Published in final edited form as:

Acta Neuropathol. 2021 July ; 142(1): 217–221. doi:10.1007/s00401-021-02331-1.

TBK1 haploinsufficiency in ALS and FTD compromises membrane trafficking

Yubing Lu^{1,2}, Sandra Almeida¹, Fen-Biao Gao¹

¹Department of Neurology, University of Massachusetts Medical School, Worcester, MA 01605, USA

²Department of Pathology, Case Western Reserve University, Cleveland, OH 44106, USA

Keywords

ALS; FTD; iPSC; TBK 1; Membrane trafficking; Neurons

TANK-binding kinase 1 (*TBK1*) haploinsufficiency causes both amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) [3, 5, 13]. TBK1 is an IKK-related serine/threonine kinase and primarily known for its involvement in inflammation and autophagy [4]. In particular, TBK1 phosphorylates several autophagy adaptor proteins, including SQSTM1/p62, optineurin, and NDP52 [6, 10, 11, 15]. However, other phosphorylation substrates of TBK1 are largely unknown.

To understand how *TBK1* haploinsufficiency causes ALS and FTD, it is essential to understand the full range of normal cellular functions of TBK1 in both neuronal and nonneuronal cells. We expressed EGFP or EGFP–TBK1 in HEK293 cells and performed an unbiased interactome analysis (Fig. 1a). We identified 302 proteins that interacted with EGFP–TBK1 but not EGFP (Supplementary Fig. 1a and Supplementary Table 1). These proteins included some known TBK1-interacting proteins such as TANK, TBKBP1, optineurin, AZI2, TRAF2, and syntaxin 17, as well as many potentially novel TBK1-interacting proteins such as several t-SNARE proteins, including syntaxins 4, 7, 12, 17, and 18 (Fig. 1b). Further analysis showed that syntaxins 4, 12, and 17 were among the top 100 TBK1-interacting proteins (Supplementary Fig. 1b). Here, we decided to focus on syntaxin 12 (also known as syntaxin 13, hereafter, STX12/13) because we previously showed that the corresponding gene is a strong genetic modifier of mutant CHMP2B toxicity associated with FTD3 [9].

To confirm STX12/13 is indeed a TBK1-interacting protein, we found EGFP–TBK1 bound to ectopically expressed STX12/13 in a co-immunoprecipitation (IP) experiment (Fig. 1c). Moreover, these two proteins were partially colocalized in transfected cells (Fig. 1d), further indicating their biochemical interaction. Either transfected TBK1 (Fig. 1e) or endogenous

[✉]Yubing Lu, yxl2282@case.edu. [✉]Fen-Biao Gao, fen-biao.gao@umassmed.edu.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00401-021-02331-1>.

TBK1 (Fig. 2b) also formed a complex with endogenous STX12/13. To examine the functional significance of their association, we found TBK1 phosphorylated several serine and threonine residues on STX12/13 (Supplementary Table 2). Thus, STX12/13 is a novel substrate for TBK1 kinase activity.

STX12/13 is primarily localized in the endosome and interacts with the v-SNARE protein VAMP3 [1, 14, 17]. Indeed, mCherry-tagged STX12/13 and EGFP-tagged VAMP3 largely colocalized in transfected cells (Fig. 2a). To study the effect of TBK1 knockdown, we identified three siRNAs that could effectively knock down the expression of TBK1 in HEK293 cells (Supplementary Fig. 2a). After TBK1 knockdown, STX12/13 remained colocalized with VAMP3 (Fig. 2a), but their interaction became stronger (Fig. 2b, c, Supplementary Fig. 2c). Thus, TBK1 may have a function in the dissociation of the STX12/13–VAMP3 complex during membrane trafficking.

To examine the functional consequence of loss of TBK1 activity, we took advantage of the involvement of VAMP3 and STX12/13 in vesicular trafficking and secretion of matrix metalloproteinases (MMPs) [7, 16]. We used an ELISA assay to measure secreted MMP2 as the representative of the cargos (Fig. 2d) and found that *TBK1* knockdown by three separate siRNAs all resulted in a decrease in secreted MMP2 level (Fig. 2e), revealing a novel role for TBK1 in vesicular trafficking and secretion of extracellular cargos. The detailed mechanism underlying this process remains to be further investigated.

To study the role of TBK1 in human neurons and pathogenic mechanisms of ALS/FTD caused by *TBK1* haploinsufficiency, we used CRISPR–Cas9 technology to generate *TBK1* knockout-induced pluripotent stem cell (iPSC) lines. Using guide RNAs targeting exon 2 (Fig. 3a), we obtained *TBK1*^{−/−} lines L3 and L4 lacking full-length TBK1 (Fig. 3b) caused by a 14-basepair deletion in one allele and a 50-base-pair deletion in the other (Fig. 3c, Supplementary Fig. 3a). To exclude off-target effects associated with guide RNAs, we used second set of guide RNAs in exon 3 (Fig. 3a) and obtained two *TBK1*^{−/+} iPSC lines, L14 and L21 as a cellular model for ALS/FTD caused by TBK1 haploinsufficiency (Fig. 3b). In these heterozygous mutant lines, an 83-basepair deletion in the mutant allele created a premature stop codon in the *TBK1* coding region and decreased protein expression (Fig. 3c, Supplementary Fig. 3a). These *TBK1*^{−/+} and *TBK1*^{−/−} iPSC lines expressed pluripotent stem cell markers (Supplementary Fig. 3b). Karyotyping analysis showed that up to 15% of *TBK1*^{−/+} and *TBK1*^{−/−} iPSCs had a tetraploid phenotype lacking in the parental iPSCs (Supplementary Fig. 4). These results are consistent with previous reports that loss of TBK1 can increase the percentage of tetraploid cells in other proliferating cells [12].

To study TBK1 haploinsufficiency and model ALS/FTD in human neurons, we differentiated one parental and two *TBK1*^{−/+} iPSC lines into postmitotic neurons. We used a previously published protocol [2, 8] with some modifications in which terminal neuronal differentiation occurs in about one month after the start of the differentiation process (Fig. 3d). After differentiation, about 80% of cells were MAP2-positive neurons (Fig. 3e, f), of which about 85% were VGLUT1-positive excitatory neurons (Fig. 3g) and 10% were inhibitory neurons (Supplementary Fig. 5). Complete or partial loss of TBK1 did not affect the percentage of MAP2-positive neurons or the relative abundance of excitatory neurons

(Fig. 3e–g) or inhibitory neurons (Supplementary Fig. 5), suggesting loss of TBK1 did not affect neuronal differentiation itself. As in HEK293 cells, partial loss of TBK1 in *TBK1*^{+/+} iPSCs enhanced the interaction between VAMP3 and STX12/13 (Supplementary Fig. 6). Moreover, human neurons with TBK1 deficiency show decreased MMP2 secretion (Fig. 3h), further supporting a novel role for TBK1 in the vesicular trafficking pathway.

The results of our interactome analysis suggest that TBK1 haploinsufficiency may contribute to ALS/FTD through more molecular pathways than previous thought, such as vesicular trafficking. Although we used MMP2 as a representative cargo, it is likely that TBK1 also regulates the levels of many other extracellular proteins. The underlying molecular mechanisms in human neurons remain to be further investigated. Our findings here provide a foundation for further analysis of the multifaceted functions of TBK1 in ALS/FTD pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the UMMS Proteomics Core for help with the mass spectrometry analysis. This work was supported by NIH grants R37NS057553 and R01NS101986 (FBG), and experiments for revision were done by YL in Dr. Xiongwei Zhu's lab and supported by NIH grant R03NS120064 (YL).

References

1. Advani RJ, Bae HR, Bock JB, Chao DS, Doung YC, Prekeris R et al. (1998) Seven novel mammalian SNARE proteins localize to distinct membrane compartments. *J Biol Chem* 273:10317–10324 [PubMed: 9553086]
2. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L (2009) Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 27:275–280 [PubMed: 19252484]
3. Cirulli ET, Lasseigne BN, Petrovski S, Sapp PC, Dion PA, Leblond CS et al. (2015) Exome sequencing in amyotrophic lateral sclerosis identifies risk genes and pathways. *Science* 347:1436–1441 [PubMed: 25700176]
4. Evans CS, Holzbaur ELF (2019) Autophagy and mitophagy in ALS. *Neurobiol Dis* 122:35–40 [PubMed: 29981842]
5. Freischmidt A, Wieland T, Richter B, Ruf W, Schaeffer V, Müller K et al. (2015) Haploinsufficiency of TBK1 causes familial ALS and fronto-temporal dementia. *Nat Neurosci* 18:631–636 [PubMed: 25803835]
6. Heo JM, Ordureau A, Paulo JA, Rinehart J, Harper JW (2015) The PINK1-PARKIN mitochondrial ubiquitylation pathway drives a program of OPTN/NDP52 recruitment and TBK1 activation to promote mitophagy. *Mol Cell* 60:7–20 [PubMed: 26365381]
7. Kean MJ, Williams KC, Skalski M, Myers D, Burtnik A, Foster D et al. (2009) VAMP3, syntaxin-13 and SNAP23 are involved in secretion of matrix metalloproteinases, degradation of the extra-cellular matrix and cell invasion. *J Cell Sci* 122:4089–4098 [PubMed: 19910495]
8. Li M, Zhao H, Ananiev GE, Musser MT, Ness KH, Maglaque DL et al. (2017) Establishment of reporter lines for detecting fragile X mental retardation (FMR1) gene reactivation in human neural cells. *Stem Cells* 35:158–169 [PubMed: 27422057]
9. Lu Y, Zhang Z, Sun D, Sweeney ST, Gao F-B (2013) Syntaxin 13, a genetic modifier of mutant CHMP2B in frontotemporal dementia, is required for autophagosome maturation. *Mol Cell* 52:264–271 [PubMed: 24095276]

10. Matsumoto G, Shimogori T, Hattori N, Nukina N (2015) TBK1 controls autophagosomal engulfment of polyubiquitinated mitochondria through p62/SQSTM1 phosphorylation. *Human Mol Genet* 24:4429–4442 [PubMed: 25972374]
11. Moore AS, Holzbaur EL (2016) Dynamic recruitment and activation of ALS-associated TBK1 with its target optineurin are required for efficient mitophagy. *Proc Natl Acad Sci USA* 113:E3349–E3358 [PubMed: 27247382]
12. Pillai S, Nguyen J, Johnson J, Haura E, Coppola D, Chellappan S (2015) Tank binding kinase 1 is a centrosome-associated kinase necessary for microtubule dynamics and mitosis. *Nat Commun* 6:10072 [PubMed: 26656453]
13. Pottier C, Bieniek KF, Finch N, van de Vorst M, Baker M, Perk-ersen R et al. (2015) Whole-genome sequencing reveals important role for TBK1 and OPTN mutations in frontotemporal lobar degeneration without motor neuron disease. *Acta Neuropathol* 130:77–92 [PubMed: 25943890]
14. Prekeris R, Klumperman J, Chen YA, Scheller RH (1998) Syntaxin 13 mediates cycling of plasma membrane proteins via tubulovesicular recycling endosomes. *J Cell Biol* 143:957–971 [PubMed: 9817754]
15. Richter B, Sliter DA, Herhaus L, Stolz A, Wang C, Beli P et al. (2016) Phosphorylation of OPTN by TBK1 enhances its binding to Ub chains and promotes selective autophagy of damaged mitochondria. *Proc Natl Acad Sci USA* 113:4039–4044 [PubMed: 27035970]
16. Sbati O, Ferhat L, Bernard A, Gueye Y, Ould-Yahoui A, Thiolloy S et al. (2008) Vesicular trafficking and secretion of matrix metal-loproteinases-2, -9 and tissue inhibitor of metalloproteinases-1 in neuronal cells. *Mol Cell Neurosci* 39:549–568 [PubMed: 18817873]
17. Tang BL, Tan AE, Lim LK, Lee SS, Low DY, Hong W (1998) Syntaxin 12, a member of the syntaxin family localized to the endosome. *J Biol Chem* 273:6944–6950 [PubMed: 9507000]

Fig. 1. Interactome analysis identifies many known and novel TBK1-interacting proteins. **a** Flowchart of the experiment to identify TBK1-interacting proteins. **b** Interactome analysis revealed a cluster of t-SNARE proteins, including STX12/13, that interact with TBK1. **c** Co-IP experiment confirming the biochemical interaction between EGFP-TBK1 and c-Myc-STX12/13 in HEK293 cells. **d** Confocal images showing co-localization of TBK1 and STX12/13 in HeLa cells. **e** Co-IP experiment confirming the interaction between EGFP-TBK1 and endogenous TBK1 in HEK293 cells. *: unwashed signal from STX12/13. Scale bar: 10 μ m

Fig. 2.

TBK1 knockdown decreases dissociation of the STX12/13–VAMP3 complex, vesicular trafficking, and MMP2 secretion. **a** TBK1 knockdown causes clustering of the STX12/13–VAMP3 puncta in HeLa cells. **b** Co-IP experiment showing that *TBK1* knockdown increases endogenous STX12/13–VAMP3 complex formation in HEK293 cells. **c** Quantification of STX12/13–VAMP3 complex formation before and after *TBK1* knockdown in HEK293 cells. **d** Standard curve of MMP2 ELISA assay. **e** TBK1 knockdown decreased vesicular trafficking and secretion of MMP2. Values are mean $\pm$ SEM by *t* test

Fig. 3.

Generation of *TBK1* mutant human iPSC lines and the effect of partial loss of TBK1 on MMP2 in iPSC-derived neurons. **a** Schematic of the genomic structure and CRISPR editing strategy in the *TBK1* locus. **b** Western blot analysis of TBK1 levels in *TBK1*^{-/+} and *TBK1*^{-/-} human iPSC lines. **c** Sequencing analyses reveals the nature of mutations in *TBK1*^{-/+} and *TBK1*^{-/-} human iPSC lines. **d** Schematic of the neuronal differentiation of *TBK1* heterozygous human iPSC lines. **e** Representative images of control and *TBK1*^{-/+} vGLUT1- and MAP2-positive neurons. Scale bar: 50 μm. **f** Percentage of MAP2-positive neurons among all cells. **g** Percentage of vGLUT1-positive neurons among MAP2-positive

neurons. **h** MMP2 levels in the medium of control and *TBKI*^{-/-} human neurons. Values are mean $\pm$ SEM *t* test

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript