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PARP1-mediated PARylation of TonEBP prevents R-loop–associated DNA damage

Byeong Jin Ye [1], Hyun Je Kang [1], Whaseon Lee-Kwon, Hyug Moo Kwon *, Soo Youn Choi *

School of Life Sciences, Ulsan National Institute of Science and Technology, Ulsan, 44919, Republic of Korea

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ABSTRACT

Lack of coordination between the DNA replication and transcription machineries can increase the frequency of transcription–replication conflicts, leading ultimately to DNA damage and genomic instability. A major source of these conflicts is the formation of R-loops, which consist of a transcriptionally generated RNA–DNA hybrid and the displaced single-stranded DNA. R-loops play important physiological roles and have been implicated in human diseases. Although these structures have been extensively studied, many aspects of R-loop biology and R-loop–mediated genome instability remain unclear. We found that in cancer cells, tonicity-responsive enhancer-binding protein (TonEBP, also called NFAT5) interacted with PARP1 and localized to R-loops in response to DNA-damaging agent camptothecin (CPT), which is associated with R-loop formation. PARP1-mediated PARylation was required for recruitment of TonEBP to the sites of R-loop–associated DNA damage. Loss of TonEBP increased levels of R-loop accumulation and DNA damage, and promoted cell death in response to CPT. These findings suggest that TonEBP mediates resistance to CPT-induced cell death by preventing R-loop accumulation in cancer cells.

1. Introduction

Maintenance of genome integrity depends on spatiotemporal coordination between the DNA replication and transcription machineries. Lack of such coordination can lead to DNA damage and genomic instability resulting from an elevated frequency of transcription–replication conflicts [1,2]. A major source of these conflicts is the formation of R-loops, which consist of a co-transcriptionally generated RNA–DNA hybrid and the displaced single-stranded DNA [3]. R-loops, which arise naturally during transcription in organisms from bacteria to mammals, play multiple roles in cellular processes, including regulation of gene expression [4], transcription termination [5], and DNA replication [6]. In addition to these physiological functions, R-loops also threaten genome integrity, resulting in deleterious effects on the cell [7–9]. Abnormal accumulation of R-loops and collisions with replication forks can increase the rate of DNA damage and genome instability [2,10]. R-loops have been implicated in many human diseases, including neurological disorders, cancer, and autoimmunity [11,12]. Accordingly, R-loops must be tightly regulated in living cells.

R-loop homeostasis is regulated by the factors and cellular processes that control the formation and resolution of these structures. In humans, proteins involved in chromatin modification and the DNA damage response play important roles in maintaining the proper R-loop balance [13–15], and aberrant expression or function of these factors can contribute to disease [16,17]. Moreover, R-loops can regulate gene expression by recruiting protein factors [13]. However, the molecular mechanisms linking these factors to R-loop biology remain unclear, and we require a clearer understanding of their identities and the mechanisms by which they are recruited.

Tonicity-responsive enhancer-binding protein (TonEBP), also known as nuclear factor of activated T-cells 5 (NFAT5), is a pleiotropic stress protein involved in the response to various types of stress [18]. TonEBP can promote physiological or pathological consequences depending on the context: for example, TonEBP-mediated responses to osmotic stress [19–22] and bacterial infection [23] have protective functions, whereas the responses to autoimmune and metabolic stresses contribute to the pathogenesis of human diseases such as rheumatoid arthritis [24,25], atherosclerosis [26], hepatocellular carcinoma (HCC) [27,28], obesity [29], and diabetes mellitus [29,30]. Although TonEBP has well-established functions in the responses to a range of cellular stresses,

* Corresponding authors.
E-mail addresses: hmkwon@unist.ac.kr (H.M. Kwon), sychoi@unist.ac.kr (S.Y. Choi).
[1] These authors contributed equally to this work.

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its role in the cellular response to DNA damage remains to be elucidated. To systematically explore the cellular functions of TonEBP, we analyzed proteins that interacted with TonEBP using tandem affinity purification-mass spectrometry. The results of that study revealed that TonEBP interacts with DNA repair proteins; in addition, TonEBP is recruited to DNA damage sites and protects cells from genotoxic stress [31]. TonEBP mediates DNA damage tolerance through ubiquitination of proliferating cell nuclear antigen (PCNA), a DNA clamp required for replication and repair. Furthermore, TonEBP is involved in the dynamic association of DNA repair proteins and their recruitment to DNA damage sites. Our interactomic data also revealed that TonEBP associates with proteins that resolve R-loop structures, such as RNA helicases and inhibitors of R-loop accumulation (31, Supplementary Fig. 1), raising the possibility that it plays a role in R-loop biology.

In this study, we explored the role of TonEBP in R-loop–associated DNA damage. We found that in cancer cells, TonEBP interacted with poly (ADP-ribose) polymerase 1 (PARP1), localized to R-loops, and prevented R-loop accumulation and DNA damage in response to an R-loop–associated DNA-damaging agent. Together, these findings suggest that TonEBP mediates resistance to DNA damage by preventing R-loop accumulation in oncogenic cells. These findings provide new insights into the molecular mechanisms underlying DNA damage and repair responses, and reveal novel functions of TonEBP in R-loop biology.

2. Results

2.1. TonEBP interacts with, and is PARylated by, PARP1

Our interactomic data from a previous study revealed that TonEBP interacts with proteins involved in R-loop resolution, including PARP1 and RNA helicases (31, Supplementary Fig. 1). Given the pivotal role of PARP1 in resolving R-loop–associated DNA damage [13], we decided to investigate the interaction between TonEBP and PARP1, and its role in R-loop biology. To this end, we first sought to determine whether TonEBP physically binds to PARP1. Co-immunoprecipitation (co-IP) experiments in HEK293 T cells revealed that endogenous TonEBP and PARP1 proteins interact (Fig. 1A). Next, we defined the molecular basis of the TonEBP–PARP1 interaction using constructs expressing several recombinant variants of TonEBP (Fig. 1B) and PARP1 (Fig. 1C). Constructs containing the Rel-homology domain (RHD), including full-length TonEBP and Yc1 (N terminus of TonEBP), interacted with PARP1, whereas a mutant lacking the RHD (ΔRHD) did not (Fig. 1D), indicating that the RHD of TonEBP is essential for its interaction with PARP1. To determine which structural elements of PARP1 are required for the interaction with TonEBP, we transfected cells with constructs expressing Yc1 and full-length PARP1 or a deletion mutant. Full-length PARP1 and the catalytic domain (CatD) both bound to Yc1, whereas the DNA-binding domain and BRCA1 C-terminal domain did not (Fig. 1E). These data indicate that the RHD of TonEBP and the CatD of PARP1 mediate the interaction between the two proteins.

In response to DNA damage, PARP1 is activated and promotes the formation of poly (ADP-ribose) polymer on its substrates [32]. In light of

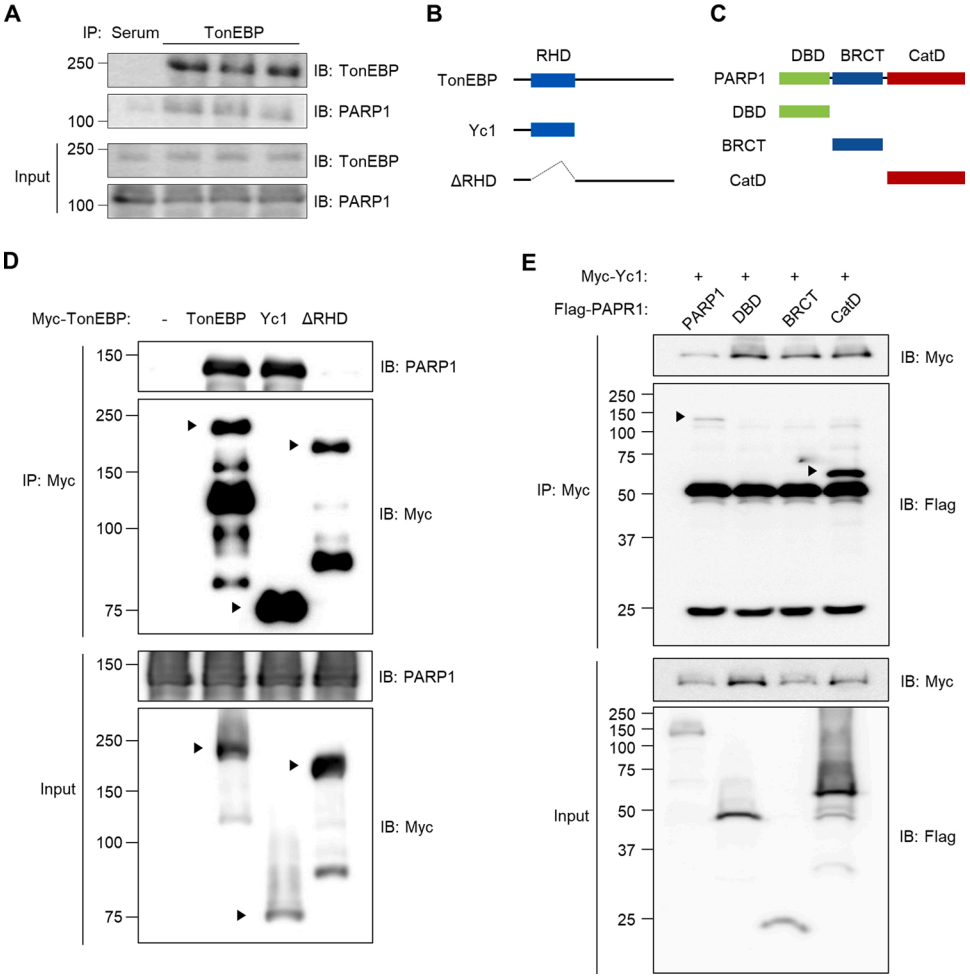


Fig. 1. TonEBP interacts with PARP1 through the Rel-homology domain. (A) HEK293 T cells were immunoprecipitated (IP) with normal serum (Serum) or anti-TonEBP antibody (TonEBP). Cell lysates (input) and precipitated proteins were immunoblotted for TonEBP and PARP1. (B) Domain structures of human TonEBP and deletion constructs Yc1 and ΔRHD. (C) Domain structures of human PARP1 and deletion constructs containing the DNA-binding domain (DBD), BRCA1 C-terminal domain (BRCT), or catalytic domain (CatD). (D) U2OS cells were transfected with a plasmid expressing Myc-tagged TonEBP, Yc1, or ΔRHD for 24 h. Cell lysates were immunoprecipitated with anti-Myc antibody. Cell lysates and precipitated proteins were immunoblotted for PARP1 and Myc. (E) U2OS cells were transfected for 24 h with plasmids expressing Myc-Yc1 in combination with FLAG tagged PARP1, DBD, BRCT, or CatD. Cell lysates were immunoprecipitated with anti-Myc antibody. Cell lysates and precipitated proteins were immunoblotted for FLAG and Myc.

the observation that TonEBP interacts with PARP1, we asked whether TonEBP was poly-ADP-ribosylated (PARylated) by PARP1 in Yc1-overexpressing cells. For these studies, we used the U2OS human osteosarcoma cell line, which has a robust and well-characterized response to DNA damage, and camptothecin (CPT), which promotes R-loop–associated DNA damage. The content of PARylated protein increased in a time-dependent manner in response to CPT (Fig. 2A, bottom panel). Notably, co-IP experiments revealed that CPT induced increased PARylation intensity and up-shifting smears of Yc1 (Fig. 2A, top panel), and this effect was abrogated by the PARP1 inhibitor olaparib (Fig. 2B). The interaction between TonEBP and PARP1 remained even after PARylation of TonEBP (Fig. 2A, top panel). Next, we examined whether PARylation of TonEBP in response to CPT was mediated by the binding. PARylation of TonEBP was dramatically reduced in the PARP1 knockout cells (Fig. 2C), whereas PARP1 overexpression induced a clear increase both in basal and CPT-induced PARylation of TonEBP (Fig. 2D). In addition, the ΔRHD mutant was not PARylated (Fig. 2E). Together, these data demonstrate that TonEBP interacts with PARP1, and that PARP1 mediates the PARylation of TonEBP in response to CPT.

2.2. PARylation of TonEBP mediated by PARP1 triggers recruitment of TonEBP to sites of DNA damage

PARylation of target proteins by PARP1 is one of the earliest steps in the cellular response to DNA lesions, leading to activation of DNA damage response pathways and facilitating DNA damage repair. Indeed, many DNA repair factors are PARylated to access DNA damage sites [32]. Our previous study provided evidence that TonEBP is recruited to DNA damage sites and promotes the DNA damage bypass pathway [31]. Hence, we investigated whether PARP1 is required for recruitment of TonEBP to DNA damage sites. To this end, we performed laser micro-irradiation to induce double-strand breaks in DNA and then examined laser damage-induced recruitment of GFP-tagged Yc1 proteins. Yc1, which contains the RHD of TonEBP, was translocated to the micro-irradiated region of the nucleus, but this translocation was not observed in olaparib- or PARP1-siRNA–treated cells (Fig. 3A and B). Furthermore, we examined DNA damage-induced foci formation of TonEBP in response to CPT and found that both pretreatment with olaparib and knockdown of PARP1 significantly inhibited the CPT-mediated formation of TonEBP foci (Fig. 3C).

CPT-induced DNA Topoisomerase 1 cleavage complex formation is the major source of DNA damage, most likely through replication-induced DNA damage [33,34] and R-loop is one of the sources of DNA

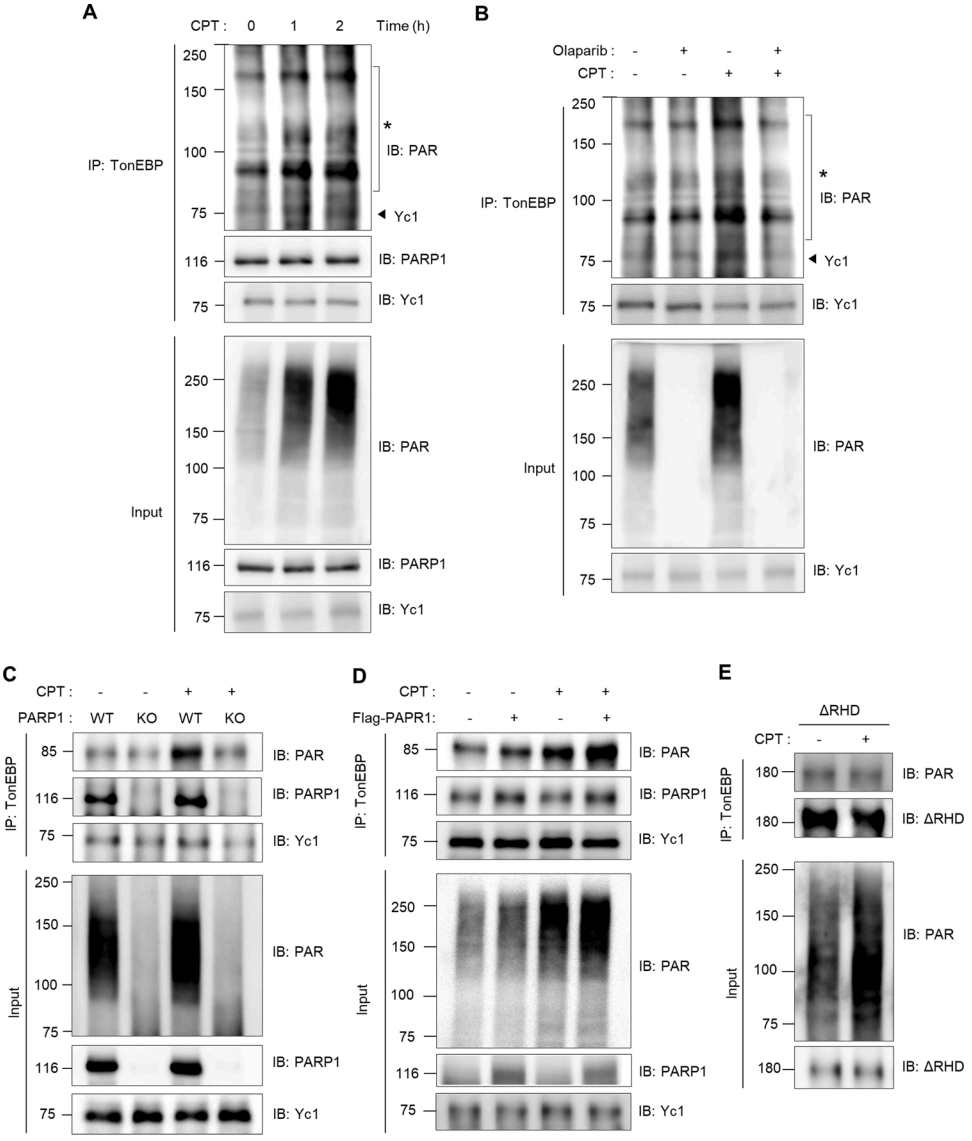


Fig. 2. PARP1 mediates PARylation of TonEBP in response to camptothecin.
(A) U2OS cells transfected with Yc1 were treated with 10 μM camptothecin (CPT) for the indicated times (0–2 h). Proteins were immunoprecipitated (IP) with anti-TonEBP antibody (TonEBP). Precipitates and cell lysates were immunoblotted for PAR (poly (ADP-ribose)), PARP1 and TonEBP. *, PARylated proteins. (B) The cells were pretreated with 1 μM olaparib for 1 h, followed by treatment with 10 μM CPT for 2 h as indicated. Cell lysates were prepared and immunoprecipitated with anti-TonEBP antibody. Precipitates and cell lysates were immunoblotted for PAR and TonEBP. (C) PARP1 wild type (WT) or knockout (KO) HEK-293 T cells transfected with Yc1 were treated with 10 μM CPT for 2 h. Cells were then immunoprecipitated with anti-TonEBP antibody. Precipitates and cell lysates were immunoblotted for PAR, PARP1 and TonEBP. (D) U2OS cells transfected with Yc1 were transfected a second time with a plasmid expressing FLAG-empty (-) or FLAG-PARP1 (+) for 24 h. The transfected cells were treated with 10 μM CPT for 2 h and then immunoprecipitated with anti-TonEBP antibody. Precipitates and cell lysates were immunoblotted for PAR, PARP1 and TonEBP. (E) U2OS cells were transfected with ΔRHD mutant of TonEBP and treated with 10 μM CPT for 2 h. Cells were then immunoprecipitated with anti-TonEBP antibody. Precipitates and cell lysates were immunoblotted for PAR and TonEBP.

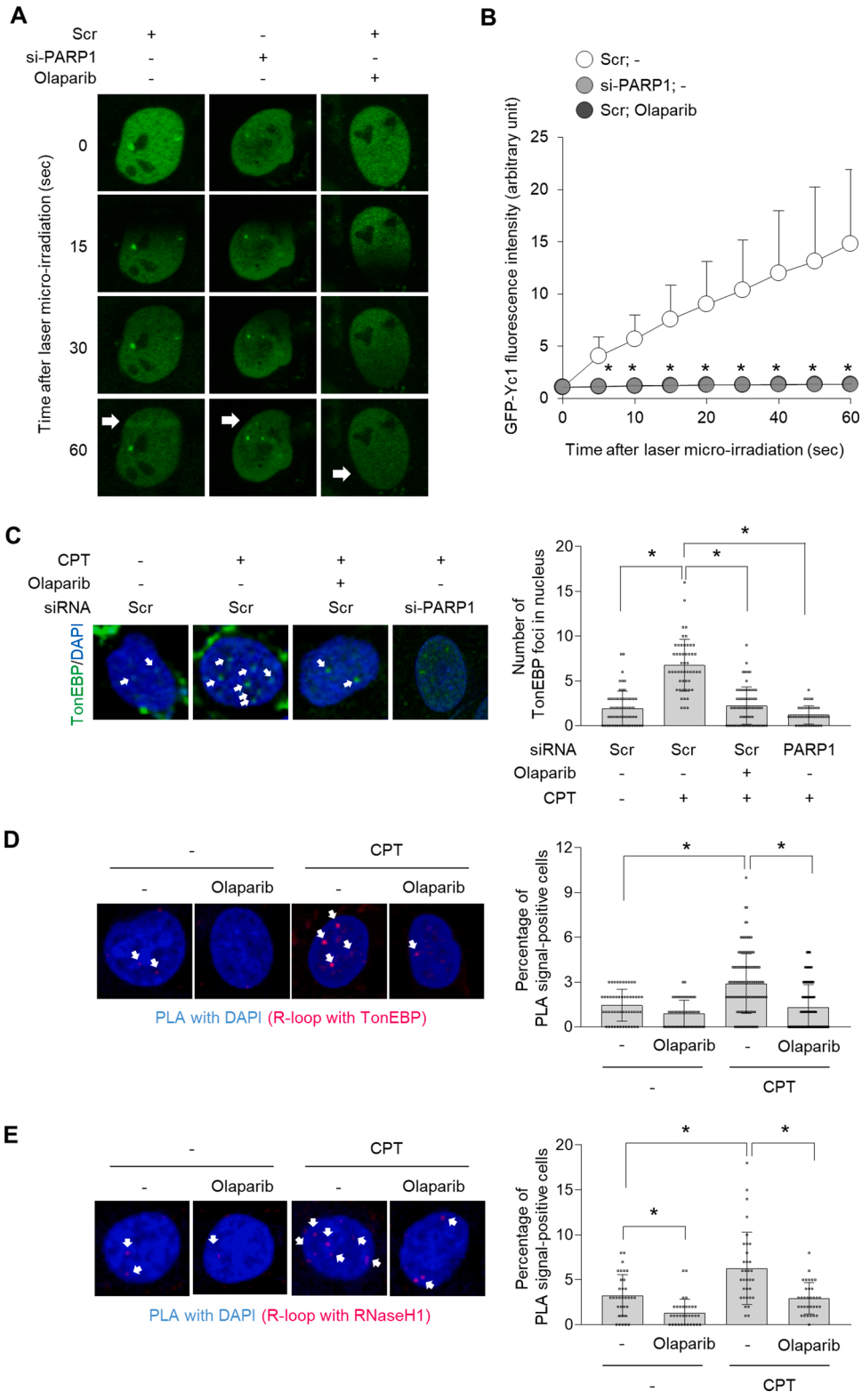


Fig. 3. PARylation of TonEBP mediated by PARP1 is required for recruitment of TonEBP to sites of R-loop-associated DNA damage.
(A and B) U2OS cells transfected with scrambled (Scr) or PARP1-targeted siRNA (si-PARP1) were transfected again with GFP-Yc1 for 24 h. The cells were then subjected to laser micro-irradiation after pretreatment with vehicle (-) or 1 μM olaparib for 30 min. (A) After irradiation, green fluorescence images were acquired at the indicated times (0–60 sec). (B) The fluorescence intensity in the micro-irradiated area at indicated time point was determined from 10 cells. Data (means ± SD) were obtained from three independent experiments ($n = 3$). * $p < 0.01$ compared to Scr; -. (C) Cells were transfected with siRNA and treated with olaparib, as in A and B. Cells were then treated with vehicle (-) or 10 μM CPT, followed by immunostaining for TonEBP. (Left) Representative images. (Right) Numbers of TonEBP foci (means ± SD) were determined from at least 50 cells in each condition. * $p < 0.01$. (D and E) Cells were pretreated with vehicle (-) or 1 μM olaparib followed by treatment with vehicle (-) or 10 μM CPT. The cells were then subjected to PLA between TonEBP and R-loops (D), or RNaseH1 and R-loops (E). (Left) Representative images. (Right) Percentages of PLA signal-positive nuclei were calculated from at least 30 cells. Data (means ± SD) are from three independent experiments ($n = 3$). * $p < 0.01$.

damage following CPT treatment [35–37]. Our recent data show that TonEBP is recruited to R-loops in response to CPT and mediates R-loop resolution via RNaseH1 recruitment [38]. Thus, we asked whether PARylation of TonEBP is required for R-loop recognition. To answer this question, we performed a proximity ligation assay (PLA) with anti-–DNA/RNA hybrid (S9.6) and anti-TonEBP antibodies, enabling us to monitor the interaction between R-loop–enriched foci and TonEBP. We observed PLA signals in the nucleus following CPT treatment but to a lesser extent in cells treated with olaparib (Fig. 3D). These data indicate that PARP1-dependent PARylation of TonEBP was essential for its recruitment to sites of R-loop–associated DNA damage in response to CPT. Furthermore, olaparib significantly inhibited recruitment of RNase H1 to R-loops at both basal and CPT-treated conditions (Fig. 3E), demonstrating that PARylation was required for recruitment of RNase

H1 to sites of R-loops.

2.3. PARP1-mediated PARylation of TonEBP prevents R-loop accumulation

Next, we investigated the potential role of TonEBP and its PARylation in the R-loop response following CPT treatment using cells in which TonEBP had been knocked down with siRNA. Immunocytochemistry experiments with S9.6 antibodies revealed that the global level of R-loops was elevated in TonEBP-depleted cells irrespective of CPT treatment (Fig. 4A and B). The R-loop signal was also elevated upon PARP inhibition with olaparib, as previously reported [13]. However, PARP inhibition did not further increase the R-loop signal in TonEBP-depleted cells, again irrespective of CPT treatment (Fig. 4A and B), suggesting that TonEBP and PARP1 act in the same pathway to suppress R-loops.

Excessive R-loop accumulation triggers DNA damage and genome instability [3,7]. Hence, we asked whether R-loop accumulation in TonEBP-depleted cells increases the rate of DNA damage. To answer this question, we analyzed γH2AX and 53BP1 foci, which are markers of DNA damage. Consistent with the accumulation of R-loops (Fig. 4A), TonEBP depleted cells had significantly increased levels of γH2AX and 53BP1 foci, irrespective of CPT treatment (Fig. 4C-F). Pretreatment with olaparib also significantly increased the intensities of γH2AX and 53BP1 (Fig. 4C–F). By contrast, in TonEBP-depleted cells, olaparib did not further increase formation of γH2AX and 53BP1 foci, irrespective of CPT treatment, suggesting that TonEBP and PARP1 are epistatic for signaling or repair of DNA damage induced by CPT.

These data, together with our data in Figs. 3 and 4, indicate that PARP1-mediated PARylation of TonEBP is required to prevent R-loop accumulation and DNA damage.

2.4. The RHD of TonEBP is required to prevent R-loop–associated DNA damage and cell survival in response to CPT

To further explore our observation that RHD of TonEBP was required

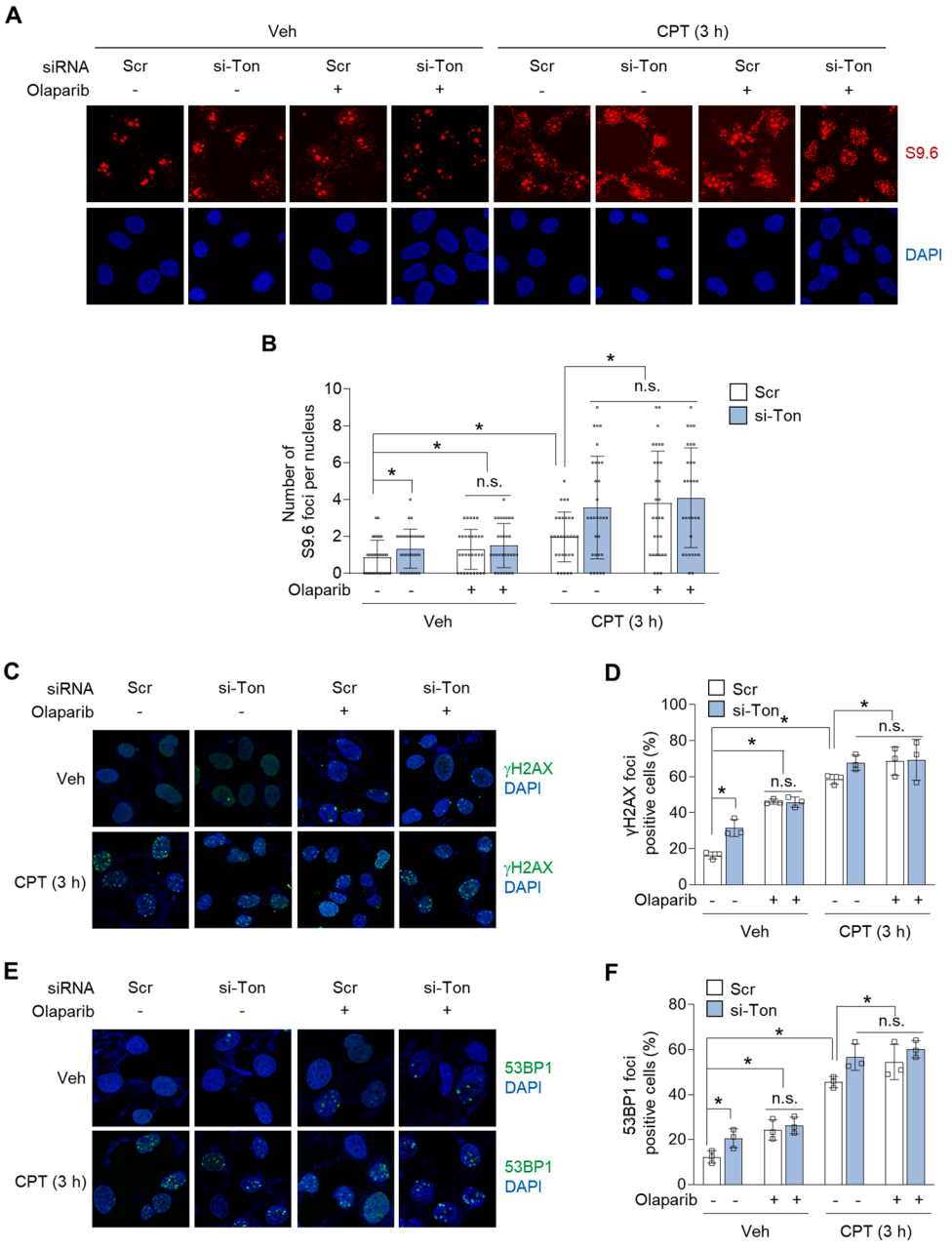
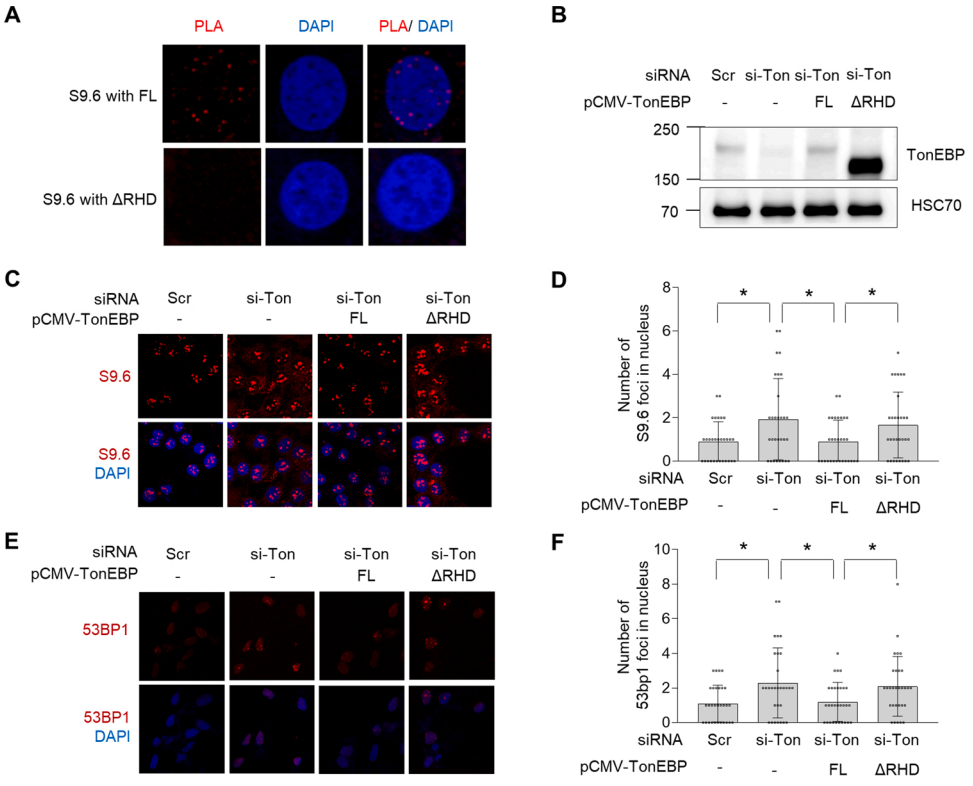


Fig. 4. PARP1-mediated PARylation of TonEBP prevents R-loop–associated DNA damage.
U2OS cells were transfected with scrambled siRNA (-) or TonEBP-targeting siRNA (si-Ton) for 24 h. The cells were then untreated (-) or pretreated (+) with 1 μM olaparib for 1 h, followed by treatment with either 10 μM CPT or vehicle alone (Veh) for 3 h. (A) R-loops and nuclei were visualized by immunostaining with S9.6 antibody and DAPI staining, respectively. (B) S9.6 foci per nucleus were counted from 30 cells (means ± SD). (C–F) Immunostaining was performed for γH2AX and 53BP1. Representative images for γH2AX (C) and 53BP1 (E) were shown. Percentages of γH2AX (D) and 53BP1 (F) foci-positive cells were calculated from at least 30 cells. Data (means ± SD) are from three independent experiments ($n = 3$). * $p < 0.01$, n.s.: not significant.

for PARylation via an interaction with PARP1 (Figs. 1 and 2), we performed rescue experiments to determine whether the RHD of TonEBP is required for R-loop resolution, maintenance of genome integrity, and cell survival. We first examined whether RHD of TonEBP is critical for R-loop binding, which is required for function of TonEBP at R-loops, using PLA in cells overexpressing intact TonEBP or ΔRHD. The PLA signals were observed in cells overexpressing intact TonEBP but not in ΔRHD mutant (Fig. 5A), indicating that the RHD of TonEBP is critical for R-loop binding. More importantly, the re-expression of intact TonEBP (Fig. 5B) rescued the increase in R-loop accumulation caused by TonEBP depletion, whereas re-expression of the ΔRHD mutant did not (Fig. 5C-D). Moreover, the ΔRHD mutant did not revert the increase in abundance of 53BP1 foci in TonEBP-depleted cells (Fig. 5E and F). Consistent with the higher levels of R-loop accumulation and DNA damage, depletion of TonEBP enhanced the dose dependent cell death by treatment with CPT (Fig. 5G and H). Overexpression of intact TonEBP abolished this



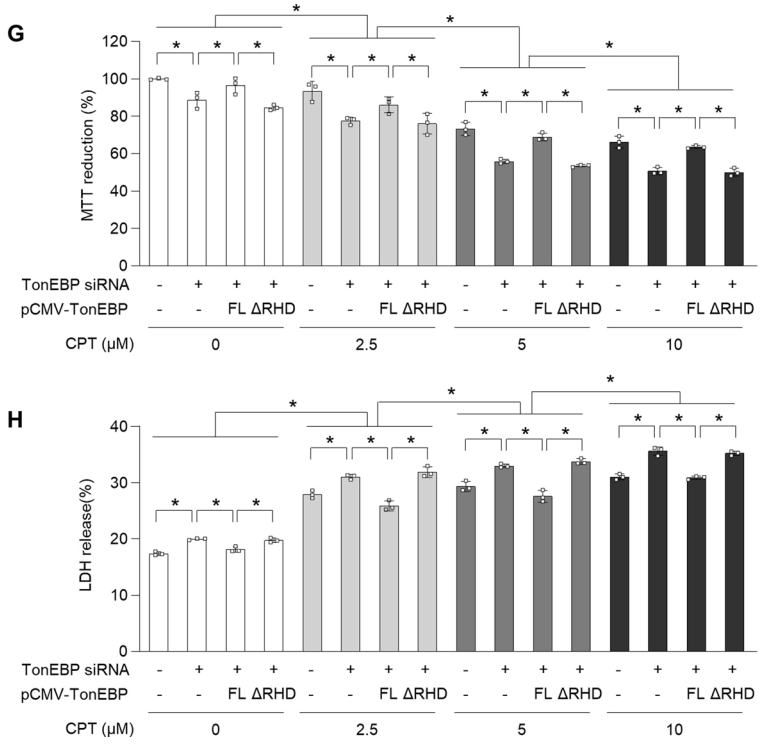


Fig. 5. RHD of TonEBP is required for prevention of R-loop–associated DNA damage and cell survival in response to CPT.
(A) U2OS cells were transfected with plasmid expressing full-length TonEBP (FL) or ΔRHD for 24 h. The cells were then subjected to PLA between TonEBP and R-loops. Representative images are shown. (B-H) U2OS cells were transfected with scrambled siRNA (Scr or -) or TonEBP-targeting siRNA (si-Ton) for 24 h. The siRNA-transfected U2OS cells were then transfected a second time with plasmid expressing full-length TonEBP (FL) or ΔRHD for 24 h. (B) Cell lysates were immunoblotted for TonEBP and HSC70. Representative images are shown. (C) R-loop and nuclei were visualized by immunostaining with S9.6 antibody and DAPI staining, respectively. (D) The number of S9.6 foci per nucleus was determined from at least 30 cells. (E) Immunostaining was performed for 53BP1. Representative images are shown. (F) Percent of 53BP1 foci-positive cells was calculated from at least 30 cells. (G and H) The transfected cells were treated with vehicle (-) or 2.5, 5, or 10 μM of CPT. Cell viability was assessed by MTT reduction (F) and LDH release (G) after 24 h. Data (means ± SD) were from three independent experiments ($n = 3$). * $p <$ 0.05.

increase, whereas overexpression of the ΔRHD mutant did not (Fig. 5G and H). Taken together, these data suggest that the RHD of TonEBP prevents R-loop accumulation and DNA damage, and promotes cell survival, following CPT treatment.

2.5. TonEBP prevents R-loop–mediated DNA damage in HepG2 human hepatoma cells

The findings described above indicated that PARP1-mediated PARylation of TonEBP promotes DNA repair and cell survival in human osteosarcoma U2OS cells. Given that TonEBP [27,28] and PARP1 [39] are key drivers of HCC tumorigenesis, and that their inhibition suppresses HCC progression, we investigated the potential role of the PARP1–TonEBP axis in R-loop–associated DNA damage in HepG2 human hepatoma cells. siRNA-mediated depletion of TonEBP in HepG2 cells increased R-loop accumulation in both the presence and absence of CPT (Fig. 6A and B), and led to a defect in the incorporation of the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) (Fig. 6C and D), indicating that TonEBP depletion promotes R-loop accumulation and DNA replication defects. Interestingly, TonEBP depletion exerted synergistic effects on olaparib-induced cell death in CPT-untreated cells (Fig. 6E and F). TonEBP depletion and olaparib increased sensitivity to cell death in response to CPT treatment (Fig. 6E and F). However, olaparib treatment of TonEBP-depleted cells did not further increase cell death in response to CPT (Fig. 6E and F), as in the case of U2OS cells. Taken together, these data indicate that the PARP1–TonEBP axis suppresses R-loop–associated DNA damage and cell death in cancer cells.

3. Discussion

TonEBP is a well-established transcriptional regulator that serves as both an activator and a repressor [18]. TonEBP regulates transcription of a large number of genes, either by direct binding to DNA via a specific motif or indirect binding via an interacting protein. Beyond its role as a transcriptional regulator, TonEBP is recruited to sites of DNA damage and promotes error-prone DNA damage bypass (controlled by PCNA) in response to genotoxic stress [31]. Given that one of the hallmarks of cancer is widespread genomic instability, this implies that TonEBP is

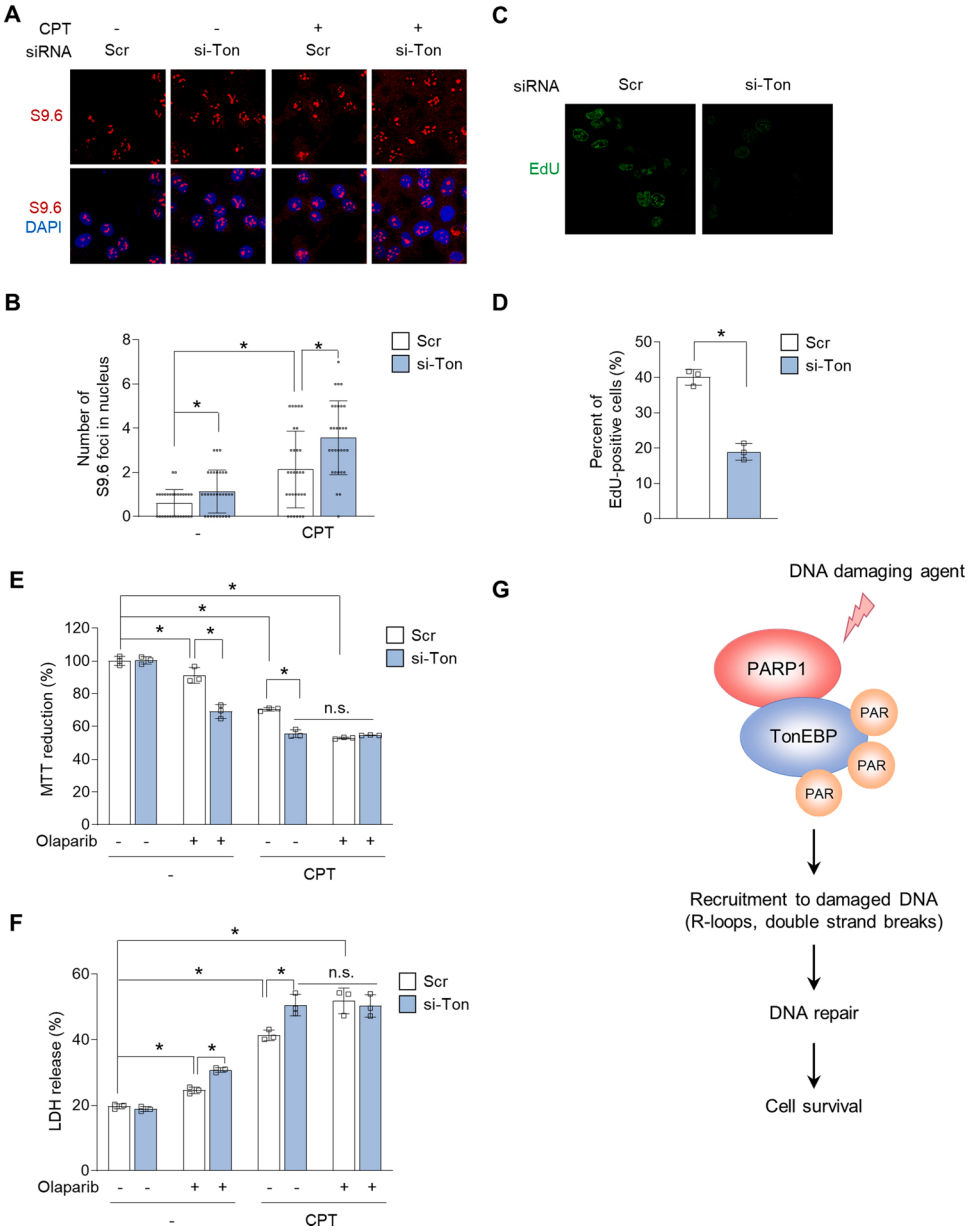


Fig. 6. TonEBP prevents R-loop–mediated DNA damage in HepG2 human hepatoma cells.
(A and B) HepG2 cells were transfected with scrambled siRNA (Scr) or TonEBP-targeting siRNA (si-Ton) for 24 h. The cells were then treated with vehicle (-) or 10 μM CPT. R-loop and nuclei were visualized by immunostaining with S9.6 antibody and DAPI staining, respectively (A). The number of S9.6 foci per nucleus was determined from at least 30 cells (B). (C and D) HepG2 cells were transfected with siRNA as above, and then subjected to Click-iT EdU assay. (C) Representative images of cells in each condition. (D) Percent of EdU positive cells were measured. Mean ± SD, n = 3. *p < 0.01. (E and F) HepG2 cells were transfected with siRNA and then treated with vehicle (-) or 1 μM olaparib for 1 h in the absence (-) or presence 10 μM CPT as indicated. Cell viability was assessed by MTT assay (E) and LDH release (F) after 24 h. Data (means ± SD) are from three independent experiments ($n = 3$). * $p <$ 0.01. n.s.: not significant. (G) Model: PARP1-mediated PARylation of TonEBP prevents R-loop–associated DNA damage.

involved in human cancer. Indeed, TonEBP facilitates DNA repair via recruitment of a prominent DNA repair protein, the ERCC1/XPF complex, and consequently promotes self-renewal and chemoresistance of liver stem cells in response to cisplatin, a DNA-damaging agent that is widely used as an anticancer drug [28]. However, it remains to be determined how TonEBP is recruited to sites of DNA damage, and which factors influence its functions in DNA damage and repair response.

Our primary finding of this study was that TonEBP interacts with PARP1, and that PARP1-mediated PARylation is necessary for recruitment of TonEBP to sites of DNA damage. PARP1 associates with TonEBP in cells exposed to high NaCl and suppresses its transcriptional activity [40]. One intriguing possibility is that in addition to promoting recruitment of TonEBP to sites of DNA damage, PARylation of TonEBP is required for regulation of its transcriptional activity. Consistent with this idea, it is already known that transcriptional activity of TonEBP is regulated by post-translational modification, including phosphorylation [11] and sumoylation [41]. Future studies should investigate the impact of the TonEBP–PARP1 interaction on other cellular responses, including transcriptional regulation.

PARP1 is an important DNA repair molecule [32], and the successful application of PARP1 inhibitors in treating cancer is a classic example of the rational development of DNA repair-targeted cancer therapy [42]. Specifically, olaparib suppresses HCC growth in mouse and HCC patient-derived xenograft models [39]. TonEBP also acts as an oncoprotein via multiple mechanisms in HCC [27,28]. TonEBP plays a critical role in tumorigenesis and tumor progression by activating the NF-κB enhanceosome and upregulating COX2 expression [27]. It also promotes stemness of liver cancer and cisplatin resistance via recruitment of the ERCC1/XPF dimer [28]. Interestingly, PARP and the ERCC1/XPF complex participate in distinct DNA damage repair pathways [43]. This study demonstrated that PARP is involved in a common repair pathway with tyrosyl-DNA phosphodiesterase 1, and that the ERCC1/XPF complex is involved in DNA repair by inducing the γH2AX response. Based on these findings, we speculate that TonEBP may be involved in tumorigenesis and tumor progression via both DNA repair pathways involving PARP and XPF-ERCC1 in liver cancer, although additional studies focusing on this issue are still required.

R-loops play important physiological roles and have been implicated in pathogenesis [13–17]. Although the factors and cellular processes associated with R-loops have been extensively studied, many aspects of R-loop biology and R-loop–mediated genome instability remain unclear. Given the multifaceted roles of TonEBP and PARP1 in cancers, we focused on the PARP1-mediated function of TonEBP in R-loop–associated DNA damage in cancer cells, and identified TonEBP as a novel regulator of R-loop–associated DNA damage. We showed that TonEBP is recruited to R-loop–forming loci in response to the R-loop–associated DNA-damaging agent CPT. The loss of TonEBP increased the levels of R-loop accumulation, DNA damage, and DNA replication defect, thereby increasing the rate of CPT-mediated cell death, demonstrating that TonEBP plays an important general role in resolving R-loop–associated DNA damage in cancer cells. This finding is supported by our recent study showing that the overexpression of catalytically-active RNaseH1, a conserved enzyme that performs R-loop resolution, recovered the increase of DNA damage and the reduction of cell proliferation induced by TonEBP depletion [38].

Similar results were obtained by knocking down or inhibiting of PARP1. However, loss of PARP1 function did not further increase R-loop–associated DNA damage in TonEBP-depleted cells, suggesting that TonEBP and PARP1 act in the same pathway to resolve replication stress, coordinate DNA repair, and promote cell survival (Fig. 6G). These findings provide new insights into the molecular mechanisms underlying cellular DNA damage and repair response, as well as the novel functions of TonEBP in R-loop biology.

In summary, we have shown that in cancer cells, TonEBP is PARylated via an interaction with PARP1, localizes to R-loops, prevents R-loop accumulation and DNA damage, and protects against cell death induced by an R-loop–associated DNA-damaging agent. These findings suggest that TonEBP mediates resistance to CPT-induced cell death by preventing R-loop accumulation in cancer cells. Therefore, TonEBP or its protein–protein interactions represent potential therapeutic targets for human disorders associated with dysregulation of R-loop structures. One important issue to be addressed is how the same DNA damage response (DDR) stimulus can lead to markedly different responses, and how complex pathways and events are orchestrated. A better understanding of DDR will open new avenues for rational disease management.

4. Materials and methods

4.1. Cells and reagents

HEK293T (ATCC CRL-3216), U2OS (ATCC HTB-96) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS; ThermoFisher Scientific, Waltham, MA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (GE Healthcare, Salt Lake City, UT, USA). Hep G2 (ATCC HB-8065) cells were maintained in modified Eagle's medium (Hyclone, South Logan, UT, USA) supplemented with 10 % FBS and penicillin–streptomycin. All cells were maintained at 37 °C in an incubator in an atmosphere containing 5% CO_2. Cells were transfected with the same concentrations of scrambled or gene-targeted siRNAs for 24 h using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). All siRNA duplexes were purchased from Integrated DNA Technologies. All plasmids were purified using an endotoxin-free purification system (Qiagen, Hilden, North Rhine-Westphalia, Germany) and transfected into cells using Lipofectamine 2000 (Invitrogen) for 24 h. Transfected cells were then cultured in fresh complete medium and analyzed as indicated in the figure legends.

4.2. Immunofluorescence and EdU staining, and image analysis

Cells were plated on LabTek chamber slides (Thermo Fisher Scientific), cultured for 1 day, and then fixed with 100 % methanol at 20 °C for 30 min. For chromatin-bound proteins, cells were pretreated with 0.5 % Triton X-100 for 2 min before fixation. For UV micro-irradiation, UVA laser (55 mW) irradiation was performed by means of a Palm MicroBeam laser microdissection workstation. The fixed cells were stained with the indicated primary antibodies overnight at 4 °C. After washes with 0.05 % Triton X-100, Alexa Fluor 488–, 568–, and 633–conjugated secondary antibodies (Invitrogen) were added and incubated for 1 h. The stained cells were mounted. EdU staining was performed by using Click-iT EdU assay kit (Invitrogen). Images were acquired on an Olympus FV1000 confocal fluorescence microscope and processed using the Olympus Fluoview or ImageJ software.

4.3. Immunoprecipitation

To prepare the total cell lysates, cells were washed three times with ice-cold PBS, and lysed and incubated with RIPA buffer on ice [37]. The lysates were incubated with the indicated antibody overnight at 4 °C under rotary agitation, followed by incubation with Protein A/G Sepharose beads (GE Healthcare Sciences) for 2 h. Bead–antibody–antigen complexes were pelleted by centrifugation at 4 °C for 1 min, and the supernatant was removed. The beads were washed three times for 10 min at 4 °C in RIPA buffer, resuspended in sample buffer, and boiled at 95 °C for 5 min. The complexes were analyzed by immunoblotting.

4.4. Immunoblotting

Cells were washed twice with cold PBS and lysed in RIPA buffer (0.01 M Tris pH 7.4, 0.15 M NaCl, 0.001 M EDTA, 0.001 M EGTA, 1 % Triton X 100; all from Sigma-Aldrich, Saint Louis, MO, USA) containing 2 μM

PMSF (Sigma-Aldrich) and protease inhibitors (Roche, Rotkreuz, Switzerland). After centrifugation of the lysate, the supernatant was used for immunoblot analysis. Protein concentration was measured using the BCA protein assay system (Pierce Biotechnology, Rockford, IL, USA). Proteins were denatured in Laemmli buffer. Equal amounts of protein from each sample were separated on SDS-PAGE gels and transferred to PVDF membranes. Blocking, incubation with primary antibody, and washing of the membrane were performed in PBS supplemented with 0.05 % Tween-20 (v/v) and 5 % (w/v) non-fat dry milk. The primary antibodies used for immunoblotting were anti-TonEBP (16; 1:3000), anti-Myc-tag (#2778), anti-PAR (#83732) (all from Cell Signaling Technology, Danvers, MA, USA), anti-FLAG tag (#F1804, Sigma-Aldrich), anti-S9.6 (#ENH001, Kerafast, Boston, MA, USA), and anti-PARP1 (#ab227244, Abcam, Cambridge, UK). All antibodies were used at a 1:1000 dilution unless stated otherwise. Horseradish peroxidase-conjugated goat anti-mouse (62-6520; Thermo Fisher Scientific) or anti-rabbit (65-6120; Thermo Fisher Scientific) secondary antibodies were diluted 1:5000. Reactive bands were detected by chemiluminescence on an ImageQuant LAS 4000 imaging system (GE Healthcare).

4.5. Proximity ligation assay

For the PLA, cells were pre-extracted with cold 0.5 % NP-40 for 3 min on ice. The cells were then fixed with 4% PFA/PBS for 15 min, washed three times with PBS, and blocked for 1 h at RT with 2% BSA/PBS. The fixed and blocked cells were incubated with primary antibody overnight at 4 °C. The next day, the cells were washed three times in PBS and incubated in a pre-mixed solution of PLA probe anti-mouse minus and PLA probe anti-rabbit plus (Sigma-Aldrich) for 1 h at 37 °C. The Duolink In Situ Detection Reagents (Green) were used to perform the PLA reaction. Slides were mounted in Duolink In Situ Mounting Medium with DAPI and imaged on a Zeiss Axioscope at 40 × . PLA foci were quantified using the ImageJ software.

4.6. Cell survival analysis

U2OS and HepG2 cells were plated in triplicate in 96-well plates and subjected to the MTT assay. Absorbance at 490 nm was measured using a multi-well plate reader. LDH release was calculated as a percentage using the following formula:

$$\text{percentage} = (\text{sample–spontaneous release/maximum release} - \text{spontaneous release}) \times 100.$$

4.7. S9.6 IP

Cells were trypsinized, washed with PBS, and resuspended in 25 mL PBS. The cells were then crosslinked in 1 % formaldehyde (Pierce Biotechnology, Rockford, IL, USA), quenched with 0.125 M glycine, and washed twice in PBS containing protease inhibitor (PI; Roche). The fixed cells were lysed with lysis buffer (50 mM HEPES pH 7.9, 140 mM NaCl, 1 mM EDTA, 10 % glycerol, 0.5 % NP-40, 0.25 % Triton X-100) with proteinase inhibitor cocktail, and chromatin was sonicated in shearing buffer (0.1 %SDS, 1 mM EDTA, 10 mM Tris pH 8.1) on a ultrasonicator (Covaris, Woburn, MA, USA) to an average size of 1 kb. Washed Protein A/G Sepharose beads (Pierce Biotechnology) were used to pre-clear chromatin for 2 h. Ten micrograms of the chromatin fraction was mixed with either 20 μg S9.6 antibody or 20 μg mouse IgG, and incubated overnight at 4 °C. Pre-washed Protein A/G Sepharose beads were then added to the chromatin/antibody mixture for 2 h. After washing three times with binding buffer (10 mM $NaPO_4$ pH 7, 140 mM NaCl, 0.05 % Triton X-100), bound beads were boiled in 30 μL 5X sample buffer (10 % SDS, 500 mM DTT, 50 % glycerol, 250 mM Tris-HCL and 0.5 % bromophenol blue dye, pH 6.8) and loaded on a 4–20 % gradient gel (Bio-Rad, Hercules, CA, USA).

4.8. Statistical analysis

Data are expressed as the mean ± standard deviation or standard error of the mean. The statistical significance of differences between two conditions was estimated using the unpaired *t*-test. Comparisons between more than two conditions were evaluated by one-way ANOVA with Tukey's post-hoc test. A p-value < 0.05 was deemed significant. All statistical analyses were performed using the GraphPad Prism 8.2 software (GraphPad, San Jose, CA, USA).

Author statement

Byeong Jin Ye: Conceptualization, Investigation, Writing-Original draft preparation, Writing - Review & Editing

Hyun Je Kang: Investigation, Writing-Original draft preparation

Whaseon Lee-Kwon: Supervision

Hyug Moo Kwon: Supervision, Writing-Original draft preparation, Project administration, Writing - Review & Editing

Soo Youn Choi: Supervision, Writing-Original draft preparation, Project administration, Writing - Review & Editing

Author contributions

B.J.Y., H.J.K., S.Y.C., and H.M.K. made substantial contributions to the conception and design, acquisition of data, analysis and interpretation of data, drafting of the article, revisions of the article critical for important intellectual content, and provided the final approval of the version to be submitted. W.L.-K. made substantial contributions to the acquisition of data, and the analysis and interpretation of data. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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