DDA-PASEF phosphoproteomics reveals TTBK2 function during ciliogenesis

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INTRODUCTION

Primary cilia are tiny organelles that organize the cellular signaling required for development and homeostasis. Tau tubulin kinase 2 (TTBK2) is a pivotal organizer of ciliogenesis, and its catalytic activity is pivotal for its proper function. Previous studies suggest that TTBK2 possesses a phosphate-priming binding site in its catalytic domain equivalent to that in related CK1 isoforms. Peptide substrate studies on RRKDLHDDEEDEAMSIyA show preference of TTBK2 towards phosphorylation of serine/threonine on -2 position of phosphotyrosine.[1] Other in-vitro studies on the TTBK2 kinase activity show serine/threonine phosphorylation on the -3 position of glutamic acid.[2] Despite identification of in-vivo protein targets based on electrophoretic mobility shifts analysis including CEP164 and other distal appendage proteins,[2] the in-vivo phosphosite determinations on these targets remain poorly understood, and the full map of TTBK2 substrates still needs to be elucidated.

Therefore, the precise function and substrates of TTBK2 during ciliogenesis remains unknown because high sensitivity is required to survey the phosphoproteomic contents of primary cilia. Here, we studied the role of TTBK2 during ciliogenesis using high sensitivity Data-Dependent Acquisition (DDA)- Parallel Accumulation–Serial Fragmentation (PASEF) phosphoproteomics. We identified both types of substrates in-vivo, revealing that TTBK2 prefers to phosphorylate serine/threonine on the -3 position of glutamic acid or -2 of phosphorylated serine.

HYPOTHESIS

It has been shown that the loss of centrosomal protein 164kDa (CEP164), a component of the ciliary distal appendage, leads to early defects in ciliogenesis, similar to the effects of TTBK2 loss. The primary function of Cep164 is to recruit TTBK2 to basal bodies. Once localized correctly, TTBK2 then functions in distal appendage assembly and primary cilia formation (Figure 1). We hypothesized that phosphoproteomic study of ciliogenesis in wild type retinal pigment epithelial (RPE) cells compared to the defective ciliogenesis of either TTBK2 KO or CEP164 KO will reveal target proteins and phosphosites of TTBK2 required for the ciliogenesis.

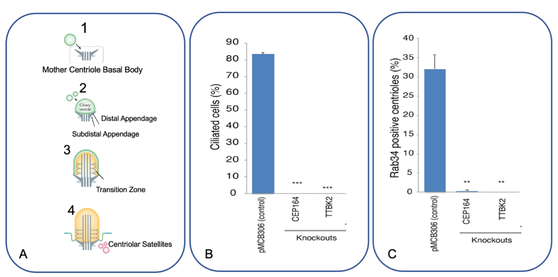


Figure 1. A) Different steps of ciligenesis. B) The ciliation defects on TTBK2 and CEP164 KOs. C) Requirement of TTBK2 and CEP164 for ciliary vesical recruitment.

METHOD

RPE cells (TTBK2 KO, CEP164 KO, andTTBK2 KO rescued with either wild-type or TTBK2 kinase-dead mutant) were serum-starved for 32 hours to induce cilium formation and then lysed, reduced, alkylated, digested with Trypsin/Lys-C overnight, desalted with STAGE tips, and enriched for phosphopeptides with both TiO2 and NTA columns. Peptides were separated sequentially with C18 nanoflow LC and by ion mobility using the TIMS device available on the Bruker timsTOF Pro. The dependent collisional energy was set between 25 to 50eV depending on the inverse ion mobility values (1/K0) from 0.85 to 1.3. The UHPLC nanoflow gradient was set at 60 min (Figure 2). Data were manually inspected by Skyline-daily 21.2.1.514 (Figure 3).

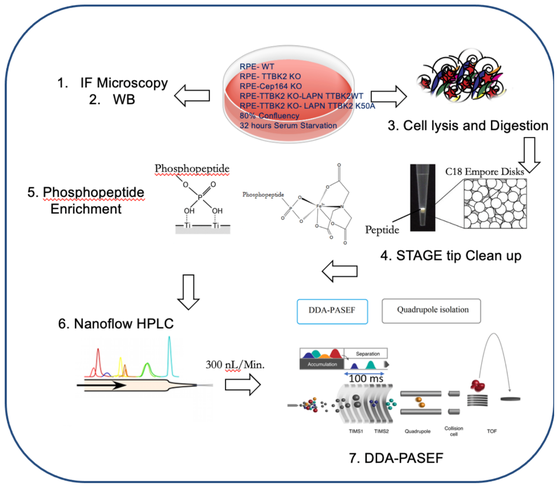


Figure 2. Workflow of different steps of sample preparation and DDA-PASEF phosphoproteomics

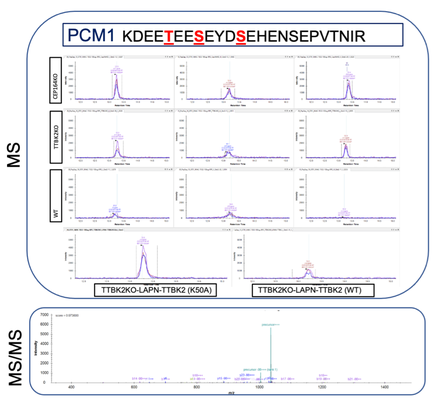


Figure 3. Data analysis and manual inspection and validation of MS2 and MS1

RESULTS

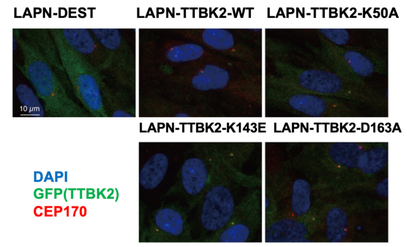


Figure 4. The localization of TTBK2 WT and kinase dead mutants

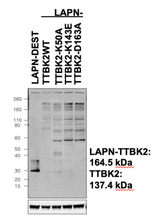


Figure 5. The expression check of LAPN - TTBK2 in TTBK2 KO cells

CEP164-S201	YTKGLLGSIYEDKTA
YWHAE-S210	IAELDTLSEESYKDS
CEP41-S99-S96	QRLEDNDSAASDPDA

TTBK2 KO ➪ PCM1 Hyperphosphorylation

PCM1	VTNDISPESSPGVGR
PCM1	KDEETEESEYDSEHENSEPVTNIR

Figure 6. In-vivo substrates of TTBK2 including CEP164, YWHAE, and CEP41. TTBK2 KO causes hyperphosphorylation of PCM1.

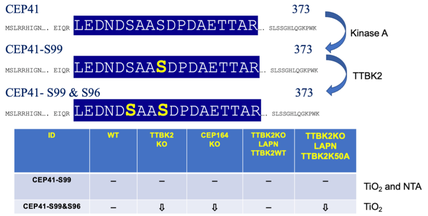


Figure 7. Priming kinase activity of TTBK2. Mono-phosphorylated CEP41-S99 remains constant between WT and dead mutant TTBK2. Doubly phosphorylated CEP41-S99&96 reduced in the TTBK2 KO, CEP164 KO and TTBK2 K50A compared to the WT.

Figure 8. Schematic representation of the domain structure of TTBK2. Underlined amino acids are in the kinase domain. White S/T indicates phosphorylations specific to the WT and absent in the TTBK2-K50A dead mutant. Red S/T are the same between the TTBK2-WT and K50A. Italic S/T shows ambiguity in the phosphorylation site with CAD fragmentation.

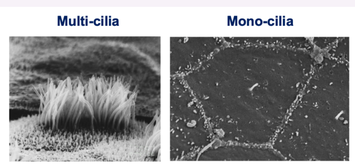
TGNDGSLTTTTTSTT
SLTTTTTSTTPQLHT
VIVEKDHSATTEPLD
RKLRSIHSFELEKRL
AATEEENSHGQANGL
DNEDEKLSRGQHCIE
TVGTSEISSRDIDPH
TTTTSTTPQLHTRLT
PQLHTRLTPAAIGIA
AIGIANATPIPGDLL
LGICKAATEEENSHGQ
FENLPGETEEKSILL
EKSILLESDNEDEKL
GSPIRVRSEITQPDR
AATEEENSHGQANGL
DNEDEKLSRGQHCIE
TVGTSEISSRDIDPH

Figure 9. Phosphorylated sites +/- 7 amino acids specific to the WT TTBK2 compared to the TTBK2-K50A dead mutant.

CONCLUSION

TTBK2 is known to bind to CEP164, a centriolar protein, and is recruited to the ciliary base by CEP164. The CEP164 and TTBK2 knockouts completely failed to assemble cilia in RPE cells after 32 hours of serum starvation, as confirmed by immunofluorescence (IF) microscopy, whereas 90 percent of WT RPE cells formed the cilium. The ciliation defect observed in TTBK2 knockout cells was efficiently rescued by wild-type but not by the kinase-dead mutant (K50A) of TTBK2, confirming the previously established importance of TTBK2 kinase activity. Identifying the ciliary and centrosomal phosphopeptides is a challenge because of their low abundance. Additional TIMS separation using parallel accumulation–serial fragmentation (PASEF) enabled high sensitivity and comprehensive analysis of phosphoproteomics of RPE cells. Our data demonstrate that ciliary proteins like CEP164, CEP41, and YWHAE are substrates of TTBK2. Furthermore, a set of phosphosites unique to WT RPE cells after ciliation suggests that TTBK2 can either function as a priming kinase and phosphorylate CEP41 at -2 position of phosphoserine or phosphorylate substrates like CEP164 at -3 position of glutamic acid. We have also mapped all phosphosites on TTBK2. The comparative analysis of phosphorylations on TTBK2 WT versus dead mutant TTBK2-K50A reveals phosphorylations specific to the wild-type TTBK2 and required for its function during ciliogenesis.

FUTURE



Phosphoproteomic analysis of ciliogenesis in multi-ciliary human nasal epithelial cells

References

1. Bouskila et al. Biochem J. 437 (2011) 157–167.
2. Bernatik et al. Mol Biol Cell. 31 (2020) 1032–1046.

Acknowledgement

NIH grants RO1DK127665 and RO1CA25486 to Peter K. Jackson.

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