Genes & Diseases (2023) 10, 1739–1742

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.keaipublishing.com/en/journals/genes-diseases

RAPID COMMUNICATION

ZRANB1 is an NBS1 deubiquitinase and a potential target to overcome radioresistance and PARP inhibitor resistance in triple-negative breast cancer

Poly (ADP-ribose) polymerase inhibitors (PARPi) are used to treat ovarian cancer and triple-negative breast cancer (TNBC) with defective homologous recombination repair pathways. However, *de novo* and acquired PARPi resistance limits clinical benefits.[1] The MRE11-RAD50-NBS1 (MRN) complex mediates the sensing, processing, and signaling of DNA double-strand breaks (DSBs) and plays important roles in the efficacy of PARPi and radiation treatment,[2] and yet the mechanisms for the regulation and degradation of the MRN complex are not well understood. ZRANB1, also known as Trabid, is a breast cancer-promoting deubiquitinase that preferentially cleaves K29-, K33-, and K63-linked ubiquitin chains,[3] but its role in therapy resistance remains unknown.

To systematically identify the potential substrates and downstream pathways of ZRANB1, we performed a BioID-based interactome analysis along with SILAC-based quantitative proteomic profiling (Fig. S1A), by using a stable MYC-BioID2-tagged ZRANB1-expressing HEK293A cell line (Fig. S1B) and ZRANB1-knockout HEK293A cells generated by CRISPR-Cas9 (Fig. S1C).[3] The top 300 interactors identified by the BioID-based analysis are presented in Table S1 and the top 500 downregulated proteins identified by SILAC are presented in Table S2. To explore the cellular pathways regulated by ZRANB1, we subjected the ZRANB1-interacting proteins and the proteins downregulated in ZRANB1-knockout cells to enrichment analysis. Interestingly, both sets of proteins were enriched in the DNA ionizing radiation (IR)-DSBs and cellular response via the ATM pathway (Fig. S1D, E). We identified 20 overlapping proteins by the STRING Functional Protein Association Network analysis of the BioID interactome and the quantitative proteome. Pathway classification of these 20 proteins revealed a network of ZRANB1 interactors consisting of DNA damage response (DDR), homologous recombination (HR), DNA IR-DSBs, and cellular response via ATM, and breast cancer pathways (Fig. S1F). In particular, in the DNA IR-DSBs and cellular response via ATM pathway, four proteins, MRE11, RAD50, NBS1, and TP53, were identified in both the BioID interactome analysis and the quantitative proteome analysis (Fig. 1A).

To validate our mass spectrometry results, we performed pulldown assays, finding that ZRANB1 interacted with MRE11, RAD50, and NBS1, but not p53, in HEK293 cells (Fig. 1B). Moreover, the protein levels, but not the mRNA levels, of MRE11, RAD50, and NBS1 were markedly downregulated in ZRANB1-knockout HEK293A cells (Fig. 1C; Fig. S2A). To determine whether the regulation of MRN complex components is due to ZRANB1's deubiquitinating activity, we generated a catalytically inactive ZRANB1 mutant (C443A) and transfected wild-type or mutant ZRANB1 into ZRANB1-knockout HEK293A cells, finding that MRN complex expression levels could be restored by wild-type ZRANB1 but not catalytically inactive ZRANB1 (Fig. S2B), which suggests that ZRANB1's deubiquitinase activity is critical for stabilizing the MRN complex. Further, ectopic expression of ZRANB1 in ZRANB1-knockout HEK293A cells substantially reduced NBS1 polyubiquitination, but not the polyubiquitination of MRE11 and RAD50 (Fig. 1D; Fig. S2C–E), suggesting that NBS1, but not MRE11 or RAD50, is a candidate substrate of ZRANB1.

By using lysine-specific mutants of ubiquitin, we found that ZRANB1 specifically downregulated K33-linked polyubiquitination of NBS1 (Fig. S2F). To confirm that ZRANB1 stabilizes NBS1, we examined the protein half-life of NBS1. As expected, the knockout of ZRANB1 in HEK293A cells shortened the half-life of endogenous NBS1 protein (Fig. S3A, B). To determine whether ZRANB1 can bind and

Peer review under responsibility of Chongqing Medical University.

https://doi.org/10.1016/j.gendis.2022.09.013

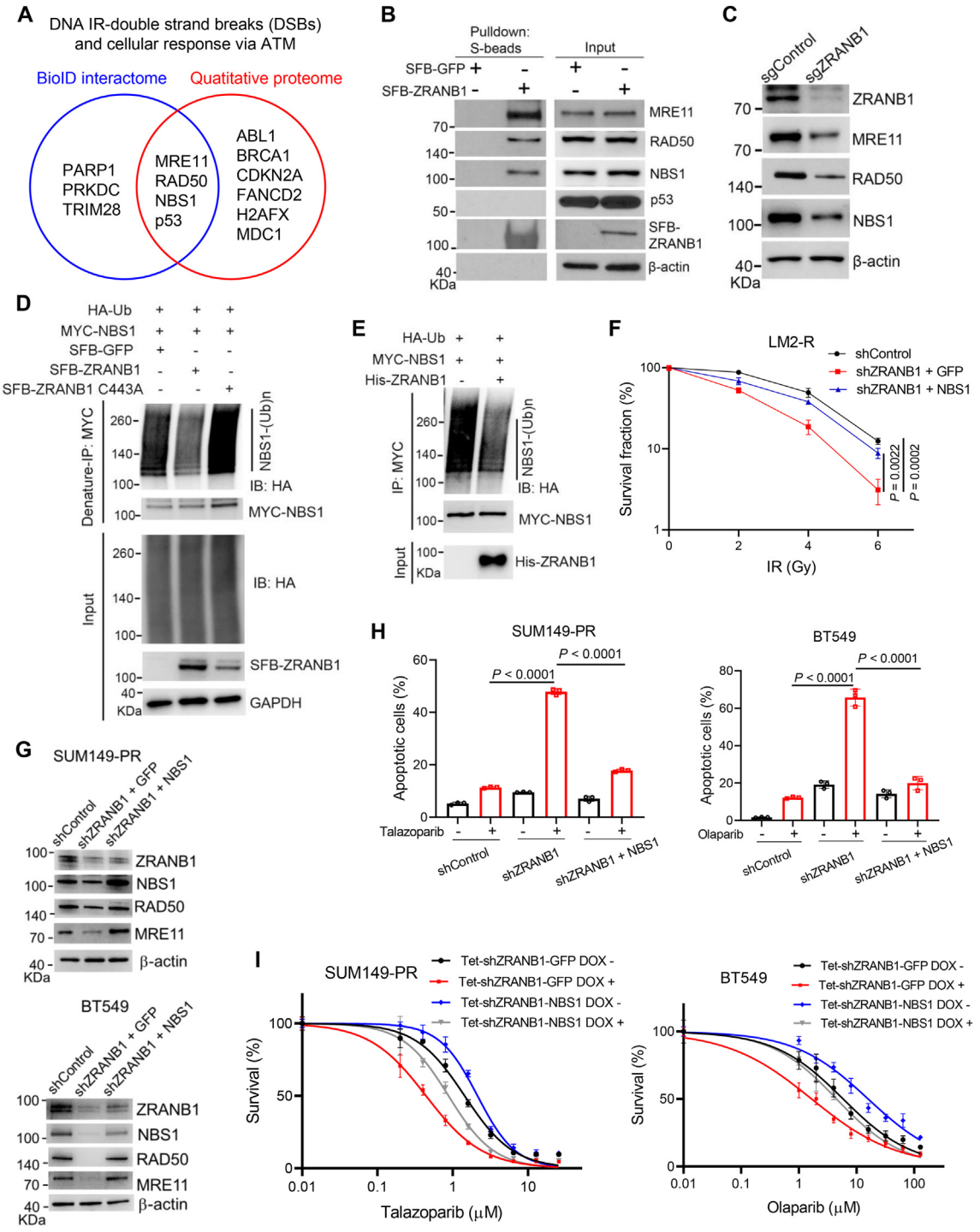

Figure 1 ZRANB1 is an NBS1 deubiquitinase and a potential target to overcome radioresistance and PARP inhibitor resistance in triple-negative breast cancer. **(A)** Venn diagram showing the overlapping proteins enriched in the DNA IR-DSBs and cellular response via ATM pathway that were identified in both the BioID interactome analysis (blue: ZRANB1-interacting proteins) and the quantitative proteome analysis (red: proteins downregulated in ZRANB1-knockout HEK293A cells). **(B)** HEK293T cells were transfected with SFB-GFP or SFB-ZRANB1, followed by pulldown with S-protein beads and immunoblotting with antibodies against MRE11, RAD50, NBS1, p53, FLAG, and β-actin. **(C)** Immunoblotting of ZRANB1, MRE11, RAD50, NBS1, and β-actin in ZRANB1-knockout HEK293A cells. **(D)** ZRANB1-knockout HEK293A cells were co-transfected with MYC-NBS1, HA-ubiquitin (Ub), and SFB-ZRANB1 or SFB-ZRANB1 (C443A), followed by immunoprecipitation with anti-MYC beads and immunoblotting with antibodies against HA and MYC. Cells were treated with 10 μM MG132 for 6 h before collection. Before immunoprecipitation, lysates were heated at 95 °C for 5 min in the presence of 1% SDS (for denaturing), followed by a 10-fold dilution with lysis buffer and sonication. **(E)** Ubiquitinated MYC-NBS1 was purified with anti-MYC beads from ZRANB1-knockout HEK293A cells co-transfected with MYC-NBS1 and HA-ubiquitin (Ub), and was incubated with purified His-ZRANB1. After the *in-vitro* deubiquitination reaction, the bound proteins were eluted by

deubiquitinate NBS1 directly, we performed *in vitro* binding assays and deubiquitination assays using purified proteins. Indeed, recombinant His-ZRANB1 directly bound to purified GST-NBS1 (Fig. S3C), and purified ZRANB1 protein markedly decreased NBS1 polyubiquitination in a cell-free system (Fig. 1E) in a K33 linkage-specific manner (Fig. S3D), indicating that NBS1 is a K33 linkage-specific substrate of ZRANB1. To our knowledge, this is the first report of the deubiquitinating enzyme of NBS1.

ZRANB1 consists of three N-terminal zinc-finger domains, a central ankyrin repeat ubiquitin-binding domain, and a C-terminal OTU domain.[3] We performed a deletion analysis by using different truncation mutants of ZRANB1 (Fig. S4A), demonstrating that the OTU domain-mediated the interaction between ZRANB1 and NBS1 (Fig. S4B). To determine the domains of NBS1 responsible for ZRANB1 binding, we co-transfected various deletion mutants of NBS1 (Fig. S4C) with HA-tagged ZRANB1, followed by co-immunoprecipitation (co-IP). Whereas wild-type NBS1 and the ΔFHA, ΔBRCT1, and ΔC mutants interacted with ZRANB1, the ΔBRCT2 mutant failed to do so (Fig. S4D), suggesting that the BRCT2 domain is required for the interaction with ZRANB1. Taken together, these results indicate that the OTU domain of ZRANB1 interacts with the BRCT2 domain of NBS1.

Non-homologous end-joining (NHEJ) and homologous recombination (HR) are the main pathways for DSB repair, and defects in either pathway are associated with elevated radiosensitivity. Interestingly, ZRANB1 was upregulated in a time-dependent manner upon IR treatment (Fig. S5A). We previously selected radioresistant subpopulations from the LM2 lung-metastatic breast cancer cell line, called LM2-R cells (Fig. S5B).[4] Compared with the parental LM2 cells, ZRANB1 was markedly upregulated in LM2-R cells (Fig. S5C). shRNA-mediated knockdown of ZRANB1 in LM2-R cells drastically reduced protein levels of the MRN complex components (Fig. S5D) and significantly decreased the survival fraction after IR treatment (Fig. S5E). Strikingly, the protein level of the DNA break marker γH2AX was much higher in ZRANB1-knockdown LM2-R cells compared with the control LM2-R cells upon irradiation (Fig. S5F).

NBS1 is known to stabilize MRE11-RAD50 protein—DNA complexes. To determine whether ZRANB1 regulates the MRN complex and radioresistance through NBS1, we overexpressed NBS1 in ZRANB1-knockout HEK293A cells and found that ectopic expression of NBS1 rescued MRE11 and RAD50 protein levels (Fig. S5G). Moreover, in ZRANB1-knockdown LM2-R cells, ectopic expression of NBS1 also rescued MRE11 and RAD50 expression (Fig. S5H) and radioresistance (Fig. 1F). To determine whether ZRANB1 regulates DSB repair through NBS1, we performed NHEJ and HR reporter assays in the LM2-DRR reporter cell line established in our recent study (Fig. S5I),[4] and we found that depletion of ZRANB1 significantly decreased HR and NHEJ repair efficiency, while the inhibition of HR was greater than that of NHEJ (Fig. S5J). Furthermore, the inhibited HR repair efficiency caused by ZRANB1 knockdown was significantly rescued by overexpression of NBS1 (Fig. S5J). Collectively, these results suggest that ZRANB1 depletion overcomes tumor cell radioresistance through, at least in part, downregulation of the MRN complex and HR repair efficiency.

PARPi, such as olaparib and talazoparib, exploits HR deficiency to induce synthetic lethality. The MRN complex is a critical regulator of HR repair, and defects in the HR pathway or the MRN complex can synergize with PARPi and improve drug efficacy. Because ZRANB1 knockdown caused MRN complex downregulation and HR deficiency (Fig. S5J), we hypothesized that ZRANB1 depletion would increase PARPi sensitivity through downregulation of MRN complex-mediated HR repair. To test this hypothesis, we examined ZRANB1 expression in the parental SUM149 human breast cancer cell line and a PARPi-resistant SUM149 cell line (hereafter named SUM149-PR) derived from talazoparib treatment,[5] and we found that ZRANB1 was upregulated in SUM149-PR cells compared with the parental SUM149 cells (Fig. S6A). Next, we established a SUM149-PR stable cell line expressing doxycycline (DOX)-inducible ZRANB1 shRNA. We found that DOX-induced ZRANB1 knockdown in SUM149-PR cells downregulated MRN complex expression and decreased the IC50 value of talazoparib from 2.02 μM to 0.82 μM (Fig. S6B, C). BT549 is a *BRCA1* wild-type, HR-proficient TNBC cell line with *de novo* olaparib resistance. Similar to the SUM149-PR cells with acquired PARPi resistance, DOX-induced ZRANB1 knockdown in BT549 cells also decreased MRN complex expression and reduced the IC50 value of olaparib from 3.65 μM to 1.9 μM (Fig. S6D, E).

The MRN complex facilitates HR repair through DNA end resection and generation of a long 3′ single-stranded DNA (ssDNA). pRPA32 (S4/8) is a surrogate marker of ssDNA. In both SUM149-PR and BT549 cells, ZRANB1 knockdown not only decreased MRN expression but also reduced PARPi-induced pRPA32 (S4/8) expression and augmented PARPi-induced γH2AX expression (Fig. S6F), suggesting that loss of ZRANB1 impairs DNA resection and leads to HR repair defects and PARPi-induced accumulation of DSBs.

boiling in Laemmli sample buffer and immunoblotted with antibodies against HA and MYC. **(F)** Clonogenic survival assays of ZRANB1-knockdown LM2-R cells with ectopic expression of V5-GFP or V5-NBS1. $n = 3$ wells per group. **(G)** ZRANB1-knockdown SUM149-PR (upper panel) and BT549 cells (lower panel) were transduced with V5-GFP or V5-NBS1, followed by immunoblotting with antibodies against ZRANB1, NBS1, RAD50, MRE11, and β-actin. **(H)** ZRANB1-knockdown SUM149-PR and BT549 cells were transduced with V5-GFP or V5-NBS1. The percentage of apoptosis in talazoparib-treated SUM149-PR cells (left panel) and olaparib-treated BT549 cells (right panel) was analyzed by flow cytometry (representative flow cytometry plots are shown in Fig. S6G). $n = 3$ wells per group. **(I)** Left panel: SUM149-PR cells stably expressing doxycycline (DOX)-inducible shZRANB1 were transduced with V5-GFP or V5-NBS1, followed by treatment with different concentrations of talazoparib with or without DOX for 6 days. Right panel: BT549 cells stably expressing doxycycline (DOX)-inducible shZRANB1 were transduced with V5-GFP or V5-NBS1, followed by treatment with different concentrations of olaparib with or without DOX for 3 days. Cell viability was measured by CCK8 assays. $n = 3$ wells per group. IC50 values are shown in Figure S7C. Statistical significance in **F** and **H** was determined by a two-tailed unpaired *t*-test (Error bars: mean ± standard deviation).

To determine whether NBS1 mediates the role of ZRANB1 in PARPi resistance, we overexpressed NBS1 in ZRANB1-knockdown SUM149-PR and BT549 cells and found that NBS1 overexpression rescued the expression of MRE11 and RAD50 (Fig. 1G). Importantly, the knockdown of ZRANB1 markedly increased talazoparib-induced apoptosis of SUM149-PR cells and olaparib-induced apoptosis of BT549 cells (Fig. 1H; Fig. S6G). The sensitization of ZRANB1-knockdown breast cancer cells to PARPi treatment was reversed by overexpression of NBS1 (Fig. 1H, I; Fig. S7A—C). Altogether, these results indicate that ZRANB1 depletion overcomes *de novo* and acquired PARPi resistance through, at least in part, downregulation of NBS1.

Consistent with our observation that ZRANB1-stabilized NBS1 maintains the stability of the MRN complex, analysis of the MRN complex protein levels of 30 breast cancer cell lines in the DepMap database and 74 breast cancer tissues in the Clinical Proteomic Tumor Analysis Consortium (CPTAC) database showed that NBS1 protein expression positively correlated with MRE11 and RAD50 protein levels (Fig. S8A—D). Furthermore, analysis of the drug sensitivity data in the DepMap database revealed that protein levels of the MRN complex positively correlated with olaparib resistance (Fig. S8E—G). Based on our findings that ZRANB1 depletion in TNBC cells reversed radioresistance and PARPi resistance through destabilization of NBS1 and downregulation of the MRN complex, ZRANB1 represents a potential target to overcome radioresistance and PARPi resistance in TNBC.

Author contributions

Y.M. and L.M. conceived and designed the study. Y.M. performed and analyzed most of the experiments. Q.H. established the LM2-R and LM2-DRR cell lines. Q.H., H.T., and F.Y. provided technical assistance. M.-K.C. and M.-C.H. provided the PARPi-resistant SUM149 cell line (SUM149-PR). Y.S. contributed protocols and provided consultation. Y.M. and L.M. wrote the manuscript with input from all other authors. L.M. supervised the study and allocated funding. All experiments were performed at MD Anderson Cancer Center.

Conflict of interests

All authors declare that there is no potential conflict of interests.

Funding

L.M. is supported by a US National Institutes of Health (NIH) grant R01CA166051 and a Cancer Prevention and Research Institute of Texas (CPRIT) grant RP190029. M.-C.H. was supported by the Sister Institution Fund of MD Anderson Cancer Center and China Medical University. The cores are supported by MD Anderson's Cancer Center Support Grant (CCSG, No. P30CA016672) from NIH.

Acknowledgements

We thank Dr. Xiang Zhang for providing the LM2 cell line, Dr. Hui-Kuan Lin for providing the NBS1 mutant plasmids, Dr. Junjie Chen for providing the HEK293A cell line and the Bio-ID plasmids, and Dr. Litong Nie for assistance with mass spectrometric analysis. We thank MD Anderson's Functional Genomics Core and Characterized Cell Line Core Facility for technical support, Ashli Nguyen-Villarrealand and Sarah Bronson for editing this manuscript, and members of the Ma Lab for useful discussion.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.09.013.

References

1. D'Andrea AD. Mechanisms of PARP inhibitor sensitivity and resistance. *DNA Repair*. 2018;71:172—176.
2. Bian L, Meng Y, Zhang M, et al. MRE11-RAD50-NBS1 complex alterations and DNA damage response: implications for cancer treatment. *Mol Cancer*. 2019;18(1):169.
3. Zhang P, Xiao Z, Wang S, et al. $ZRANB_1$ is an EZH2 deubiquitinase and a potential therapeutic target in breast cancer. *Cell Rep*. 2018;23(3):823—837.
4. Hang Q, Zeng L, Wang L, et al. Non-canonical function of DGCR8 in DNA double-strand break repair signaling and tumor radioresistance. *Nat Commun*. 2021;12(1):4033.
5. Chu YY, Yam C, Chen MK, et al. Blocking c-Met and EGFR reverses acquired resistance of PARP inhibitors in triple-negative breast cancer. *Am J Cancer Res*. 2020;10(2):648—661.

Ying Mei [a], Qinglei Hang [a], Hongqi Teng [a], Fan Yao [a,d], Mei-Kuang Chen [b], Mien-Chie Hung [b], Yutong Sun [b], Li Ma [a,c,*]

[a] *Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA*
[b] *Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA*
[c] *The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences, Houston, TX 77030, USA*
[d] *Hubei Hongshan Laboratory, College of Biomedicine and Health, College of Life Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, China*

*Corresponding author. Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA.
E-mail address: lma4@mdanderson.org (L. Ma)

17 September 2022
Available online 15 October 2022