EFFECTS OF *TPRKB* DEFICIENCY ON THE EXPRESSION OF NEURONAL TRANSCRIPTION FACTORS IN ZEBRAFISH

A Synopsis Submitted By

Dr. Meghna C Rao

(US No. NU24BSBM05)

To

NITTE
(Deemed to be University)

(Estd under Section 3, UGC Act 1956)
(Placed under Category 'A' by MHRD., Govt. of India; Accredited as 'A+' Grade University by NAAC)

For registration of Synopsis of Dissertation

MASTER OF SCIENCE IN BIOMEDICAL SCIENCES

DEPARTMENT OF MOLECULAR GENETICS & CANCER

NITTE UNIVERSITY CENTRE FOR SCIENCE EDUCATION & RESEARCH

DERALAKATTE, MANGALURU-575018

JULY 2025

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Under the Guidance of
Dr. Gunimala Chakraborty

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CONTENTS

1. LIST OF ABBREVIATIONS

bHLH	Basic helix loop helix
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
GAMOS	Galloway-Mowat Syndrome
HRMA	High Resolution Melting Analysis
LAGE3	L Antigen Family Member 3
KEOPS	Kinase, Endopeptidase and Other Proteins of Small size
OSGEP	O-Sialoglycoprotein Endopeptidase (Syn: KAE1)
PRPK	p53- related protein kinase
TPRKB	TP53RK Binding Protein
TP53RK	Tumor Protein P53 Regulating Kinase

2. BACKGROUND

The KEOPS (Kinase, Endopeptidase and Other Proteins of small Size) complex is a highly conserved protein assembly important for the biosynthesis of a universal tRNA modification, N6-threonylcarbamoyladenosine (t6A), at position 37 of ANN-decoding tRNAs. This modification is needed for translational fidelity and efficiency. The TPRKB (TP53RK Binding Protein), a non-catalytic component of the KEOPS complex, acts as a stabilizer and facilitator of the enzymatic activity of the complex, specifically in complex formation and conformational integrity [1].

Recent studies have highlighted that mutations in KEOPS components, including TPRKB, OSGEP, LAGE3, GON7, and YRDC, lead to a spectrum of not just rare but also severe neurodevelopmental and renal disorders, especially Galloway-Mowat Syndrome (GAMOS) and microcephaly syndromes [2,3,4]. The proposed mechanism underlying these disorders lies in disrupted t6A modification, leading to defective protein translation, activation of integrated stress response (ISR), and cellular dysfunction. These findings suggest a direct link between KEOPS dysfunction and neural development, supporting the importance of investigating its role in neuronal gene regulation.

While broad studies in mammalian systems have shown the functional roles of KEOPS complex subunits, much less is known about the specific transcriptional changes, mainly those involving neuronal transcription factors, that occur as a result of *tprkb* gene disruption. This study aims to investigate how loss of the *tprkb* gene affects neuronal transcription factors in zebrafish by proposing to generate a stable *tprkb* knockout zebrafish line and assess changes. The zebrafish (*Danio rerio*) presents as an ideal model to explore this, given its conserved neurodevelopmental pathways, transparent embryonic development, and genetic tractability [5].

3. REVIEW OF LITERATURE

Loss-of-function mutations in KEOPS subunits have been closely linked to clinical phenotypes marked by neurodevelopmental delays, microcephaly, and renal abnormalities. Mutations in TPRKB and other KEOPS genes cause GAMOS, with zebrafish models confirms the conservation of disease phenotypes, especially microcephaly [1]. Further, pathogenic mutations in KEOPS subunits result in severe neurological manifestations, implicating neuronal dysfunction as a primary pathology [4,6].

The 2019 study by Arrondel et al. focused on GON7 and YRDC mutations but indirectly emphasized that loss of t6A modification, irrespective of the subunit mutated, leads to translational stress and impaired neurogenesis [2]. This agrees with the broader understanding on how defective tRNA modifications can impair nervous system development and lead to neurodegeneration [7].

The Jobst-Schwan et al. (2018) study has succeeded in demonstrating that zebrafish knockdowns of KEOPS complex genes, including *tprkb*, accurately reproduce the human microcephaly phenotype [8]. Using multi-sgRNA CRISPR-mediated knockdowns, the study found that zebrafish embryos exhibited reduced brain size, abnormal morphology, and reduced proliferation of neural progenitors, which mirrors the human condition. This validates the zebrafish model as both phenotypically and functionally homologous to humans in the context of KEOPS gene dysfunction.

Importantly, the phenotypes in zebrafish were observed even in acute knockdown models, suggesting that early disruption of KEOPS function during development is sufficient to impair neurogenesis, possibly by downregulating neural transcription factors or disrupting their translation altogether.

Though close connections between TPRKB and specific neuronal transcription factors have not been completely clarified, the after-effects of KEOPS dysfunction can be hypothesized. The loss of t6A modification is known to affect codon-anticodon interactions and cause ribosome stalling, which specifically affects mRNAs that have certain pattern of codon use [7]. Transcription factors with high translational demand or sensitivity to stress (e.g., NeuroD, Neurog1/2, Neurogenin3) could be especially vulnerable [9,10].

Key bHLH transcription factors like NeuroD and Neurogenin3 in zebrafish that are essential for neuronal fate determination and differentiation in the CNS [9,10]. Distrubances in their expression due to disruptions in the early steps of translation, as expected in *tprkb* mutants, could explain the reduced neurogenesis and smaller brain size observed in KEOPS-compromised zebrafish [8].

Stem cell function under stress, is tightly regulated by protein synthesis, suggesting that *tprkb*-related translational defects can impair neural stem cell maintenance and lineage commitment [11].

While zebrafish do not possess identical gene orthologs to humans for all pathways, the KEOPS complex is very conserved. Structural studies by Beenstock and Sicheri (2021) show that TPRKB interacts similarly with PRPK and OSGEP in zebrafish and humans, implying functional equivalency [1]. Therefore, phenotypes like microcephaly, as reported in both systems, possibly share a conserved mechanistic basis involving tRNA modification, ISR activation, and translational collapse [3,8].

However, certain limitations must be acknowledged, zebrafish models may not exhibit complex cognitive or behavioural phenotypes seen in humans with GAMOS. But at the molecular level, especially during early neurogenesis, zebrafish present a valid and informative proxy for dissecting the transcriptional consequences of *tprkb* loss.

Despite these advances, there remains a significant knowledge gap regarding how *tprkb* loss affects specific transcriptional programs in neurons, specially via regulation of transcription factors. This study proposes to bridge that gap by creating a stable *tprkb*-null zebrafish line using CRISPR-Cas9 and then evaluating the expression patterns of neuron-specific transcription factors.

By combining genome editing with in situ hybridization, qPCR, and immunostaining for key markers (e.g., NeuroD, Neurog1/2, Neurogenin3), this work aims to bring to light whether transcriptional regulation is directly or indirectly disrupted by *tprkb* loss, providing mechanistic insight into KEOPS-associated neurodevelopmental disease.

4. RESEARCH QUESTION

Will zebrafish *tprkb* mutants partially recapitulate the *TPRKB* loss-of-function phenotype of Galloway-Mowat Syndrome patients?

5. AIMS & OBJECTIVES

1. To generate a stable *tprkb* mutant zebrafish using CRISPR-Cas9 gene editing.
2. To evaluate the expression of neuronal transcription factors in the mutant zebrafish.

6. METHODOLOGY

Task 1: Generation of *tprkb* loss-of-function Zebrafish

Task 1.1: **Determination of Gene and Protein homology between human *TPRKB* and zebrafish *tprkb***

Zebrafish has two *tprkb* transcripts (tprkb-201 and tprkb-202). The first task will be to determine the sequence similarities of these two transcripts and to identify the canonical one. The next step will be to determine the sequence similarities between zebrafish *tprkb* and human *TPKRB* to determine the conserved nature of the mutated codons identified in humans.

Task 1.2: **Identification of CRISPR target sites**

The designing of oligos against zf tprkb-201 transcript will be done using online software E-CRISP ((e-crisp.org). To determine which target would be best suited, two CRISPR targets, in two different exons, will be designed, and the similarities of these two regions at the nucleotide and protein level in both transcripts will be ascertained. Considering that the CRISPR technique introduces indels, the target that is likely to introduce a premature stop codon in the coding region will be chosen. Following the identification of CRISPR sites, forward and reverse oligos will be designed, and the process of gRNA preparation, which includes ligation in the guide RNA vector, cloning, and confirmation by colony PCR and sequencing, will be carried out. Once the insertion is confirmed, the gRNA containing the spacer sequence (CRISPR recognition sequence) will be linearized and will be *in vitro* transcribed using mMessage mMachine kit. In parallel, the Cas9 mRNA will be *in vitro* synthesised using the linearised Cas9 mRNA vector. The guide RNA containing the spacer sequence and the Cas9 mRNA will be co-injected at the 1-cell-stage zebrafish embryos.

Task 1.3: **Injection, confirmation and morphological studies**

Briefly, gRNA containing the CRISPR sites and the Cas9 mRNA will be co-injected into one-cell stage embryos of wild-type AB zebrafish. Determination of recombination efficiency will be ascertained by sequencing of the target region using total DNA from pooled primary injected embryos. The recombination will be further confirmed by HRMA and HD assays. The founder fish with germline-transmitted indels will be outcrossed to wild-type to establish F1 heterozygotes, which will be intercrossed to generate stable *tprkb* mutants. The heterozygous mutants will be crossed to obtain the null mutants, and extensive phenotypic characterization of the mutants will be carried out using a 28-point developmental anomaly chart. All animal procedures will adhere to institutional animal ethical guidelines. Embryos will be staged according to hours post-fertilization (hpf) or days post-fertilization (dpf) and raised at 28.5°C.

Task 2: Evaluation of neuronal transcription factors in the mutant.

Task 2.1: **Whole mount in situ hybridization *(WISH)*.**

Since GM patients display microcephaly as a prominent phenotype, the expression pattern of the neuronal transcription factors will be undertaken to determine if the brain-specific phenotype is due to the loss of neuronal cell abundance or cell fate. Whole mount in situ hybridization (WISH) will be carried out to determine the localization of transcription factors (*sox2*, *otx2*, *neurod1*, *pax2*, *pax6a*, and *krox20*) that are important for brain development using digoxigenin-labeled antisense RNA probes.

Task 2.2: **RNA extraction and cDNA synthesis**

Total RNA from null mutants and their heterozygous and wild-type siblings will be extracted using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA will be synthesized and qPCR will be performed for the neuronal transcription factors (*sox2*, *otx2*, *neurod1*, *pax2*, *pax6a*, and *krox20*), normalized to the expression of reference genes such as elf1a or beta-actin.

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8. BUDGET REQUIREMENTS

The total cost of the project would be approximately Rs 50,000, which will be covered from the institutional research budget.

9. ETHICAL CONSIDERATIONS

The proposal has minimal biosafety issues. It does not involve the use of any samples from humans. All experiments using zebrafish will be carried out in a zebrafish facility with proper protocols in place pertaining to care and disposal of materials. Institutional animal ethical clearance will be obtained.

10. JUSTIFICATION/IMPLICATIONS

The *tprkb* mutants generated in this study will be important *in vivo* tools to understand the pathogenicity in GM syndrome. The model could be utilized to determine the pathogenicity of *TPRKB* mutations identified from GM patients by performing functional studies. In addition, the model could also be used to screen novel compounds that could alleviate the morphological phenotypes due to loss of *tprkb* function. Such an approach could lead to the identification of novel therapeutics for GM patients.

11. WORK PLAN

Month / **Activity**	August 2025	Sept-Dec 2025	Jan-Mar 2026	April-June 2026
Synopsis preparation and literature survey				
Design sgRNAs targeting *tprkb* Order synthetic sgRNAs and Cas9 protein Generate F0 mosaic founder embryos				
Raise injected F0 embryos to adulthood under standard zebrafish husbandry				

conditions.				
Generate F1 heterozygous fish: Cross F0 with WT adults. Collect F1 embryos and raise them separately. Genotype F1 heterozygotes. Obtain F2 Homozygous(null) *tprkb* mutant. Isolate total RNA from pooled or individual embryos of each genotype. Perform WISH & cDNA synthesis for downstream gene expression studies. Use RT-qPCR to quantify the expression of neuron-specific transcription factors.				

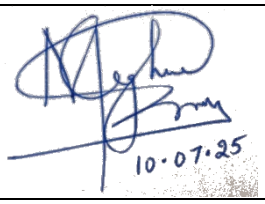
Data compilation and thesis submission				

12. RELEVANT APPENDICES AND ATTACHMENTS

None

PROTOCOL FOR REGISTRATION OF M.Sc. SYNOPSIS OF DISSERTATION

1	NAME OF CANDIDATE AND UNIVERSITY SEAT NUMBER	Dr. MEGHNA C RAO NU24BSBM05
2	NAME OF INSTITUTION AND ADDRESS	NITTE UNIVERSITY CENTRE FOR SCIENCE EDUCATION AND RESEARCH PANEER CAMPUS, DERALAKATTE, MANGALURU- 575018
3	PROGRAM OF STUDY AND COURSE	MASTER OF SCIENCE BIOMEDICAL SCIENCES
4	MONTH AND YEAR OF JOINING THE PROGRAM	AUGUST 2024

5	TITLE OF THE TOPIC	EFFECTS OF *TPRKB* DEFICIENCY ON THE EXPRESSION OF NEURONAL TRANSCRIPTION FACTORS IN ZEBRAFISH.
6	DOES THE STUDY REQUIRE ANY INVESTIGATIONS OR INTERVENTIONS TO BE CONDUCTED ON PATIENTS / HUMANS /ANIMALS IF SO, PLEASE DESCRIBE BRIEFLY	The study requires investigations or interventions to be conducted on animals (*Danio rerio*)
7	ETHICS COMMITTEE CLEARANCE BEEN OBTAINED FROM YOUR INSTITUTION?	Institutional animal ethical Clearance will be obtained
8	SIGNATURE OF THE CANDIDATE WITH DATE	
9	REMARKS OF THE CO-GUIDE	
10	NAME AND DESIGNATION OF CO-GUIDE	Dr. ANIRBAN CHAKRABORTY Director Dean- Faculty of Biological Sciences Nitte University Centre for Science Education and Research, Nitte (Deemed to Be University)
11	SIGNATURE OF THE CO-GUIDE WITH DATE	

12	REMARKS OF THE GUIDE	

**Before submission to EC please write as: submitted to EC for clearance

Before submission to university please write: Yes (mention IEC letter No. and Date)

13	NAME AND DESIGNATION OF THE GUIDE	Dr. GUNIMALA CHAKRABORTY Associate Professor, Dept. Of Molecular Genetics & Cancer Nitte University Centre for Science Education and Research, Nitte (Deemed to Be University)
14	SIGNATURE OF GUIDE WITH DATE	
15	NAME OF THE HEAD OF THE DEPARTMENT	Dr. GUNIMALA CHAKRABORTY
16	SIGNATURE OF THE HEAD OF THE DEPARTMENT WITH DATE	
17	REMARKS OF DIRECTOR/DEAN	
18	NAME AND SIGNATURE OF DIRECTOR/DEAN WITH DATE	Dr. ANIRBAN CHAKRABORTY