hCINAP regulates the DNA-damage response and mediates the resistance of acute myelocytic leukemia cells to therapy

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Acute myeloid leukemia (AML) is a genetically heterogeneous malignant disorder of the hematopoietic system, characterized by the accumulation of DNA-damaged immature myeloid precursors. Here, we find that hCINAP is involved in the repair of double-stranded DNA breaks (DSB) and that its expression correlates with AML prognosis. Following DSB, hCINAP is recruited to damage sites where it promotes SENP3-dependent deSUMOylation of NPM1. This in turn results in the dissociation of RAP80 from the damage site and CTIP-dependent DNA resection and homologous recombination. NPM1 SUMOylation is required for recruitment of DNA repair proteins at the early stage of DNA-damage response (DDR), and SUMOylated NPM1 impacts the assembly of the BRCA1 complex. Knockdown of hCINAP also sensitizes a patient-derived xenograft (PDX) mouse model to chemotherapy. In clinical AML samples, low hCINAP expression is associated with a higher overall survival rate in patients. These results provide mechanistic insight into the function of hCINAP during the DNA-damage response and its role in AML resistance to therapy.
Maintenance of genomic stability is critical for the proper functioning of organisms. The genome of a cell is continually under attack from various DNA-damaging agents, and double strand break (DSB) is the most deleterious of such events. To maintain genomic stability following DSBs, cells have developed two major DSB-repair pathways, classical non-homologous end-joining (c-NHEJ), and homologous recombination (HR), both of which are indispensable to the DNA-damage response (DDR) system. However, if damage to the DNA is too severe to repair, the cell will undergo apoptosis. To understand the mechanisms involved in the DDR, identification of regulatory proteins that participate in and control these repair pathways is of primary importance.

In response to DNA-damage, a series of repair proteins sequentially accumulate at the damaged site and function to induce signal transduction pathways that initiate the subsequent repair of DSBs. These proteins are finely modulated by dynamic and reversible posttranslational modifications. Protein modifications via phosphate and ubiquitin have already been recognized as important regulators of the DDR. Increasing evidence also indicates that SUMO is involved in the DNA-repair mechanisms of higher organisms. During DNA-damage or replication stress, components of the SUMO pathway accumulate at DSB sites within the nucleus, including E1 SUMO-activating enzyme, E2-conjugating enzyme, and E3 SUMO ligases. The reversibility of SUMOylation is conferred by the Sin3/SUMO-specific proteases (SENP)s.

Acute myeloid leukemia (AML) is a serious hematological malignancy. Nucleophosmin (NPM1) gene mutations represent the most-frequent genetic lesions in patients with AML. The standard induction chemotherapy for AML relies on a combination of the nucleoside analog cytarabine (Ara-C) and an anthracycline, such as daunorubicin (DNB). Although most patients meet the remission expectations after initial chemotherapy treatment, relapse frequently occurs. As such, the global prognosis for these patients remains poor. Generally, the mechanism of action of the chemotherapeutic drugs used for AML treatment relies on the inhibition of DNA synthesis and the induction of DNA DSB in cancer cells, eventually leading to cell apoptosis or cell death. Very high rates of genomic instability and apoptosis have been associated with an improved prognosis in patients with AML. Importantly, induction of the DDR is one of the main consequences of taking these drugs, leading to the induction of DNA repair via the c-NHEJ and HR pathways. However, the cellular effectors influencing these two repair pathways in AML have not been clearly identified.

Human colin-interacting nuclear ATPase protein (hCINAP), also known as adenylate kinase 6, is highly conserved in eukaryotes. In human cells, hCINAP participates in the formation of Cajal bodies, affects p53 activity via the HDM2-p53 pathway, regulates 18S rRNA processing, and determines self-renewal of colorectal cancer stem cells by modulating the Warburg effect. However, the physiological function of hCINAP in the DDR and maintenance of genome stability has not been elucidated.

Here, we determine that hCINAP is involved in the relatively late stage of the DDR by inhibiting NPM1 SUMOylation in a SENP3-dependent manner. DNA-damage-induced BRCA1 recruitment and the choice of DNA repair pathways are fine-tuned by hCINAP. These findings suggest that hCINAP is a potential and promising target for overcoming resistance towards chemotherapy and radiotherapy in patients with AML.

Results

hCINAP is essential for genome stability and is associated with AML. To explore whether hCINAP is involved in the DDR, U2OS cells were treated with ionizing radiation (IR) to induce DSBs. In response to damage stimuli, hCINAP translocated from the cytoplasm to the nucleus in immunofluorescence assays (Fig. 1a, b). Consistent with these observations, nuclear hCINAP increased, whereas cytoplasmic hCINAP decreased following IR treatment (Fig. 1c). These results indicated that hCINAP functions in a spatiotemporal manner in the process of DNA repair.

Next, a laser micro-irradiation system was utilized to generate localized DNA-damage in GFP-hCINAP expressing U2OS cells. GFP-hCINAP was recruited to the laser-induced DNA-damage tracks after micro-irradiation. The fluorescent signals began to intensify ~2 min after micro-irradiation, reaching a maximum after ~8 min, and decreased thereafter, receding to pre-damage levels by ~40 min after irradiation (Fig. 1d), suggesting that the recruitment of hCINAP to DNA-damage sites was a chronological process. The recruitment of endogenous hCINAP to the damaged sites was also detected (Supplementary Fig. 1a). We deduced that hCINAP does not function during the relatively early damage response, as it is recruited to DNA-damage sites after a subsequent lag period following irradiation.

In addition, we constructed an hCINAP−/− cell line using the CRISPR/Cas9 system (Supplementary Fig. 1b, c) and evaluated the global effects of hCINAP on DNA-damage via neutral comet assays. Knockout of hCINAP caused the appearance of tails, photocopying DNA damage caused by genome instability (Fig. 1e, f). The hCINAP−/− cells showed higher basal DNA-damage levels than wild-type cells. Similar comet tail length was observed 1 h after irradiation between the two groups. However, in the relatively later stage, after a 12-hour recovery following irradiation, hCINAP−/− cells still had longer tails than wild-type cells. Furthermore, hCINAP-depleted cells accumulated chromosome breaks and showed chromosome instability phenotypes (Fig. 1g). Loss of hCINAP in cells induced a higher frequency (5.65%) of chromosome rearrangements compared with the 2.87% total breaks per chromosome in hCINAP wild-type cells (Supplementary Fig. 1d), which is similar to that of p53 reported previously.

Collectively, these results indicate that hCINAP functions at a relatively late stage in the DDR pathway and is essential for maintaining genome stability.

AML is a serious hematological malignancy with well-known radiotherapy and chemotherapy resistance, and high rates of genomic instability in AML cells have been associated with improved prognosis in patients with AML. Considering the indispensable role of hCINAP in maintaining genomic stability, we wanted to investigate whether hCINAP expression affects AML diagnosis and therapy. Using the TCGA and GTEx databases, we observed that hCINAP expression levels were frequently downregulated in AML compared with healthy controls (Fig. 1h). We collected the peripheral blood (PB) of patients with AML and healthy controls without any sign of hematological malignancies and detected low expression levels of hCINAP in AML patients (Fig. 1i, j). To verify the role of hCINAP in maintaining genomic stability, we performed neutral comet assays on three samples: healthy control 13 with the highest hCINAP expression level, AML 10 with moderate hCINAP expression, and AML 11 with the lowest level of hCINAP expression. As expected, healthy control 13 had the...
lowest rate of genomic instability, whereas the highest genomic instability frequency was observed in AML sample 11 (Fig. 1k, Supplementary Fig. 1e). These results support the observation that hCINAP is essential for genomic stability. Furthermore, we detected chromosome morphology abnormalities, using a metaphase spread assay, in PB cells from healthy control 13, AML 10, and AML 11 (Supplementary Fig. 1f). Low hCINAP expression in PB cells from AML patients induced a higher frequency of chromosome rearrangements. The AML PB cells and KG-1α cells with lower abundance of hCINAP accumulated...
subjected to neutral comet assay. The olive tail moments were quantified by generating blots from three independent experiments using ImageJ.

As hCINAP interacts with NPM1, we examined how hCINAP regulates NPM1 deSUMOylation during DSB repair. First, we found that overexpression of hCINAP in DSB. Next, we explored whether the inhibition of NPM1 SUMOylation depends on SENP3. As hCINAP is an adenylate kinase but not a SUMO protease16, we questioned how hCINAP could inhibit NPM1 SUMOylation. To assess the involvement of the enzymatic activity of hCINAP in NPM1 SUMOylation, its pull-down assays were performed with the enzymatic mutants, hCINAP-D77G and hCINAP-H79G (Supplementary Fig. 3f). Both wild-type hCINAP and hCINAP-Δ mutant OC1-AML2 cell line (Supplementary Fig. 2a) and NPM1 mutant OC1-AML3 cell line (Supplementary Fig. 2b). We also determined that the C-terminal nucleic-acid-binding domain was critical for its binding to hCINAP (Fig. 2d). Collectively, these data demonstrated that hCINAP direct interacts with NPM1.

hCINAP regulates NPM1 deSUMOylation during DSB repair. As hCINAP interacts with NPM1, we examined how hCINAP regulates NPM1 in DSB. First, we found that overexpression of hCINAP did not significantly affect NPM1 expression (Supplementary Fig. 3a). A previous study showed that NPM1 is modified by phosphoric acid, ubiquitin, and SUMO chains25. Therefore, we tested whether hCINAP could affect such modifications in relation to NPM1. Overexpression of hCINAP did not affect NPM1 phosphorylation levels (Supplementary Fig. 3a). His-ubiquitin pull-down analysis also showed that hCINAP was dispensable for NPM1 ubiquitination (Supplementary Fig. 3b). His-SUMO pull-down assays, however, indicated that endogenous NPM SUMOylation could be detected only when the damage occurred, and this changed dynamically during the DDR (Fig. 3a, b). The SUMOylation of endogenous NPM1 increased in response to damage and reached its peak at 1 h after irradiation. After 1 h, the level of NPM1 SUMOylation decreased and was barely detectable 12 h after irradiation (Fig. 3b). In the post IR period, such as 8 h after IR treatment, hCINAP obviously inhibited IR-induced NPM1 SUMOylation (Fig. 3c), whereas in hCINAP−/− cells, the level of NPM1 SUMOylation was maintained at a high level, especially in the late DDR stage (8 h after IR) (Fig. 3d, e). These results indicate that the increased IR-induced NPM1 SUMOylation cannot return to its basal level in the absence of hCINAP.

UV treatment has been shown to promote NPM1 phosphorylation26. Thus, we investigated the cross-talk between different NPM1 posttranslational modifications. We overexpressed hCINAP with either a SUMOylation deficient mutation (K263R) or a phosphorylation deficient mutation (T199A) and detected phosphorylation or SUMOylation changes, respectively (Supplementary Fig. 3c–e). We observed no cross-talk between NPM1 SUMOylation and phosphorylation. Therefore, in the following studies, we mainly focused on the function of hCINAP with regard to the regulation of NPM1 SUMOylation and its association within the DDR. We assessed the timing of recruitment of these two proteins to damage sites following micro-irradiation. NPM1 accumulated at irradiated sites within 10 s after micro-irradiation, whereas hCINAP accumulated at irradiated sites within 2 minutes after micro-irradiation (Fig. 3f, g), suggesting that NPM1 and hCINAP may function at different time points during the DDR process.
and RNF8, RNF168 recruitment\(^{26}\). We found that NPM1 and its phosphorylation has been shown to in regulation of the recruitment of DNA-damage proteins. NPM1 phosphorylation is correlated with the occurrence of DSBs and rapidly SUMOylation in a SENP3-dependent manner. Taken together, these results suggest that hCINAP inhibits NPM1 much longer than that of wild type (Supplementary Fig. 2e). Based on the above discoveries that NPM1 SUMOylation is important for the recruitment of repair proteins, we constructed an NPM1 knockdown U2OS cell line and examined the formation of BRCA1 foci at different time lags following IR treatment. NPM1 depletion reduced the recruitment of BRCA1 to the damage sites and could be rescued by introduction of wild-type NPM1, but not the NPM1 K263R mutant, a mutant dysfunctional at the major SUMOylation catalytic site (Fig. 4c). Similar changes in BRCA1 foci were observed at 1 h after IR treatment. We further confirmed the role of NPM1 SUMOylation in the formation of BRCA1 foci and the interaction between NPM1 and BRCA1 by immunofluorescence and co-IP experiments. The NPM1 K263R mutant no longer localized to the nucleus and, consequently, the number of IR-induced BRCA1 foci decreased significantly in comparison to NPM1 WT cells (Fig. 4d). Meanwhile, BRCA1 only interacted with NPM1 WT but not NPM1 K263R (Fig. 4e). Moreover, a proximity ligation assay (PLA), which enables detection of protein interaction in situ with high specificity and sensitivity\(^{27,28}\), was performed showing that NPM1 WT and BRCA1 interacted mainly in the nucleus (Fig. 4f). These results indicate that NPM1 SUMOylation is necessary for the interaction between NPM1 and BRCA1 and promotes the accumulation of BRCA1 foci induced by DSB.

**NPM1 SUMOylation promotes DSB-induced BRCA1 accumulation.** Based on the above discoveries that NPM1 SUMOylation is correlated with the occurrence of DSBs and rapidly increased following DNA damaging stimuli, we questioned whether NPM1 SUMOylation was necessary for the dynamic regulation of the recruitment of DNA-damage proteins. NPM1 phosphorylation has been shown to influence H2A ubiquitination and RNF8, RNF168 recruitment\(^{26}\). We found that NPM1 and its SUMOylation showed no effect on H2A ubiquitination (Fig. 3k). Moreover, no significant changes in the foci formation of MDC1, RNF8, RNF168, and FLC2 were observed in the NPM1 K263R mutant or hCINAP-depleted cell lines (Supplementary Fig. 4b-e and Fig. 5a-d). These data indicate that NPM1 SUMOylation does not influence H2A ubiquitination and subsequent recruitment of the DNA-damage repair proteins.

We then identified repair proteins including BRCA1 that were potentially associated with NPM1 (Fig. 4a). Co-IP assays demonstrated that endogenous NPM1 indeed interacted with BRCA1 in vivo (Fig. 4b). To determine whether NPM1 and its SUMOylation were important for the recruitment of repair proteins, we constructed an NPM1 knockdown U2OS cell line and examined the formation of BRCA1 foci at different time lags following IR treatment. NPM1 depletion reduced the recruitment of BRCA1 to the damage sites and could be rescued by introduction of wild-type NPM1, but not the NPM1 K263R mutant, a mutant dysfunctional at the major SUMOylation catalytic site (Fig. 4c). Similar changes in BRCA1 foci were observed at 1 h after IR treatment. We further confirmed the role of NPM1 SUMOylation in the formation of BRCA1 foci and the interaction between NPM1 and BRCA1 by immunofluorescence and co-IP experiments. The NPM1 K263R mutant no longer localized to the nucleus and, consequently, the number of IR-induced BRCA1 foci decreased significantly in comparison to NPM1 WT cells (Fig. 4d). Meanwhile, BRCA1 only interacted with NPM1 WT but not NPM1 K263R (Fig. 4e). Moreover, a proximity ligation assay (PLA), which enables detection of protein interaction in situ with high specificity and sensitivity\(^{27,28}\), was performed showing that NPM1 WT and BRCA1 interacted mainly in the nucleus (Fig. 4f). These results indicate that NPM1 SUMOylation is necessary for the interaction between NPM1 and BRCA1 and promotes the accumulation of BRCA1 foci induced by DSB.

**Loss of hCINAP leads to defects in error-free DSB repair HR.** As HR and NHEJ are the two main DSB repair pathways\(^{29}\), we next evaluated the effects of hCINAP on these pathways. Depletion of hCINAP resulted in a significant decrease in the
percentage of GFP-positive cells (Fig. 5a), demonstrating that loss of hCINAP function impairs the proficiency of error-free HR repair. We assessed the abundance of BRCA1 foci in hCINAP<sup>WT</sup> and hCINAP<sup>−/−</sup> U2OS cells at different time points following IR treatment. The hCINAP<sup>−/−</sup> U2OS cells had much more BRCA1 foci than hCINAP<sup>WT</sup> cells in the resting state. After exposure to irradiation, BRCA1 foci in hCINAP<sup>−/−</sup> cells were more abundant than in hCINAP<sup>WT</sup> cells. At 1 h after irradiation, hCINAP<sup>−/−</sup>...
cells contained slightly more BRCA1 foci at the lesion points than hCINAPWT cells, suggesting that hCINAP moderately regulates IR-induced DNA-damage foci during the early DNA repair period. Strikingly, after 12 h of recovery, hCINAPΔΔ cells still had much more BRCA1 foci than hCINAPWT cells (Fig. 5b), suggesting that hCINAP affects the dissociation of BRCA1 from the lesion sites at a relatively late stage after damage repair.

To verify the regulation of BRCA1 recruitment in AML, we isolated white cells from the PB of AML patients and knocked down hCINAP expression. We then examined the effect of hCINAP depletion on BRCA1 foci formation. Knockdown of hCINAP increased the number of BRCA1 foci in leukocytes isolated from AML patients (Supplementary Fig. 6a, b). Collectively, these results indicate that hCINAP affects BRCA1 foci dissociation from lesion points in the DSB repair process, especially in the relatively late stage.

We next investigated whether hCINAP-regulated BRCA1 foci recruitment via its regulation of NPM SUMOylation. Indeed, when NPM1 was knocked down, BRCA1 foci remained at very low levels even in the damaged state. In addition, hCINAP no longer influenced BRCA1 foci recruitment (Fig. 5c). These results indicate that hCINAP inhibits BRCA1 recruitment in the damaged state in an NPM1-dependent manner.

Normally, the recruitment of BRCA1 is considered a landmark event in the HR pathway5. However, the data above showed that depletion of hCINAP reduced HR efficiency but increased the abundance of BRCA1 foci. To explain this seemingly controversial observation, we further examined the effects of hCINAP on foci formation of RAP80 and CtIP. RAP80 and CtIP are the key factors in the BRCA1-A and BRCA1-C complexes, respectively29. As indicated in Fig. 5d, the formation of the BRCA1-C complex containing CtIP and BRCA1 promotes end resection and the subsequent process of HR, whereas the Abraxas/RAP80/BRCA1-containing BRCA1-A complex has the opposite effect on HR29. Consistent with the pattern in Fig. 5d, knockdown of hCINAP increased RAP80 foci recruitment but reduced CtIP foci (Fig. 5e–g). These observations indicate that hCINAP regulates the end resection process by selectively affecting the repair proteins RAP80 and CtIP of the BRCA1 complexes, thus affecting the efficiency of HR.

RAD51 has a central role in the HR pathway. After a DSB occurs, RAD51 promotes invasion of ssDNA into undamaged homologous dsDNA30. We further assessed the role of hCINAP in the end resection process by examining its influence on IR-induced RAD51 protein recruitment. Deficiency of hCINAP blocked RAD51 foci formation (Fig. 5h, i), which is consistent with its effect on HR repair efficiency (Fig. 5a). Furthermore, we found that RAD51 only bound to NPM1 WT but not NPM1 K263R (Fig. 5i). Thus, in NPM1 K263R cells, the number of IR-induced RAD51 foci decreased significantly (Fig. 5k, Supplementary Fig. 12). These results indicate that NPM1 SUMOylation promotes RAD51 recruitment.

CHK1 is one of the ATR substrates, and CHK1 phosphorylation is critical for end resection. We next examined the effect of NPM1 SUMOylation on CHK1 phosphorylation. Comparison of the abundance of p-CHK1 in K263R mutant cells with that of NPM1 WT cells revealed that IR treatment promoted CHK1 phosphorylation, which was inhibited by NPM1 knockdown. Furthermore, re-expression of NPM1 WT, but not the K263R mutant, significantly attenuated the abundance of p-CHK1 (Supplementary Fig. 7a), suggesting that only NPM1 WT could rescue the reduced IR-induced CHK1 phosphorylation. Consistent with this data, measurement of HR efficiency showed that endogenous NPM1 expression and its SUMOylation are necessary for efficient HR repair (Supplementary Fig. 7b). Taken together, hCINAP functions in the HR pathway through regulation of NPM1 SUMOylation.

**SUMOylated NPM1 directly binds to RAP80.** Considering NPM1 SUMOylation is beneficial for the recruitment of repair proteins, such as RAP80 (Fig. 5d), we next investigated if NPM1 SUMOylation is required for recruitment of RAP80. In NPM1-depleted cells, we reintroduced NPM1 WT or the NPM1 K263R mutant, and found that IR treatment enhanced the interaction between wild-type NPM1 and RAP80, whereas NPM1 K263R mutant showed no interaction with RAP80 (Fig. 6a). PLA analysis also showed increased interacting PLA signals in cells with NPM1 WT and RAP80 compared with cells with NPM1 K263R-RAP80 (Fig. 6b, c). As SUMO and UB modifications are coordinated to recruit RAP80 and BRCA1 to DNA-damage sites31,32, we constructed two expression vectors, RAP80 full-length (FL) and SIM domain-deleted RAP80 (ΔSIM), and examined whether SUMOylated NPM1 directly interacted with the SIM domain of RAP80 in vivo and in vitro. Deletion of the SIM domain abrogated the interaction between NPM1 and RAP80 with/without IR (Fig. 6d). Consistently, GST-NPM1-SUMO2 could directly bind to His-RAP80 FL but not His-RAP80 ΔSIM, and no interaction was observed between unSUMOylated GST-NPM1 and His-RAP80 FL or His-RAP80 ΔSIM (Fig. 6e). These data suggest that SUMOylated NPM1 directly interacts with the SIM domain of RAP80 (Fig. 6f). These results indicate that the SIM domain of
Knockout of hCINAP was associated with a dramatic upregulation in the relative percentage of GFP-positive cells by about 45% (Fig. 7a). Furthermore, we elucidated the effect of hCINAP on the dynamic foci formation of 53BP1 and DNA-PKcs, two specific markers for NHEJ repair. The hCINAP+/− cells had more 53BP1 foci than wild-type cells in the resting state. At 1 h after IR treatment, the number of 53BP1 foci increased similarly in both wild-type and hCINAP+/− cells. When the cells were treated with IR followed by a 12-hour recovery, hCINAP−/− cells presented...
Fig. 5 Loss of hCINAP leads to a defect in error-free HR pathway. **a** hCINAPWT and hCINAP−/− HEK293T cells were subjected to HR assays. The data are presented as the mean ± SEM of three replicates. More than 1000 cells were counted in each group, two-tailed students’ t test, ***P < 0.001. **b** hCINAPWT and hCINAP−/− U2OS cells treated with or without IR (10 Gy) were subjected to immunoﬂuorescence assays. Representative immunofluorescence images are shown (left), and statistics of BRCA1 foci numbers in each group are shown (right). ****P < 0.0001. e NPM1 knockdown U2OS cells transfected with the indicated plasmids were treated with IR (10 Gy) and released for 1 h. The accumulation of BRCA1 was then assessed. Scale bar, 10 μm. **d** Schematic of the role of different BRCA1 complex in end resection. e-g hCINAPWT and hCINAP−/− U2OS cells were immunostained with anti-RAP80 or anti-CtIP antibodies. Representative immunofluorescence images are shown in e, and BRCA1 foci numbers in cells were counted in f and g. Scale bar, 10 μm. ****P < 0.0001. h, I Immunofluorescence h and its quantification i showing that hCINAP deﬁciency blocked the RAD51 foci recruitment. Representative images and percentages of cells with > 10 RAD51 foci were counted. The U2OS cells were treated with 4 Gy IR and released 4 h before ﬁxing. Scale bar, 10 μm. ***P < 0.001. j HEK293T cells transfected with the indicated vectors were subjected to co-IP analysis using the indicated antibodies. k Cells were exposed to 10 Gy IR and then recovered for 1 h before being subjected to immunofluorescence. Scale bar, 10 μm. Additional cell images and quantiﬁcation of RAD51 foci are shown in Supplementary Fig. 12. For e, f, g, the results are shown as the mean ± SEM from three independent experiments. Data were analyzed using Student’s t test. More than 100 cells were counted per group. Unprocessed scans of blots are provided in Supplementary Fig. 13.
with more 53BP1 foci (Fig. 7b, c). These results indicate that hCINAP inhibits 53BP1 foci dissociation in the post IR stage.

Consistent with this finding, we also found that knockout of hCINAP had no influence on the total protein level of DNA-PKcs, but promoted its Ser2056 phosphorylation. Re-expression of hCINAP rescued the inhibitory effect on DNA-PKcs phosphorylation (Fig. 7d). This result was confirmed by IR-induced immunofluorescence foci quantification (Fig. 7e, f). These results demonstrate that loss of hCINAP impaired HR proficiency, which was compensated by an increase in NHEJ repair. Together these results suggest that hCINAP functions in the efficient repair of DSBs.

AML PDX mice with depleted hCINAP show higher drug sensitivity. The above data indicate that hCINAP is essential for maintaining genomic stability, and its expression level is associated with the prognosis of patients with AML (Fig. 1h). Thus, we explored its biological significance by constructing patient-derived xenograft (PDX) AML mouse models with depleted hCINAP or wild-type hCINAP (Fig. 8a). PB leukocytes from AML patients were collected and injected into NOD/SCID donor mice to induce AML mice with depleted hCINAP or wild-type hCINAP (scrambled control), respectively. To mimic the clinical medication practice, at D10 after injection, we administered the 7 + 3 chemotherapy to the mice (Fig. 8a) and investigated the effects of hCINAP depletion on cell survival, apoptosis, spleen morphology, and mice survival in response to the chemotherapy.

At D20 after injection, PB cells were collected to detect cell apoptosis by TdT-mediated dUTP Nick-End Labeling (TUNEL) fluorescence assay. In the AML mice, hCINAP knockdown resulted in increased cell death (TUNEL+) (Fig. 8b). At D26, two mice in each group were euthanized and the spleen samples were collected for immunohistochemistry to investigate the expression of γ-H2AX and Ki67 (Fig. 8c). The mice induced with knocked down hCINAP AML showed decreased cell proliferation (Ki67+) and increased γ-H2AX. These results indicate that hCINAP depletion results in higher levels of DNA damage and renders cells more sensitive to chemotherapeutic drugs. Two mice in each group were sacrificed and the femur BM cells were isolated at D26 and subjected to fluorescence-activated cell sorting (FACS) to analyze the proliferation of xenografted GFP-tagged BM cells. Malignant BM cell proliferation and metastases decreased in hCINAP knockdown mice (Fig. 8d). At the end of the experiment, the spleens of AML mice were photographed and weighed (Fig. 8e, f). In comparison with the hCINAP shRNA AML mice, larger and heavier spleens in the control shRNA group revealed that the AML mice with hCINAP knockdown had significantly longer survival (Fig. 8g).

We investigated the effects of hCINAP knockdown on KG-1a cell survival in response to IR and the representative AML therapeutic agents, DNR and HD-Ara-C. Soft agar assays showed...
that knockdown of hCINAP in AML KG-1a suspended cells resulted in a significant decrease in cell survival rate, and exposing cells to an increased dosage of IR severely aggravated this phenotype (Supplementary Fig. 8a–c). We next performed the apoptosis proportion analyses by using the three samples (healthy control 13, AML 10, and AML 11) mentioned in Fig. 1i. The results showed that healthy control 13 possessed the highest level of hCINAP, with the lowest apoptosis rate, whereas the highest apoptosis rate was observed in AML sample 11 (Fig. 8h). Of note, the level of DSB breaks was elevated by a relatively low dose (4 Gy) IR treatment, indicating that the necrotic white cells from the AML samples not only possessed lower genomic stability but were also more sensitive to IR radiotherapy. The same experiment was performed in the AML KG-1a cell line. After IR treatment, low hCINAP expression in KG-1a resulted in an increased apoptosis rate and more sensitivity to DDR stimuli (Supplementary Fig. 1g). Consistent with this observation, the Kaplan–Meier analysis of overall survival of patients with AML also indicated that the superior curative overall survival ratio was negatively correlated with hCINAP abundance (Fig. 8i).

Altogether, our results indicate that depletion of hCINAP combined with chemotherapy increases DNA-damage, genomic instability, and drug sensitivity in AML PDX mice and AML patient PB cells, promoting regression of AML. Moreover, we examined NPM1 SUMOylation, NPM1-SENP3 interaction, and BRCA1 foci in PB cells and spleen sections from the AML PDX mice. In agreement with our aforementioned molecular mechanism, the NPM1 SUMOylation level is higher in AML mice PB cells and spleen specimens with depleted hCINAP than that of the control group (Fig. 8j, k, Supplementary Fig. 9a). Meanwhile, using spleen sections, PLA staining revealed that knockdown of hCINAP attenuated the interaction between NPM1 and SENP3 (Fig. 8k, Supplementary Fig. 9a); and the immunoassay showed that the number of BRCA1 foci positively correlated with the levels of SUMOylated NPM1 (Fig. 8k, l and Supplementary Fig. 9a). Meanwhile, the number of BRCA1 foci positively correlated with the levels of SUMOylated NPM1 (Fig. 8k, l and Supplementary Fig. 9a). These data support our model that hCINAP regulates NPM1 SUMOylation and affects BRCA1 protein recruitment, thereby enhances radiotherapy and chemotherapy resistance.

**Discussion**

The DDR is a complex system that enables cells to survive and maintain genomic integrity. This process is fine-tuned by many types of posttranslational protein modifications. In this study, we propose a spatiotemporal working model in which NPM1 is SUMOylated in response to DSBs, which is beneficial for BRCA1 recruitment. Meanwhile, hCINAP translocates from the cytosol to the nucleus, where it is recruited to the damaged sites and promotes SENP3-dependent deSUMOylation of NPM1. DeSUMOylation of NPM1 causes dissociation of RAP80 from the damage site and facilitates DNA resection and homologous recombination (Fig. 8m).

Here, we demonstrate that NPM1 SUMOylation responds to IR-induced DSB and is beneficial for maintaining chromosome...
morphology. The NPM1-depleted AML KG-1α cells that were reintroduced with an NPM1 K263R mutant showed more chromosome breaks than that of KG-1α cells rescued with wild-type NPM1 (Supplementary Fig. 10a). NPM1 SUMOylation is required for the recruitment of DNA repair proteins. Immediately after DNA-damage occurs, NPM1 accumulates at the damage sites and promotes repair. Knockdown of NPM1 leads to a significant decrease in cell survival. Interestingly, we showed that SUMOylation of NPM1 does not affect recruitment of itself to the DSBs (Supplementary Fig. 10b) and the regulatory function of NPM1 SUMOylation in the DDR is independent of NPM1 phosphorylation. These observations suggest that SUMOylation...
and phosphorylation of NPM1 work independently in response to different types of stimuli and that NPM1 SUMOylation is a responsive regulator of DSBs required for DNA repair and cell survival.

Notably, we identified hCINAP as a negative regulator of NPM1 SUMOylation, which functions at different time points from that of NPM1 in the DDR process. At the later stage of the DDR, hCINAP is recruited to the damage sites and prevents excessive repair by downregulation of NPM1 SUMOylation. Although deletion of hCINAP has no effect on the recruitment of NPM1 to the damage sites (Supplementary Fig. 10c), hCINAP reduces the level of NPM1 SUMOylation and recruitment of repair proteins by promoting the interaction between SENP3 and NPM1. Thus, hCINAP has a critical role in promoting genomic stability.

The role of hCINAP has largely been attributed to its “onco-gene” function, as it is upregulated in solid tumors and correlated with a poor prognosis. However, the functions of hCINAP in hematologic malignancies remained unclear. In this study, we found that excessively low hCINAP protein expression was detrimental in cases of AML. It is notable that hCINAP expression levels are lower in samples collected from AML patients relative to the control group. According to the Oncomine AML database, those patients with AML demonstrating lower hCINAP expression levels show a better prognosis. These results are consistent with the survival analysis of our AML PDX mouse model, supporting the hypothesis that lower expression of hCINAP in AML may confer higher error-prone NHEJ repair efficiency and lower error-free HR repair efficiency, to increase the sensitivity to DNR chemotherapy or radiotherapy. The conventional AML therapy is to kill all the leukocytes and then re-establish the blood circulation system by hemopoietic stem cell transplantation. In line with this idea, high levels of drug sensitivity and genomic instability have been associated with improved prognosis. Our results (Fig. 8) reveal that AML cells with depleted hCINAP show higher sensitivity to chemotherapeutics, increased rates of DNA damage and cell death, and alleviated AML progression. Finally, considering the low expression of hCINAP in patients with AML, a combination of an hCINAP regulator and chemotherapy may provide a beneficial approach toward more-efficient AML therapies.

It is worth mentioning that after treatment with different concentrations of DNR or Ara-C for 24 h, the abundance of hCINAP in the leukocytes isolated from PB of AML patients and healthy people changed in distinct patterns. The hCINAP level increased in AML patients, but not healthy people, after drug treatment (Supplementary Fig. 11a, b); yet the level of increased hCINAP was still well below that of the healthy people. As hCINAP is important for maintaining genome integrity, AML cells with low abundance of hCINAP are more sensitive to the drug treatment and then undergo apoptosis. On the other hand, cells might increase chemotherapy resistance by promoting hCINAP expression. Meanwhile, the different response of hCINAP to the chemotherapy reagents in normal and AML cells suggest that hCINAP is a potential therapeutic target.

The discovery of SUMOylation/deSUMOylation pathways that function to control DNA-damage repair highlights the possibility of modulating these PTM activities to protect healthy cells from the effects of genotoxic anticancer therapies, while till eliminating the cancer cells. As drugs can easily target the ubiquitin-like modification system, such pleiotropic mechanisms can be of substantial use in cancer treatments, offering a number of possibilities for future applications.

**Methods**

**Cell lines and clinical samples.** HEK293T (CRL-11268), HeLa (CCL-2), and U2OS (HTB-96) cells (purchased from ATCC, USA) were cultured in Dulbecco’s Modified Eagle Medium medium (Gibco, USA). KG-1a (CCL-246.1) cells (purchased from ATCC, USA) were cultured in Iscove’s Modified Dulbecco’s medium (Gibco, USA). OCI-AML2 and OCI-AML3 cells were obtained from Peking University People’s Hospital. All cell lines were tested by PCR to make sure there was no mycoplasma contamination. All knockout cell lines used in this study were generated using the CRISPR-Cas9 gene editing approach. For CRISPR/Cas9 knockout of human SENP3 in HEK293T and U2OS cells, the following two sgRNAs were used: sgSENP3-1: 5′-GGATCGTGGGAGGG-3′; sgSENP3-2: 5′-CCAGGCATTGAGCTCCGG-3′. For CRISPR/Cas9 knockout of human hCINAP in HEK293T and U2OS cells, the following two sgRNAs were used: sgCINAP-1: 5′-CAGGTACACCGGGTTGGG-3′; sgCINAP-2: 5′-ATGCGTGGGAGGG-3′. The sgRNA sequences were cloned into the U6-Cas9 plasmid (a gift from Wensheng Wei at Peking University). The sgRNA/Cas9 expression constructs were transiently transfected into HeLa, U2OS, or HEK293T cell lines. Twelve hours after transfection, cells were filtered via FACS and plated to acquire individual clones. Gene knockout cells were validated and confirmed by immunoblotting and sequencing.

The PB samples were collected the first time when the patients were diagnosed with AML at the Peking University People’s Hospital according to the guidelines of the ethics committees. The PB samples of healthy patients without any sign of hematological malignancies were collected at Peking University Hospital. Informed consent was obtained from all the patients. The investigation was performed under approval by the Ethics Committees of Peking University and Peking University People’s Hospital. Total samples of AML patients included six females and seven males aged from 24 to 80. Total samples of the control group included eight females and five males aged from 19 to 78. White blood cells were separated from the PB using Ficoll-Paque PLUS (GE, USA) and Red Blood Cell Lysis Buffer (Tiangen, China).

**Pharmacological inhibitors, reagents, plasmids, and antibodies.** Daunorubicin-hydrochloride (DNR) and HA-Cytarabine (HA-Ara-C) were purchased from Pharmabiology (USA). Duolink In Situ Red Starter Kit Mouse/Rabbit kit used for detecting proximity ligation was purchased from Sigma (USA). SUMOylation Assay Kit was purchased from Abcam (USA). Protease Inhibitor Cocktail was purchased from TOPSCIENCE, TargetMol (USA), Catalog No.C0001.

The expression plasmids pRK-Flag-hCINAP and pRK-HA-NPM1 full-length as well as their corresponding enzymatic mutations and deletion mutants were...
constructed by inserting hCINAP or NP-M1 into the pRK vector. His-SUMO2 were inserted into the pEF-BH1 vector.

Antibodies, including anti-Myc (M047-3, WB: 1:1000), anti-GST (M071-3, WB: 1:1000), anti-β-actin (PM053, WB: 1:2000), anti-Lamin B1 (PM064, WB: 1:2000), anti-His (D291-3, WB: 1:1000), and anti-a-Tubulin (M175-3, WB: 1:2000), were purchased from MBL (USA). Monoclonal anti-Flag (M2, F3165, WB: 1:1000; IF: 1:100) and anti-HA (HA-7; 19668, WB: 1:1000) were purchased from Sigma (USA). Phospho-Histone H2A.X (Ser139, 059836, WB: 1:5000; IF: 1:100) was purchased from Merck Millipore (USA). DNA-PKcs (sc-5282, WB: 1:1000; IF: 1:100), RNFSF (sc-271462, IF: 1:50), RNFLF8 (sc-101125, IF: 1:100), RAD51 (sc-53428, IF: 1:1000), 53BP1 (sc-27609, IF: 1:100), and IFN (sc-31401, IF: 1:100) were purchased from Santa Cruz. Phospho-DNA-PKcs (See2056, WB: 1:1000; IF: 1:100) was purchased from Cell Signaling Technology. IRDye 800CW goat anti-mouse (926−32120, 1:1000) and IRDye 800CW goat anti-rabbit (926−32211, 1:1000) were purchased from LI-COR Bioscience (USA). Phospho-DNA-PKcs (sc-5282, IF: 1:50), 53BP1 (sc-22760, IF: 1:100), and BRCA1 (sc-135731, IF: 1:1000) were purchased from Merck Millipore (USA). Phospho-Histone H2A.X (Ser139, 059636, WB: 1:5000; IF: 1:100) was purchased from Cell Signaling Technology. IRDye 800CW goat anti-mouse (926−32120, 1:1000) and IRDye 800CW goat anti-rabbit (926−32211, 1:1000) were purchased from LI-COR Bioscience (USA). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG (IF: 1:5000, 1:1000) were purchased from ZSGB-Bio (China). Rabbit polyclonal anti-hCINAP was generated by immunizing a rabbit with the purified hCINAP protein.

**Immunofluorescence**

The recruitment of repair proteins to the damage site (BRCA1, RAP80, ChiP, RAD51, 53BP1, and the phosphorylation of 53BP1) under different IR treatment lengths was investigated in U2OS cells and PB leukocytes from patients with AML. U2OS cells were treated with or without IR at 10 Gy and collected after resting for 1 h or 12 h. AML patient leukocyte samples were adhered to glass slides using a Thermo Scientific Cytospin 4 Cytocentrifuge. Images were visualized with a confocal laser-scanning microscope (Zeiss LSM-710 NLO and DuoScan, Germany) using a ×63 oil objective lens. The number and intensity of IR-induced nuclear foci were quantified using the Imaris 7.6 software (Bitplane, UK). The DNA-damage marker, γ-H2AX, was used to indicate the lesion points. Nuclear DNA was stained with 4',6-diamidino-2-phenylindole (DAPI; 1 g/mL).

**Histology and immunohistochemistry**

Spleens excised from mice were fixed in 10% paraformaldehyde overnight and embedded in paraffin. The maximum cross sections (3 μm thick) were picked; primary antibodies were used for the following staining hCINAP, SUMO2/3, K67, and γ-H2AX. HRPC-conjugated secondary antibodies were used, and the signal was visualized using DAB (3, 3-diaminobenzidine). Apoptosis was detected by TUNEL using the In situ Cell Death Kit, Fluorescein following the manufacturer’s instructions (Beyotime, China).

**Laser-induced DNA-damage and live cell imaging**

Laser micro-irradiation is a specialized method to observe DSB repair protein recruitment. U2OS cells were grown in a glass-bottomed petri dish and topically irradiated with a 365-nm pulsed nitrogen laser. Laser-induced DNA-damage and live cell imaging (Olympus, Japan) was performed on an epifluorescence microscope (Olympus, Japan). Quantification was performed on 60–100 nuclei from each gel using Comet Assay IV software (Perceptive Instruments, UK). Olive tail moment was used as the parameter of this assay instead of tail length or DNA content, since it is independent of the comet shape and can better represent DNA-damage status.

**DNA repair assays**

In NHEJ assays, hCINAPWT and hCINAPΔ–3′ cells were transected with linearized pcDNA3.1/puromycin (Invitrogen) and the pEG-FP-CI plasmid. After 36 h, the cells were collected, counted, and plated on two plates. The plates were fixed and stained with 1 × PBS 48 h after transfection. Green (EGFP) and red (DsRed) fluorescence were measured by FACs on a FACSVerses instrument (BD Biosciences, USA). The ratio of red and DsRed double-positive cells was taken as the repair efficiency. The results were normalized to those of hCINAPWT cells. Samples were analyzed using Flowjo software to determine GFP-positive cells relative to cells expressing DsRed. U2OS-DR-GFP cells that were only transected with DsRed but lacked I-SceI were considered as the negative control (background level of HR). Repair frequencies were normalized to those of hCINAPWT.

**PDsA AML mice**

All animal experiments were approved by the Peking University Laboratory Animal Center. The donor mouse NOD/SCID mice (6–8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology and housed at Peking University Laboratory Animal Center following the ‘Principles for the Utilization and Care of Vertebrate Animals’ and ‘Guidance for the Care and Use of Laboratory Animals’. The serial number of production license for laboratory animals (SCXK) is SCXK-2016-0010. The serial number of use license for laboratory animals (SYXK) is SYXK-2016-0028. MR cells were isolated from the donor mice. In vitro proliferation and induction of the BM cells were performed, followed by GFP-tagged lentiviral infection to get two types of AML-BM hematopoietic stem cells, the CINAP knockdown and the control group. The two cell types were transfused intravascularly into 28 recipient mice (14 per group, 106 cells transplanted in each mouse) at Day0 (D0). In the next 10 days, leukemia development was monitored daily by physical appearance. To mimic the clinical medication practice, “7 + 3” chemotherapy (DNR from D10 to D12, HA-Ara-C from D10 to D16) was administered by tail vein injection. PB was analyzed by FACs at D10 and D20. The leukemia cell load (CD45+ cells) in the caudal vein, BM, and spleen were determined by TUNEL, FACS, and immunohistochemistry analyses at D26. The death date of every mouse in the two groups was recorded and the survival rate of each group was calculated at the end.

**Bioinformatics analysis**

RNA-seq analysis was performed by ANNONROAD. In brief, the total RNA from wild-type and knockout hCINAP U2OS cells was extracted, and sequencing was performed with Illumina Solexa Ultrasequencing. The downstream analysis includes differential expression analysis and KEGG pathway enrichment. For analyzing the expression level of hCINAP in patients with AML, the recompeted gene expression data sets of TCGA LAML cancer type and GTEx BM that were based on the gencode v23 gene model from UCSC Xena (http://xena.ucsc.edu/) were downloaded. The clinical data of TCGA LAML cancer type were downloaded from the GDC Data Portal (https://gdc-portal.nci.nih.gov/). The expression unit was TPM (transcript per million) and the survival rate of each patient was determined. For analyzing the expression data sets of TCGA LAML cancer and GTEx BM tissue for differential expression and survival analyses, the sample set included 243 samples (including survival information of 173 patients and 70 normal samples).

**Statistical analysis**

The statistical results were obtained from at least three independent biological replicates. Detailed n values for each panel in the Figs. are stated in the corresponding legends. All results were presented as mean ± SEM unless otherwise stated. P values were obtained via the Student’s t-test (two-tailed), Mann–Whitney test (for two group comparisons) or one-way ANOVA using GraphPad Prism 8.0 software. *P < 0.05, **P < 0.01, ***P ≤ 0.001. Survival curves were obtained from Kaplan–Meier estimates and validated with the log-rank test.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Data availability

A reporting summary for this Article is available as a Supplementary Information file. Unprocessed scans of western blots are provided in Supplementary Fig. 13. The source data underlying Figs. 1h, 2a, 4a, and Supplementary Fig. 1h are deposited in the figshare (https://doi.org/10.6084/m9.figshare.c.4438148.v2). RNA-sequencing data are available under the accession code GSE134342 at Gene Expression Omnibus (GEO). All data are available from the corresponding author upon reasonable request.

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Author contributions

R.X. designed the experiments, analyzed the data, and wrote the manuscript; S.Y., D.Z., X.H., J.Z., and Y.T. carried out the experiments. Y.R., Z.T., and X.Z. statistically analyzed the public data sets; X.Z. designed and conceptualized the research, supervised the experimental work, analyzed data, and wrote the manuscript.

Additional information

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