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RAPID COMMUNICATION

Super-enhancer-associated TTC8 alters the nucleocytoplasmic distribution of PHOX2B and activates MAPK signaling in neuroblastoma

Super-enhancers are regions of the mammalian genome comprising multiple enhancers in close proximity and often enriched at cancer genes.[1] Recent studies suggest the emerging roles of super-enhancer-associated genes in underlying the cell identity of neuroblastoma (NB)[2] which rises from neural crest-derived cells and is one of the most common malignant solid tumors in children. Tetratricopeptide repeat domain 8 (TTC8), a member of the BBS family of proteins involved in ciliary transport,[3] is our newly identified super-enhancer-associated gene in NB, and its roles in tumorigenesis remain elusive. Herein, TTC8 expression levels were significantly upregulated in NB and correlated with poor prognosis in patients through integrative analysis of public datasets and microarrays. Further investigations show that TTC8 drives the growth and aggressiveness of NB cell lines both *in vitro* and *in vivo*. Mechanistically, TTC8 interacts with Paired-like homeobox 2B (PHOX2B), a master transcription factor in NB,[4] to regulate the nucleocytoplasmic distribution of PHOX2B, thereby activating the MAPK signaling pathway, promoting the occurrence and development of NB. In summary, we find that TTC8 might be a potential target for the treatment of NB, warranting further exploration of its specific mechanisms of action.

Super-enhancers can be identified by extensive active histone marks, such as histone 3 lysine 27 acetylation (H3K27Ac).[1] To identify genes associated with super-enhancers in NB, we performed ChIP-seq analysis for H3K27ac in 4 NB cases and 5 NB cell lines from published ChIP-seq data sets. Super-enhancers are defined as those that showed high H3K27ac enrichment (Fig. 1A; Fig. S1A).[1] The result showed that TTC8 and a number of known NB oncogenes such as PHOX2B, MEIS2, GATA3 and HAND2 were enriched (Table. S2).[2] By re-analyzing the publicly available Hi-C data of the SK-N-DZ cell line, we confirmed extensive interactions between TTC8 enhancers and promoters (indicated by connecting lines above the ChIP-seq profiles in Fig. 1B). Luciferase reporter assay showed robust luciferase reporter activities of E2 and E3, but weak activity of E1 (Fig. S1C), consistent with the H3K27ac signal. BRD4 is a transcriptional and epigenetic regulator known to be involved in super-enhancer organization and transcriptional control in malignancies. GNE987 and MZ1 are two BRD4 inhibitors that have been developed. As expected, the expression of TTC8 and the other known super-enhancer-associated genes significantly decreased in SK-N-BE(2) cells treated with GNE987 or MZ1 (Fig. S1D, E). Taken together, TTC8 is our newly identified super-enhancer-associated gene in NB.

Further analysis of The Cancer Cell Line Encyclopedia (CCLE) and three different R2 databases revealed that TTC8 is highly expressed in NB and associated with disease progression (Fig. 1C; Fig. S2A–C and Table S4). To determine the protein levels of TTC8 in clinical samples, we analyzed the protein expression of TTC8 in human NB samples and found that TTC8 protein expression was significantly higher in neuroblastoma tissues compared to peripheral nerve tissues (Fig. 1D; Fig. S2D). Together, these findings strongly suggest that TTC8 is upregulated and might act as a potential oncogene in NB. Hence, we further explored the function of TTC8 in NB.

As TTC8 was found to be expressed highly in neuroblastoma and to be associated with poor prognosis, we explored the function of TTC8 by knocking down its expression in NB cell lines both *in vitro* and *in vivo*. The results indicated that TTC8 could promote the growth and aggressiveness of NB cells *in vitro* (Fig. S3A–I). To

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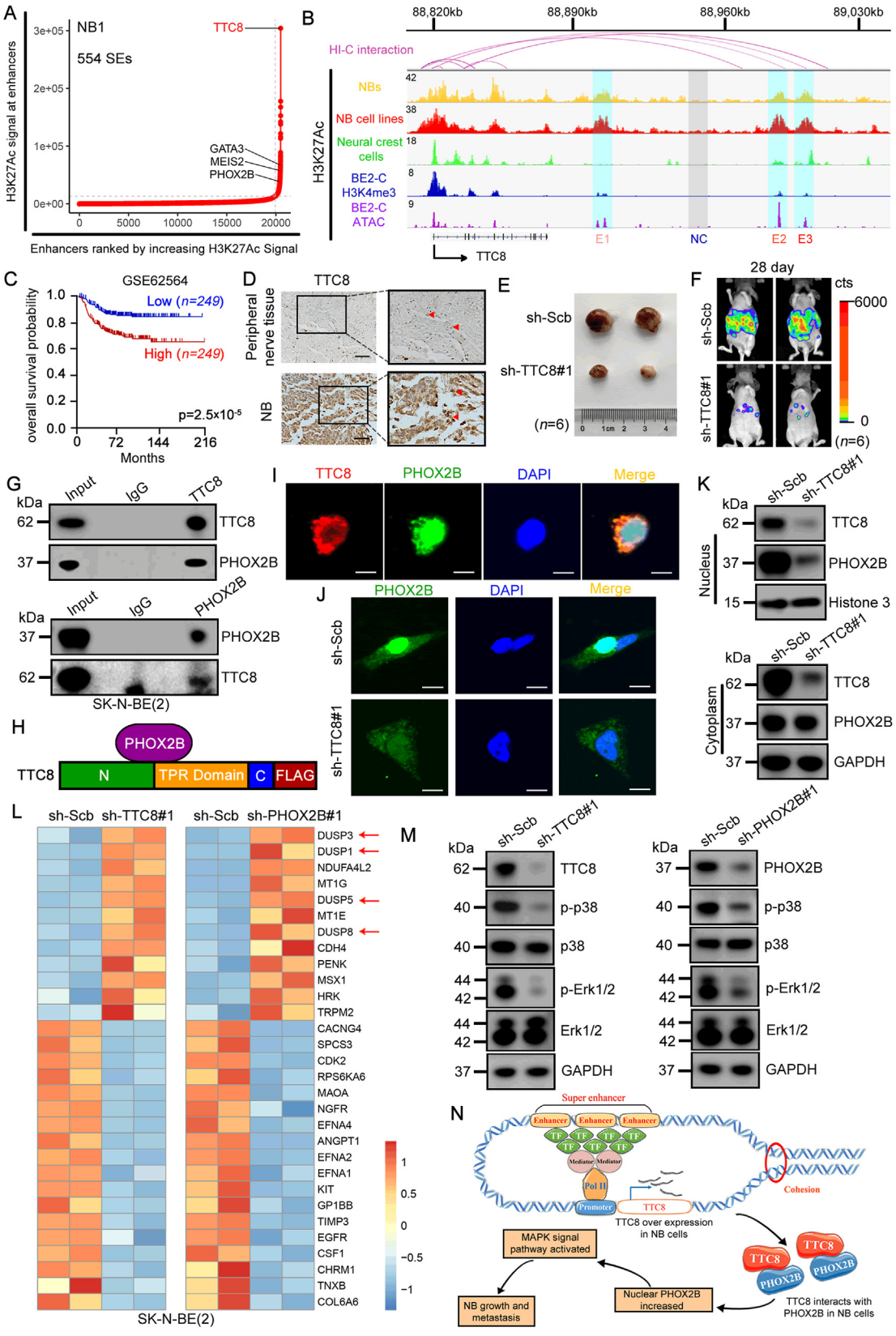

Figure 1 Super-enhancer-associated TTC8 alters the nucleocytoplasmic distribution of PHOX2B and activates MAPK signaling in neuroblastoma. **(A)** The hockey stick plots shown genes associated with super-enhancers. **(B)** The ChIP-seq gene tracks represent the H3K27Ac signal in NB tumors, NB cell lines, and neural crest cells, while the last two tracks represent H3K4me3 and ATAC signals in BE(2)-C at the TTC8 gene loci. Hi-C interactions were re-analyzed from the Hi-C data of SK-N-DZ cell line. **(C)** Kaplan–Meier curves indicating the survival of NB patients with high or low TTC8 expression. **(D)** Representative immunohistochemistry

investigate the effects of TTC8 knockdown on NB growth *in vivo*, we established subcutaneous xenograft mouse model. During the experiment, we observed that the tumors with TTC8 knockdown were significantly smaller and with fewer blood vessels (Fig. 1E; Fig. S4A–I). In the NB metastasis model, more widespread metastasis was observed in the control group (Fig. 1F). Moreover, mice in the TTC8 knockdown group had a higher survival rate (Fig. S5B).

To identify the proteins with which TTC8 interacts, we scanned the public geneMANIA database and found PHOX2B (Fig. S7A), a transcription factor, which is known to play important roles in NB.[4] Co-IP indicated the interaction between TTC8 and PHOX2B proteins, and the N-terminus and TPR domains of TTC8 were found to be crucial for its binding to PHOX2B (Fig. 1G, H; Fig. S7C). Protein transport is a key function of BBS family proteins.[3] For example, BBS7 has a dynamic cellular localization pattern and has the capacity to enter the nucleus. The localization of the BBS7 protein is changed from the cytoplasm to the nuclear membrane or the nucleus when a part of it interacts with the nuclear protein RNF2. As an important transcription factor in neuroblastoma, PHOX2B is mainly located in the nucleus and needs to enter the nucleus after its protein synthesis to regulate the transcription of NB-specific oncogenes. Given that TTC8 acts as a protein transporter,[3] we speculated that it could also act as a nucleoplasmic shuttle protein to assist PHOX2B in entering the nucleus and modulating transcription. First, immunofluorescence co-localization assay showed that the binding site of TTC8 and PHOX2B was mainly around the nuclear membrane (Fig. 1I). Afterwards, using immunofluorescence assay followed by confocal imaging and Western blotting, we confirmed that knocking down the expression of TTC8 reduced the level of nuclear PHOX2B (Fig. 1J, K). Together, these data suggested that TTC8 might modulate to the nuclear distribution of PHOX2B to promote its transcription-regulating activity.

We next studied the relationship between the expression levels of these two proteins. PHOX2B expression was decreased in cells after TTC8 knockdown, but no significant changes in the expression of TTC8 after knocking down PHOX2B were observed (Fig. S7D). In our study, we proved that overexpression of PHOX2B ameliorates the tumor suppressive effects of TTC8 deficiency in NB cells (Fig. S8A–L). Collectively, these data highlight PHOX2B as an important functional target of TTC8 and strongly supports the notion that the TTC8–PHOX2B signaling axis is involved in the regulation of NB growth and aggressiveness.

To examine the molecular mechanisms by which TTC8 contributes to tumorigenesis and metastasis, we performed RNA-seq to screen for differentially expressed genes in NB cells after silencing TTC8 or PHOX2B. Analysis of RNA-seq data revealed that expression of a number of negative regulators of MAPK pathway, such as DUSP1, DUSP3, DUSP5 and DUSP8,[5] were prominently altered in both TTC8 and PHOX2B knockdown cells (Fig. 1L). MAPKs are a highly conserved serine/threonine protein kinase family that plays an important role in the occurrence and development of tumors. Further analysis of Western blotting suggested that phosphorylation levels of Erk1/2 and p38 were decreased in both TTC8 and PHOX2B knockdown cells (Fig. 1M; Fig. S9C). These findings indicate that TTC8 regulates MAPK signaling pathways through interactions with PHOX2B.

In summary, we find that TTC8 might function as an oncogene through interactions with PHOX2B to increase the distribution of PHOX2B into the nucleus and activate the MAPK signaling pathway in NB (Fig. 1N). Our study could favor better understanding of the mechanisms underlying NB progression, and suggests TTC8–PHOX2B–MAPK axis as a potential target for NB therapy.

Conflict of interests

The authors declare that they have no conflict of interests.

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staining of NB tissue microarray slides showing the expression of TTC8. Scale bars: 50 μm. **(E)** Representative nude mice images of xenografts formed by subcutaneous injection of SK-N-BE(2) cells through the armpit of nude mice ($n = 6$ per group). **(F)** Representative images of nude mice treated with tail vein injection of SK-N-BE(2) cells ($n = 6$ per group). **(G–I)** Co-IP, Western blot assays and immunofluorescence indicating the interaction between TTC8 and PHOX2B proteins. Scale bars, 10 μm. **(J, K)** Western blot and immunofluorescence confocal images showing the subcellular distribution of PHOX2B in SK-N-BE(2) cells transfected with sh-Scb or sh-TTC8 for 72 h. Scale bars, 10 μm. **(L)** Heatmap showing the expression of the cancer-related genes differentially expressed in both TTC8-knockdown and PHOX2B-knockdown SK-N-BE(2) cells. **(M)** Western blot analysis of p-38 and p-Erk expression in TTC8-knockdown and PHOX2B-knockdown SK-N-BE(2) cells. **(N)** A proposed model of the mechanism by which super-enhancer-associated TTC8 promotes neuroblastoma progression.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.07.014.

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