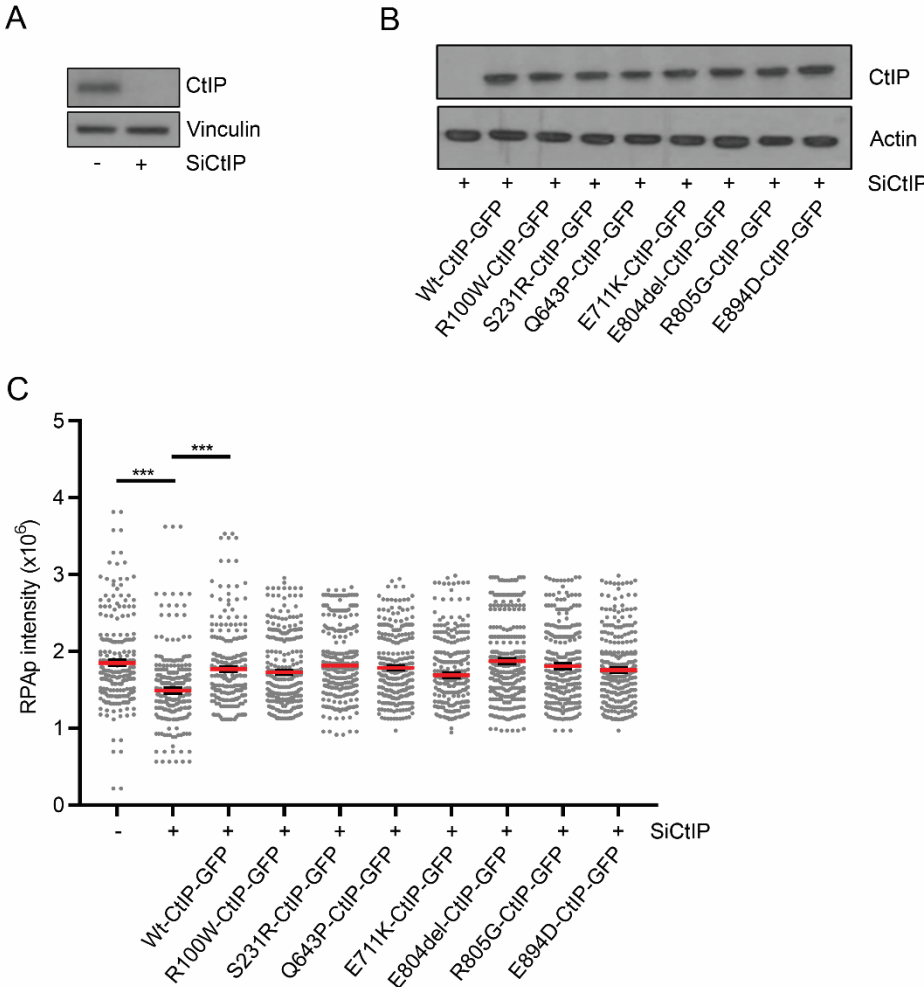


Figure 2. Subset of *RBBP8*/CtIP variants display a genome maintenance defect. (A-B) Western blot analysis of CtIP siRNA, GFP CtIP variants, Actin and Vinculin expression in MCF7 cells. Actin and Vinculin were used as loading controls. (C) The relative intensity of phosphorylated RPA (S4/8) was examined in the total population of Wt or its mutated CtIP variants 3 h post exposure to IR (15 Gy). Cells were fixed and stained for pRPA (S4/8). Each of the variants was compared to Wt-CtIP-GFP, but no significant changes were observed. The displayed data represents three independent biological replicates and per sample $n \geq 280$ nuclei were analyzed. Holm-corrected multiple testing was performed of ranked data fitted by a linear mixed model, comparing all CtIP variants to Wt-CtIP-GFP.

Figure 3. CtIP prevents ssDNA accumulation after replication stress.

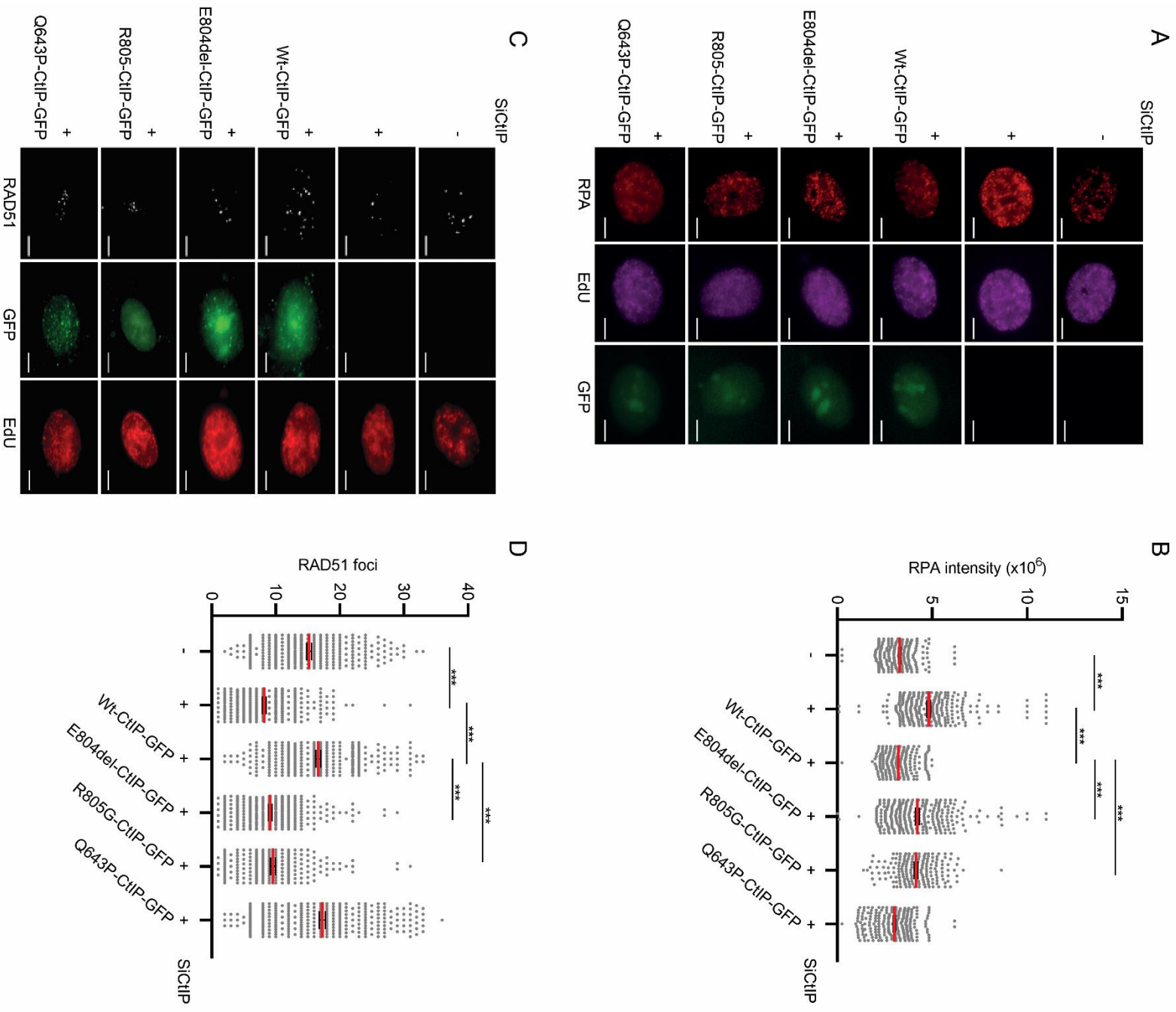


Figure 3: CtIP prevents ssDNA accumulation after replication stress. (A) Representative images displaying RPA in HU-treated EdU-positive cells. Scale bar= 20 μ m. (B) MCF7 cells were transfected with the indicated siRNA and 24 h later, cells were transfected with Wt or its mutated CtIP variants. Afterwards cells were pulsed with 10 μ M EdU for 20 min prior to addition of 4 mM HU. Cells in S phase (EdU+) at the time of HU treatment were Click-IT labeled with an Alexa Fluor 594 azide and RPA intensity in EdU-positive cells were enumerated using Image J/Fiji. The displayed data represents three independent biological replicates and per sample $n \geq 174$ nuclei were analyzed. Holm-corrected multiple testing was performed of ranked data fitted by a linear mixed model, comparing all CtIP variants to Wt-CtIP-GFP. (C) Representative images displaying RAD51 in HU-treated EdU-positive cells. Scale bar= 20 μ m. (D) MCF7 cells were transfected with the indicated siRNA and 24 h later, cells were transfected with Wt or mutated CtIP variants. Afterwards cells were pulsed with 10 μ M EdU for 20 min prior to addition of 4 mM HU. Cells in S phase (EdU+) at the time of HU treatment were Click-IT labeled with an Alexa Fluor 594 azide and RAD51 foci in EdU-positive cells were enumerated using Image J/Fiji. The displayed data represent three independent biological replicates and per sample $n \geq 207$ nuclei were analyzed. Holm-corrected multiple testing was performed of ranked data fitted by a linear mixed model, comparing all CtIP variants to Wt-CtIP-GFP.

Figure 4. CtIP promotes fork protection through FBH1.

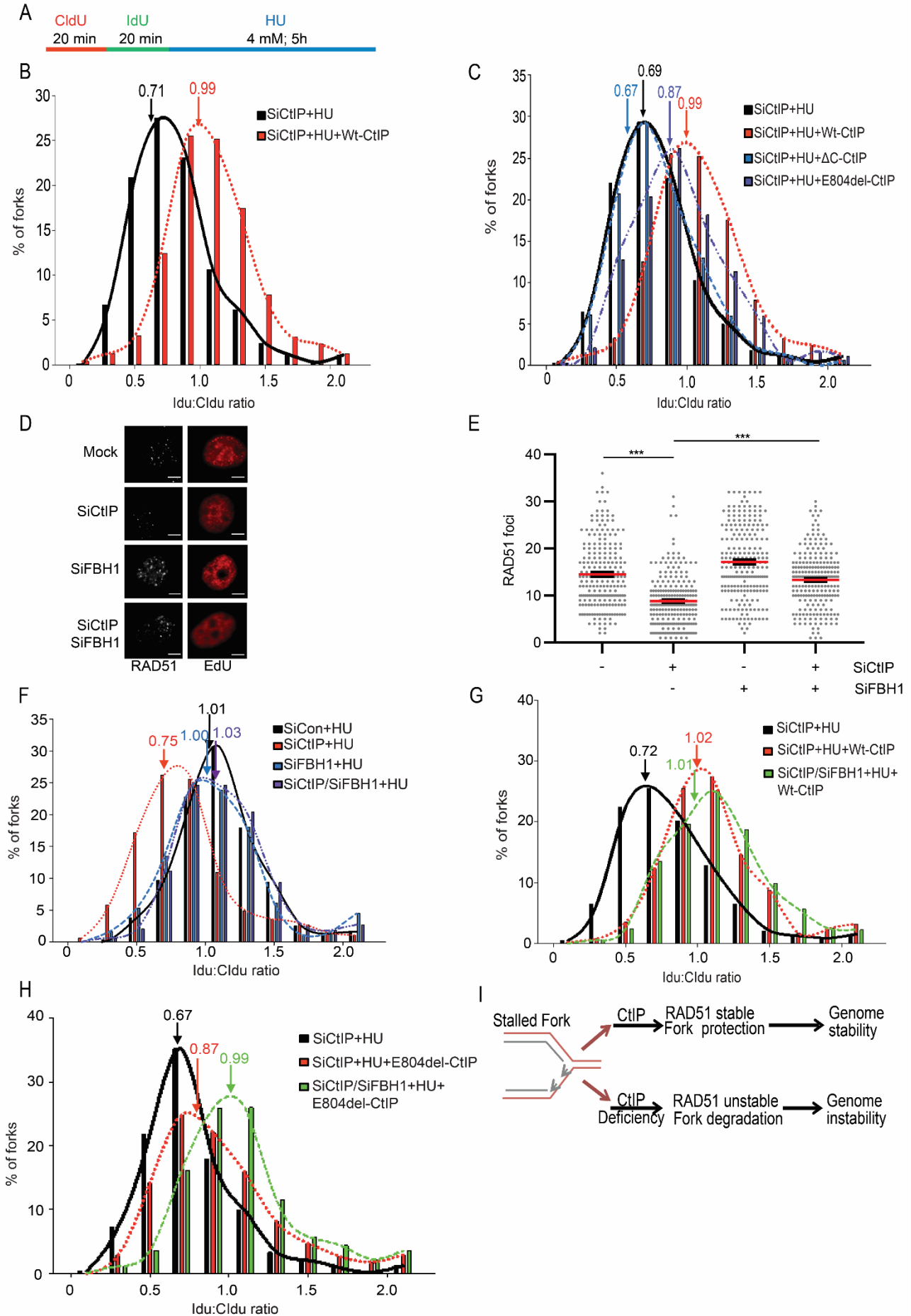


Figure 4: CtIP promotes replication fork protection through FBH1. (A) Experimental scheme of dual labeling of DNA fibres in DOX inducible U-2-OS cells stably expressing the siRNA resistant full-length Wt, E804del or Δ C CtIP. Cells were sequentially pulse-labeled with CldU and IdU, then treated with 4 mM HU for 5 h. (B-C) Loss of CtIP results in replication fork instability in response to replication stress. DOX-inducible U-2-OS cells were transfected with either UNC (negative control) or CtIP siRNA and 24 h later, cells were induced with DOX for 24 h. IdU:CldU ratios are given. (D-E) Representative images displaying RAD51 in HU-treated EdU-positive cells, scale bar= 20 μ m. MCF7 cells were transfected with the indicated siRNAs. Cells were pulsed with 10 μ M EdU for 20 min prior to addition of 4 mM HU. Cells in S phase (EdU+) at the time of HU treatment were Click-IT labeled with an Alexa Fluor 594 azide and RAD51 foci in EdU-positive cells were enumerated using Image J/Fiji. The displayed data represents three independent biological replicates and per sample n=224 nuclei were analyzed. Holm-corrected multiple testing was performed of ranked data fitted by a linear mixed model. (F) U-2-OS cells were transfected with the indicated siRNAs and exposed to 4 mM HU for 5 h. IdU:CldU ratios are given. (G) U-2-OS cells were transfected with the indicated siRNAs and exposed to 4 mM HU for 5 h. IdU:CldU ratios are given. (H) U-2-OS cells were transfected with the indicated siRNAs and exposed to 4 mM HU for 5 h. IdU:CldU ratios are given. (I) Schematic model for the role of CtIP at stalled forks. CtIP regulates RAD51 stability at stalled forks, counteracting the dissolution of the RAD51 filament by FBH1. Loss of CtIP leads to DNA damage accumulation and enhanced chromosomal instability.