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TopBP1 assembles nuclear condensates to switch on ATR signalling.

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Summary

ATR checkpoint signalling is crucial for cellular responses to DNA replication impediments. Using an optogenetic platform, we show that TopBP1, the main activator of ATR, self-assembles extensively to yield micron-sized condensates. These opto-TopBP1 condensates are functional entities organized in tightly packed clusters of spherical nano-particles. TopBP1 condensates are reversible, occasionally fuse and co-localise with TopBP1 partner proteins. We provide evidence that TopBP1 condensation is a molecular switch that amplifies ATR activity to phosphorylate checkpoint kinase 1 (Chk1) and slowdown replication forks. Single amino acid substitutions of key residues in the intrinsically disordered ATR-activation domain disrupt TopBP1 condensation and, consequently, ATR/Chk1 signalling. In physiologic salt concentration and pH, purified TopBP1 undergoes liquid-liquid phase separation in vitro. We propose that the actuation mechanism of ATR signalling is the assembly of TopBP1 condensates driven by highly regulated multivalent and cooperative interactions.
The primary structure of DNA is subjected to constant chemical alterations caused by spontaneous decay, endogenous metabolites and environmental genotoxic agents (Friedberg et al., 2006; Lindahl, 1993), hence organisms have evolved multiple DNA repair mechanisms to ensure genome integrity and survival (Ciccia and Elledge, 2010; Jackson and Bartek, 2009; Tubbs and Nussenzweig, 2017). DNA damage sensors, protein scaffolds and DNA processing activities accumulate at DNA damage sites to form spatially defined and reversible structures commonly referred to as nuclear foci (Garcia-Higuera et al., 2001; Lisby et al., 2001; Maser et al., 1997; Park et al., 1996). Much still remains to be understood about the molecular forces that drive the formation of DNA damage foci and their functional consequences in the DNA damage response.

In recent years, application of the principles of polymer chemistry to biological molecules has accelerated spectacularly our understanding of the assembly mechanism and functions of membraneless compartments (Banani et al., 2017; Bracha et al., 2019; Hyman and Simons, 2012; Shin and Brangwynne, 2017; Soding et al., 2020). These self-organized micron-scale structures, called biomolecular condensates, assemble via multiple weak, cooperative and dynamic interactions and yield a rich repertoire of higher-order structures with diverse physical properties, variable size, and no defined stoichiometry of constituent proteins. Whereas nucleic acids can serve a seeding platform for the self-organization of soluble proteins (Mao et al., 2011; McSwiggen et al., 2019), soluble bridging factors can cross-link chromatin segments to compartmentalize chromatin via a process of polymer-polymer phase separation (Erdel et al., 2020). Increasing evidence indicates that diverse multivalent protein scaffolds self-organize via liquid-liquid phase separation, a process of de-mixing that yields a condensed phase enriched in the protein and a dilute phase (Banani et al., 2017; Shin and Brangwynne, 2017). Biomolecular
condensates are functional hubs implicated in diverse cellular processes, including innate immune signalling (Du and Chen, 2018), microtubule nucleation (Woodruff et al., 2017), transcription (Boija et al., 2018; Kwon et al., 2013; Lu et al., 2018; Sabari et al., 2018) and adaptative stress responses (Franzmann and Alberti, 2019; Franzmann et al., 2018; Riback et al., 2017). Protein phase separation can occur at DNA damage sites. Upon laser micro-irradiation, the activity of poly(ADP-ribose) polymerase 1 seeds the condensation of the prototypical liquid-liquid phase separation protein FUS at damaged chromatin (Altmeyer et al., 2015; Patel et al., 2015). 53BP1 phase separates at double-strand DNA breaks (Kilic et al., 2019; Pessina et al., 2019), and the condensation of 53BP1 promotes induction of p53 and p21 (Kilic et al., 2019).

To explore the mechanisms and functional consequences of biomolecular condensates in the DNA damage response (DDR), we studied Topoisomerase IIβ-binding protein (TopBP1), an essential factor in the DDR pathway and a prototype of protein scaffolds composed of multiple modular interaction domains. TopBP1 features nine repetitions of a well-folded protein-protein interaction motif, the BRCA1 C terminus domain (BRCT), and an ATR activation domain (AAD), located between BRCT6 and BRCT7, which is intrinsically disordered. TopBP1 brings together different sets of proteins to form distinct protein complexes involved in DNA replication initiation (Hashimoto and Takisawa, 2003; Makiniemi et al., 2001), DNA replication stress signalling (Kumagai et al., 2006; Mordes et al., 2008), DNA repair (Broderick et al., 2015; Leimbacher et al., 2019; Liu et al., 2017; Moudry et al., 2016) and transcription regulation (Liu et al., 2009; Wright et al., 2006). TopBP1 is the main activator of the master checkpoint kinase ATR (Kumagai et al., 2006; Mordes et al., 2008). ATR and its effector kinase Chk1 are crucial for cellular responses to DNA damage and DNA replication impediments (Ciccia and Elledge, 2010; Marechal and Zou, 2013; Saldivar et al., 2017). ATR/Chk1 signalling ensures cell and organismal survival.
through coordination of DNA repair and DNA replication with physiological processes (Saldivar et al., 2017). Studies using Xenopus egg extracts have largely contributed to defining the orchestrated set of events leading to ATR activation (Acevedo et al., 2016; Byun et al., 2005; Duursma et al., 2013; Kumagai et al., 2006; Van et al., 2010). ATR is recruited to DNA lesions and replication intermediates via the ATR interacting protein ATRIP, which binds RPA-covered single-stranded DNA (Zou and Elledge, 2003). TopBP1 interacts with ATRIP, with ATR and with the RAD9-RAD1-HUS1 (9-1-1) clamp and activates ATR (Delacroix et al., 2007; Duursma et al., 2013; Kumagai et al., 2006; Mordes et al., 2008; Yan and Michael, 2009). Here, we used the conceptual framework born from studies of biomolecular condensates to gain fresh insights into the activation mechanism of ATR/Chk1 signalling. Using a combination of optogenetic and biochemical approaches, we show that TopBP1 self-assembles extensively to yield functional biomolecular condensates, and that purified TopBP1 has an intrinsic capacity to undergo liquid phase separation. We provide evidence that TopBP1 self-organization into micron-sized compartments in living cells activates ATR/Chk1 signalling and slows down the progression of replication forks. Our data indicate that essential responses to DNA replication impediments emerge from TopBP1-driven assembly of nuclear condensates.
Results

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

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

A conserved tryptophane in the ATR activation domain of TopBP1 at position 1138 in *Xenopus Laevis*, 1147 in mice, and 1145 in human, is essential for the capacity of TopBP1 to activate ATR (Kumagai et al., 2006) (Zhou et al., 2013). In mice, the substitution of W1147 for an arginine
residue is embryonic lethal, indicating that the ATR-activating function of TopBP1 is essential (Zhou et al., 2013). The W1148R substitution does not disrupt the association of xTopBP1 with xATR (Kumagai et al., 2006). Here, we analyzed the impact of W1145R on the capacity of TopBP1 to assemble higher order structures. W1145R TopBP1 expressing cells exhibited a markedly reduced number of optogenetic TopBP1 condensates in comparison with wild-type TopBP1 (Figure 1B), suggesting that this aromatic residue plays an important role in TopBP1 higher-order assembly or in the growth of TopBP1 condensates.

Ewing’s tumor associated antigen 1 (ETAA1) is required for Chk1 basal activity and stability (Michelena et al., 2019), and for the regulation of mitotic ATR signalling (Bass and Cortez, 2019). Like TopBP1, ETAA1 is endowed with an intrinsically disordered ATR activation domain (AAD) (Bass and Cortez, 2019; Bass et al., 2016; Haahr et al., 2016; Lee et al., 2016). Considering the conserved function of these disordered AADs, we replaced the AAD of TopBP1 with the AAD of ETAA1, and then tested the capacity of the chimeric opto-TopBP1ETAA1-AAD protein to form condensates. In comparison with TopBP1, the TopBP1ETAA1-AAD chimeric protein exhibited reduced capacity to form condensates (Figure 1C). Consistent with this, optogenetic activation of ETAA1 fused to Cry2 did not yield foci (Figure S1C). Moreover, the AAD of TopBP1 did not form optogenetic condensates (Figure 1D). Collectively, these data indicate that TopBP1 can self-assemble into micron-sized condensates and that the TopBP1 AAD has unique characteristic features that are necessary but not sufficient for TopBP1 higher-order assembly.

Optogenetic and endogenous TopBP1 condensates share similar properties.

A three-dimensional analysis revealed that optogenetically-induced TopBP1 condensates have a spherical shape with an aspect ratio close to one (Figure 2A + Suppl. video), suggesting that the
interface of these structures may be subjected to surface tension. Opto-TopBP1 condensates occasionally fused, hence TopBP1 can undergo dynamic clustering (Figure 2B). These structures dissolved within 15 minutes (Figure 2C + Figure S2A), while Cry2 oligomers disassemble spontaneously within 1-2 minutes (Shin et al., 2017). The relative stability of opto-TopBP1 condensates reflects the role of TopBP1 multivalent cooperative interactions in the formation of micron-sized protein assemblies. Next, we assessed the permeability of TopBP1 condensates to the surrounding milieu by fluorescence redistribution after photobleaching. We expressed eGFP-TopBP1 in U-2-OS cells with doxycycline for 24 hours (Sokka et al., 2015). In these experimental conditions, overexpressed eGFP-TopBP1 spontaneously forms foci that accumulate in nucleoli (Sokka et al., 2015). After photobleaching, eGFP-TopBP1 bodies recovered fluorescence signal within seconds, which reflects the rapid exchange of eGFP-TopBP1 molecules between the nucleoplasm and TopBP1 nuclear condensates (Figure S2B). We conclude that the boundaries of TopBP1 compartments are permeable. We noted also that eGFP-TopBP1 bodies were sensitive to hexanediol (Figure S2C), an aliphatic alcohol that disrupts weak hydrophobic interactions (Kroschwald et al., 2017).

The cooperative interactions that drive the formation of biomolecular condensates are influenced by the properties of the surrounding milieu. Changes in osmotic concentration by addition of sorbitol or sucrose in the cell culture medium disrupt 53BP1 phase separation (Kilic, 2019). Likewise, osmotic stress inhibited both the assembly of TopBP1 condensates induced by optogenetic activation (Figure 2D + Figure S2D), and the formation of endogenous TopBP1 foci induced by treatment with the inhibitor of ribonucleotide reductase hydroxyurea (Figure 2E + Figure S2E). This suggests that electrostatic forces drive the assembly of both synthetic (optogenetic) and endogenous TopBP1 condensates. Recombinant opto-TopBP1 also formed
nuclear foci in cells exposed to hydroxyurea (Figure S2F), indicating that like endogenous 
TopBP1, opto-TopBP1 engages with endogenous components in response to stalled replication 
forks. Whereas TopBP1 condensates appear as homogenous structures by conventional 
fluorescence microscopy, stimulated emission depletion (STED) nanoscopy with an anti-mCherry 
antibody revealed the underlying sub-structure of optogenetic TopBP1 condensates, which consist 
in clusters of spherical, nanometer-sized particles (Figure 2F, left panel). TopBP1 clusters had a 
very tight appearance when detected with an anti-TopBP1 antibody that recognizes both 
endogenous and recombinant TopBP1 (Figure 2F, right panel). Likewise, high-resolution imaging 
of endogenous TopBP1 in cells treated with hydroxyurea revealed numerous sub-structured 
clusters of nano-condensates (Figure 2G). The data suggest that whether seeded by DNA 
replication impediments or Cry2 oligomerisation, the driving forces and the organization of 
TopBP1 nuclear condensates are similar.

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

To test if TopBP1 has the capacity to phase separate, we expressed and purified the carboxy- 
terminal half of TopBP1 (amino acids 884-1522). This portion of TopBP1, hereafter called b6-8, 
includes the BRCT 6 to 8 and the AAD (Figure 3A + Figure S3B). TopBP1\(^{b6-8}\) mediates directly 
the activation of ATR, independently of its amino-terminal portion (Hashimoto et al., 2006; 
Kumagai et al., 2006). TopBP1\(^{b6-8}\) formed optogenetically-induced foci in live cells (Figure S3A). 
In the presence of the crowding agent Polyethylene Glycol (2%), purified TopBP1\(^{b6-8}\)-GFP formed 
spherical condensates detected by fluorescence microscopy (Figure 3B, upper panel and Figure 
S3C). Consistent with optogenetic experiments (Figure 1B), the W1145R substitution abolished 
the phase separation of TopBP1\(^{b6-8}\) (Figure 3B, lower panel). Addition of TopBP1\(^{b6-8}\)-RFP to pre-
formed TopBP1^{b6-8}.GFP condensates yielded yellow compartments, indicating that soluble TopBP1 molecules are recruited to TopBP1 condensates. By contrast, W1145R TopBP1^{b6-8}.RFP did not stably associate with TopBP1^{b6-8}.GFP condensates (Figure 3C). TopBP1 condensates were permeable to DNA, as revealed by the partitioning of double-strand DNA fragments into pre-formed TopBP1 condensates (Figure 3D). In the presence of a circular DNA plasmid (2.9 kb), TopBP1^{b6-8}.GFP formed clusters of nano-condensates that were reminiscent, yet not equivalent, to high-resolution images of cellular TopBP1 condensates (Figure 3E). This observation suggests that the long anionic DNA polymer strongly influences the assembly of TopBP1 molecules. In the absence of PEG, purified TopBP1^{b6-8}.GFP (10\mu M) did not phase separate (Figure 3B minus PEG, and 3F). To recapitulate partially the complex environment of the nucleus, we spiked “Dignam and Roeder” nuclear extracts with recombinant TopBP1^{b6-8}.GFP in physiologic salt concentration and pH. After incubation of the reaction mixture at 37\degree C, fluorescence microscopy analyses revealed \mu m-scaled TopBP1^{b6-8}.GFP condensates (Figure 3F) co-localizing with endogenous DNA (Figure S3D). Hence, TopBP1 foci-like structures that depend on the integrity of the AAD are recapitulated with purified TopBP1 in the presence of a crowding agent or nuclear extracts. We conclude that TopBP1 has intrinsic capacity to form micron-sized condensates, both in vitro and in living cells.

Consequence of TopBP1 condensation on TopBP1-associated protein network.

To test if recombinant TopBP1 condensates mimic endogenous TopBP1 foci, we analyzed the composition of TopBP1 condensates assembled in live cells. We used a biotin labelling approach to gain a panoramic view of TopBP1 proximal proteins. A doxycycline-inducible cDNA encoding WT or W1145R TopBP1 fused to the mutated biotin ligase BirA* and the Flag epitope was stably
integrated in Flp-In HEK293 cells (Figure 4A). We overexpressed Flag-BirA*-TopBP1 to a level that induces constitutive BirA*-TopBP1 condensates (Figure S4), in the absence of DNA damaging agents. We labelled TopBP1 proximal proteins with biotin for 3 hours. More than 500 TopBP1 proximal proteins were identified by mass spectrometry with high confidence and reproducibility (Supplemental Excel sheet). Proteins were ranked according to their iBAQ value (intensity-based absolute quantification), a proxy for protein abundance (Figure 4B). Among abundant TopBP1 proximal proteins, we identified known TopBP1 partners, including TOP2A, FANCJ, BRCA1, MRE11, MDC1, 53BP1 and BLM (Figure 4B). Only five proteins showed differences in abundance between WT and W1145R TopBP1 (Log2 diff >2 with a p-value ≤ 0.05) (Figure 4C). Among them, only the nucleolar protein NOL11 was detected at high level, and, therefore, considered as a significant difference between WT and W1145R TopBP1. This is consistent with a previous report demonstrating that upregulated TopBP1 accumulates on ribosomal chromatin, segregates nucleolar components and yields nucleolar caps (Sokka et al., 2015). By contrast, W1145R substitution abrogates TopBP1 nucleolar segregation (Sokka et al., 2015). Consistent with this, W1145R TopBP1 had lost proximity with the nucleolar protein NOL11 (Figure 4D). Immunoblot analysis of TopBP1 proximal proteins confirmed that the substitution W1145R in the AAD of TopBP1 does not alter the proximity of TopBP1 with partner proteins implicated in ATR signalling, including ATR, BRCA1, MRE11 and FANCJ (Figure 4D). More generally, W1145R did not alter significantly the network of TopBP1 proximal proteins. In conclusion, at a low temporal resolution, whereas the composition of recombinant TopBP1 assemblies recapitulates endogenous TopBP1 foci, TopBP1 condensation does not increase significantly the association of TopBP1 with partner proteins.
Functional consequences of TopBP1 condensation.

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

Of note, we observed the spontaneous activation of endogenous Chk1 in “Dignam and Roeder” nuclear extracts after 10 minutes incubation at 37°C (Figure S5A), in the absence of recombinant TopBP1 protein and without an exogenous ATR-activating DNA structures. The level of phospho Chk1 (Ser345) signals was significantly higher than background signals observed when reaction mixtures were incubated at 4°C, or at 37°C in the presence of the ATR and mTOR inhibitor ETP-46464 (Figure S5A), confirming that the reaction was the product of ATR activity and occurred in vitro. Phosphorylation of Chk1 was blocked when nuclear extracts were pre-
incubated with ethidium bromide, which disrupts protein-DNA interaction (Figure S5B), indicating that endogenous DNA fragments present in the extracts are required for ATR activation. The activation of endogenous ATR in nuclear extracts suggests that when a critical concentration of reactants is reached, a productive supramolecular ATR signalling complex assembles spontaneously.

Once activated by ATR through Ser345 phosphorylation, Chk1 auto-phosphorylates on Ser296. This step is required for Chk1 to induce downstream molecular events leading to cell cycle arrest (Kasahara et al., 2010). Light-induced TopBP1 condensation yielded Chk1 Ser296 phospho-signals, confirming that Chk1 is active (Figure S6A). UCN-01, an inhibitor of Chk1, blocked Chk1 auto-phosphorylation on Ser296, but had no major impact on Chk1 phosphorylation on Ser345 by ATR (Figure S6A). This confirms that the Chk1 phospho Ser296 signal is a product of Chk1 activity. To explore further the impact of TopBP1 condensation on the interaction of TopBP1 with partner proteins, we took advantage from the optogenetic tool shown in Figure 1A. The TopBP1-mCherry-Cry2 used for light-induced foci formation was tagged at its N-terminus with TurboID, an optimized biotin ligase that can biotinylate proteins within minutes (Branon et al., 2018). To detect ATR activity within optogenetic TopBP1 condensates, we induced TopBP1 condensation by 488 nm light in the presence of biotin in the cell culture medium, and then purified biotinylated proteins with streptavidin-coated beads. We enriched phospho ATR (Thr1989) and phospho TopBP1 (Ser1138) signals from cells expressing WT TopBP1 after optogenetic activation, specifically (Figure 5C). Furthermore, this biotin labelling strategy allowed to capture some Chk1 phosphorylation events occurring within TopBP1 condensates (Figure S6B). By contrast, we did not isolate biotinylated phospho ATR, phospho TopBP1 and phospho Chk1 in proximity to W1145R TopBP1 (Figure 5C and Figure S6B). These data indicates that the condensation of
TopBP1 promotes the phosphorylation of ATR target proteins. In fluorescence microscopy, optogenetic TopBP1 condensates co-localised with RAD9 and with phospho ATR (Figure S6C-D). By contrast, Chk1 Ser345 phospho-signals rarely co-localised with TopBP1 condensates per se, but were detected in cells positive for TopBP1-mCherry condensates (Figure S6E), consistent with Chk1 high mobility (Liu et al., 2006). Chk1 is not retained physically at DNA damage sites, allowing signal transmission from DNA damage sites to the rest of the cell (Liu et al., 2006).

Super-resolution STED imaging revealed phospho ATR (Thr1989) signals intertwined with TopBP1 nano-condensates in TopBP1 clusters (Figure 5D). These observations indicate that TopBP1 condensates function as reaction hubs.

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

Regulation of TopBP1 condensation

TopBP1 is highly phosphorylated in response to DNA replication stress (Munk et al., 2017), and the activation of ATR/Chk1 signalling is dependent on TopBP1 phosphorylation (Burrows and...
We reasoned that the basal kinase activity of ATR, which is independent of TopBP1 (Liu et al., 2011), may play a role in the condensation process. To test this, we pre-incubated cells with the ATR inhibitors VE-821 (Charrier et al., 2011), or ETP-46464 (Llona-Minguez et al., 2014), for 1 hour, and then exposed opto-TopBP1 expressing cells to blue light for 3 minutes. In these experimental conditions, the formation of TopBP1 condensates was inhibited and the phosphorylation of Chk1 on the ATR site Ser345 was blocked (Figure 6A + Figure S7A). By contrast, pre-incubation of the cells with the Chk1 inhibitor UCN-01 did not affect the formation of optogenetic TopBP1 condensates (Figure S7B). To explore further the role of ATR in TopBP1 condensation, we mutated key amino acids in the AAD (Figure 6B). We substituted TopBP1 phenylalanine at position 1071 with alanine. This substitution locates within a predicted coiled coil in TopBP1 AAD and partially destabilizes the interaction of TopBP1 with ATR (Thada and Cortez, 2019). The F1071A substitution severely reduced the optogenetic induction of TopBP1 condensates (Figure 6C, left panel + Figure S7C). Biotin labelling revealed, however, that F1071A TopBP1 remained proximal to ATR and basal phosphorylation on Ser1138 was detected (Figure 6D). The Ser1138 phospho signal was induced upon optogenetic activation of WT TopBP1 condensates, but not in cells expressing F1071A TopBP1, consistent with a condensation defect (Figure 6D). Several contact points maintain TopBP1 in proximity with the ATR-ATRIP complex (Thada and Cortez, 2019). Hence, the reduction of the strength of the association of F1071A TopBP1 with ATR-ATRIP does not fully account for the severe condensation defect observed in these experimental conditions (Figure 6C).

In *Xenopus Laevis*, the phosphorylation of Ser1131 enhances the capacity of TopBP1 to activate ATR (Yoo et al., 2009). XTopBP1Ser1131 corresponds to Ser1138 in human TopBP1. Ser1138A partially inhibited the optogenetic activation of TopBP1 condensates, whereas the phospho mimic
S1138D stimulated TopBP1 condensation (Figure 6C, left panel + Figure S7C). The partial suppression of TopBP1 condensates by S1138A suggests that additional phospho-sites contribute to the cooperative assembly of TopBP1 condensates. In blue-light exposed cells expressing F1071A TopBP1 or S1138A TopBP1, the level of Chk1 phosphorylation on Ser345 was reduced in comparison with cells expressing WT TopBP1 (Figure 6C, right panel). Furthermore, phospho Ser1138 TopBP1 and phospho Thr1989 ATR signals were barely detectable in streptavidin pulldowns (Figure 6D). By contrast, the phosphomimetic substitution S1138D in TopBP1 yielded phospho Ser345 Chk1 signals upon optogenetic activation (Figure 6C, right panel) and phospho Thr1989 ATR was enriched in proximity of TopBP1 (Figure 6D). Collectively, the data suggest an amplification mechanism for activation of ATR/Chk1 signalling, whereby TopBP1 phosphorylation by ATR induces TopBP1 condensation, and TopBP1 condensation unleashes its capacity to activate ATR.
In this study, we provide evidence that TopBP1 is a protein scaffold that can self-assemble extensively to yield tight clusters of nano-condensates, and that the association of TopBP1 with ATR yields a positive feedback loop. We propose that TopBP1 condensation is a molecular switch that triggers checkpoint responses to DNA replication impediments. TopBP1 condensation depends on the basal kinase activity of ATR and on the phosphorylation of TopBP1 on serine 1138. Thus, we propose a refined model of ATR activation (Figure 7). In the early stages of ATR signalling, ATR-ATRIP and TopBP1 congregates on RPA-coated single-stranded DNA, the 9-1-1 complex is loaded at single to double strand DNA junctions and stabilizes TopBP1. In later stages, the phosphorylation of TopBP1 induces its higher-order assembly into micron-sized clusters of nano-condensates that propel ATR signal transduction. The yeast homologue of ATR, Mec1, has been proposed to be activated via an allosteric mechanism (Wang et al., 2017). We surmise that the compartmentation of ATR signalling proteins creates a reaction hub where the probability of molecular interactions required for ATR activation is increased. A TopBP1 clustering mechanism for ATR activation is reminiscent of Ras clusters that assemble transiently on the plasma membrane, and function as high-gain amplifiers critical for MAPK signal transduction (Prior et al., 2003; Tian et al., 2007).

The data shown here suggest that the molecular forces driving TopBP1 condensation are multiple weak and highly cooperative interactions. First, whether seeded by hydroxyurea-induced replication stress or Cry2 oligomerisation, TopBP1 nuclear condensates were dissolved upon addition of sorbitol or sucrose in the cell culture medium. These compounds destabilize weak electrostatic interactions involved in protein phase separation. The aliphatic alcohol hexanediol also dissolves TopBP1 condensates, suggesting that hydrophobic interactions contribute to
TopBP1 higher order assembly. Second, purified TopBP1 undergoes liquid-liquid phase separation, a characteristic feature of multivalent protein scaffolds that underpin the formation of membrane-less compartments. TopBP1 phase separation in vitro occurred also in the complex environment of a nuclear extract, where multiple homotypic and heterotypic interactions could influence the capacity of TopBP1 to self-assemble. Third, TopBP1 condensation was highly sensitive to key amino acids substitutions and post-translational modifications in the AAD. These modifications typically change the cooperative molecular forces that organize protein condensation. Last, our results suggest that the BRCT6-8 also contributes to TopBP1 higher-order assembly, consistent with data showing that BRCT7/8 promotes TopBP1 oligomerisation (Chowdhury et al., 2014). We observed that both optogenetic and endogenous TopBP1 form tight clusters of nano-condensates. Interestingly, in mitosis, TopBP1 forms filamentous structures that bridge MDC1 foci (Leimbacher et al., 2019), suggesting that specific molecular associations dictate the organization of TopBP1 higher-order structures.

The function of TopBP1 condensation described here explains previous observations. In Saccharomyces cerevisiae, artificial co-localization of the 9-1-1 complex and Ddc2^{ATRIP}-Mec1^{ATR} via tethering to an array of 256 LacO repeats bypasses the requirement for DNA damage to activate Mec1^{ATR} (Bonilla et al., 2008). In Schizosaccharomyces pombe, artificial tethering of either one of Rad3^{ATR}, RAD4^{TopBP1} or RAD9 to a LacO array triggers a checkpoint response that utilizes the endogenous proteins (Lin et al., 2012). Furthermore, TopBP1 activates ATR in vitro and in cells when artificially tethered to DNA (Lindsey-Boltz and Sancar, 2011). Based on the findings described here, we surmise that the artificial tethering of checkpoint proteins to LacO arrays is nucleating the condensation of endogenous proteins, which switches on checkpoint signalling.
The transient and reversible nature of the molecular forces that underpin the formation of functional TopBP1 nuclear condensates appears well adapted to cellular regulation and optimal responsivity to DNA replication impediments, as opposed to the stable interaction of proteins that characterize molecular machines with defined stoichiometry. The formation of functional micron-sized condensates through the regulated self-assembly of multivalent protein scaffolds may represent a fundamental principle underlying the formation of functional nuclear foci in response to DNA damage.

Limitations

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

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Author contributions


Declaration of Interests

The authors declare no competing interests.
Main Figure Legends

Figure 1. Optogenetic activation of TopBP1 condensates. A) Schematic representation of the TopBP1 optogenetic platform B) Optogenetic activation of wild-type and W1145R TopBP1. The opto-module denotes mcherry-Cry2. Light ON: 3 min cycles of 4s light (488nm)-10s resting. Representative fluorescence images are shown. DNA is stained with Hoechst 33258. Scale bars: 10μm. Violin plot represents distribution of number of condensates per cell. Median and quartile values are represented by continues and dashed lines, respectively. The statistical significance between samples is represented by *. Number of biological replicates = 3. See Figure S1A for protein expression levels. (C) Optogenetic activation of opto-TopBP1ETAA1-AAD. D) Optogenetic activation of opto-AAD-NLSTopBP1. FL stands for Full Length.

Figure 2. Properties of TopBP1 condensates. A) 3D analysis of opto-TopBP1 condensates by IMARIS software. Sphericity = area_{sphere}/area_{particle}. The 3D projection and the spherical value for particle 1 (P1) is represented. Other particles were analyzed for their spherical values (P2=0.932587; P3=0.918362; P4=0.949144; P5=0.935103). Scale bar: 2μm. B) Time laps microscopy images of activated opto-TopBP1 condensates. C) Histograms representing the mean of mCherry-labelled condensates per cell. Activated TopBP1 WT condensates were incubated in the dark for the indicated time. D) Violin plot representing the number of optogenetic TopBP1 condensates in cells were pre-treated with 0.5M sucrose or 0.4M sorbitol for 1h, as indicated. E) Violin plot representing the number of endogenous TopBP1 foci per cell in cells treated with 5mM HU and 0.5M sucrose or 0.4M sorbitol, as indicated. The values correspond to the sum of three independent experiments. C-D-E) Number of biological replicates = 3. See Figure S2A, S2D and S2E for representative images. F) Representative super-resolution STED images of optogenetic
TopBP1 condensates identified with anti-mCherry (left panel) or anti-TopBP1 (right panel). G) STED image of endogenous TopBP1 induced by hydroxyurea (left panel). Right panel: untreated control cells. Scale bars: 2µm for CONFOCAL images and 1µm for STED images.

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

**Figure 4. Composition of TopBP1 condensates.** A) Schematic representation of the Flag-BirA*-TopBP1 platform. See Figure S4 for representative images of BirA*-TopBP1 condensates. B-C) Proteins identified by mass spectrometry were ranked according to their iBAQ value (B) or
compared in a Volcano plot (C). A standard t-test was used to evaluate differences in protein abundance between samples. D) Streptavidin pulldowns of proteins biotinylated by WT and W1145R Flag-BirA*-TopBP1 were probed for the indicated proteins by immunoblotting. When indicated (+), expression of Flag-BirA*-TopBP1 was induced with 1µg/ml doxycycline.

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

**Figure 6. Regulation of TopBP1 condensation.** A) Impact of ATR inhibitors on TopBP1 condensation. Violin plot represents the number of light-induced TopBP1 condensates in cells pretreated with 10µM ATR inhibitors ETP-46464 or VE-822, as indicated. Cells used for optogenetic experiments were probed for the indicated proteins by immunoblotting. See Figure S7A for representative images. B) Alignment of ATR Activation Domain (AAD) sequences from Human,
Mouse and Xenopus. C) Violin plot represents the number of light-induced condensates in cells expressing the indicated mutant proteins. Cells were probed for the indicated proteins by immunoblotting. See Figure S7C for representative images. D) Streptavidin pulldowns of proteins biotinylated by WT or mutants opto-TopBP1 proteins were probed for the indicated proteins by immunoblotting. A-C-D) Number of biological replicates = 3.

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

STAR Methods Text

RESOURCE AVAILABILITY

Lead Contact

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

Materials Availability

Plasmids generated in this study are available upon request.

Data and Code Availability

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

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human Cell lines

Flp-In™T-REx™293 and HEK293 cell lines were grown under standard conditions (37°C, 5% CO₂) in Dulbecco’s modified Eagle’s medium (Merck-Sigma-Aldrich, D5796). For Flp-In™T-REx™293 the medium was supplemented with 10% fetal bovine serum (FBS), 100µg/ml Zeocin
and 15µg/ml Blasticidin. *Flp-In*™-*REx*™293 transfected cells were selected and maintained with 15µg/mL Blasticidin and 150µg/mL Hygromycin.

U-2-OS Tet-On cell lines expressing eGFP-TopBP1 WT and RPE1 cell lines were grown under standard conditions (37°C, 5% CO₂) in modified McCoy’s 5a medium (Merck-Sigma-Aldrich, M9309) supplemented with 10% fetal bovine serum (FBS), 100µg/ml of Hygromycin and 200µg/ml of G418 as selective antibiotics (Sokka et al., 2015). Expression of eGFP-TopBP1 WT was induced with 1µg/ml of doxycycline for 24 hours.

**Sf9 insect cells**

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

**METHOD DETAILS**

**Plasmid constructs**

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

For pCDNA5_FRT-TO_mCherry-Cry2 (opto-module#), the Flag-BirA* fragment was deleted from the pCDNA5_FRT-TO_FlagBirA* using *KpnI/AscI* enzymes and replaced with the mCherry-Cry2 fragment amplified by PCR from the plasmid pH-mCherry-Cry2 (a kind gift from...
Brangwynne’s lab) with primers 4 and 5. In the second step, TopBP1 amino acids 884 to 1522 fragment (BRCT6-AAD-BRCT7-8 WT or W1145R) was amplified with primers 6 and 7, digested with KpnI/NheI and inserted into pCDNA5_FRT-TO_mCherry-Cry2 to produce opto-TopBP1<sup>b6</sup>WT and opto-TopBP1<sup>b6-8</sup>W1145R. For pCDNA5_FRT-TO_TurboID-mCherry-Cry2 construction (opto-module), the TurboID fragment was amplified by PCR from the 3xHA-TurboID-NLS_pCDNA3 plasmid (Addgene #107171) with primers 8 and 9, digested with AflII/KpnI enzymes and inserted into the opto-module# to produce the opto-module. In the second step, TopBP1 WT and W1145R were amplified by PCR with primers 10 and 11, digested with PmlI/KpnI enzymes and inserted into the opto-module construct. Mutations in the AAD of TopBP1 were generated using the “QuickChange Multi Site-directed mutagenesis kit” (Agilent technologies, C200515): W1145R with primer 3, F1071A with primer 12, S1138A with primer 13, S1138D with primer 14. pCDNA5_FRT-TO_TurboID-AAD-NLS<sup>TopBP1</sup>-mCherry-Cry2 was generated by PCR amplification of TopBP1 AAD (primers 15-16) and TopBP1 NLS (primers 17-18). Fragments were inserted in 2 steps into the opto-module digested with AflII/KpnI (TopBP1 AAD) and KpnI/NheI (TopBP1 NLS) enzymes respectively. pCDNA5_FRT-TO_TurboID-ETAA1-mCherry-Cry2 was generated by PCR amplification of ETAA1 with primers 19 and 20 on pCDNA5_FRT-TO_<GFP>_ETAA1 (a kind gift from Mailand’s lab) and insertion of the fragments into the opto-module digested with KpnI/NheI enzymes. pCDNA5_FRT-TO_TurboID-RAD9A-mCherry-Cry2 was generated by PCR amplification of RAD9 with primers 21 and 22 on pDONR223-RAD9A (obtained through MGC Montpellier Genetic Collections) and fragment was cloned into the KpnI-digested opto-module following the In-Fusion HD Cloning Kit protocol. The plasmid with the chimeric constructs of TopBP1 carrying the AAD of ETAA1 (pCDNA5_FRT-TO_TurboID-TopBP1<sup>ETAA1-AAD</sup>-mCherry-Cry2) was generated using the NEBuilder HiFi DNA
assembly Master Mix (New England Biolabs, E2621L). This kit was used to assemble multiple DNA fragments with 30 bp-overlap and replace 1530 bp inside TopBP1. DNA fragments were produced by PCR: oligos 23-24 were used to amplify TopBP1 sequence before the AAD (PCR on any plasmid containing full-length TopBP1), oligos 25-26 were specific for the AAD of ETAA1 (PCR on pCDNA5_FRT-TO_GFP-ETAA1) and oligos 27-28 were specific for TopBP1 portion after the AAD (PCR on any plasmid containing full-length TopBP1). To obtain the chimeric constructs, PCR products were assembled according to the manufacturer’s instruction and ligated into pCDNA5_FRT-TO_TurboID-TopBP1-mCherry-Cry2 digested with EcoNI/SbfI.

pFastBac1 plasmids containing WT and W1145R BRCT 6 to 8 fragments of TopBP1 were synthesized by GeneScript after codon optimization for expression in insect cells (sequence available upon request), and sub-cloned into the 6His-MBP_3C_MCS_TEV_mRFP and 6His-MBP_3C_MCS_TEV_mGFP cassette using the restriction sites EcoRI/KpnI.

Western Blotting

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

Affinity capture of biotinylated proteins: BioID
Flp-In\textsuperscript{TM}T-REx\textsuperscript{TM}293 cell lines stably transfected with Flag-BirA\textsuperscript{*}-TopBP1 WT or W1145R grown to 75% confluence were incubated with 1\(\mu\)g/ml of doxycycline (Clontech, 631311) for 16h and with 50\(\mu\)M biotin for 3 or 16 hours. Cells were washed with PBS and lysed with lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% NP-40, 0.2% SDS, 0.5% Sodium deoxycholate) supplemented with 1X complete protease inhibitor (Roche) and 250U benzonase (Sigma, CE1014). Lysed cells were incubated on a rotating wheel for 1h at 4°C prior sonication on ice (40% amplitude, 3 cycles 10sec sonication- 2sec resting). After 30min centrifugation (7750 rcf) at 4°C, the cleared supernatant was transferred to a new tube and total protein concentration was determined by Bradford protein assay (BioRad, C500-0205). For each condition, 300\(\mu\)g of proteins were incubated with 30\(\mu\)l of Streptavidin-agarose beads (Sigma, CS1638) on a rotating wheel at 4°C for 3hr. After 1min centrifugation (400 rcf), beads were washed, successively, with 1ml of lysis buffer, 1ml wash buffer 1 (2% SDS in H\textsubscript{2}O), 1ml wash buffer 2 (0.2% sodium deoxycholate, 1% Triton X-100, 500mM NaCl, 1mM EDTA, and 50mM Hepes pH 7.5), 1ml wash buffer 3 (250mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM EDTA, 500mM NaCl and 10mM Tris pH 8) and 1ml wash buffer 4 (50mM Tris pH 7.5 and 50mM NaCl). Bound proteins were eluted from the magnetic beads using 80\(\mu\)l of 2X Laemmli Sample buffer and incubated at 95°C for 10min. 10% of the sample was used for Western blot analysis. For the Flp-In\textsuperscript{TM}T-REx\textsuperscript{TM}293 cell lines stably transfected with the doxycycline-inducible TurboID-TopBP1WT-mCherry-Cry2 or the mutated forms of TopBP1, cells were simultaneously incubated with 500\(\mu\)M of biotin and exposed to blue light for 10min of light-dark cycles (4s light followed by 30s dark). Biotin proximity labelling of light-induced TopBP1 partners were pulled-down using streptavidin-coated beads as described before and probed by immunoblotting to detect proteins that are associated with TopBP1 clusters, in absence of DNA damage.
Sample digestion was essentially performed as described (Shevchenko et al., 2006). Briefly, proteins were loaded on a SDS-PAGE (BioRad, 456-1034) and, after short migration, a single band was excised. Proteins in the excised band were digested with Trypsin (Promega, V5280). The resulting peptides were analyzed online by nano-flow HPLC-nanoelectrospray ionization using a Qexactive HFX mass spectrometer (Thermo Fisher Scientific) coupled to a nano-LC system (Thermo Fisher Scientific, U3000-RSLC). Desalting and preconcentration of samples were performed online on a Pepmap® precolumn (0.3 x 10mm; Fisher Scientific, 164568). A gradient consisting of 0% to 40% B in A (A: 0.1% formic acid [Fisher Scientific, A117], 6% acetonitrile [Fisher Scientific, A955], in H₂O [Fisher Scientific, W6], and B: 0.1% formic acid in 80% acetonitrile) for 120min at 300nl/min was used to elute peptides from the capillary reverse-phase column (0.075 x 250mm, Pepmap®, Fisher Scientific, 164941). Data were acquired using the Xcalibur software (version 4.0). A cycle of one full-scan mass spectrum (375–1,500m/z) at a resolution of 60000 (at 200m/z) followed by 12 data-dependent MS/MS spectra (at a resolution of 30000, isolation window 1.2m/z) was repeated continuously throughout the nanoLC separation. Raw data analysis was performed using the MaxQuant software (version 1.5.5.1) with standard settings. Used database consist of Human entries from Uniprot (reference proteome UniProt 2018_09) and 250 contaminants (MaxQuant contaminant database). Graphical representation and statistical analysis were performed using Perseus (version 1.6.1.1). A standard t-test was used to evaluate protein abundance difference between samples.

**TopBP1 expression and purification**
Plasmids for protein expression in insect cells using baculoviruses are listed in Key Resources Table of Star Methods. For the production of bacmids, 50ng of pFastbac plasmids were transformed into MultiBac DH10 cells (Invitrogen, 10361-012) and positive clones were selected on LB Ampicillin (100μg/ml) plates supplemented with 40μg/ml IPTG, 100μg/ml XGal and 7μg/ml Gentamycin. Blue colonies were screened for the presence of inserts by colony PCR using pUC/M13 Forward and Reverse oligos (Bac-to-Bac® Baculoviruses Expression System_Invitrogen user guide). To generate baculoviruses, 12x10^6 Sf9 cells (1ml) were transfected with 5μg of purified bacmid using 15μl of Cellfectin™ (Invitrogen, P/N 58760). After 5h incubation, 9ml of medium were added to Sf9 cells and cultures were incubated for 2.5 days. The supernatant (P1) was collected by centrifugation (400 rcf 10min) and a 1/100 dilution was used to infect 4x10^6 cells/ml Sf9 cells. Cells were incubated for 2 days and the supernatant (P2) was collected by centrifugation. Expression of fluorescent proteins was verified by Western Blotting and microscopy. For protein expression, a 1/10 dilution of freshly prepared P2 was added to 2x10^6 cell/ml Sf9 culture and incubated for 48 to 72 hours.

Infected cells were collected and lysed mechanically using a HTU-DIGI-French-Press (10000 PSI) in 15X packed cell weight hypertonic lysis buffer (50mM Na₂H/NaH₂PO₄ pH 8.0, 500mM NaCl, 1% glycerol, 0.1% CHAPS) supplemented with protease inhibitors. Lysate was clarified by centrifugation (7750 rcf, 40min, 4°C), filtered and loaded on a 5ml HisTrap HP column (GE Healthcare, 71-5027-68 AF) equilibrated with 5CV (Column Volumes) of buffer A (lysis buffer + 0.1 mM PMSF, 5mM imidazole). The column was washed with 5CV of buffer A and TopBP1^b6-8 was eluted stepwise using 5CV of buffer A + 30mM, 50mM, 75mM, 125mM and 500mM imidazole. Peak TopBP1^b6-8 fractions (eluted with 50mM and 75mM imidazole) were desalted (HiTrap™ or HiPrep 26/10, GE Healthcare) in physiological buffer (10mM Na₂H/NaH₂PO₄ pH
7.6, 150mM KOAc, 0.1mM MgOAc, 0.5mM DTT, 2.5% glycerol), snap frozen and stored at -
80°C. Protein concentration was estimated by stain-free gel quantification using Image Lab
Software.

**Nuclear extract preparation**

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

**TopBP1 phase separation assay**

Phase separation of purified TopBP1\textsubscript{b6-8} was performed in physiological buffer C (10mM Na\textsubscript{2}H/NaH\textsubscript{2}PO\textsubscript{4} pH 7.6, 150mM KOAc, 0.1mM MgOAc, 0.5mM DTT, 2.5% Glycerol). Purified TopBP1\textsubscript{b6-8} WT and W1145R were digested with PreScission 3C enzyme (GenScript, Z03092-500) for 3h at 16°C to remove the 6-His and MBP tag before phase separation in reaction mixtures in buffer C containing 10μM of WT or W1145R TopBP1\textsubscript{b6-8}-GFP and 2% of PEG4000 (Merck-Sigma-Aldrich, 95904). Reaction mixtures were mixed by gently tapping the Eppendorf.

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

TopBP1 phase separation in human protein extracts was performed in reaction mixtures containing 0.2μg/μl of human nuclear extract, 2μM of WT or W1145R TopBP1\textsubscript{b6-8}-GFP in ATR activation
buffer (10mM Hepes-KOH pH 7.6, 50mM KCl, 0.1mM MgCl₂, 1mM PMSF, 0.5mM DTT, 1mM ATP, 10μg/ml creatine kinase, 5mM phosphocreatine). Reaction mixtures were incubated for 10min at 4°C or 37°C, as indicated. 5μl of reaction mixtures were used for analyses by immunofluorescence microscopy. Fluorescence microscopy analyses were performed using PEG silanized glass slides prepared as described (Alberti et al., 2018), and coverslips were sealed with nail polish. DNA was stained with Hoechst 33258. Images were captured on an inverted microscope using a 63x objective (NA 1.4 oil).

**Immunofluorescence staining**

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

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

For FRAP experiments, *U-2-OS Tet-On* cells were seeded into µ-Dish³⁵ mm, high (Ibidi, 81156) and incubated 24h in the presence of 1μg/ml doxycycline to induce expression of eGFP-TopBP1. Imaging was realized using a 63x objective (NA 1.4). eGFP-TopBP1 bodies were photo-bleached
and the GFP signal intensity of 23 spots was measured before and during 5min following photo-bleaching with an imaging frequency of 60 images/min.

**Opto-TopBP1 activation**

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

**Live cell microscopy**

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

For Figure 2F, Flp-In\textsuperscript{TM}-Rex\textsuperscript{TM}293 cells expressing opto-TopBP1 were exposed to blue light (488nm) to induce TopBP1 foci, as described before. In Figure 2G, endogenous TopBP1 foci were analysed in RPE1 cells after HU-induced replication stress (2h 5mM), untreated cells were used as control. For STED microscopy, immunolabelling of mCherry was realized using an anti-mCherry antibody followed by a secondary antibody coupled to Atto-647N fluorochrome. Confocal and STED imaging was performed using a quad scanning STED microscope (Expert Line, Abberior Instruments, Germany) equipped with a PlanSuperApo 100x/1.40 oil immersion objective (Olympus, Japan). Atto-647N was excited at 640nm with a dwell time of 10\(\mu\)s and STED was performed at 775nm. Images were collected in line accumulation mode (5 lines accumulation). Fluorescence was detected using avalanche photo diodes and spectral detection (650-750nm). The pinhole was set to 1.0 Airy units and a pixel size of 10nm was used for all acquisitions. A gating of 8ns was applied. Sphericity of opto-TopBP1 condensates was assessed using IMARIS software (Bitplane). For dual color STED imaging, Atto-647N and Abberior STAR Orange were used and respectively imaged at 640 and 561nm excitation. Detection was set to 650-750nm for Atto-647N and 570-630nm for Abberior STAR Orange. Other acquisition parameters were the same described as above.

**DNA fibers**

Neo-synthesized DNA was sequentially pulse labelled with two halogenated thymidine analogs, 5-Iodo-2’-deoxyuridine (IdU at 25\(\mu\)M – Sigma # I7125) and 5-Chloro-2’-deoxyuridine (CldU at 50\(\mu\)M – Sigma # C6891), for 20min. After IdU incorporation, cells were washed two times before
CldU addition. CldU incorporation (20min total) was conducted in the presence of 3min of light-dark cycles (4s light followed by 10s dark) each 7min, to assure opto-TopBP1 condensates formation and persistence. Cells were trypsinized and washed with ice cold PBS. 2000 cells were drop on top of a microscope slide and let dry at least 5min before lyse with 7µL of spreading buffer (200mM Tris.HCl pH7.5, 50mM EDTA, 0.5% SDS) during 3min. DNA was spread by tilting the slide and letting the drop running down slowly. Once air-dry, DNA spreads were fixed in methanol/acetic acid 3:1 for 10min, denatured with 2.5M HCl during 1h and blocked in PBS/1% BSA/0.1% Tween. DNA spreads were immunostained with mouse anti-BrdU, rat anti-BrdU and mouse anti-ssDNA antibodies to detect IdU, CldU and intact DNA fibers respectively. Corresponding secondary antibodies conjugated to Alexa Fluor dyes were used in a second step. Images were captured using a 40x objective (NA 1.4 oil). The acquired DNA fiber images were analyzed by using FIJI Software and statistical analysis of at least 150 IdU and CldU tracks on intact fibers was performed with GraphPad Prism 8. Mean values were indicated by red lines. One-way ANOVA analysis was applied to compare means of samples in a group.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

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

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

All microscopy images were analyzed by OME Remote Objects (OMERO) software. Sphericity of opto-TopBP1 condensates was assessed using IMARIS software (Bitplane). STED microscopy and DNA fiber images were analyzed using FIJI software.
A Cell Profiler (version 2.2.0) pipeline was used to quantify nuclear mCherry foci. All statistical analysis were performed with GraphPad Prism 8. For DNA fibers One-way ANOVA analysis was applied to compare means of samples in a group. To compare mCherry spot/cell distributions between samples we used a non-parametric t-test (Mann-Whitney).

**KEY RESOURCES TABLE**

**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.

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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) ETTAA1 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<sup>b6-8</sup> 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<sup>b6-8</sup>-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: 10μm. 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: 10μm. 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 (10μM), 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 10μM UCN-01. B) Immunoblotting analysis of the indicated biotinylated proteins isolated with streptavidin beads in WT and W1145R opto-TopBP1 expressing cells 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: 10μm. 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 10μM 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.

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Park, M.S., Knauf, J.A., Pendergrass, S.H., Coulon, C.H., Strniste, G.F., Marrone, B.L., and MacInnes, M.A.
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mitosis to reduce transmission of DNA damage to G1 daughter cells. J Cell Biol 210, 565-582.

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# KEY RESOURCES TABLE

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**Experimental Models: Cell Lines**

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**Oligonucleotides**

See Table S1 for the full list of oligonucleotides used in this study

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**Deposited Data**

Raw and analyzed data: This paper

http://dx.doi.org/10.17632/6nf7b7ff7b.1
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Software and Algorithms
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Figure 1
**Figure 2**

A. Diagram showing the ratio of areas: \( \frac{\text{area}_S}{\text{area}_P} = 0.940223 \)

B. Series of images showing temporal changes in mCherry and TopBP1 spots under different treatments.

C. Graph showing the mean mCherry spots/cell over time post light activation.

D. Graph showing the distribution of mCherry and TopBP1 spots/cell under light ON and 5mM HU conditions.

F. Confocal and STED images of anti-mCherry and anti-TopBP1 signals.

G. Confocal and STED images of untreated and 2h 5mM HU treated samples.
Figure 3

A

TopBP1<sup>wt</sup> - 8

WT

2% PEG

- PEG

B

2% PEG

- PEG

C

Preformed TopBP1<sup>wt</sup> - GFP

digested ΦX174 RFII

300 nM

Preformed TopBP1<sup>wt</sup> - GFP

+ ΦX174 RFII

Hoechst

30 130 110 100 80 50 20

D

Preformed TopBP1<sup>wt</sup> - GFP

300 nM

Preformed TopBP1<sup>wt</sup> - GFP

+ ΦX174 RFII

GFP

E

TopBP1<sup>wt</sup> 2.9 kb plasmid DNA

F

Nuclear Extract

TopBP1<sup>wt</sup>  WT

TopBP1<sup>wt</sup>  W1145R

-  

TopBP1<sup>wt</sup>  WT (10 µM)
Figure 4

A

Proximal biotinylation

B

TopBP1 WT

Relative abundance (log2) Proteins ranked by iBAQ

TopBP1 W1145R

C

Relative abundance (log2) Proteins ranked by iBAQ

D

Input IP Streptavidin

TopBP1 WT W1145R WT W1145R

Dox - + - + - +

TopBP1 ATR

BRCA1

MRE11

FANCJ

NOL11
**Figure 5**

**A**

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- **opto-TopBP1**
- **endogenous TopBP1**
- **pChk1 (Ser345)**
- **Chk1**

**B**

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- **pTopBP1 (ser1138)**
- **TopBP1**
- **pChk1 (Ser345)**
- **Chk1**

**C**

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- **Dox**
- **Light**

- **pTopBP1 (Ser1138)**
- **TopBP1**
- **pATR (Thr1989)**
- **ATR**

**D**

- **TopBP1-mCherry**
- **pATR (Thr1989)**
- **Merge**

- **E**

- 488 nm light
- CldU/IdU

- **ODU/IdU**

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Figure 6
TopBP1 nucleation
DNA replication impediments

ATR/Chk1 signaling

ATR
ATRIP
RPA
MRN
9-1-1

TopBP1 condensation

Signal amplification

Figure 7_model
Suppl Figure 1_related to Figure 1
Suppl Figure 3 related to Figure 3
Suppl Figure 4_related to Figure 4
Suppl Figure 5

A

B

37°C

4°C

- EtBr

pChk1 (Ser345)

Chk1

HEK293 nuclear extract

ATP

4°C

37°C

- EtBr

pChk1 (Ser345)

Chk1

µg/µl Nuclear Extract

- 0.1 0.2 0.3 0.4 0.8 1.5 3

- + ETP - 46464

pChk1 (Ser345)

Chk1

pChk1 (Ser345)

Chk1
Suppl Figure 6-related to Figure 5
Suppl Figure 7_related to Figure 6
**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.

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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 \textit{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: 10μm. 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: 10μm. 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 (10μM), 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
10µM UCN-01. B) Immunoblotting analysis of the indicated biotinylated proteins isolated with streptavidin beads in WT and W1145R opto-TopBP1 expressing cells 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: 10µm. 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 10µM 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.