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1 **TopBP1 assembles nuclear condensates to switch on ATR signalling.**

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13

14 **Summary**

15

16 **ATR checkpoint signalling is crucial for cellular responses to DNA replication impediments.**

17 **Using an optogenetic platform, we show that TopBP1, the main activator of ATR, self-**

18 **assembles extensively to yield micron-sized condensates. These opto-TopBP1 condensates**

19 **are functional entities organized in tightly packed clusters of spherical nano-particles.**

20 **TopBP1 condensates are reversible, occasionally fuse and co-localise with TopBP1 partner**

21 **proteins. We provide evidence that TopBP1 condensation is a molecular switch that amplifies**

22 **ATR activity to phosphorylate checkpoint kinase 1 (Chk1) and slowdown replication forks.**

23 **Single amino acid substitutions of key residues in the intrinsically disordered ATR-activation**

24 **domain disrupt TopBP1 condensation and, consequently, ATR/Chk1 signalling. In**

25 **physiologic salt concentration and pH, purified TopBP1 undergoes liquid-liquid phase**

26 **separation *in vitro*. We propose that the actuation mechanism of ATR signalling is the**

27 **assembly of TopBP1 condensates driven by highly regulated multivalent and cooperative**

28 **interactions.**

29 **Introduction**

30 The primary structure of DNA is subjected to constant chemical alterations caused by spontaneous
31 decay, endogenous metabolites and environmental genotoxic agents (Friedberg et al., 2006;
32 Lindahl, 1993), hence organisms have evolved multiple DNA repair mechanisms to ensure
33 genome integrity and survival (Ciccia and Elledge, 2010; Jackson and Bartek, 2009; Tubbs and
34 Nussenzweig, 2017). DNA damage sensors, protein scaffolds and DNA processing activities
35 accumulate at DNA damage sites to form spatially defined and reversible structures commonly
36 referred to as nuclear foci (Garcia-Higuera et al., 2001; Lisby et al., 2001; Maser et al., 1997; Park
37 et al., 1996). Much still remains to be understood about the molecular forces that drive the
38 formation of DNA damage foci and their functional consequences in the DNA damage response.

39 In recent years, application of the principles of polymer chemistry to biological molecules
40 has accelerated spectacularly our understanding of the assembly mechanism and functions of
41 membraneless compartments (Banani et al., 2017; Bracha et al., 2019; Hyman and Simons, 2012;
42 Shin and Brangwynne, 2017; Soding et al., 2020). These self-organized micron-scale structures,
43 called biomolecular condensates, assemble via multiple weak, cooperative and dynamic
44 interactions and yield a rich repertoire of higher-order structures with diverse physical properties,
45 variable size, and no defined stoichiometry of constituent proteins. Whereas nucleic acids can
46 serve a seeding platform for the self-organization of soluble proteins (Mao et al., 2011;
47 McSwiggen et al., 2019), soluble bridging factors can cross-link chromatin segments to
48 compartmentalize chromatin via a process of polymer-polymer phase separation (Erdel et al.,
49 2020). Increasing evidence indicates that diverse multivalent protein scaffolds self-organize via
50 liquid-liquid phase separation, a process of de-mixing that yields a condensed phase enriched in
51 the protein and a dilute phase (Banani et al., 2017; Shin and Brangwynne, 2017). Biomolecular

52 condensates are functional hubs implicated in diverse cellular processes, including innate immune
53 signalling (Du and Chen, 2018), microtubule nucleation (Woodruff et al., 2017), transcription
54 (Boija et al., 2018; Kwon et al., 2013; Lu et al., 2018; Sabari et al., 2018) and adaptative stress
55 responses (Franzmann and Alberti, 2019; Franzmann et al., 2018; Riback et al., 2017). Protein
56 phase separation can occur at DNA damage sites. Upon laser micro-irradiation, the activity of
57 poly(ADP-ribose) polymerase 1 seeds the condensation of the prototypical liquid-liquid phase
58 separation protein FUS at damaged chromatin (Altmeyer et al., 2015; Patel et al., 2015). 53BP1
59 phase separates at double-strand DNA breaks (Kilic et al., 2019; Pessina et al., 2019), and the
60 condensation of 53BP1 promotes induction of p53 and p21 (Kilic et al., 2019).

61 To explore the mechanisms and functional consequences of biomolecular condensates in
62 the DNA damage response (DDR), we studied Topoisomerase II β -binding protein (TopBP1), an
63 essential factor in the DDR pathway and a prototype of protein scaffolds composed of multiple
64 modular interaction domains. TopBP1 features nine repetitions of a well-folded protein-protein
65 interaction motif, the BRCA1 C terminus domain (BRCT), and an ATR activation domain (AAD),
66 located between BRCT6 and BRCT7, which is intrinsically disordered. TopBP1 brings together
67 different sets of proteins to form distinct protein complexes involved in DNA replication initiation
68 (Hashimoto and Takisawa, 2003; Makiniemi et al., 2001), DNA replication stress signalling
69 (Kumagai et al., 2006; Mordes et al., 2008), DNA repair (Broderick et al., 2015; Leimbacher et
70 al., 2019; Liu et al., 2017; Moudry et al., 2016) and transcription regulation (Liu et al., 2009;
71 Wright et al., 2006). TopBP1 is the main activator of the master checkpoint kinase ATR (Kumagai
72 et al., 2006; Mordes et al., 2008). ATR and its effector kinase Chk1 are crucial for cellular
73 responses to DNA damage and DNA replication impediments (Ciccia and Elledge, 2010; Marechal
74 and Zou, 2013; Saldivar et al., 2017). ATR/Chk1 signalling ensures cell and organismal survival

75 through coordination of DNA repair and DNA replication with physiological processes (Saldivar
76 et al., 2017). Studies using *Xenopus* egg extracts have largely contributed to defining the
77 orchestrated set of events leading to ATR activation (Acevedo et al., 2016; Byun et al., 2005;
78 Duursma et al., 2013; Kumagai et al., 2006; Van et al., 2010). ATR is recruited to DNA lesions
79 and replication intermediates via the ATR interacting protein ATRIP, which binds RPA-covered
80 single-stranded DNA (Zou and Elledge, 2003). TopBP1 interacts with ATRIP, with ATR and with
81 the RAD9-RAD1-HUS1 (9-1-1) clamp and activates ATR (Delacroix et al., 2007; Duursma et al.,
82 2013; Kumagai et al., 2006; Mordes et al., 2008; Yan and Michael, 2009). Here, we used the
83 conceptual framework born from studies of biomolecular condensates to gain fresh insights into
84 the activation mechanism of ATR/Chk1 signalling. Using a combination of optogenetic and
85 biochemical approaches, we show that TopBP1 self-assembles extensively to yield functional
86 biomolecular condensates, and that purified TopBP1 has an intrinsic capacity to undergo liquid
87 phase separation. We provide evidence that TopBP1 self-organization into micron-sized
88 compartments in living cells activates ATR/Chk1 signalling and slows down the progression of
89 replication forks. Our data indicate that essential responses to DNA replication impediments
90 emerge from TopBP1-driven assembly of nuclear condensates.

91 **Results**

92 **TopBP1 drives the formation of micron-sized nuclear condensates.**

93 TopBP1 forms nuclear foci upon entry into mitosis (Leimbacher et al., 2019; Pedersen et al., 2015),
94 and in cells exposed to hydroxyurea, ultraviolet light or ionizing radiations (Cescutti et al., 2010;
95 Greer et al., 2003). To probe the capacity of TopBP1 to self-organize into biomolecular
96 condensates, we fused TopBP1 to cryptochrome 2 (Cry2) of *Arabidopsis thaliana*, a protein that
97 oligomerises upon exposure to 488nm light and seeds the formation of biomolecular condensates
98 (Bugaj et al., 2013; Kilic et al., 2019; Shin et al., 2017; Zhang et al., 2019) (Figure 1A). This
99 optogenetic system allows to control the nucleation of biomolecular condensates in space and time
100 (Bracha et al., 2018; Shin et al., 2017; Shin et al., 2018), and evaluate the functional consequences
101 of protein condensation (Kilic et al., 2019; Sabari et al., 2018). We induced the expression of
102 TopBP1 fused to mCherry and Cry2 (named opto-TopBP1) with doxycycline in Flp-In HEK293
103 cells. Overall, the expression level of recombinant opto-TopBP1 was similar to the level of
104 endogenous TopBP1 (Figure S1A). At this level of expression, opto-TopBP1 remained in a diffuse
105 state and was not directly detectable by fluorescence microscopy (Figure 1B, light OFF panel).
106 Upon exposure of these cells to an array of blue-light LEDs during 3 minutes of light-dark cycles
107 (4s light followed by 10s dark), we observed multiple and distinct opto-TopBP1 foci in the nuclei,
108 specifically (Figure 1B, light ON panel). By contrast, we did not detect any foci in blue-light
109 exposed cells expressing the opto-module (mCherry-Cry2) alone, nor in cells expressing the
110 checkpoint clamp subunit opto-RAD9 (RAD9-mCherry-Cry2) (Figure S1B).

111 A conserved tryptophane in the ATR activation domain of TopBP1 at position 1138 in *Xenopus*
112 *Laevis*, 1147 in mice, and 1145 in human, is essential for the capacity of TopBP1 to activate ATR
113 (Kumagai et al., 2006) (Zhou et al., 2013). In mice, the substitution of W1147 for an arginine

114 residue is embryonic lethal, indicating that the ATR-activating function of TopBP1 is essential
115 (Zhou et al., 2013). The W1148R substitution does not disrupt the association of xTopBP1 with
116 xATR (Kumagai et al., 2006). Here, we analyzed the impact of W1145R on the capacity of
117 TopBP1 to assemble higher order structures. W1145R TopBP1 expressing cells exhibited a
118 markedly reduced number of optogenetic TopBP1 condensates in comparison with wild-type
119 TopBP1 (Figure 1B), suggesting that this aromatic residue plays an important role in TopBP1
120 higher-order assembly or in the growth of TopBP1 condensates.

121 Ewing's tumor associated antigen 1 (ETAA1) is required for Chk1 basal activity and
122 stability (Michelena et al., 2019), and for the regulation of mitotic ATR signalling (Bass and
123 Cortez, 2019). Like TopBP1, ETAA1 is endowed with an intrinsically disordered ATR activation
124 domain (AAD) (Bass and Cortez, 2019; Bass et al., 2016; Haahr et al., 2016; Lee et al., 2016).
125 Considering the conserved function of these disordered AADs, we replaced the AAD of TopBP1
126 with the AAD of ETAA1, and then tested the capacity of the chimeric opto-TopBP1^{ETAA1-AAD}
127 protein to form condensates. In comparison with TopBP1, the TopBP1^{ETAA1-AAD} chimeric protein
128 exhibited reduced capacity to form condensates (Figure 1C). Consistent with this, optogenetic
129 activation of ETAA1 fused to Cry2 did not yield foci (Figure S1C). Moreover, the AAD of TopBP1
130 did not form optogenetic condensates (Figure 1D). Collectively, these data indicate that TopBP1
131 can self-assemble into micron-sized condensates and that the TopBP1 AAD has unique
132 characteristic features that are necessary but not sufficient for TopBP1 higher-order assembly.

133

134 **Optogenetic and endogenous TopBP1 condensates share similar properties.**

135 A three-dimensional analysis revealed that optogenetically-induced TopBP1 condensates have a
136 spherical shape with an aspect ratio close to one (Figure 2A + Suppl. video), suggesting that the

137 interface of these structures may be subjected to surface tension. Opto-TopBP1 condensates
138 occasionally fused, hence TopBP1 can undergo dynamic clustering (Figure 2B). These structures
139 dissolved within 15 minutes (Figure 2C + Figure S2A), while Cry2 oligomers disassemble
140 spontaneously within 1-2 minutes (Shin et al., 2017). The relative stability of opto-TopBP1
141 condensates reflects the role of TopBP1 multivalent cooperative interactions in the formation of
142 micron-sized protein assemblies. Next, we assessed the permeability of TopBP1 condensates to
143 the surrounding milieu by fluorescence redistribution after photobleaching. We expressed eGFP-
144 TopBP1 in U-2-OS cells with doxycycline for 24 hours (Sokka et al., 2015). In these experimental
145 conditions, overexpressed eGFP-TopBP1 spontaneously forms foci that accumulate in nucleoli
146 (Sokka et al., 2015). After photobleaching, eGFP-TopBP1 bodies recovered fluorescence signal
147 within seconds, which reflects the rapid exchange of eGFP-TopBP1 molecules between the
148 nucleoplasm and TopBP1 nuclear condensates (Figure S2B). We conclude that the boundaries of
149 TopBP1 compartments are permeable. We noted also that eGFP-TopBP1 bodies were sensitive to
150 hexanediol (Figure S2C), an aliphatic alcohol that disrupts weak hydrophobic interactions
151 (Kroschwald et al., 2017).

152 The cooperative interactions that drive the formation of biomolecular condensates are
153 influenced by the properties of the surrounding milieu. Changes in osmotic concentration by
154 addition of sorbitol or sucrose in the cell culture medium disrupt 53BP1 phase separation (Kilic,
155 2019). Likewise, osmotic stress inhibited both the assembly of TopBP1 condensates induced by
156 optogenetic activation (Figure 2D + Figure S2D), and the formation of endogenous TopBP1 foci
157 induced by treatment with the inhibitor of ribonucleotide reductase hydroxyurea (Figure 2E +
158 Figure S2E). This suggests that electrostatic forces drive the assembly of both synthetic
159 (optogenetic) and endogenous TopBP1 condensates. Recombinant opto-TopBP1 also formed

160 nuclear foci in cells exposed to hydroxyurea (Figure S2F), indicating that like endogenous
161 TopBP1, opto-TopBP1 engages with endogenous components in response to stalled replication
162 forks. Whereas TopBP1 condensates appear as homogenous structures by conventional
163 fluorescence microscopy, stimulated emission depletion (STED) nanoscopy with an anti-mCherry
164 antibody revealed the underlying sub-structure of optogenetic TopBP1 condensates, which consist
165 in clusters of spherical, nanometer-sized particles (Figure 2F, left panel). TopBP1 clusters had a
166 very tight appearance when detected with an anti-TopBP1 antibody that recognizes both
167 endogenous and recombinant TopBP1 (Figure 2F, right panel). Likewise, high-resolution imaging
168 of endogenous TopBP1 in cells treated with hydroxyurea revealed numerous sub-structured
169 clusters of nano-condensates (Figure 2G). The data suggest that whether seeded by DNA
170 replication impediments or Cry2 oligomerisation, the driving forces and the organization of
171 TopBP1 nuclear condensates are similar.

172

173 **TopBP1 undergoes liquid-liquid phase separation *in vitro*.**

174 To test if TopBP1 has the capacity to phase separate, we expressed and purified the carboxy-
175 terminal half of TopBP1 (amino acids 884-1522). This portion of TopBP1, hereafter called b6-8,
176 includes the BRCT 6 to 8 and the AAD (Figure 3A + Figure S3B). TopBP1^{b6-8} mediates directly
177 the activation of ATR, independently of its amino-terminal portion (Hashimoto et al., 2006;
178 Kumagai et al., 2006). TopBP1^{b6-8} formed optogenetically-induced foci in live cells (Figure S3A).
179 In the presence of the crowding agent Polyethylene Glycol (2%), purified TopBP1^{b6-8}-GFP formed
180 spherical condensates detected by fluorescence microscopy (Figure 3B, upper panel and Figure
181 S3C). Consistent with optogenetic experiments (Figure 1B), the W1145R substitution abolished
182 the phase separation of TopBP1^{b6-8} (Figure 3B, lower panel). Addition of TopBP1^{b6-8}-RFP to pre-

183 formed TopBP1^{b6-8}-GFP condensates yielded yellow compartments, indicating that soluble
184 TopBP1 molecules are recruited to TopBP1 condensates. By contrast, W1145R TopBP1^{b6-8}-RFP
185 did not stably associate with TopBP1^{b6-8}-GFP condensates (Figure 3C). TopBP1 condensates were
186 permeable to DNA, as revealed by the partitioning of double-strand DNA fragments into pre-
187 formed TopBP1 condensates (Figure 3D). In the presence of a circular DNA plasmid (2.9 kb),
188 TopBP1^{b6-8}-GFP formed clusters of nano-condensates that were reminiscent, yet not equivalent,
189 to high-resolution images of cellular TopBP1 condensates (Figure 3E). This observation suggests
190 that the long anionic DNA polymer strongly influences the assembly of TopBP1 molecules. In the
191 absence of PEG, purified TopBP1^{b6-8}-GFP (10 μ M) did not phase separate (Figure 3B minus PEG,
192 and 3F). To recapitulate partially the complex environment of the nucleus, we spiked “Dignam
193 and Roeder” nuclear extracts with recombinant TopBP1^{b6-8}-GFP in physiologic salt concentration
194 and pH. After incubation of the reaction mixture at 37°C, fluorescence microscopy analyses
195 revealed μ m-scaled TopBP1^{b6-8}-GFP condensates (Figure 3F) co-localizing with endogenous
196 DNA (Figure S3D). Hence, TopBP1 foci-like structures that depend on the integrity of the AAD
197 are recapitulated with purified TopBP1 in the presence of a crowding agent or nuclear extracts.
198 We conclude that TopBP1 has intrinsic capacity to form micron-sized condensates, both *in vitro*
199 and in living cells.

200

201 **Consequence of TopBP1 condensation on TopBP1-associated protein network.**

202 To test if recombinant TopBP1 condensates mimic endogenous TopBP1 foci, we analyzed the
203 composition of TopBP1 condensates assembled in live cells. We used a biotin labelling approach
204 to gain a panoramic view of TopBP1 proximal proteins. A doxycycline-inducible cDNA encoding
205 WT or W1145R TopBP1 fused to the mutated biotin ligase BirA* and the Flag epitope was stably

206 integrated in Flp-In HEK293 cells (Figure 4A). We overexpressed Flag-BirA*-TopBP1 to a level
207 that induces constitutive BirA*-TopBP1 condensates (Figure S4), in the absence of DNA
208 damaging agents. We labelled TopBP1 proximal proteins with biotin for 3 hours. More than 500
209 TopBP1 proximal proteins were identified by mass spectrometry with high confidence and
210 reproducibility (Supplemental Excel sheet). Proteins were ranked according to their iBAQ value
211 (intensity-based absolute quantification), a proxy for protein abundance (Figure 4B). Among
212 abundant TopBP1 proximal proteins, we identified known TopBP1 partners, including TOP2A,
213 FANCI, BRCA1, MRE11, MDC1, 53BP1 and BLM (Figure 4B). Only five proteins showed
214 differences in abundance between WT and W1145R TopBP1 ($\text{Log}_2 \text{diff} > 2$ with a $p\text{-value} \leq 0.05$)
215 (Figure 4C). Among them, only the nucleolar protein NOL11 was detected at high level, and,
216 therefore, considered as a significant difference between WT and W1145R TopBP1. This is
217 consistent with a previous report demonstrating that upregulated TopBP1 accumulates on
218 ribosomal chromatin, segregates nucleolar components and yields nucleolar caps (Sokka et al.,
219 2015). By contrast, W1145R substitution abrogates TopBP1 nucleolar segregation (Sokka et al.,
220 2015). Consistent with this, W1145R TopBP1 had lost proximity with the nucleolar protein
221 NOL11 (Figure 4D). Immunoblot analysis of TopBP1 proximal proteins confirmed that the
222 substitution W1145R in the AAD of TopBP1 does not alter the proximity of TopBP1 with partner
223 proteins implicated in ATR signalling, including ATR, BRCA1, MRE11 and FANCI (Figure 4D).
224 More generally, W1145R did not alter significantly the network of TopBP1 proximal proteins. In
225 conclusion, at a low temporal resolution, whereas the composition of recombinant TopBP1
226 assemblies recapitulates endogenous TopBP1 foci, TopBP1 condensation does not increase
227 significantly the association of TopBP1 with partner proteins.

228

229 **Functional consequences of TopBP1 condensation.**

230 In response to DNA replication impediments, ATR activates the effector checkpoint kinase 1
231 (Chk1) by phosphorylation on Ser345. To establish a functional link between TopBP1
232 condensation and ATR/Chk1 signalling, we exposed a cell culture dish to an array of blue-light
233 LEDs for 3 minutes and then probed cell extracts for ATR mediated Chk1 phosphorylation on
234 Ser345 by western blotting. Optogenetic condensation of TopBP1 induced robust phosphorylation
235 of Chk1 Ser345 (Figure 5A) and of TopBP1 on Ser1138 (Figure 5B, t3), within 3 minutes, in the
236 absence of an exogenous source of DNA damage. By contrast, the clustering defective W1145R
237 TopBP1 mutant protein did not activate ATR/Chk1 signalling (Figure 5A). Consistent with the
238 reversible dissolution of optogenetically-induced TopBP1 foci, phospho-Chk1 (Ser345)
239 immunoblotting signals disappeared 20 minutes after optogenetic activation (Figure 5B, t23). Re-
240 activation of TopBP1 condensates with blue light re-induced Chk1 phosphorylation (Figure 5B,
241 t26), until dissolution of the reactivated TopBP1 condensates (Figure 5B, t46). We reiterated the
242 process four times (Figure 5B). The data indicate that Chk1 activation by ATR intersects precisely
243 with TopBP1 condensation, suggesting that TopBP1 condensation operates as a switch-like
244 mechanism that amplifies ATR activity above a threshold required for Chk1 phosphorylation.

245 Of note, we observed the spontaneous activation of endogenous Chk1 in “Dignam and
246 Roeder” nuclear extracts after 10 minutes incubation at 37°C (Figure S5A), in the absence of
247 recombinant TopBP1 protein and without an exogenous ATR-activating DNA structures. The
248 level of phospho Chk1 (Ser345) signals was significantly higher than background signals observed
249 when reaction mixtures were incubated at 4°C, or at 37°C in the presence of the ATR and mTOR
250 inhibitor ETP-46464 (Figure S5A), confirming that the reaction was the product of ATR activity
251 and occurred *in vitro*. Phosphorylation of Chk1 was blocked when nuclear extracts were pre-

252 incubated with ethidium bromide, which disrupts protein-DNA interaction (Figure S5B),
253 indicating that endogenous DNA fragments present in the extracts are required for ATR activation.
254 The activation of endogenous ATR in nuclear extracts suggests that when a critical concentration
255 of reactants is reached, a productive supramolecular ATR signalling complex assembles
256 spontaneously.

257 Once activated by ATR through Ser345 phosphorylation, Chk1 auto-phosphorylates on
258 Ser296. This step is required for Chk1 to induce downstream molecular events leading to cell cycle
259 arrest (Kasahara et al., 2010). Light-induced TopBP1 condensation yielded Chk1 Ser296 phospho-
260 signals, confirming that Chk1 is active (Figure S6A). UCN-01, an inhibitor of Chk1, blocked Chk1
261 auto-phosphorylation on Ser296, but had no major impact on Chk1 phosphorylation on Ser345 by
262 ATR (Figure S6A). This confirms that the Chk1 phospho Ser296 signal is a product of Chk1
263 activity. To explore further the impact of TopBP1 condensation on the interaction of TopBP1 with
264 partner proteins, we took advantage from the optogenetic tool shown in Figure 1A. The TopBP1-
265 mCherry-Cry2 used for light-induced foci formation was tagged at its N-terminus with TurboID,
266 an optimized biotin ligase that can biotinylate proteins within minutes (Branon et al., 2018). To
267 detect ATR activity within optogenetic TopBP1 condensates, we induced TopBP1 condensation
268 by 488 nm light in the presence of biotin in the cell culture medium, and then purified biotinylated
269 proteins with streptavidin-coated beads. We enriched phospho ATR (Thr1989) and phospho
270 TopBP1 (Ser1138) signals from cells expressing WT TopBP1 after optogenetic activation,
271 specifically (Figure 5C). Furthermore, this biotin labelling strategy allowed to capture some Chk1
272 phosphorylation events occurring within TopBP1 condensates (Figure S6B). By contrast, we did
273 not isolate biotinylated phospho ATR, phospho TopBP1 and phospho Chk1 in proximity to
274 W1145R TopBP1 (Figure 5C and Figure S6B). These data indicates that the condensation of

275 TopBP1 promotes the phosphorylation of ATR target proteins. In fluorescence microscopy,
276 optogenetic TopBP1 condensates co-localised with RAD9 and with phospho ATR (Figure S6C-
277 D). By contrast, Chk1 Ser345 phospho-signals rarely co-localised with TopBP1 condensates *per*
278 *se*, but were detected in cells positive for TopBP1-mCherry condensates (Figure S6E), consistent
279 with Chk1 high mobility (Liu et al., 2006). Chk1 is not retained physically at DNA damage sites,
280 allowing signal transmission from DNA damage sites to the rest of the cell (Liu et al., 2006).
281 Super-resolution STED imaging revealed phospho ATR (Thr1989) signals intertwined with
282 TopBP1 nano-condensates in TopBP1 clusters (Figure 5D). These observations indicate that
283 TopBP1 condensates function as reaction hubs.

284 As optogenetic TopBP1 condensates were chromatin bound (Figure S6F), we analyzed the
285 consequences of TopBP1 condensation on the progression of DNA replication forks using a DNA
286 fiber labelling approach. We induced TopBP1 condensation during the CldU pulse using two
287 cycles of 3 minutes blue-light illumination, in order to actuate and maintain TopBP1 foci during
288 the 20 minutes labelling period (Figure 5E). DNA replication tracks labelled in the presence of
289 optogenetic TopBP1 condensates were shorter than DNA replication tracks labelled in the absence
290 of optogenetic activation. By contrast, blue-light illumination did not alter the progression of
291 replication forks in cells that express the condensation defective mutant W1145R TopBP1. We
292 conclude that TopBP1 condensates are functional entities. Mechanistically, TopBP1 condensation
293 triggers activation of ATR/Chk1 signalling.

294

295 **Regulation of TopBP1 condensation**

296 TopBP1 is highly phosphorylated in response to DNA replication stress (Munk et al., 2017), and
297 the activation of ATR/Chk1 signalling is dependent on TopBP1 phosphorylation (Burrows and

298 Elledge, 2008; Hashimoto et al., 2006; Yoo et al., 2007). We reasoned that the basal kinase activity
299 of ATR, which is independent of TopBP1 (Liu et al., 2011), may play a role in the condensation
300 process. To test this, we pre-incubated cells with the ATR inhibitors VE-821 (Charrier et al., 2011),
301 or ETP-46464 (Llona-Minguez et al., 2014), for 1 hour, and then exposed opto-TopBP1 expressing
302 cells to blue light for 3 minutes. In these experimental conditions, the formation of TopBP1
303 condensates was inhibited and the phosphorylation of Chk1 on the ATR site Ser345 was blocked
304 (Figure 6A + Figure S7A). By contrast, pre-incubation of the cells with the Chk1 inhibitor UCN-
305 01 did not affect the formation of optogenetic TopBP1 condensates (Figure S7B). To explore
306 further the role of ATR in TopBP1 condensation, we mutated key amino acids in the AAD (Figure
307 6B). We substituted TopBP1 phenylalanine at position 1071 with alanine. This substitution locates
308 within a predicted coiled coil in TopBP1 AAD and partially destabilizes the interaction of TopBP1
309 with ATR (Thada and Cortez, 2019). The F1071A substitution severely reduced the optogenetic
310 induction of TopBP1 condensates (Figure 6C, left panel + Figure S7C). Biotin labelling revealed,
311 however, that F1071A TopBP1 remained proximal to ATR and basal phosphorylation on Ser1138
312 was detected (Figure 6D). The Ser1138 phospho signal was induced upon optogenetic activation
313 of WT TopBP1 condensates, but not in cells expressing F1071A TopBP1, consistent with a
314 condensation defect (Figure 6D). Several contact points maintain TopBP1 in proximity with the
315 ATR-ATRIP complex (Thada and Cortez, 2019). Hence, the reduction of the strength of the
316 association of F1071A TopBP1 with ATR-ATRIP does not fully account for the severe
317 condensation defect observed in these experimental conditions (Figure 6C).

318 In *Xenopus Laevis*, the phosphorylation of Ser1131 enhances the capacity of TopBP1 to activate
319 ATR (Yoo et al., 2009). XTopBP1Ser1131 corresponds to Ser1138 in human TopBP1. Ser1138A
320 partially inhibited the optogenetic activation of TopBP1 condensates, whereas the phospho mimic

321 S1138D stimulated TopBP1 condensation (Figure 6C, left panel + Figure S7C). The partial
322 suppression of TopBP1 condensates by S1138A suggests that additional phospho-sites contribute
323 to the cooperative assembly of TopBP1 condensates. In blue-light exposed cells expressing
324 F1071A TopBP1 or S1138A TopBP1, the level of Chk1 phosphorylation on Ser345 was reduced
325 in comparison with cells expressing WT TopBP1 (Figure 6C, right panel). Furthermore, phospho
326 Ser1138 TopBP1 and phospho Thr1989 ATR signals were barely detectable in streptavidin
327 pulldowns (Figure 6D). By contrast, the phosphomimetic substitution S1138D in TopBP1 yielded
328 phospho Ser345 Chk1 signals upon optogenetic activation (Figure 6C, right panel) and phospho
329 Thr1989 ATR was enriched in proximity of TopBP1 (Figure 6D). Collectively, the data suggest
330 an amplification mechanism for activation of ATR/Chk1 signalling, whereby TopBP1
331 phosphorylation by ATR induces TopBP1 condensation, and TopBP1 condensation unleashes its
332 capacity to activate ATR.

333 **Discussion**

334 In this study, we provide evidence that TopBP1 is a protein scaffold that can self-assemble
335 extensively to yield tight clusters of nano-condensates, and that the association of TopBP1 with
336 ATR yields a positive feedback loop. We propose that TopBP1 condensation is a molecular switch
337 that triggers checkpoint responses to DNA replication impediments. TopBP1 condensation
338 depends on the basal kinase activity of ATR and on the phosphorylation of TopBP1 on serine
339 1138. Thus, we propose a refined model of ATR activation (Figure 7). In the early stages of ATR
340 signalling, ATR-ATRIP and TopBP1 congregates on RPA-coated single-stranded DNA, the 9-1-
341 1 complex is loaded at single to double strand DNA junctions and stabilizes TopBP1. In later
342 stages, the phosphorylation of TopBP1 induces its higher-order assembly into micron-sized
343 clusters of nano-condensates that propel ATR signal transduction. The yeast homologue of ATR,
344 Mec1, has been proposed to be activated via an allosteric mechanism (Wang et al., 2017). We
345 surmise that the compartmentation of ATR signalling proteins creates a reaction hub where the
346 probability of molecular interactions required for ATR activation is increased. A TopBP1
347 clustering mechanism for ATR activation is reminiscent of Ras clusters that assemble transiently
348 on the plasma membrane, and function as high-gain amplifiers critical for MAPK signal
349 transduction (Prior et al., 2003; Tian et al., 2007).

350 The data shown here suggest that the molecular forces driving TopBP1 condensation are
351 multiple weak and highly cooperative interactions. First, whether seeded by hydroxyurea-induced
352 replication stress or Cry2 oligomerisation, TopBP1 nuclear condensates were dissolved upon
353 addition of sorbitol or sucrose in the cell culture medium. These compounds destabilize weak
354 electrostatic interactions involved in protein phase separation. The aliphatic alcohol hexanediol
355 also dissolves TopBP1 condensates, suggesting that hydrophobic interactions contribute to

356 TopBP1 higher order assembly. Second, purified TopBP1 undergoes liquid-liquid phase
357 separation, a characteristic feature of multivalent protein scaffolds that underpin the formation of
358 membrane-less compartments. TopBP1 phase separation *in vitro* occurred also in the complex
359 environment of a nuclear extract, where multiple homotypic and heterotypic interactions could
360 influence the capacity of TopBP1 to self-assemble. Third, TopBP1 condensation was highly
361 sensitive to key amino acids substitutions and post-translational modifications in the AAD. These
362 modifications typically change the cooperative molecular forces that organize protein
363 condensation. Last, our results suggest that the BRCT6-8 also contributes to TopBP1 higher-order
364 assembly, consistent with data showing that BRCT7/8 promotes TopBP1 oligomerisation
365 (Chowdhury et al., 2014). We observed that both optogenetic and endogenous TopBP1 form tight
366 clusters of nano-condensates. Interestingly, in mitosis, TopBP1 forms filamentous structures that
367 bridge MDC1 foci (Leimbacher et al., 2019), suggesting that specific molecular associations
368 dictate the organization of TopBP1 higher-order structures.

369 The function of TopBP1 condensation described here explains previous observations. In
370 *Saccharomyces cerevisiae*, artificial co-localization of the 9-1-1 complex and Ddc2^{ATRIP}-Mec1^{ATR}
371 via tethering to an array of 256 LacO repeats bypasses the requirement for DNA damage to activate
372 Mec1^{ATR} (Bonilla et al., 2008). In *Schizosaccharomyces pombe*, artificial tethering of either one
373 of Rad3^{ATR}, RAD4^{TopBP1} or RAD9 to a LacO array triggers a checkpoint response that utilizes the
374 endogenous proteins (Lin et al., 2012). Furthermore, TopBP1 activates ATR *in vitro* and in cells
375 when artificially tethered to DNA (Lindsey-Boltz and Sancar, 2011). Based on the findings
376 described here, we surmise that the artificial tethering of checkpoint proteins to LacO arrays is
377 nucleating the condensation of endogenous proteins, which switches on checkpoint signalling.

378 The transient and reversible nature of the molecular forces that underpin the formation of
379 functional TopBP1 nuclear condensates appears well adapted to cellular regulation and optimal
380 responsivity to DNA replication impediments, as opposed to the stable interaction of proteins that
381 characterize molecular machines with defined stoichiometry. The formation of functional micron-
382 sized condensates through the regulated self-assembly of multivalent protein scaffolds may
383 represent a fundamental principle underlying the formation of functional nuclear foci in response
384 to DNA damage.

385

386 **Limitations**

387 We did not deplete endogenous TopBP1 in our tractable optogenetic system because the ATR-
388 activating function of TopBP1 is essential. As TopBP1 self-interacts, we must consider that
389 endogenous TopBP1 enters into the composition of optogenetic TopBP1 condensates, and may
390 attenuate the impact of amino acids substitutions on the properties of recombinant TopBP1. This
391 study does not reveal the underlying molecular organization of TopBP1 condensates. Accurate
392 energetic and structural analyses will be necessary to understand how point mutations in the ATR
393 activation domain affect the kinetics of TopBP1 condensation. Single molecule tracking and
394 quantitative measurements of the dynamics of molecules at the interface between the dense and
395 the dilute phases will give insights into the biophysical properties of TopBP1 condensates.

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404

405 **Author contributions**

406 Conceptualization: C.F., J.B. and A.C; Methodology: C.F., J.B. and A.C.; Investigation: C.F.,
407 A.P., S.V., E.A., M.L., M-P.B., S.U. and J.B.; Data curation: C.F., A.P. J.B and A.C. Writing-
408 original draft: C.F., J.B. and A.C. Writing-Review & Editing: C.F., A.P., J.B. and A.C.
409 Supervision: J.B. and A.C. Project Administration: A.C. Funding Acquisition: A.C.

410

411 **Declaration of Interests**

412 The authors declare no competing interests.

413 **Main Figure Legends**

414 **Figure 1. Optogenic activation of TopBP1 condensates.** **A)** Schematic representation of the
415 TopBP1 optogenetic platform **B)** Optogenetic activation of wild-type and W1145R TopBP1. The
416 opto-module denotes mcherry-Cry2. Light ON: 3 min cycles of 4s light (488nm)-10s resting.
417 Representative fluorescence images are shown. DNA is stained with Hoechst 33258. Scale bars:
418 10 μ m. Violin plot represents distribution of number of condensates per cell. Median and quartile
419 values are represented by continuous and dashed lines, respectively. The statistical significance
420 between samples is represented by *. Number of biological replicates = 3. See Figure S1A for
421 protein expression levels. **(C)** Optogenetic activation of opto-TopBP1^{ETAA1-AAD}. **(D)** Optogenetic
422 activation of opto-AAD-NLS^{TopBP1}. FL stands for Full Length.

423

424 **Figure 2. Properties of TopBP1 condensates.** **A)** 3D analysis of opto-TopBP1 condensates by
425 IMARIS software. Sphericity = $\text{area}^{\text{sphere}}/\text{area}^{\text{particle}}$. The 3D projection and the spherical value for
426 particle 1 (P1) is represented. Other particles were analyzed for their spherical values
427 (P2=0.932587; P3=0.918362; P4=0.949144; P5=0.935103). Scale bar: 2 μ m. **B)** Time laps
428 microscopy images of activated opto-TopBP1 condensates. **C)** Histograms representing the mean
429 of mCherry-labelled condensates per cell. Activated TopBP1 WT condensates were incubated in
430 the dark for the indicated time. **(D)** Violin plot representing the number of optogenetic TopBP1
431 condensates in cells were pre-treated with 0.5M sucrose or 0.4M sorbitol for 1h, as indicated. **(E)**
432 Violin plot representing the number of endogenous TopBP1 foci per cell in cells treated with 5mM
433 HU and 0.5M sucrose or 0.4M sorbitol, as indicated. The values correspond to the sum of three
434 independent experiments. **C-D-E)** Number of biological replicates = 3. See Figure S2A, S2D and
435 S2E for representative images. **F)** Representative super-resolution STED images of optogenetic

436 TopBP1 condensates identified with anti-mCherry (left panel) or anti-TopBP1 (right panel). **G)**
437 STED image of endogenous TopBP1 induced by hydroxyurea (left panel). Right panel: untreated
438 control cells. Scale bars: 2 μ m for CONFOCAL images and 1 μ m for STED images.

439

440 **Figure 3. Purified TopBP1^{b6-8} phase separates.** **A)** Schematic representation of recombinant WT
441 and W1145R TopBP1 (TopBP1^{b6-8}), tagged with 6xHistidine and maltose binding protein (MBP)
442 at the N-terminus, and monomeric Green Fluorescent Protein (GFP) at the C-terminus. PreScission
443 (3C) and TEV protease sites are indicated. See Figure S3B for stain-free gel of recombinant
444 proteins. **B)** Representative images of TopBP1 (10 μ M) incubated in physiological buffer and,
445 when indicated, 2% PEG. Scale bars: 10 μ m. See also Figure S3C. **C-D)** Schematic and
446 representative images of TopBP1^{b6-8}-RFP (1 μ M) (**C**) and double-stranded DNA (300nM) (**D**)
447 trapping by pre-formed TopBP1^{b6-8}-GFP (10 μ M) droplets. DNA stained with Hoechst 33258 (**D**).
448 Scale bars: 10 μ m. Line scan of GFP-RFP (**C**) and GFP-Hoechst (**D**) signals is used to analyze co-
449 localisation. **E)** Confocal image of purified (2.5 μ M) WT TopBP1^{b6-8} and 40ng 2.9 kb circular
450 DNA plasmid DNA marked with Hoechst 33258. Scale bar: 2 μ m. **F)** Fluorescence microscopy
451 image of condensates in suspension after incubation of purified (2 μ M) WT and W1145R
452 TopBP1^{b6-8} in nuclear extracts (0.2 μ g/ μ l). Control images of TopBP1^{b6-8}-GFP (10 μ M) alone and
453 of protein extract (0.2 μ g/ μ l) are shown. Scale bars: 10 μ m. See Figure S3D for co-localization with
454 endogenous DNA. **B-C-D-E-F)** Number of biological replicates = 3.

455

456 **Figure 4. Composition of TopBP1 condensates.** **A)** Schematic representation of the Flag-BirA*
457 TopBP1 platform. See Figure S4 for representative images of BirA*-TopBP1 condensates. **B-C)**
458 Proteins identified by mass spectrometry were ranked according to their iBAQ value (**B**) or

459 compared in a Volcano plot (C). A standard t-test was used to evaluate differences in protein
460 abundance between samples. **D)** Streptavidin pulldowns of proteins biotinylated by WT and
461 W1145R Flag-BirA*-TopBP1 were probed for the indicated proteins by immunoblotting. When
462 indicated (+), expression of Flag-BirA*-TopBP1 was induced with 1µg/ml doxycycline.

463

464 **Figure 5. TopBP1 condensation activates ATR/Chk1 signaling.** **A)** The indicated proteins were
465 probed by immunoblotting after light activation of cells expressing the opto-module control, WT
466 TopBP1 or W1145R TopBP1, as indicated. **B)** Reiterative activation of ATR/Chk1 signaling.
467 Resting and optogenetic activation times are indicated in red and green, respectively. **C)**
468 Streptavidin pulldowns of biotinylated proteins before and after optogenetic activation, as
469 indicated. See Figure S6B for biotinylated phospho-Chk1 signal. **D)** Representative super-
470 resolution STED image of opto-TopBP1 and pATR (Thr1989) fluorescent signals. Co-localisation
471 is indicated by line scan. Scale bar: 1µm. **E)** Replication tracks were labelled with two consecutive
472 20' pulses of CldU and IdU. Dot plots represent CldU/IdU incorporation ratio of cells expressing
473 WT and W1145R opto-TopBP1, before (-) and after (+) optogenetic activation. Mean values are
474 represented by red lines and the statistical significance among them is represented by *. Number
475 of biological replicates = 3.

476

477 **Figure 6. Regulation of TopBP1 condensation.** **A)** Impact of ATR inhibitors on TopBP1
478 condensation. Violin plot represents the number of light-induced TopBP1 condensates in cells pre-
479 treated with 10µM ATR inhibitors ETP-46464 or VE-822, as indicated. Cells used for optogenetic
480 experiments were probed for the indicated proteins by immunoblotting. See Figure S7A for
481 representative images. **B)** Alignment of ATR Activation Domain (AAD) sequences from Human,

482 Mouse and Xenopus. **C)** Violin plot represents the number of light-induced condensates in cells
483 expressing the indicated mutant proteins. Cells were probed for the indicated proteins by
484 immunoblotting. See Figure S7C for representative images. **D)** Streptavidin pulldowns of proteins
485 biotinylated by WT or mutants opto-TopBP1 proteins were probed for the indicated proteins by
486 immunoblotting. **A-C-D)** Number of biological replicates = 3.

487

488 **Figure 7. Model of ATR activation.** TopBP1 condensation enables ATR signal transduction.

489

490 **STAR Methods Text**

491 **RESOURCE AVAILABILITY**

492 ***Lead Contact***

493 Further information and requests for resources and reagents should be directed to and will be
494 fulfilled by the Lead Contact, Angelos Constantinou (angelos.constantinou@igh.cnrs.fr).

495

496 ***Materials Availability***

497 Plasmids generated in this study are available upon request.

498

499

500 ***Data and Code Availability***

501 Original data for figures in the paper are available at Mendeley
502 (<http://dx.doi.org/10.17632/6nf7b7ffb7.1>).

503

504 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

505 ***Human Cell lines***

506 *Flp-InTMT-RExTM293* and *HEK293* cell lines were grown under standard conditions (37°C, 5%

507 CO₂) in Dulbecco's modified Eagle's medium (Merck-Sigma-Aldrich, D5796). For *Flp-InTMT-*

508 *RExTM293* the medium was supplemented with 10% fetal bovine serum (FBS), 100µg/ml Zeocin

509 and 15µg/ml Blasticidin. *Flp-In*^{TMT}-*REx*^{TMT293} transfected cells were selected and maintained with
510 15µg/mL Blasticidin and 150µg/mL Hygromycin.
511 U-2-OS Tet-On cell lines expressing eGFP-TopBP1 WT and RPE1 cell lines were grown under
512 standard conditions (37°C, 5% CO₂) in modified McCoy's 5a medium (Merck-Sigma-Aldrich,
513 M9309) supplemented with 10% fetal bovine serum (FBS), 100µg/ml of Hygromycin and
514 200µg/ml of G418 as selective antibiotics (Sokka et al., 2015). Expression of eGFP-TopBP1 WT
515 was induced with 1µg/ml of doxycycline for 24 hours.

516

517 *Sf9 insect cells*

518 Sf9 cells were grown in EX-CELL® 420 Serum-free medium (Sigma-Aldrich, 14420C). Cells
519 were maintained between 2x10⁶ and 1x10⁷ cells/ml at 28°C in flasks (agitation 140 rpm).

520

521 **METHOD DETAILS**

522 *Plasmid constructs*

523 Oligonucleotides used for plasmids construction are listed in Supplementary Table S1. For
524 pCDNA5_FRT-TO_Flag-BirA*-TopBP1 WT or W1145R, TopBP1 full-length cDNA (a kind gift
525 from Lee Zou) was amplified by PCR with primers 1 and 2 using Phusion® High-Fidelity DNA
526 Polymerase (New England Biolabs, CM0530). The forward and reverse primers contain *AscI* and
527 *NotI* sites, respectively. The amplified PCR was inserted into the pCDNA5_FRT-TO_Flag-BirA*
528 (a kind gift from Biran Raught) linearised with *AscI/NotI* digestion.

529 For pCDNA5_FRT-TO_mCherry-Cry2 (opto-module#), the Flag-BirA* fragment was deleted
530 from the pCDNA5_FRT-TO_FlagBirA* using *KpnI/AscI* enzymes and replaced with the
531 mCherry-Cry2 fragment amplified by PCR from the plasmid pHR-mCherry-Cry2 (a kind gift from

532 Brangwynne's lab) with primers 4 and 5. In the second step, TopBP1 amino acids 884 to 1522
533 fragment (BRCT6-AAD-BRCT7-8 WT or W1145R) was amplified with primers 6 and 7, digested
534 with *KpnI/NheI* and inserted into pCDNA5_FRT-TO_mCherry-Cry2 to produce opto-TopBP1^{b6-}
535 ⁸WT and opto-TopBP1^{b6-8}W1145R. For pCDNA5_FRT-TO_TurboID-mCherry-Cry2
536 construction (opto-module), the TurboID fragment was amplified by PCR from the 3xHA-
537 TurboID-NLS_pCDNA3 plasmid (Addgene #107171) with primers 8 and 9, digested with
538 *AflIII/KpnI* enzymes and inserted into the opto-module# to produce the opto-module. In the second
539 step, TopBP1 WT and W1145R were amplified by PCR with primers 10 and 11, digested with
540 *PmlI/KpnI* enzymes and inserted into the opto-module construct. Mutations in the AAD of TopBP1
541 were generated using the "QuickChange Multi Site-directed mutagenesis kit" (Agilent
542 technologies, C200515): W1145R with primer 3, F1071A with primer 12, S1138A with primer
543 13, S1138D with primer 14. pCDNA5_FRT-TO_TurboID-AAD-NLS^{TopBP1}-mCherry-Cry2 was
544 generated by PCR amplification of TopBP1 AAD (primers 15-16) and TopBP1 NLS (primers 17-
545 18). Fragments were inserted in 2 steps into the opto-module digested with *AflIII/KpnI* (TopBP1
546 AAD) and *KpnI/NheI* (TopBP1 NLS) enzymes respectively. pCDNA5_FRT-TO_TurboID-
547 ETAA1-mCherry-Cry2 was generated by PCR amplification of ETAA1 with primers 19 and 20
548 on pCDNA5_FRT-TO_GFP-ETAA1 (a kind gift from Mailand's lab) and insertion of the
549 fragments into the opto-module digested with *KpnI/NheI* enzymes. pCDNA5_FRT-TO_TurboID-
550 RAD9A-mCherry-Cry2 was generated by PCR amplification of RAD9 with primers 21 and 22 on
551 pDONR223-RAD9A (obtained through MGC Montpellier Genetic Collections) and fragment was
552 cloned into the *KpnI*-digested opto-module following the *In-Fusion HD Cloning Kit* protocol. The
553 plasmid with the chimeric constructs of TopBP1 carrying the AAD of ETAA1 (pCDNA5_FRT-
554 TO_TurboID-TopBP1^{ETAA1-AAD}-mCherry-Cry2) was generated using the *NEBuilder HiFi DNA*

555 *assembly Master Mix* (New England Biolabs, E2621L). This kit was used to assemble multiple
556 DNA fragments with 30 bp-overlap and replace 1530 bp inside TopBP1. DNA fragments were
557 produced by PCR: oligos 23-24 were used to amplify TopBP1 sequence before the AAD (PCR on
558 any plasmid containing full-length TopBP1), oligos 25-26 were specific for the AAD of ETAA1
559 (PCR on pCDNA5_FRT-TO_GFP-ETAA1) and oligos 27-28 were specific for TopBP1 portion
560 after the AAD (PCR on any plasmid containing full-length TopBP1). To obtain the chimeric
561 constructs, PCR products were assembled according to the manufacturer's instruction and ligated
562 into pCDNA5_FRT-TO_TurboID-TopBP1-mCherry-Cry2 digested with *EcoNI/SbfI*.
563 pFastBac1 plasmids containing WT and W1145R BRCT 6 to 8 fragments of TopBP1 were
564 synthesized by GeneScript after codon optimization for expression in insect cells (sequence
565 available upon request), and sub-cloned into the 6His-MBP_3C_MCS_TEV_mRFP and 6His-
566 MBP_3C_MCS_TEV_mGFP cassette using the restriction sites *EcoRI/KpnI*.

567

568 ***Western Blotting***

569 Whole cell extracts were lysed with 1X Laemmli Sample buffer (Biorad, C161-0737) and heated
570 5min at 95°C. Cell extracts were resolved using pre-cast SDS-PAGE (7.5% and 10%) from BioRad
571 and transferred to nitrocellulose membrane using a transfer apparatus according to the
572 manufacturer's instructions (BioRad). Membranes were saturated with 10% non-fat milk diluted
573 in TBS-0.2% Tween 20 (TBS-T), incubated with primary antibodies overnight at 4°C and with
574 anti-mouse-HRP or anti-rabbit-HRP secondary antibodies for 1h. Blots were developed with ECL
575 according to the manufacturer's instructions.

576

577 ***Affinity capture of biotinylated proteins: BioID***

578 *Flp-InTMT-RExTM293* cell lines stably transfected with Flag-BirA*-TopBP1 WT or W1145R
579 grown to 75% confluence were incubated with 1µg/ml of doxycycline (Clontech, 631311) for 16h
580 and with 50µM biotin for 3 or 16 hours. Cells were washed with PBS and lysed with lysis buffer
581 (50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% NP-40, 0.2% SDS, 0.5%
582 Sodium deoxycholate) supplemented with 1X complete protease inhibitor (Roche) and 250U
583 benzonase (Sigma, CE1014). Lysed cells were incubated on a rotating wheel for 1h at 4°C prior
584 sonication on ice (40% amplitude, 3 cycles 10sec sonication- 2sec resting). After 30min
585 centrifugation (7750 rcf) at 4°C, the cleared supernatant was transferred to a new tube and total
586 protein concentration was determined by Bradford protein assay (BioRad, C500-0205). For each
587 condition, 300µg of proteins were incubated with 30µl of Streptavidin-Agarose beads (Sigma,
588 CS1638) on a rotating wheel at 4°C for 3hr. After 1min centrifugation (400 rcf), beads were
589 washed, successively, with 1ml of lysis buffer, 1ml wash buffer 1 (2% SDS in H₂O), 1ml wash
590 buffer 2 (0.2% sodium deoxycholate, 1% Triton X-100, 500mM NaCl, 1mM EDTA, and 50mM
591 Hepes pH 7.5), 1ml wash buffer 3 (250mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM
592 EDTA, 500mM NaCl and 10mM Tris pH 8) and 1ml wash buffer 4 (50mM Tris pH 7.5 and 50mM
593 NaCl). Bound proteins were eluted from the magnetic beads using 80µl of 2X Laemmli Sample
594 buffer and incubated at 95°C for 10min. 10% of the sample was used for Western blot analysis.
595 For the *Flp-InTMT-RExTM293* cell lines stably transfected with the doxycycline-inducible TurboID-
596 TopBP1WT-mCherry-Cry2 or the mutated forms of TopBP1, cells were simultaneously incubated
597 with 500µM of biotin and exposed to blue light for 10min of light-dark cycles (4s light followed
598 by 30s dark). Biotin proximity labelling of light-induced TopBP1 partners were pulled-down using
599 streptavidin-coated beads as described before and probed by immunoblotting to detect proteins
600 that are associated with TopBP1 clusters, in absence of DNA damage.

601

602 ***Mass Spectrometry***

603 Sample digestion was essentially performed as described (Shevchenko et al., 2006). Briefly,
604 proteins were loaded on a SDS-PAGE (BioRad, 456-1034) and, after short migration, a single band
605 was excised. Proteins in the excised band were digested with Trypsin (Promega, V5280). The
606 resulting peptides were analyzed online by nano-flow HPLC-nanoelectrospray ionization using a
607 Qexactive HFX mass spectrometer (Thermo Fisher Scientific) coupled to a nano-LC system
608 (Thermo Fisher Scientific, U3000-RSLC). Desalting and preconcentration of samples were
609 performed online on a Pepmap® precolumn (0.3 x 10mm; Fisher Scientific, 164568). A gradient
610 consisting of 0% to 40% B in A (A: 0.1% formic acid [Fisher Scientific, A117], 6% acetonitrile
611 [Fisher Scientific, A955], in H₂O [Fisher Scientific, W6], and B: 0.1% formic acid in 80%
612 acetonitrile) for 120min at 300nl/min was used to elute peptides from the capillary reverse-phase
613 column (0.075 x 250mm, Pepmap®, Fisher Scientific, 164941). Data were acquired using the
614 Xcalibur software (version 4.0). A cycle of one full-scan mass spectrum (375–1,500m/z) at a
615 resolution of 60000 (at 200m/z) followed by 12 data-dependent MS/MS spectra (at a resolution of
616 30000, isolation window 1.2m/z) was repeated continuously throughout the nanoLC separation.
617 Raw data analysis was performed using the MaxQuant software (version 1.5.5.1) with standard
618 settings. Used database consist of Human entries from Uniprot (reference proteome UniProt
619 2018_09) and 250 contaminants (MaxQuant contaminant database). Graphical representation and
620 statistical analysis were performed using Perseus (version 1.6.1.1). A standard t-test was used to
621 evaluate protein abundance difference between samples.

622

623 ***TopBP1 expression and purification***

624 Plasmids for protein expression in insect cells using baculoviruses are listed in Key Resources
625 Table of Star Methods. For the production of bacmids, 50ng of pFastbac plasmids were
626 transformed into MultiBac DH10 cells (Invitrogen, 10361-012) and positive clones were selected
627 on LB Ampicilin (100µg/ml) plates supplemented with 40µg/ml IPTG, 100µg/ml XGal and
628 7µg/ml Gentamycin. Blue colonies were screened for the presence of inserts by colony PCR using
629 pUC/M13 Forward and Reverse oligos (Bac-to-Bac® Baculoviruses Expression
630 System_ *invitrogen user guide*). To generate baculoviruses, 12×10^6 Sf9 cells (1ml) were transfected
631 with 5µg of purified bacmid using 15µl of Cellfectin™ (Invitrogen, P/N 58760). After 5h
632 incubation, 9ml of medium were added to Sf9 cells and cultures were incubated for 2.5 days. The
633 supernatant (P1) was collected by centrifugation (400 rcf 10min) and a 1/100 dilution was used to
634 infect 4×10^6 cells/ml Sf9 cells. Cells were incubated for 2 days and the supernatant (P2) was
635 collected by centrifugation. Expression of fluorescent proteins was verified by Western Blotting
636 and microscopy. For protein expression, a 1/10 dilution of freshly prepared P2 was added to 2×10^6
637 cell/ml Sf9 culture and incubated for 48 to 72 hours.

638 Infected cells were collected and lysed mechanically using a HTU-DIGI-French-Press (10000 PSI)
639 in 15X packed cell weight hypertonic lysis buffer (50mM Na₂H/NaH₂PO₄ pH 8.0, 500mM NaCl,
640 1% glycerol, 0.1% CHAPS) supplemented with protease inhibitors. Lysate was clarified by
641 centrifugation (7750 rcf, 40min, 4°C), filtered and loaded on a 5ml HisTrap HP column (GE
642 Healthcare, 71-5027-68 AF) equilibrated with 5CV (Column Volumes) of buffer A (lysis buffer +
643 0.1 mM PMSF, 5mM imidazole). The column was washed with 5CV of buffer A and TopBP1^{b6-8}
644 was eluted stepwise using 5CV of buffer A + 30mM, 50mM, 75mM, 125mM and 500mM
645 imidazole. Peak TopBP1^{b6-8} fractions (eluted with 50mM and 75mM imidazole) were desalted
646 (HiTrap™ or HiPrep 26/10, GE Healthcare) in physiological buffer (10mM Na₂H/NaH₂ PO₄ pH

647 7.6, 150mM KOAc, 0.1mM MgOAc, 0.5mM DTT, 2.5% glycerol), snap frozen and stored at -
648 80°C. Protein concentration was estimated by stain-free gel quantification using Image Lab
649 Software.

650

651 *Nuclear extract preparation*

652 Nuclear extracts were prepared as previously described (Vidal-Eychenie et al., 2013). HeLa S3
653 cells were grown to $\leq 80\%$ confluence, collected by scrapping, centrifuged (200 rcf, 3min, 4°C)
654 and washed twice in PBS 1X. Cell pellet was incubated on ice for 5min in 5X packed cell volume
655 of hypotonic buffer A (10mM Hepes-KOH pH 7.9, 10mM KCl, 1.5mM MgCl₂, 0.5mM DTT,
656 0.5mM PMSF) supplemented with protease (complete, EDTA free; Roche, 31075800) and
657 phosphatase inhibitors (Fisher Scientific). Cells were then spun down (500 rcf, 5min), suspended
658 in 2X packed cell volume of buffer A and lysed by dounce homogenization using a tight-fitting
659 pestle. Nuclei were collected by centrifugation (4000 rcf) for 5min at 4°C, extracted in one nuclei
660 pellet volume of buffer C (20mM Hepes-KOH pH 7.9, 600mM KCl, 1.5mM MgCl₂, 0.2mM
661 EDTA, 25% glycerol, 0.5mM DTT, 0.5mM PMSF) supplemented with cocktails of protease and
662 phosphatase inhibitors, and mixed on a rotating wheel at 4°C for 30min. Nuclear extracts
663 (supernatants) were recovered by centrifugation (16000 rcf, 15min, 4°C) and dialyzed using Slide-
664 A-Lyzer Dialysis Cassettes (3,500-D protein molecular weight cutoff; Fisher Scientific, 68035)
665 against buffer D (20mM Hepes-KOH pH 7.9, 100mM KCl, 0.2mM EDTA, 20% glycerol, 0.5mM
666 DTT, and 0.5mM PMSF). Dialyzed nuclear extracts were centrifuged (100000 rcf, 30min, 4°C) to
667 eliminate residual precipitates. The protein concentration of the clear supernatant was determined
668 by Bradford (BioRad, C500-0205) protein assay, and aliquots were snap frozen and stored at -

669 80°C. Important to note that the Dignam & Roeder extract preparation contain DNA fragments
670 after centrifugation.

671

672 ***TopBP1 phase separation assay***

673 Phase separation of purified TopBP1^{b6-8} was performed in physiological buffer C (10mM
674 Na₂H/NaH₂ PO₄ pH 7.6, 150mM KOAc, 0.1mM MgOAc, 0.5mM DTT, 2.5% Glycerol). Purified
675 TopBP1^{b6-8} WT and W1145R were digested with PreScission 3C enzyme (GenScript, Z03092-
676 500) for 3h at 16°C to remove the 6-His and MBP tag before phase separation in reaction mixtures
677 in buffer C containing 10µM of WT or W1145R TopBP1^{b6-8}-GFP and 2% of PEG4000 (Merck-
678 Sigma-Aldrich, 95904). Reaction mixtures were mixed by gently tapping the Eppendorf.

679 TopBP1 droplet permeability assay was performed in two steps: first, TopBP1^{b6-8}-GFP droplets
680 were formed in reaction mixtures containing 10µM of WT TopBP1^{b6-8}-GFP supplemented with
681 2% PEG in physiological buffer C, and then we added either 1µM of WT or W1145R TopBP1^{b6-8}-
682 RFP or *StuI/SacII* digested pX174 RFII DNA (300nM), as indicated. To study the role of DNA
683 in the organization of TopBP1 condensates, we incubated 2.5µM of WT TopBP1^{b6-8} with 40ng 2.9
684 kb circular DNA plasmid DNA at 37°C during 10min in physiological buffer C. The ratio purified
685 TopBP1:DNA was decided according to Choi JH et al., 2008. Samples were imaged on a LSM780
686 confocal microscope (Leica, Germany) using a 63x oil immersion objective (N.A. 1.4). DAPI and
687 GFP fluorescence were excited at 405 and 488nm respectively, and emitted fluorescence were
688 collected sequentially at 415-460nm and 500-550nm respectively. The pinhole size was set to 1
689 Airy unit.

690 TopBP1 phase separation in human protein extracts was performed in reaction mixtures containing
691 0.2µg/µl of human nuclear extract, 2µM of WT or W1145R TopBP1^{b6-8}-GFP in ATR activation

692 buffer (10mM Hepes-KOH pH 7.6, 50mM KCl, 0.1mM MgCl₂, 1mM PMSF, 0.5mM DTT, 1mM
693 ATP, 10µg/ml creatine kinase, 5mM phosphocreatine). Reaction mixtures were incubated for
694 10min at 4°C or 37°C, as indicated. 5µl of reaction mixtures were used for analyses by
695 immunofluorescence microscopy. Fluorescence microscopy analyses were performed using PEG
696 silanized glass slides prepared as described (Alberti et al., 2018), and coverslips were sealed with
697 nail polish. DNA was stained with Hoechst 33258. Images were captured on an inverted
698 microscope using a 63x objective (NA 1.4 oil).

699

700 ***Immunofluorescence staining***

701 Cells grown on coverslips were fixed with PBS/4% paraformaldehyde (PFA) for 15min at RT
702 followed by a 10min permeabilization step in PBS/0.2% Triton X-100-PBS and blocked in
703 PBS/3% BSA for 30min. For immunofluorescence staining, primary antibodies and appropriate
704 secondary antibodies coupled to fluorochrome were diluted in blocking solution and incubated for
705 1h at RT. To detect endogenous TopBP1, cells were pre-treated with Cytoskeleton (CSK) buffer
706 before fixation. DNA was stained with Hoechst 33258 (Invitrogen, Cat H21491) and coverslips
707 were mounted on glass slides with Prolong Gold antifade reagent (Invitrogen, Cat P36930). Images
708 were captured using a 63x objective (NA 1.46 oil).

709

710 ***Fluorescence Redistribution After Photo-bleaching (FRAP)***

711 For FRAP experiments, *U-2-OS Tet-On* cells were seeded into µ-Dish^{35 mm, high} (Ibidi, 81156) and
712 incubated 24h in the presence of 1µg/ml doxycycline to induce expression of eGFP-TopBP1.
713 Imaging was realized using a 63x objective (NA 1.4). eGFP-TopBP1 bodies were photo-bleached

714 and the GFP signal intensity of 23 spots was measured before and during 5min following photo-
715 bleaching with an imaging frequency of 60 images/min.

716

717 ***Opto-TopBP1 activation***

718 Cells were plated at around 70% confluency in DMEM. Expression of opto-TopBP1 was induced
719 for 16h with 2 μ g/ml doxycycline. For light activation, plates were transferred into a custom-made
720 illumination box containing an array of 24 LEDs (488nm) delivering 10mW/cm² (light intensity
721 measured using a ThorLabs-PM16-121-power meter). Cry2 oligomerisation was induced using
722 3min of light-dark cycles (4s light followed by 10s dark). Images were captured using a 63x
723 objective (NA 1.46 oil). A Cell Profiler (version 2.2.0) pipeline was used to quantify nuclear
724 mCherry foci. Values were represented via violin plots or histograms elaborated in Graph Pad
725 Prism 8. Median and quartile values were indicated by continues and dashed lines respectively. A
726 non-parametric t-test (Mann-Withney) was used to compare mCherry spot/cell distributions
727 between samples. For each experiment, we performed three biological replicates and we show one
728 representative experiment.

729

730 ***Live cell microscopy***

731 Live imaging of opto-TopBP1 WT overexpressing cells was performed on an inverted microscope
732 (AxioObserver, Carl Zeiss, Germany) using a 63x oil immersion objective (NA 1.4). Fluorescence
733 was detected on a CMOS camera (ORCA-Flash4.0, Hamamatsu) with an exposure time set to
734 100ms and at frame rate of 2 images/min. All recordings were carried out at 37°C under 5% CO₂.
735 For Cry2 activation, cells were treated as explained before, under the microscope, and imaged
736 immediately after light activation.

737

738 ***Stimulated emission depletion (STED) super-resolution microscopy***

739 For Figure 2F, *Flp-InTMT-RExTM293* cells expressing opto-TopBP1 were exposed to blue light
740 (488nm) to induce TopBP1 foci, as described before. In Figure 2G, endogenous TopBP1 foci were
741 analysed in *RPE1* cells after HU-induced replication stress (2h 5mM), untreated cells were used
742 as control. For STED microscopy, immunolabelling of mCherry was realized using an anti-
743 mCherry antibody followed by a secondary antibody coupled to Atto-647N fluorochrome.
744 Confocal and STED imaging was performed using a quad scanning STED microscope (Expert
745 Line, Abberior Instruments, Germany) equipped with a PlanSuperApo 100x/1.40 oil immersion
746 objective (Olympus, Japan). Atto-647N was excited at 640nm with a dwell time of 10 μ s and STED
747 was performed at 775nm. Images were collected in line accumulation mode (5 lines accumulation).
748 Fluorescence was detected using avalanche photo diodes and spectral detection (650-750nm). The
749 pinhole was set to 1.0 Airy units and a pixel size of 10nm was used for all acquisitions. A gating
750 of 8ns was applied. Sphericity of opto-TopBP1 condensates was assessed using IMARIS software
751 (Bitplane). For dual color STED imaging, Atto-647N and Abberior STAR Orange were used and
752 respectively imaged at 640 and 561nm excitation. Detection was set to 650-750nm for Atto-647N
753 and 570-630nm for Abberior STAR Orange. Other acquisition parameters were the same described
754 as above.

755

756 ***DNA fibers***

757 Neo-synthesized DNA was sequentially pulse labelled with two halogenated thymidine analogs,
758 5-Iodo-2'-deoxyuridine (IdU at 25 μ M – Sigma # I7125) and 5-Chloro-2'-deoxyuridine (CldU at
759 50 μ M – Sigma # C6891), for 20min. After IdU incorporation, cells were washed two times before

760 CldU addition. CldU incorporation (20min total) was conducted in the presence of 3min of light-
761 dark cycles (4s light followed by 10s dark) each 7min, to assure opto-TopBP1 condensates
762 formation and persistence. Cells were trypsinized and washed with ice cold PBS. 2000 cells were
763 drop on top of a microscope slide and let dry at least 5min before lyse with 7 μ L of spreading buffer
764 (200mM Tris.HCl pH7.5, 50mM EDTA, 0.5% SDS) during 3min. DNA was spread by tilting the
765 slide and letting the drop running down slowly. Once air-dry, DNA spreads were fixed in
766 methanol/acetic acid 3:1 for 10min, denatured with 2.5M HCl during 1h and blocked in PBS/1%
767 BSA/0.1% Tween. DNA spreads were immunostained with mouse anti-BrdU, rat anti-BrdU and
768 mouse anti-ssDNA antibodies to detect IdU, CldU and intact DNA fibers respectively.
769 Corresponding secondary antibodies conjugated to Alexa Fluor dyes were used in a second step.
770 Images were captured using a 40x objective (NA 1.4 oil). The acquired DNA fiber images were
771 analyzed by using FIJI Software and statistical analysis of at least 150 IdU and CldU tracks on
772 intact fibers was performed with GraphPad Prism 8. Mean values were indicated by red lines. One-
773 way ANOVA analysis was applied to compare means of samples in a group.

774

775 **QUANTIFICATION AND STATISTICAL ANALYSIS**

776 For Mass Spectrometry, raw data analysis was performed using the MaxQuant software
777 (version 1.5.5.1) with standard settings. Graphical representation and statistical analysis were
778 performed using Perseus (version 1.6.1.1). A standard t-test was used to evaluate protein
779 abundance difference between samples.

780 Purified protein concentration was estimated by stain-free gel quantification using Image
781 Lab Software (version 5.2.1).

782 All microscopy images were analyzed by OME Remote Objects (OMERO) software.
783 Sphericity of opto-TopBP1 condensates was assessed using IMARIS software (Bitplane). STED
784 microscopy and DNA fiber images were analyzed using FIJI software.

785 A Cell Profiler (version 2.2.0) pipeline was used to quantify nuclear mCherry foci.

786 All statistical analysis were performed with GraphPad Prism 8. For DNA fibers One-way
787 ANOVA analysis was applied to compare means of samples in a group. To compare mCherry
788 spot/cell distributions between samples we used a non-parametric t-test (Mann-Withney).

789

790 KEY RESOURCES TABLE

791 SUPPLEMENTAL INFORMATION

792 **Supplementary video. Related to Figure 2A.** 3D projection of opto-TopBP1 condensates.

793

794 **Table S1.** Oligonucleotides used in this study. Related to Key Resources Table and Method
795 Details.

Primers	Sequence
Primer 1 (Fw)	TTGGCGCGCCAtccagaaatgacaaagaaccgt
Primer 2 (Rev)	TTTTCCTTTTGCGGCCGcttagtgactctaggtcgttg
Primer 3 (antisense)	GTAGGGTCATCCCTAATGATCTGTTCATTTTGGGAAGGC
Primer 4 (Fw)	GGGGTACCATCGATGCTAGCatggtgtctaaaggcgagga
Primer 5 (Rev)	GGCGCGCCtcagtcacgcattgacaggt
Primer 6 (Fw)	GGGGTACCACCATGctttctgccagccctca
Primer 7 (Rev)	CTAGCTAGCgtgtactctaggtcgtttgatt
Primer 8 (Fw)	gggCTTAAGgccaccATGaaagacaatactgtgcctctg
Primer 9 (Rev)	cggGGTACCCACGTGcttttcggcagaccgcaga
Primer 10 (Fw)	gggCACGTGtccagaaatgacaaagaaccg
Primer 11 (Rev)	cggGGTACCgtgtactctaggtcgtttgat
Primer 12 (sense)	gagatgagagagaacgctcagaagcagttacag
Primer 13 (sense)	gtcaacacagagcctgcccaaatgaacagatc
Primer 14 (sense)	gtcaacacagagcctgaccaaatgaacagatc
Primer 15 (Fw)	CTAGCGTTTAAACTTAAGATGgctgtgtcttcaaaaggatg

Primer 16 (Rev)	cctaCATGGTACCCatcatgtaaaggctttgaaaagg
Primer 17 (Fw)	tacacGGTACCATGaggaaagctcctacag
Primer 18 (Rev)	cacatGCTAGCtctaggtcgttgatttta
Primer 19 (Fw)	AGCTTGGTACCATGAGTCGGCGAAGGAAACATGATG
Primer 20 (Fw)	ccgaaaagCACGTGGGTACCAAGTGCCTGGTCACGGG
Primer 21 (Rev)	atGCTAGCATCGATGGTACCGCCTTCACCCTCACTGTCTTCC
Primer 22 (Rev)	cacatGCTAGCAAGAAATGAAGTGGGAGCTGC
Primer 23 (Fw)	tacacctgataccatcaaaattcctgccaaggacaac
Primer 24 (Rev)	CTTTGGTGTTCATAtgagagtagtcgactattacagagccg
Primer 25 (Fw)	agtcgactactctcaTATGAAACACCAAAGAGAGCGCTGA
Primer 26 (Rev)	agcaatttctgaatcTGACCACATCTGTATATTATCTTTATAAATTAATTAATAAATCTCTGTTTCT
Primer 27 (Fw)	ATACAGATGTGGTCAgattcagaaattgctaaacaggctgtct
Primer 28 (Rev)	ttcctgcacgaagtgtccagcagctcctgcaggctcaaggta

796

797 **Supplementary Figure Legends**

798 **Figure S1. Related to Figure 1. A)** Immunoblotting of TopBP1. **B)** (Left) Representative
799 fluorescence images of cells expressing opto-RAD9 under control of doxycycline. Light ON:
800 488nm light. (Right) Immunoblotting of the cells with the indicated antibody. **C)** ETAA1 does not
801 assemble condensates. The construct, representative fluorescence images, and Violin plot
802 representation of quantified condensates are shown, as described in Figure 1.

803

804 **Figure S2. Related to Figure 2. A)** Representative fluorescence images of cells used for
805 experiments in Figure 2C. DNA stained with Hoechst 33258. Scale bars: 10µm. **B)** (Left) FRAP
806 recordings of two individual eGFP-TopBP1 condensates are reported as examples. Scale bars:
807 10µm. (Right) Histogram representation of the mean of immobile and mobile fractions per nucleus
808 (13 nuclei, 23 individual spots). **C)** Representative fluorescence images of eGFP-TopBP1

809 expressing U-2-OS cells treated, when indicated, with 3% of 1,6 Hexanediol. **D-E)** Representative
810 fluorescence images of cells used for experiments in Figure 2D (**D**) and 2E (**E**). DNA stained with
811 Hoechst 33258. Scale bars: 10 μ m. **F)** Violin plot representing the number of mCherry foci per cell
812 in opto-TopBP1 expressing cells treated with 5mM HU. TopBP1 foci were identified with anti-
813 mCherry antibody after CSK treatment. **A-B-C-D-E-F)** Number of biological replicates = 3.

814

815 **Figure S3. Related to Figure 3.** **A)** Representative fluorescence images of cells expressing opto-
816 TopBP1^{b6-8} WT before (Light OFF) and after (Light ON) optogenetic activation. DNA stained
817 with Hoechst 33258. Western blotting of the indicated proteins is shown. Violin plot represents
818 the number of mCherry foci per cell. **(B)** Stain-free gel of purified TopBP1^{b6-8}-GFP WT and
819 W1145R used for phase separation assays. **(C)** Representative fluorescence microscopy images of
820 TopBP1 condensates assembled *in vitro*. The concentration of TopBP1 is indicated **(D)**
821 Representative fluorescent images of TopBP1 condensates in suspension. DNA stained with
822 Hoechst 33258. Line scan of GFP-Hoechst signals are shown. **A-C-D)** Scale bars: 10 μ m. Number
823 of biological replicates = 3.

824

825 **Figure S4. Related to Figure 4.** Immunofluorescence staining of Flag-BirA*-TopBP1 WT using
826 anti-Flag antibody. Biotin conjugates were revealed using AlexaFluor streptavidin. DNA stained
827 with Hoechst 33258. Scale bars: 10 μ m. Number of biological replicates = 3.

828

829 **Figure S5. Activation of endogenous ATR in nuclear extracts.** **A)** Nuclear extracts at the
830 indicated protein concentration were incubated in the presence of the ATR inhibitor ETP-46464
831 (10 μ M), as indicated. Chk1 activation is revealed by immunoblotting with the indicated

832 antibodies. * represents unspecific bands. **B)** Immunoblotting of Chk1/pChk1 (Ser345) in reaction
833 mixtures. When indicated nuclear extracts were pre-incubated with ethidium bromide (EtBr). **A-**
834 **B)** Number of biological replicates = 3.

835

836 **Figure S6. Related to Figure 5.** **A)** Immunoblotting analyses of the indicated proteins before and
837 after optogenetic activation of TopBP1 condensates. When indicated, cells were pre-treated with
838 10 μ M UCN-01. **B)** Immunoblotting analysis of the indicated biotinylated proteins isolated with
839 streptavidin beads in WT and W1145R opto-TopBP1 expressing cells exposed exposed to 488 nm
840 light, when indicated. **C-D-E)** Immunofluorescence staining with the indicated antibodies of opto-
841 TopBP1 expressing cells activated by light. Line scans are shown. DNA stained with Hoechst
842 33258. Scale bars: 10 μ m. Violin plot (**E-right**) representing spot of pChk1 in cells with (dashed
843 white line) or without (solid white line) opto-TopBP1 foci. **F)** Histograms representing the mean
844 of mCherry foci per cell. Cells expressing opto-TopBP1 WT were exposed to 488 nm light, and,
845 when indicated, nuclear soluble proteins were extracted with Cytoskeleton (CSK) buffer. **A-B-C-**
846 **D-E-F)** Number of biological replicates = 3.

847

848 **Figure S7. Related to Figure 6.** **A)** Representative fluorescence images of cells used for
849 optogenetic experiment in Figure 6A. **B)** Violin plot representing the number of mCherry
850 condensates per cell in opto-TopBP1 expressing cells pre-treated for 1h with 10 μ M UCN-01, when
851 indicated. **C)** Representative fluorescence images of cells used for optogenetic experiment in
852 Figure 6C. **A-B-C)** Number of biological replicates = 3.

853

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1052

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
ATR	Bethyl/euromedex	Cat# A300-137A; RRID:AB_185544
BRCA1 (C-20)	Santa Cruz Biotechnology	Cat# sc-642; RRID:AB_630944
Chk1	Santa Cruz Biotechnology	Cat# sc-8408; RRID:AB_627257
FANCJ/BRIP1	Novus Biologicals	Cat# NB100-416; RRID:AB_2066307
Flag-M2	Sigma-Aldrich	Cat# F1804; RRID:AB_262044
mCherry	Elabscience	Cat# E-AB-20087
MRE11	Novus Biologicals	Cat# NB100-142; RRID:AB_10077796
NOL11	Sigma-Aldrich	Cat# HPA022010; RRID:AB_2267158
pATR (T1989)	GeneTex	Cat# GTX128145; RRID:AB_2687562
pChk1 (Ser296)	Cell Signaling Technology	Cat# 2349; RRID:AB_2080323
pChk1 (Ser345)	Cell Signaling Technology	Cat# 2348; RRID:AB_331212
pTopBP1 (Ser1138)	Interchim	Cat# orb140434
Streptavidin, Alexa Fluor 488 conjugate	Molecular Probes	Cat# S32354; RRID:AB_2315383
Tubulin (B-5-1-2)	Sigma-Aldrich	Cat# T5168; RRID:AB_477579
TopBP1	Bethyl	Cat# A300-111A; RRID:AB_2272050
Alexa Fluor 546 goat anti-mouse IgG	Molecular Probes	Cat# A-11030; RRID:AB_144695
Alexa Fluor 546 goat anti-rabbit IgG	Molecular Probes	Cat# A-11010; RRID:AB_2534077
Alexa Fluor 488 goat anti-mouse IgG2b	Molecular Probes	Cat# A-21141; RRID:AB_141626
Alexa Fluor 488 goat anti-rabbit IgG	Molecular Probes	Cat# A-21141; RRID:AB_141626
Anti-rabbit IgG Atto-647N	Sigma-Aldrich	Cat# 40839; RRID:AB_1137669
Anti-mouse IgG Atto-647N	Sigma-Aldrich	Cat# 50185; RRID:AB_1137661
Anti-mouse IgG Abberior STAR 580	Sigma-Aldrich	Cat# 38377
Anti-mouse IgG, HRP linked Antibody	Cell Signaling Technology	Cat# 7076; RRID:AB_330924
Anti-rabbit IgG, HRP linked Antibody	Cell Signaling Technology	Cat# 7074; RRID:AB_2099233
Bacterial and Virus Strains		
5-alpha Competent <i>E. coli</i> (High Efficiency)	NEB	Cat# C2987
MAX Efficiency™ DH10Bac Competent Cells	ThermoFisher Scientific	Cat# 10361012
Chemicals, Peptides, and Recombinant Proteins		
Biotin	Sigma-Aldrich	Cat# B4501; CAS: 58-85-5
Doxycycline	Clontech	Cat# 631311; CAS: 10592-13-9
Blasticidin	InvivoGen	Cat# ant-bl
Hygromycin	Sigma-Aldrich	Cat# H3274; CAS: 31282-04-9.
Zeocin	ThermoFisher Scientific	Cat# R25001

Ampicilin	Sigma-Aldrich	Cat# A9518; CAS: 69-52-3
Penicillin streptomycin	Sigma-Aldrich	Cat# P0781; ID 329820056
Gentamicin Sulfate	Sigma-Aldrich	Cat# G1914; CAS: 1405-41-0
X-Gal	Merck	Cat# 10745740001; CAS: 7240-90-6
IPTG	Sigma-Aldrich	Cat# I6758; CAS: 367-93-1
cOmplete, EDTA free	Roche	Cat# 4693159001
Halt phosphatase inhibitor cocktail	ThermoFisher Scientific	Cat# 78427
Benzonase Nuclease	Sigma-Aldrich	Cat# E1014; CAS: 9025-65-4
Ethidium bromide solution	Sigma-Aldrich	Cat# E1510; CAS: 1239-45-8
Adenosine 5'-triphosphate disodium salt hydrate	Sigma-Aldrich	Cat# A7699; CAS:34369-07-8
Creatine kinase	Sigma-Aldrich	Cat# C9858; MDL: MFCD00151687
Phosphocreatine di(tris) salt	Sigma-Aldrich	Cat# P1937; CAS: 108321-17-1
5-Iodo-2'-deoxyuridine	Sigma-Aldrich	Cat# I7125; CAS: 54-42-2
5-Chloro-2'-deoxyuridine	Sigma-Aldrich	Cat# C6891; CAS: 50-90-8
ATR Inhibitor III, ETP-46464	Merck	Cat# 5005080001; CAS: 1345675-02-7
VE-821	TINIB-TOOLS	Cat# V134; CAS: 1232410-49-9
UCN-01	Sigma-Aldrich	Cat# U6508; CAS: 112953-11-4
Streptavidin-Agarose	Sigma-Aldrich	Cat# S1638; MDL: MCFD00082035
Dulbecco's Modified Eagle's Medium - high glucose	Sigma-Aldrich	Cat# D5796
McCoy's 5A Medium	Sigma-Aldrich	Cat# M9309; MDL: MFCD00217560
EX-CELL [®] 420 Serum-Free Medium for Insect Cells	Sigma-Aldrich	Cat# 14420c; MDL: MFCD01634638
BioWest - Fetal Bovine Serum	Eurobio Scientific	Cat# S1810
Critical Commercial Assays		
Quick Start [™] Bradford 1x Dye Reagent	Bio-Rad	Cat# 500-0205
Clarity western ECL substrate	Bio-Rad	Cat# 170-5061

Clarity max western ECL substrate	Bio-Rad	Cat# 1705062
Criterion TGX stain free gel 7,5%	Bio-Rad	Cat# 5678024
Criterion TGX stain free gel 4-15%	Bio-Rad	Cat# 5678084
Criterion TGX stain free gel 10%	Bio-Rad	Cat# 5671034
Mini-PROTEAN TGX Stain Free Gels, 7.5%	Bio-Rad	Cat# 4568023
Mini-PROTEAN TGX Stain Free Gels, 4-15%	Bio-Rad	Cat# 4568083
Mini-PROTEAN TGX Stain Free Gels, 10%	Bio-Rad	Cat# 4568033
Trans-Blot Turbo Transfer Pack 0,2µm Nitrocellulose Midi, 10 pack	Bio-Rad	Cat# 1704159
Trans-Blot Turbo Transfer Pack 0,2µm Nitrocellulose Mini, 10 pack	Bio-Rad	Cat# 1704158
Color Prestained Protein Standard, Broad Range	BioLABs	Cat# P7712S
HisTrap HP column	GE Healthcare	Cat# 71-5027-68 AF
HiTrap™ Desalting column	GE Healthcare	Cat# 71-7154-00 AM
HiPrep 26/10	GE Healthcare	Cat# 28-4026-52 AB
Cellfectin	ThermoFisher Scientific	Cat# P/N 58760
Lipofectamine 2000	ThermoFisher Scientific	Cat# 11668-019
SnakeSkin Dialysis Tubing	Thermo Fisher Scientific	Cat# 68700
Precision 3C	GenScript	Cat# Z03092-500
Deposited Data		
Raw and analyzed data	This paper	http://dx.doi.org/10.17632/6nf7b7ffb7.1
Experimental Models: Cell Lines		
Flp-In™ T-REx™ 293	Invitrogen	Cat# R78007; RRID:CVCL_U427
RPE1 HTRT	Gift from Dr. Krzysztof Rogowski	N/A
U2OS	Gift from Dr. Mikko Sokka	N/A
Hela S3	ATCC	Cat# CCL-2.2; RRID:CVCL_0058
Sf9 Insect Cells - Novagen	Sigma-Aldrich	Cat# 71104-M
Oligonucleotides		
See Table S1 for the full list of oligonucleotides used in this study	This paper	N/A
Recombinant DNA		
pOG44 Flp-Recombinase Expression Vector	ThermoFisher Scientific	Cat# V600520
3xHA-TurboID-NLS_pCDNA3	Addgene	DOI: 10.1038/nbt.4201
pCDNA5_FRT-TO_Flag-BirA*	Gift from Dr. Brian Raught	N/A

pHR-mCherry-Cry2	Gift from Dr. Clifford Brangwynne	N/A
pCDNA5_FRT-TO_GFP-ETAA1	Gift from Dr. Niels Mailand	N/A
pDONR223-RAD9A	Montpellier Genomic Collection (MGC)	N/A
pCDNA5_FRT-TO_TurboID-mCherry-Cry2 (opto-module)	This paper	N/A
pCDNA5_FRT-TO_TurboID-TopBP1WT-mCherry-Cry2	This paper	N/A
pCDNA5_FRT-TO_TurboID-TopBP1W1145R-mCherry-Cry2	This paper	N/A
pCDNA5_FRT-TO_TurboID-ETAA1-mCherry-Cry2	This paper	N/A
pCDNA5_FRT-TO_TurboID-TopBP1 ^{ETAA1} -AAD-mCherry-Cry2	This paper	N/A
pCDNA5_FRT-TO_TurboID-RAD9A-mCherry-Cry2	This paper	N/A
pFastBac1_6His_MBP_TopBP1WT ^{b6-8} -mGFP	This paper	N/A
pFastBac1_6His_MBP_TopBP1W1145R ^{b6-8} -mGFP	This paper	N/A
pFastBac1_6His_MBP_TopBP1WT ^{b6-8} -mRFP	This paper	N/A
pFastBac1_6His_MBP_TopBP1W1145R ^{b6-8} -mRFP	This paper	N/A
pCDNA5_FRT-TO_Flag-BirA*-TopBP1WT	This paper	N/A
pCDNA5_FRT-TO_Flag-BirA*-TopBP1W1145R	This paper	N/A
pCDNA5_FRT-TO_mCherry-Cry2 (opto-module#)	This paper	N/A
pCDNA5_FRT-TO_TopBP1WT ^{b6-8} -mCherry-Cry2	This paper	N/A
pCDNA5_FRT-TO_TopBP1W1145R ^{b6-8} -mCherry-Cry2	This paper	N/A
pCDNA5_FRT-TO_AAD-NLS ^{TopBP1} -mCherry-Cry2	This paper	N/A
pCDNA5_FRT-TO_TurboID-TopBP1F1071A-mCherry-Cry2	This paper	N/A
pCDNA5_FRT-TO_TurboID-TopBP1S1138A-mCherry-Cry2	This paper	N/A
pCDNA5_FRT-TO_TurboID-TopBP1S1138D-mCherry-Cry2	This paper	N/A
Software and Algorithms		

Image Lab™ Software (Version 5.2.1)	Bio-Rad	http://www.bio-rad.com/fr-fr/product/image-lab-software?ID=KRE6P5E8Z
Cell Profiler 2.2.0	Cell image analysis software	DOI:10.1093/bioinformatics/btr095
Prism 8 (Version 8.4.2)	Graphpad Software	https://www.graphpad.com/scientific-software/prism/
Omero	OME Remote Objects software	DOI:10.1038/nmeth.1896.
MaxQuant (Version 1.5.5.1)	MaxQuant software	https://pubmed.ncbi.nlm.nih.gov/19029910/
IMARIS	Bitplane	https://imaris.oxinst.com
FIJI	Open source	https://imagej.net/Fiji

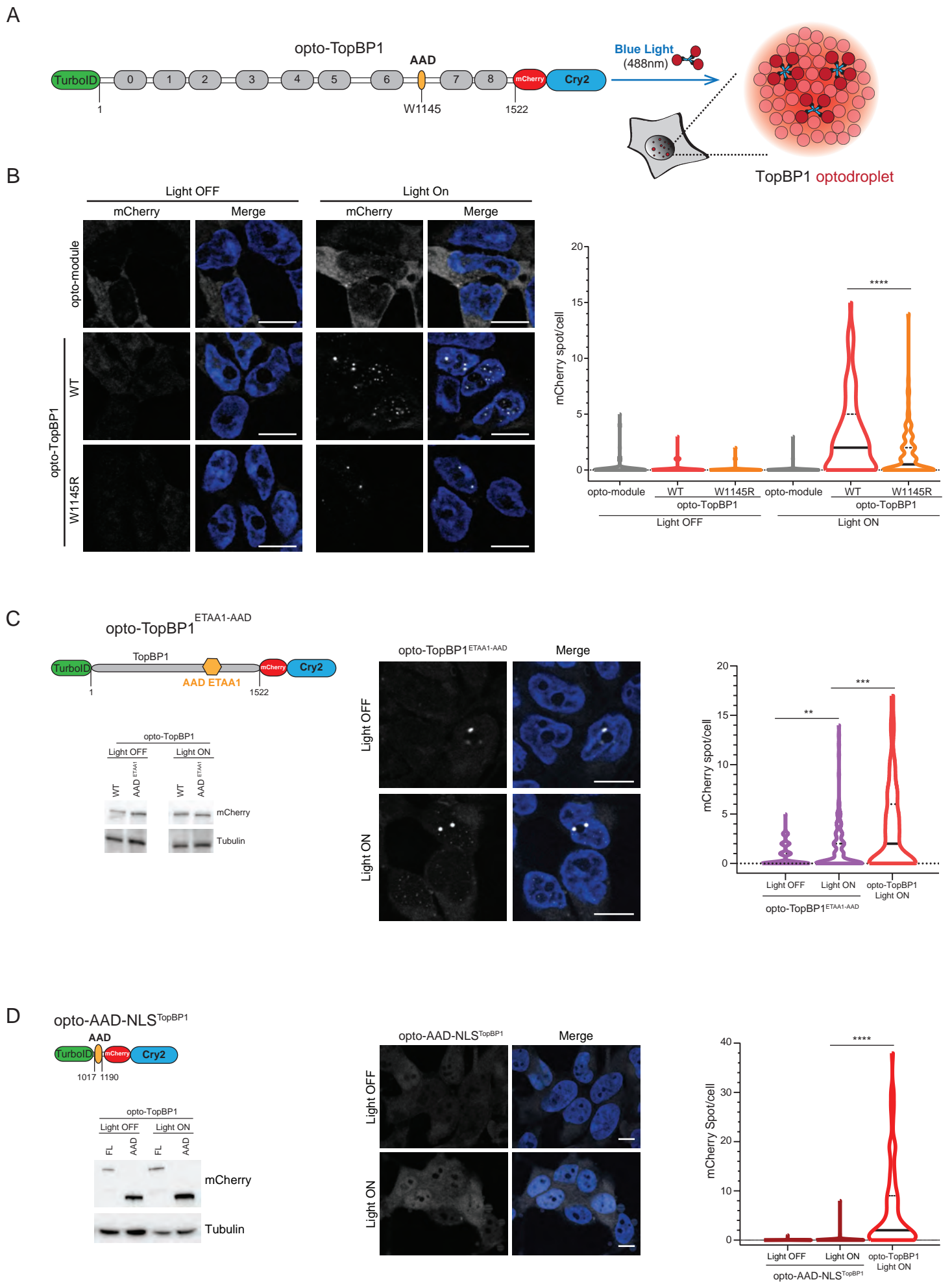


Figure 1

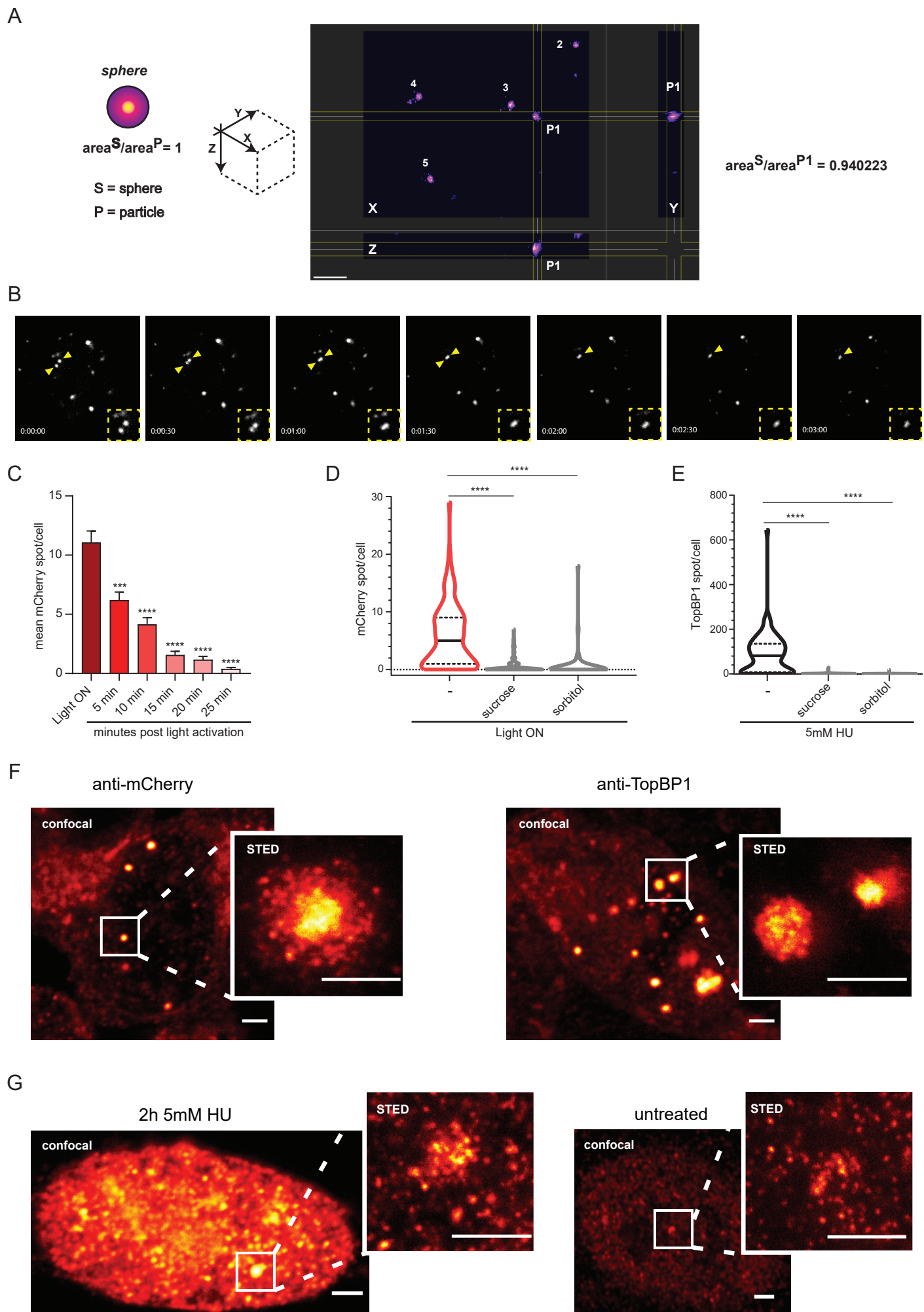


Figure 2

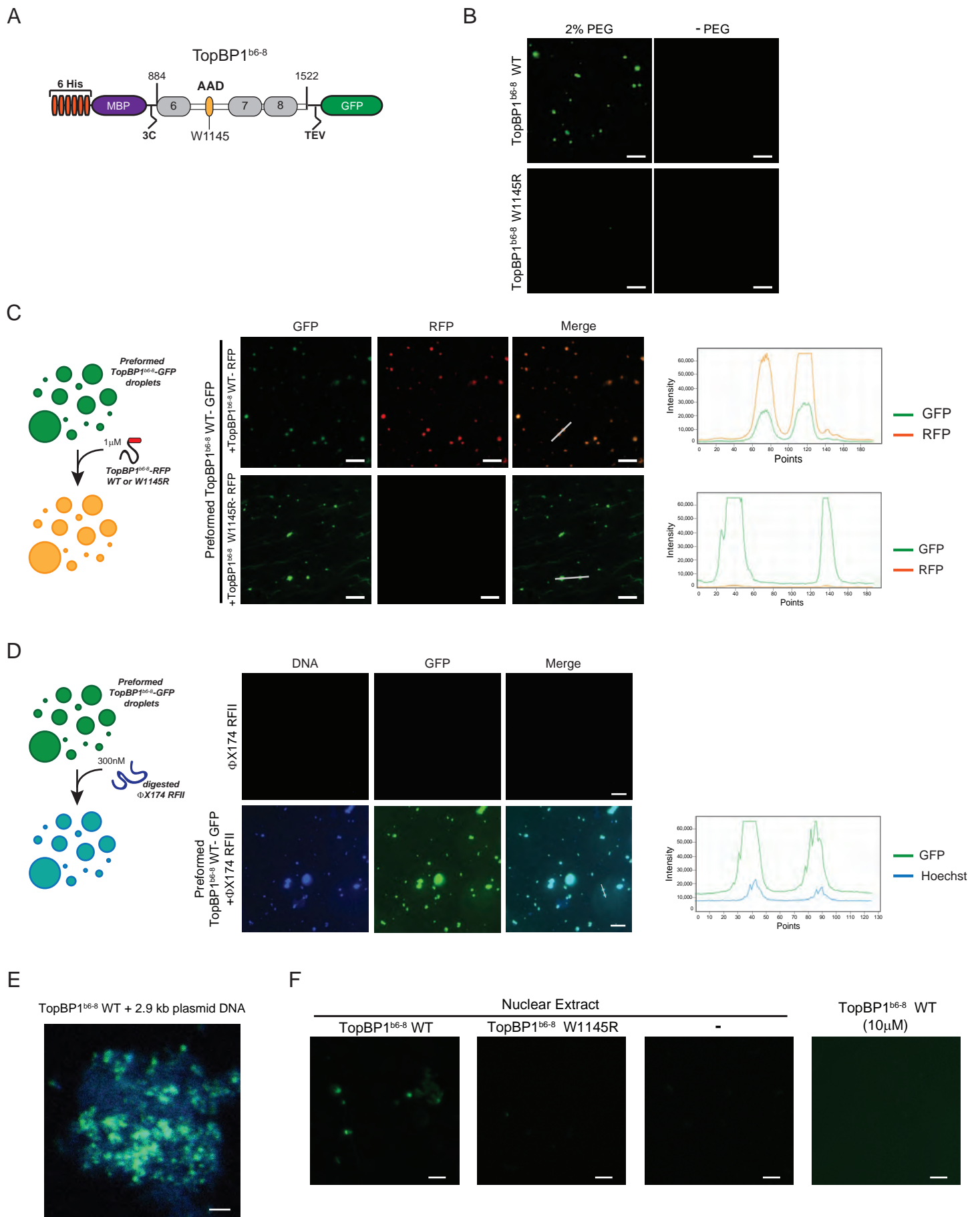


Figure 3

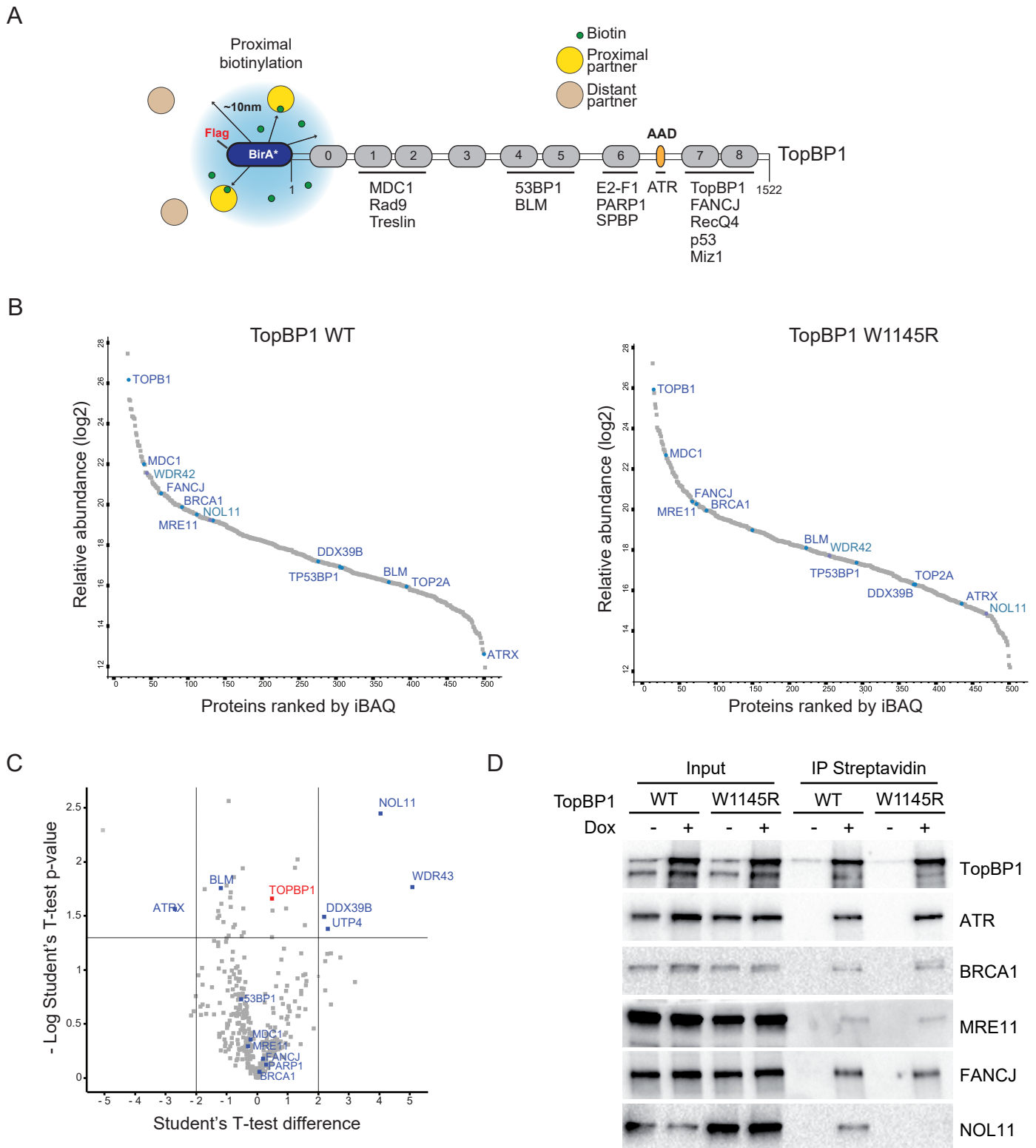


Figure 4

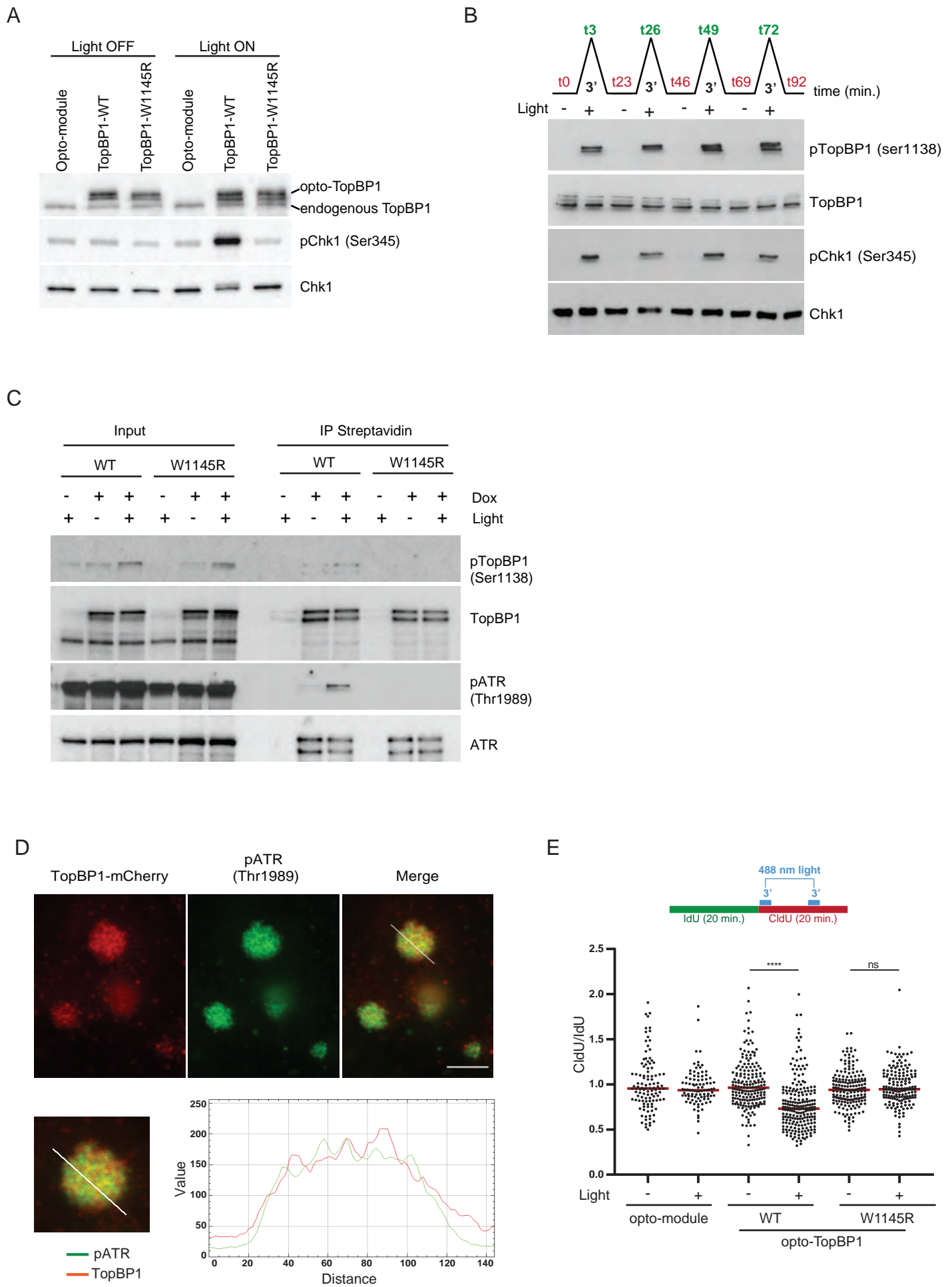


Figure 5

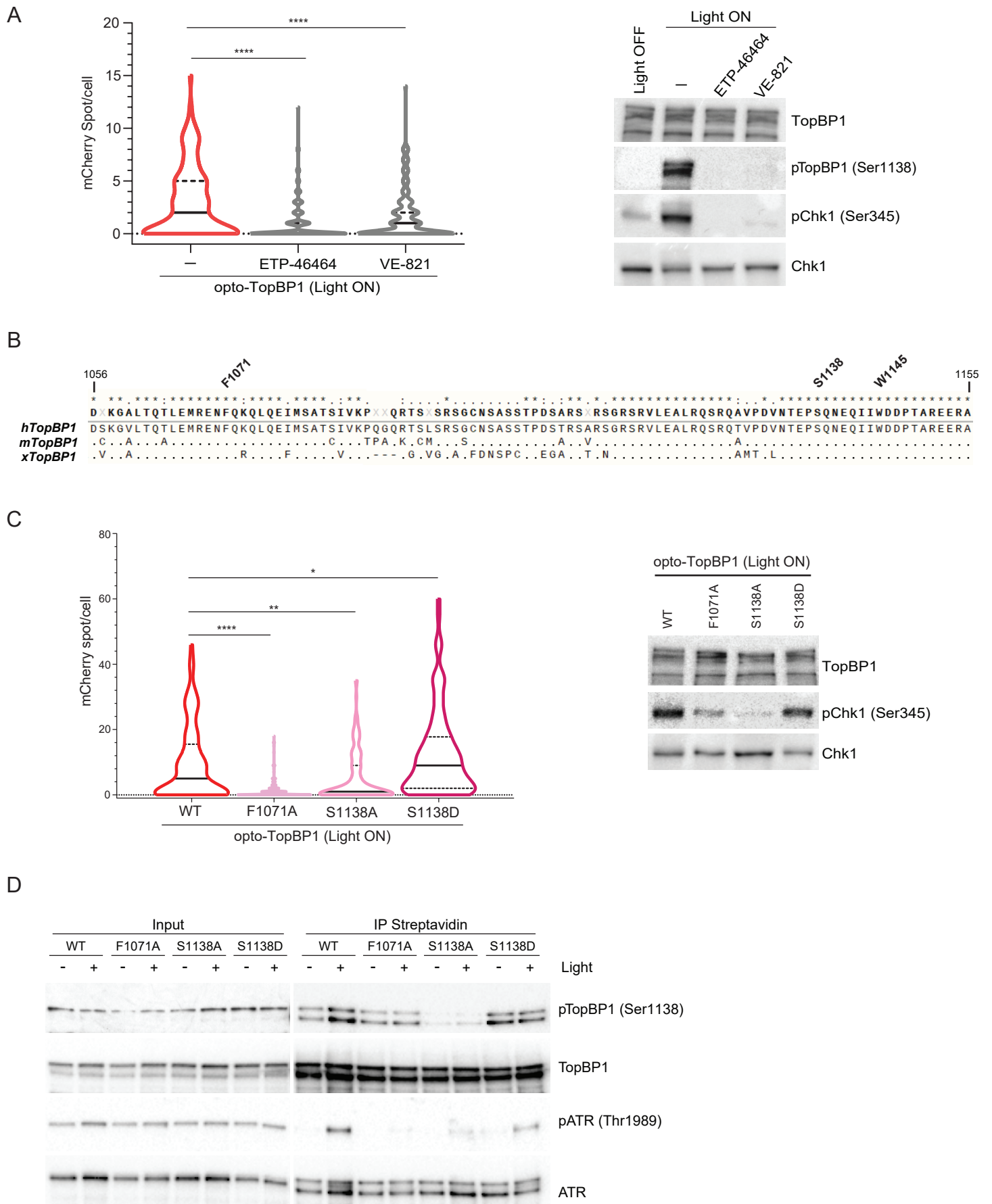


Figure 6

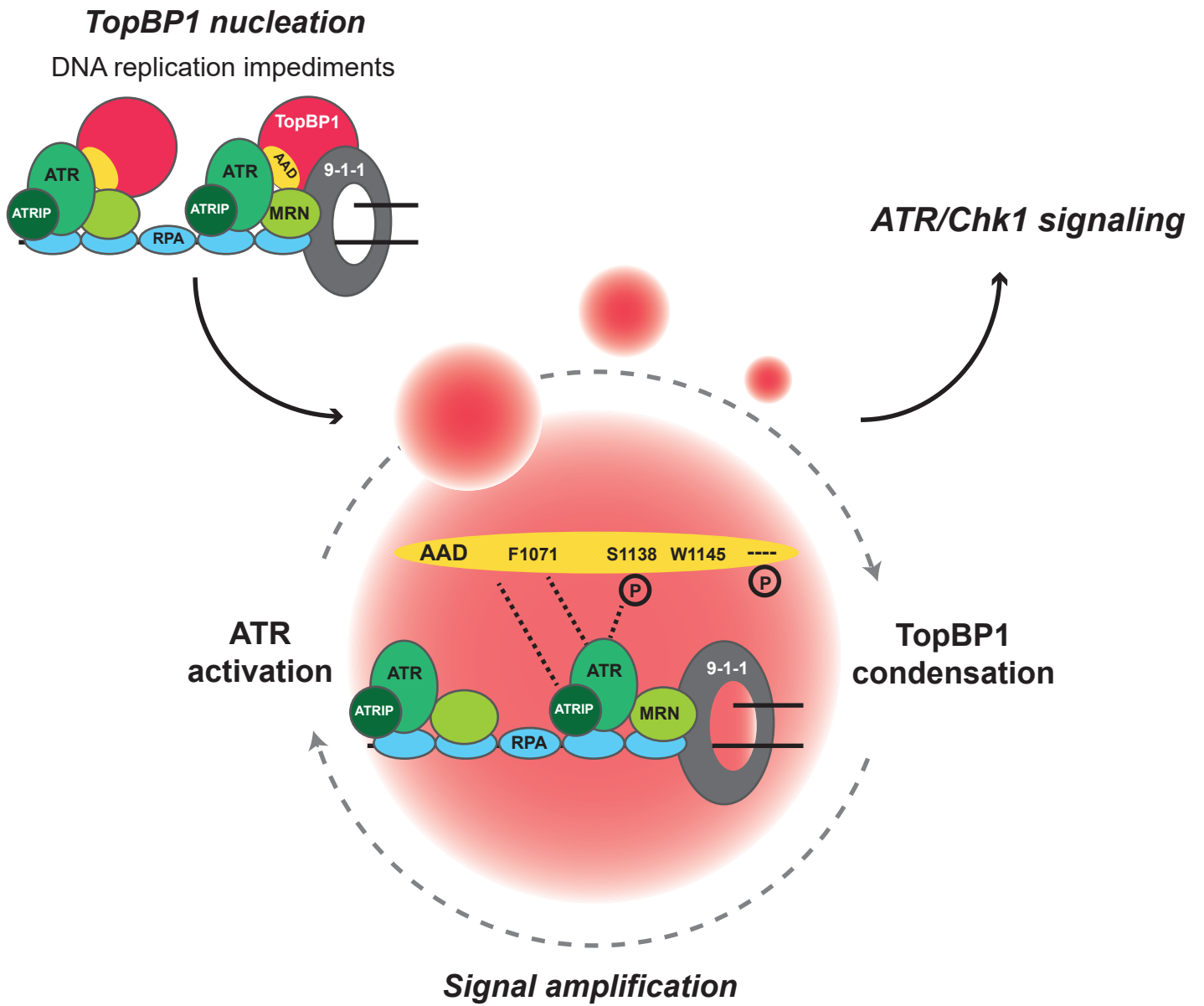
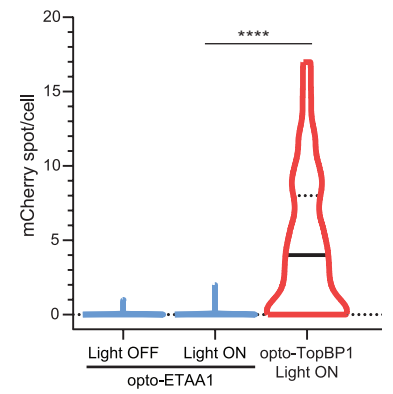
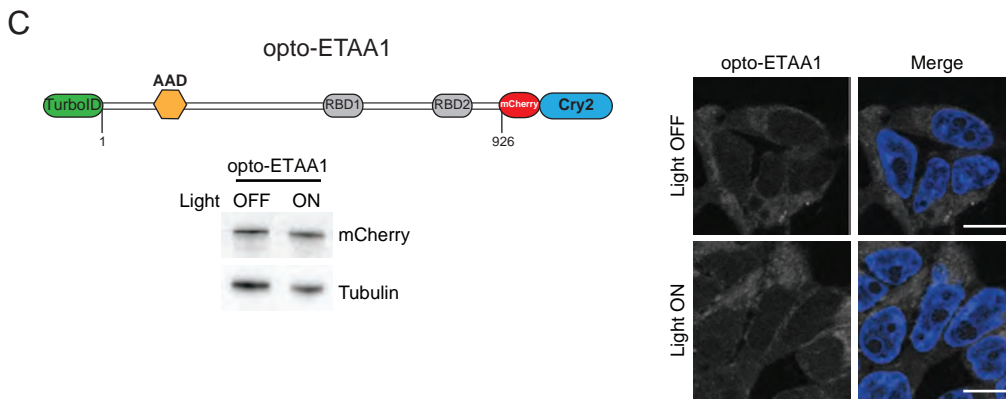
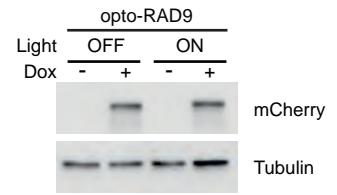
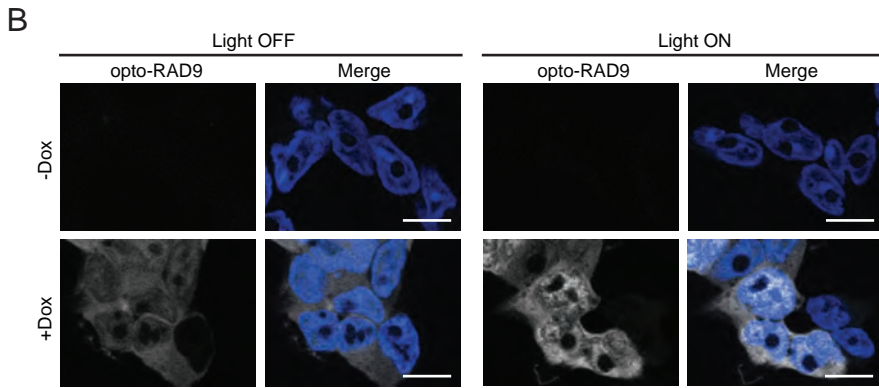
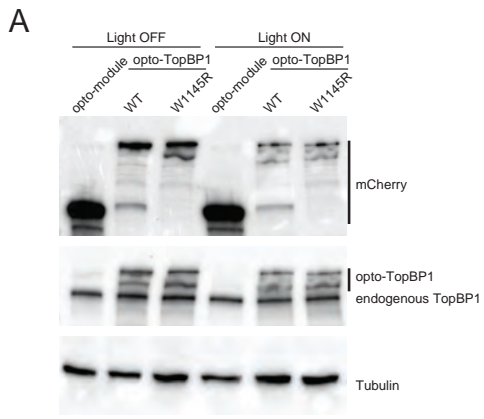
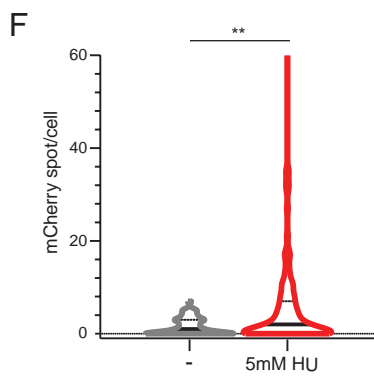
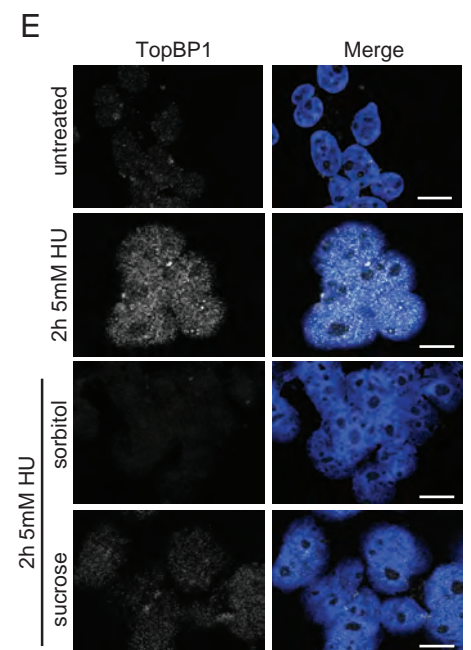
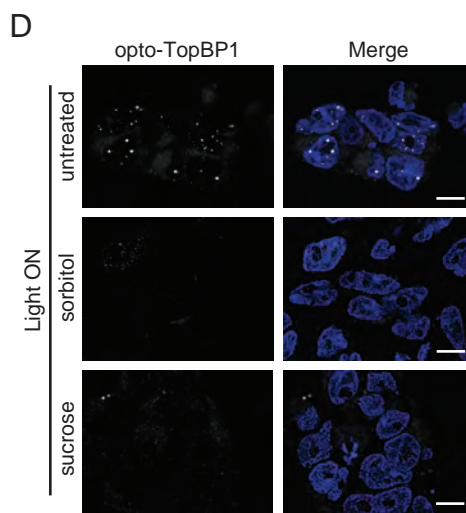
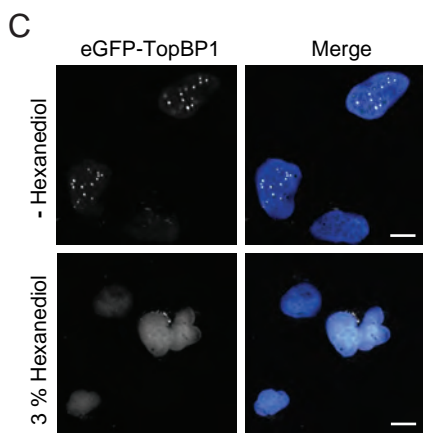
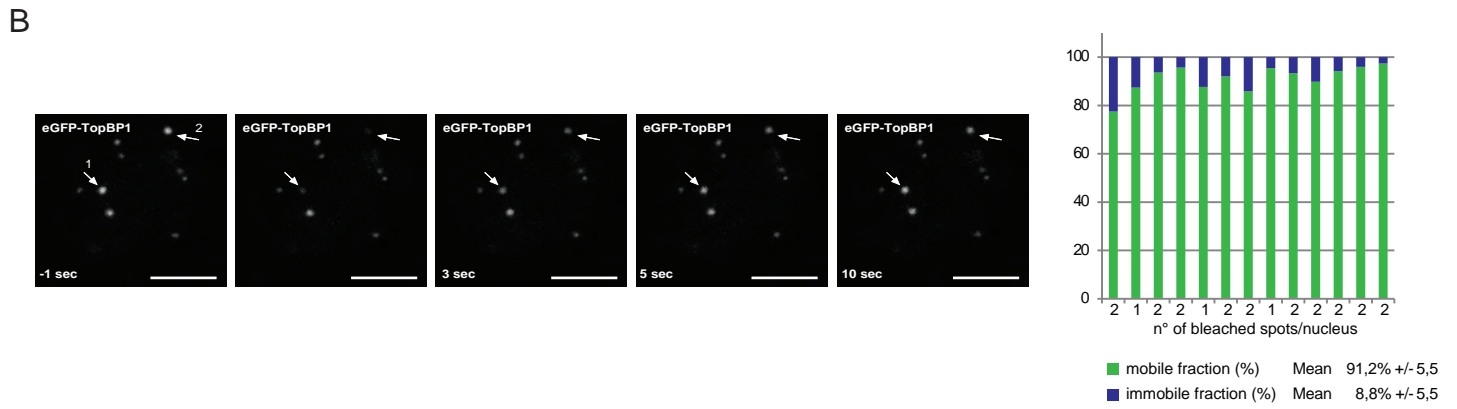
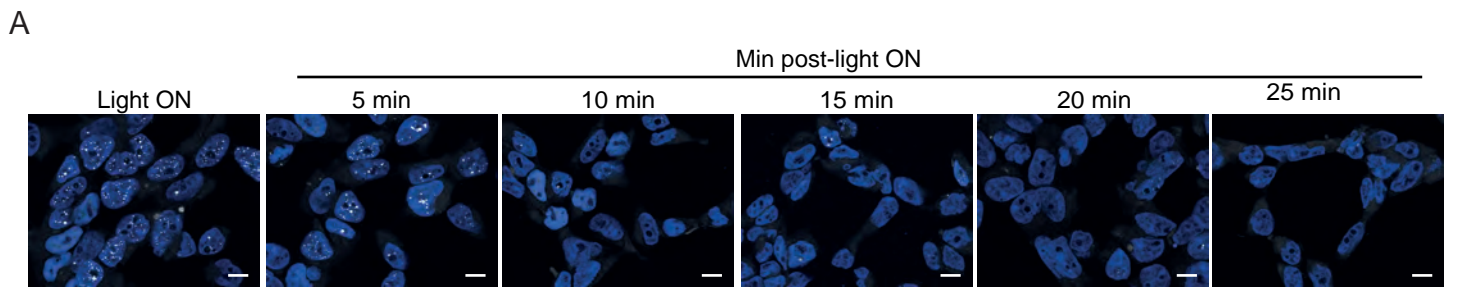
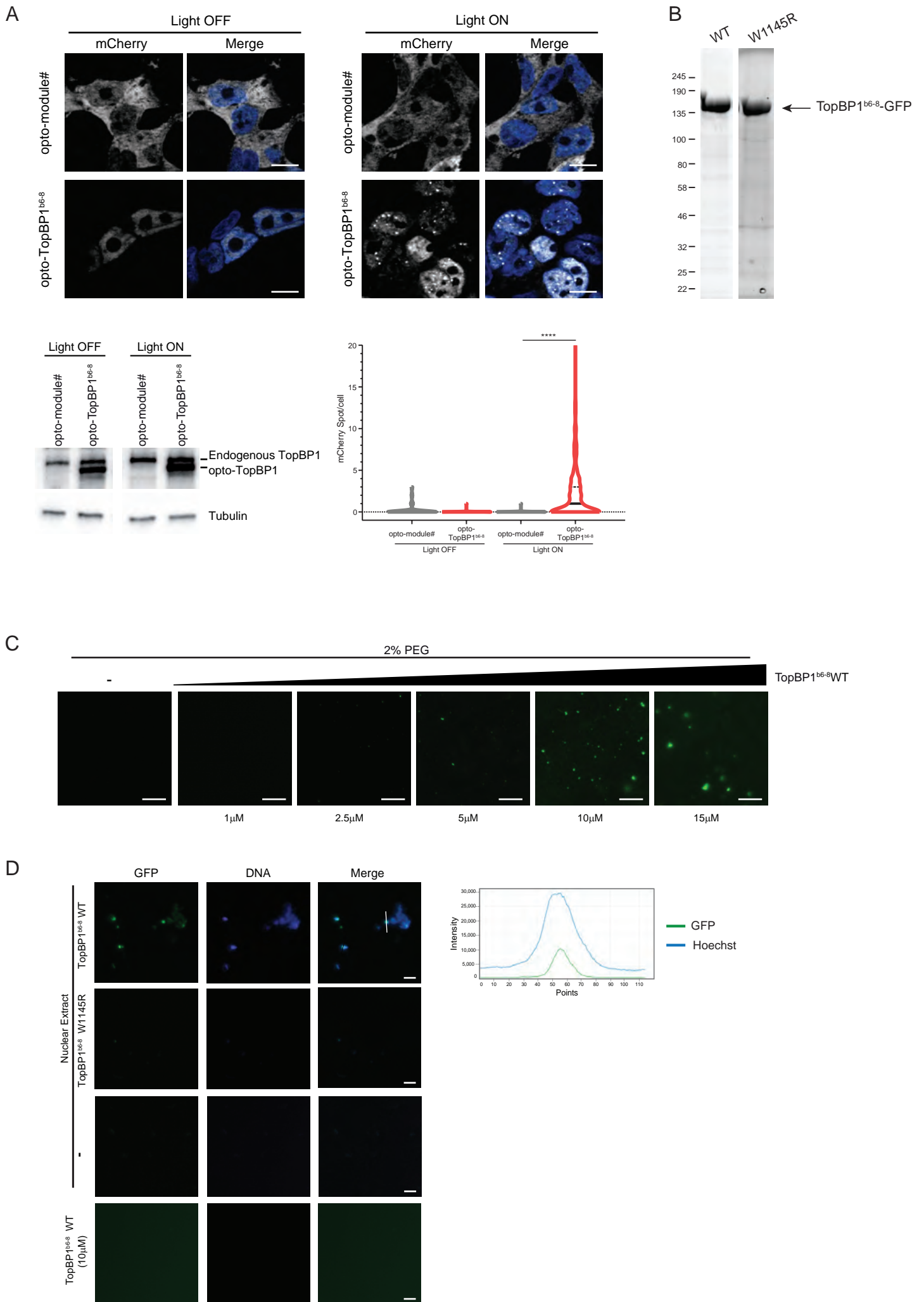


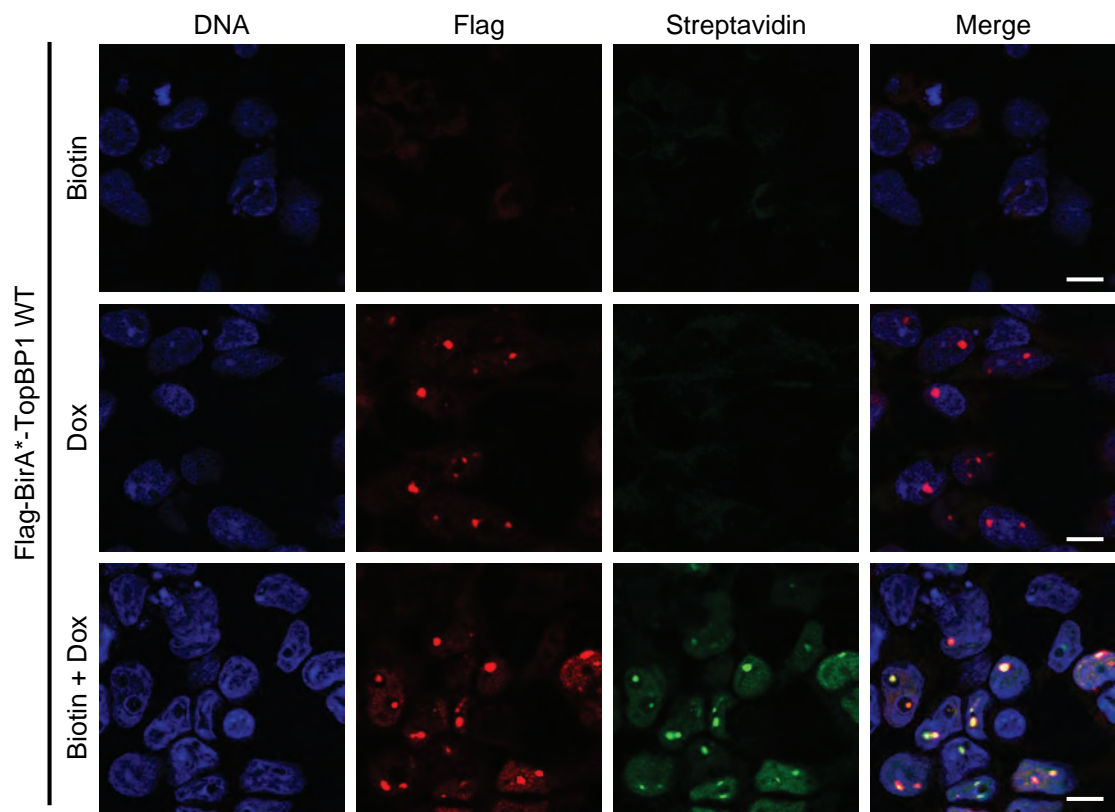
Figure 7_model



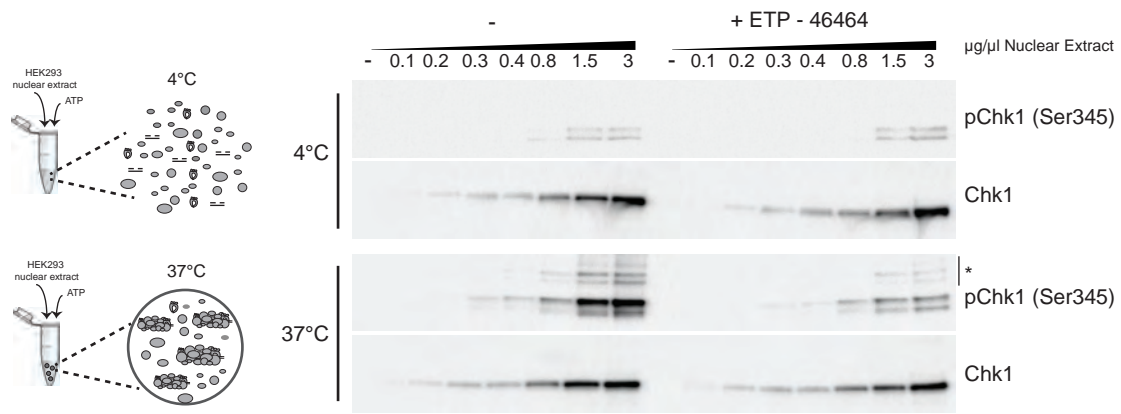




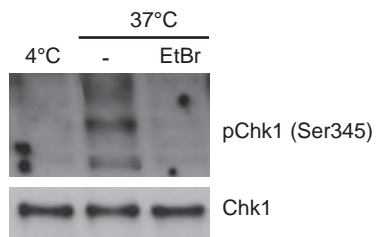
Suppl Figure 3_related to Figure 3

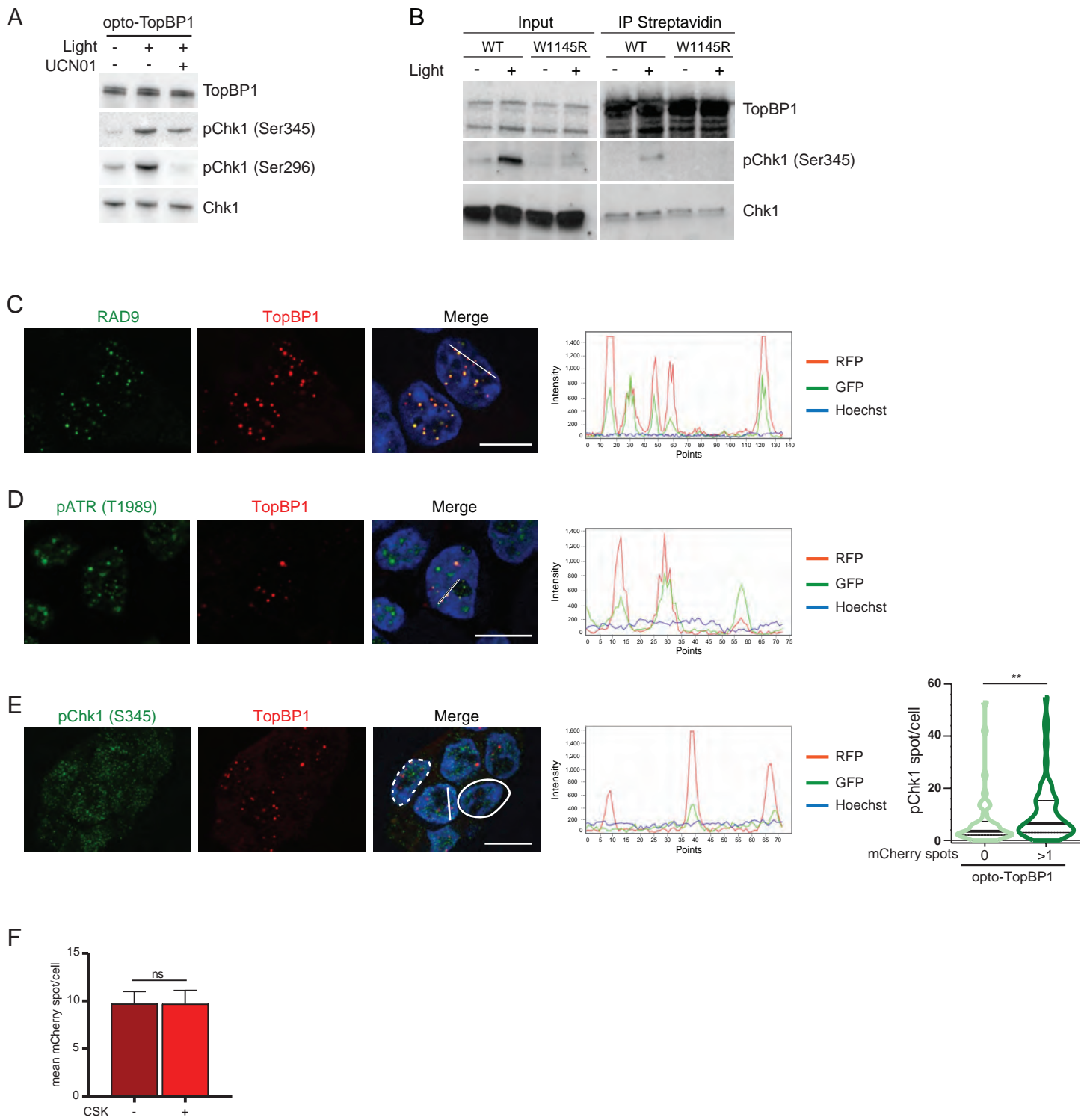


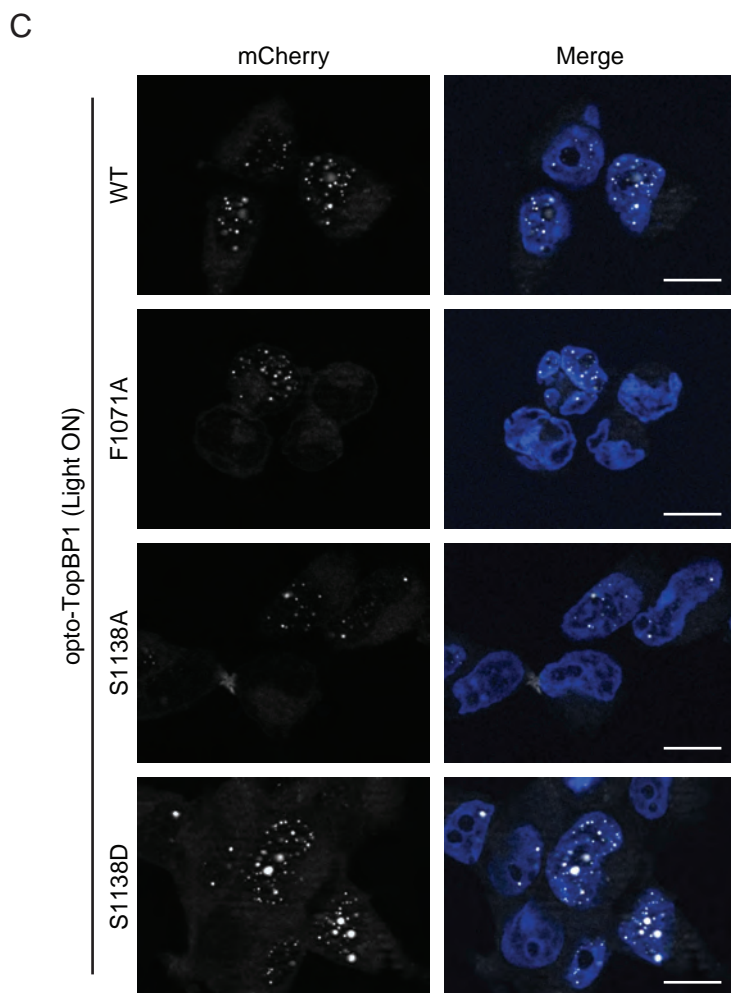
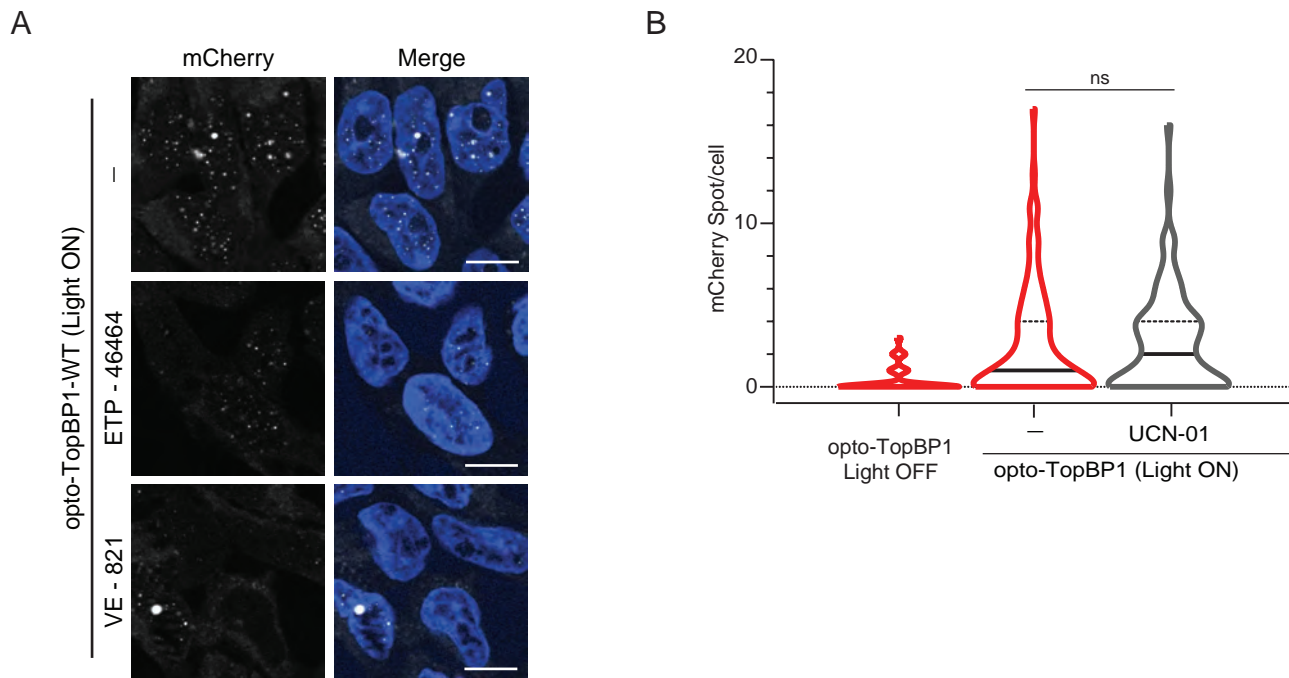
A



B







SUPPLEMENTAL INFORMATION

Supplementary video. Related to Figure 2A. 3D projection of opto-TopBP1 condensates.

Table S1. Oligonucleotides used in this study. Related to Key Resources Table and Method Details.

Primers	Sequence
Primer 1 (Fw)	TTGGCGCGCCAtccagaaatgacaaagaaccgt
Primer 2 (Rev)	TTTTCCTTTTGCGGCCGCttagtgtactctaggtcgtttg
Primer 3 (antisense)	GTAGGGTCATCCCTAATGATCTGTTCATTTTGGGAAGGC
Primer 4 (Fw)	GGGGTACCATCGATGCTAGCatggtgtctaaaggcgagga
Primer 5 (Rev)	GGCGCGCCtcagtcacgcatgttcaggt
Primer 6 (Fw)	GGGGTACCACCATGctttctgccagccctca
Primer 7 (Rev)	CTAGCTAGCgtgtactctaggtcgtttgatt
Primer 8 (Fw)	gggCTTAAGgccaccATGaaagacaatactgtgcctctg
Primer 9 (Rev)	cggGGTACCCACGTGcttttcggcagaccgcaga
Primer 10 (Fw)	gggCACGTGtccagaaatgacaaagaaccg
Primer 11 (Rev)	cggGGTACCgtgtactctaggtcgtttgat
Primer 12 (sense)	gagatgagagagaacgctcagaagcagttacag
Primer 13 (sense)	gtcaacacagagcctgcccaaaatgaacagatc
Primer 14 (sense)	gtcaacacagagcctgaccaaaatgaacagatc
Primer 15 (Fw)	CTAGCGTTTTAAACTTAAGATGgctgtgtcttcaacaaaggatg
Primer 16 (Rev)	cctaCATGGTACCatcatgtaaaggcttttgaagagg
Primer 17 (Fw)	tacacGGTACCATGaggaaagctctacag
Primer 18 (Rev)	cacatGCTAGCtctaggtcgtttgatttta
Primer 19 (Fw)	AGCTTGGTACCATGAGTCGGCGAAGGAAACATGATG
Primer 20 (Fw)	ccgaaaagCACGTGGGTACCAAGTGCCTGGTCACGGG
Primer 21 (Rev)	atGCTAGCATCGATGGTACCGCCTTCACCCTCACTGTCTTCC
Primer 22 (Rev)	cacatGCTAGCAAGAAATGAAGTGGGAGCTGC

Primer 23 (Fw)	tacacctggatacaccatcaaaattcctgtccaaggacaaac
Primer 24 (Rev)	CTTTGGTGTTCATAtgagagtagtcgactattacagagccg
Primer 25 (Fw)	agtcgactactctcaTATGAAACACCAAAGAGAGCGCTGA
Primer 26 (Rev)	agcaatttctgaatcTGACCACATCTGTATATTATCTTTATAAATACTTAAAATCTCTGTTTCT
Primer 27 (Fw)	ATACAGATGTGGTCAgattcagaaattgctaaacaggctgtct
Primer 28 (Rev)	ttcctgcacgaagtgtccagcagtcctgcaggcttcaaggta

Supplementary Figure Legends

Figure S1. Related to Figure 1. **A)** Immunoblotting of TopBP1. **B)** (Left) Representative fluorescence images of cells expressing opto-RAD9 under control of doxycycline. Light ON: 488nm light. (Right) Immunoblotting of the cells with the indicated antibody. **C)** ETAA1 does not assemble condensates. The construct, representative fluorescence images, and Violin plot representation of quantified condensates are shown, as described in Figure 1.

Figure S2. Related to Figure 2. **A)** Representative fluorescence images of cells used for experiments in Figure 2C. DNA stained with Hoechst 33258. Scale bars: 10µm. **B)** (Left) FRAP recordings of two individual eGFP-TopBP1 condensates are reported as examples. Scale bars: 10µm. (Right) Histogram representation of the mean of immobile and mobile fractions per nucleus (13 nuclei, 23 individual spots). **C)** Representative fluorescence images of eGFP-TopBP1 expressing U-2-OS cells treated, when indicated, with 3% of 1,6 Hexanediol. **D-E)** Representative fluorescence images of cells used for experiments in Figure 2D (**D**) and 2E (**E**). DNA stained with Hoechst 33258. Scale bars: 10µm. **F)** Violin plot representing the number of mCherry foci per cell in opto-TopBP1 expressing cells treated with 5mM HU. TopBP1 foci were identified with anti-mCherry antibody after CSK treatment. **A-B-C-D-E-F)** Number of biological replicates = 3.

Figure S3. Related to Figure 3. **A)** Representative fluorescence images of cells expressing opto-TopBP1^{b6-8} WT before (Light OFF) and after (Light ON) optogenetic activation. DNA stained with Hoechst 33258. Western blotting of the indicated proteins is shown. Violin plot represents the number of mCherry foci per cell. **(B)** Stain-free gel of purified TopBP1^{b6-8}-GFP WT and W1145R used for phase separation assays. **(C)** Representative fluorescence microscopy images of TopBP1 condensates assembled *in vitro*. The concentration of TopBP1 is indicated **(D)** Representative fluorescent images of TopBP1 condensates in suspension. DNA stained with Hoechst 33258. Line scan of GFP-Hoechst signals are shown. **A-C-D)** Scale bars: 10 μ m. Number of biological replicates = 3.

Figure S4. Related to Figure 4. Immunofluorescence staining of Flag-BirA*-TopBP1 WT using anti-Flag antibody. Biotin conjugates were revealed using AlexaFluor streptavidin. DNA stained with Hoechst 33258. Scale bars: 10 μ m. Number of biological replicates = 3.

Figure S5. Activation of endogenous ATR in nuclear extracts. **A)** Nuclear extracts at the indicated protein concentration were incubated in the presence of the ATR inhibitor ETP-46464 (10 μ M), as indicated. Chk1 activation is revealed by immunoblotting with the indicated antibodies. * represents unspecific bands. **B)** Immunoblotting of Chk1/pChk1 (Ser345) in reaction mixtures. When indicated nuclear extracts were pre-incubated with ethidium bromide (EtBr). **A-B)** Number of biological replicates = 3.

Figure S6. Related to Figure 5. **A)** Immunoblotting analyses of the indicated proteins before and after optogenetic activation of TopBP1 condensates. When indicated, cells were pre-treated with

10 μ M UCN-01. **B)** Immunoblotting analysis of the indicated biotinylated proteins isolated with streptavidin beads in WT and W1145R opto-TopBP1 expressing cells exposed exposed to 488 nm light, when indicated. **C-D-E)** Immunofluorescence staining with the indicated antibodies of opto-TopBP1 expressing cells activated by light. Line scans are shown. DNA stained with Hoechst 33258. Scale bars: 10 μ m. Violin plot (**E-right**) representing spot of pChk1 in cells with (dashed white line) or without (solid white line) opto-TopBP1 foci. **F)** Histograms representing the mean of mCherry foci per cell. Cells expressing opto-TopBP1 WT were exposed to 488 nm light, and, when indicated, nuclear soluble proteins were extracted with Cytoskeleton (CSK) buffer. **A-B-C-D-E-F)** Number of biological replicates = 3.

Figure S7. Related to Figure 6. **A)** Representative fluorescence images of cells used for optogenetic experiment in Figure 6A. **B)** Violin plot representing the number of mCherry condensates per cell in opto-TopBP1 expressing cells pre-treated for 1h with 10 μ M UCN-01, when indicated. **C)** Representative fluorescence images of cells used for optogenetic experiment in Figure 6C. **A-B-C)** Number of biological replicates = 3.