Role Of TOR (Target Of Rapamycin) In Cellular Metabolism And Morphological Transformation In *Naegleria* Ameoba

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ABSTRACT

Naegleria fowleri is a pathogenic unicellular protist free living ameba commonly found in warm freshwater. Depending on the environment, it undergoes three stages of life cycle - trophozoite (infectious), flagellate (motile), cyst (dormant). The trophozoite form infects the central nervous system by entering the brain through the olfactory nerve in the nasal cavity, causing primary amoebic meningoencephalitis (PAM). The infection is rapid and fatal, with a mortality rate of 97%. Our central idea is to promote cyst formation in *Naelgeria*, followed by targeting drugs to treat PAM. In this study, regulatory role of target of rapamycin (TOR) kinase in Naegleria gruberi, a non-pathogenic species, was assessed. Phosphoproteomics analysis identified phosphorylation of glycolytic enzyme enolase and enolase-associating proteins when TOR was activated, making it an attractive candidate as a downstream target of TOR. Therefore, GFP-tagged Naegleria enolase was engineered and expressed in *N. gruberi* and murine MK4 cells, and subsequently, point mutations were introduced to the TOR-sensitive phosphorylation sites. We are currently investigating the effects of the mutations on enolase subcellular localization.

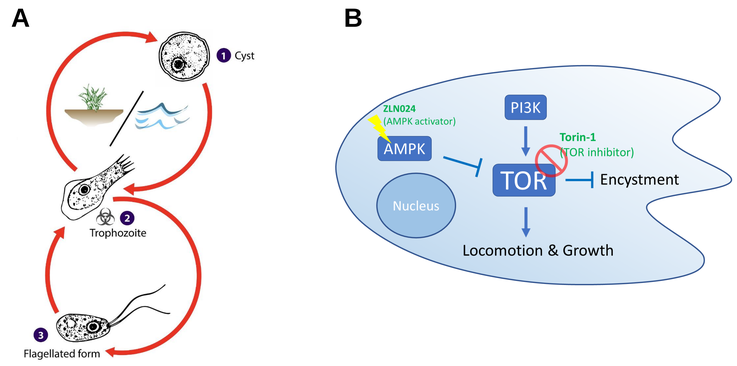

Figure 1. Life Cycle of *Naegleria* and Hypothetical Model
(A) The schematic shows three life stages of *Naegleria* (https://www.cdc.gov/parasites/naegleria/index.html). ① *Naegleria* in cyst stage is metabolically dormant to withstand the unfavorable environment, forming cell wall, ② the vegetative state trophozoite is amoeboid and can be infectious to humans, ③ Flagellated form uses two flagella for locomotion to swim away from the unfavorable environment. (B) "Master Regulator"- TOR signal transduction pathway. Inhibition of TOR kinase activity stops cell growth and locomotion while directing encystment.

MATERIALS AND METHODS

Cell culture *N. gruberi* (strain NEG-M, ATCC#30224) was cultured and maintained in petri dish at room temperature in media that consists of 2% Bacto™ casitone, 10% FBS, and 1% yeast extract.

TOR inhibition Torin-1 at 1.5 µM was introduced to 25,000 *N. gruberi* cells in a petri dish, while DMSO solvent was served as a control. The cell numbers were counted by hemocytometer on days 2 and 6.

Phosphoproteomics and STRING Analysis One hundred million *N. gruberi* cells were cultured in Chalkley's medium and starved for five hours. The cells were then treated with either DMSO or Torin-1 (1.5µM) for an hour, followed by incubation with complete media (15 ml) for 30 min. Proteins were extracted from the cells in lysis buffer (8 M urea, 1% protease inhibitor, 1% phosphatase inhibitor), and eight hundred micrograms of protein were trypsinized and underwent phosphopeptide enrichment by Fe-IMAC beads prior to the nanoflow UHPLC run. The MS/MS scan was done by a Q-Exactive HF mass spectrometer. The 228 proteins phosphorylated only in complete media were analyzed for functional and physical protein interaction by STRING (https://string-db.org).

Construction of expression vector Ubiquitin promoter element (1874 bp) of *N. gruberi* was synthesized and cloned in the pcDNA3.1 expression vector, resulting in pcDNA3-Ubi1874. Subsequently, GFP-tagged *N. gruberi* enolase gene was subcloned to the expression vector and termed pcDNA3-Ubi1874-GFP-enolase.

Transfection A million *N. gruberi* cells were transfected with 4 µg of pcDNA3-Ubi1874-GFP-enolase in Chalkley's solution with 2% FBS in a cuvette (4 mm gap) by electroporator (BioRad Gene Pulser xCell) with the setting 175 V, 500 µF, 400 Ω. Transfected *N. gruberi* cells were cultured in three conditions, complete media, complete media with 1.5 µl Torin-1, and Chalkley's solution (starvation) in a four-well chamber coverslip. For MK4 cell (murine transformed embryonic kidney cell) transfection, 1 µg of pcDNA3-GFP-enolase was transfected into 50,000 murine MK4 cells with TransIT 293 reagent (Mirus). The cells were observed two days post-transfection.

Microscopy The expression of GFP-enolase in *N. gruberi* cells were observed with Leica DMi8 Widefield Fluorescence Microscope and MK4 cells under Zeiss LSM710 Live Duo Confocal Microscope.

RESULTS

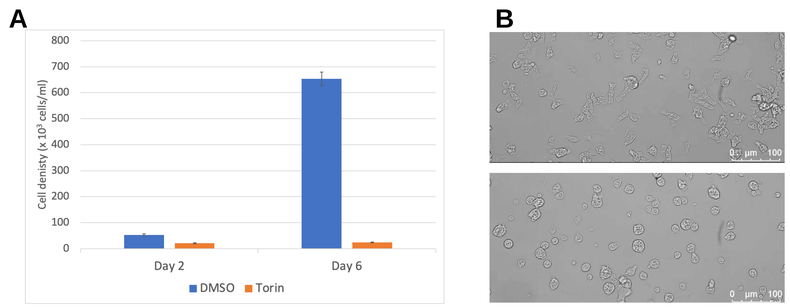

Figure 2. TOR inhibition by Torin 1 suppresses *Naegleria* proliferation and induces cyst-like morphology
(A) Torin 1 treated *N. gruberi* cells showed reduced cell growth (effect of TOR inhibition), whereas cells treated with DMSO continuously proliferated. (B) Micrographs of *N. gruberi* cells after being treated with DMSO (top) or 1.5 µM Torin 1 (bottom) for 30 min. Torin 1 treated cells manifest reduced cell locomotion and form cyst-like morphology.

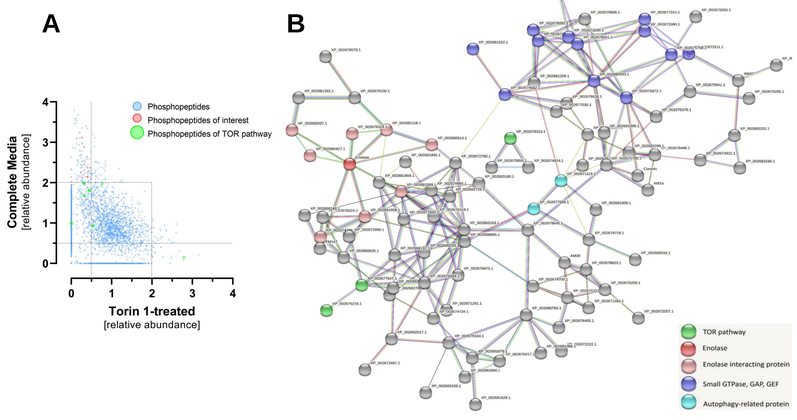

Figure 3. Phosphoproteomics and STRING analyses identified Torin-1-sensitive phosphoproteins
(A) LC-MS/MS phosphoproteomics analysis detected 3,884 phosphorylation sites in 2084 phosphoproteins out of 15,727 predicted proteins. The scatter plot shows the relative abundance of phosphopeptides in complete media vs. torin 1 treatment. (B) STRING analysis identified enolase and eight enolase-associated proteins as torin 1 and starvation-sensitive phosphoproteins, indicating that enolase is a relevant candidate for a downstream target of TOR kinase.

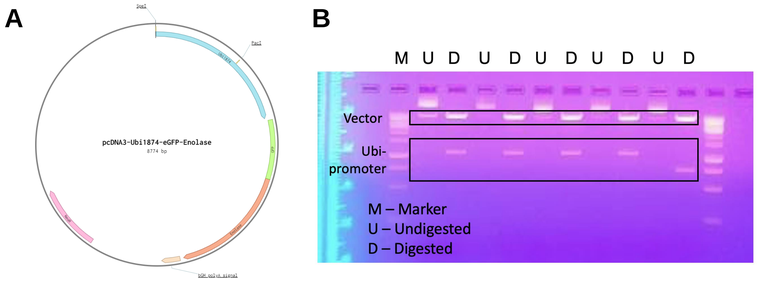

Figure 4. Construction of *Naegleria* expression vector
(A) The vector map of *Naegleria* expression plasmid, which carries *N. gruberi*-derived ubiquitin promoter (1874 bp) and enolase (GFP-tagged). (B) Restriction analysis on miniprep products of pcDNA3-Ubi1874-GFP-enolase constructs with SpeI and PacI restriction enzymes shows the expected sizes of DNA fragments. The right orientation of Ubi-promoter presents a 1096 bp fragment, while the reversed oriented Ubi-promoter gives a 784 bp fragment.

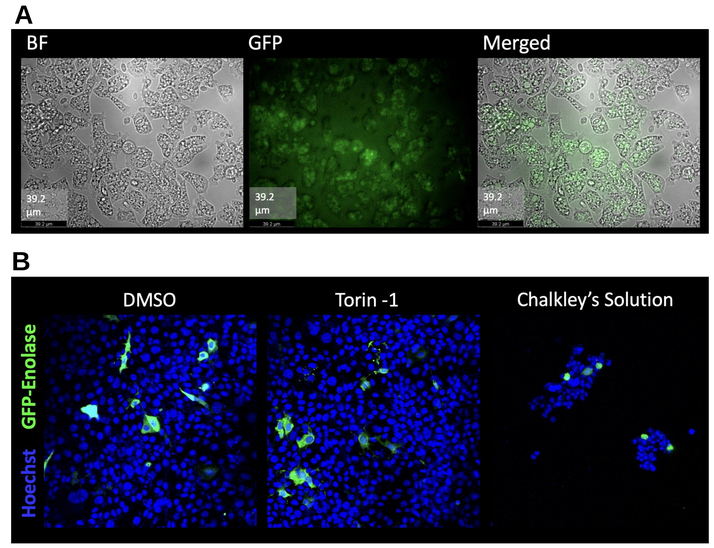

Figure 5. Expression of GFP-enolase in *N. gruberi* and MK4 cells
(A) pcDNA3-Ubi-GFP-enolase transfected *N. gruberi* cells showed weak expression of GFP. (B) Confocal micrographs showing GFP-enolase expression (pcDNA3-CMV-GFP-enolase) in MK4 cells cultured in complete media with DMSO (left), Torin 1 (middle), and Chalkley's Solution (right).

CONCLUSIONS

- STRING analysis of phosphoproteomics data identified enolase as a candidate for a downstream target of TOR
- Four regulated phosphorylation sites in enolase were identified
- Enolase constructs were successfully transfected into *N. gruberi* and MK4 cells and GFP expression were observed

FUTURE DIRECTION

Following the expression of enolase in the cells, our next goal is to assess the localization of mutated enolase in different culture conditions as described in Fig 5. Four phosphosites (serine residues) of enolase will be mutated to alanine, and the localization of mutated enolase will be monitored by confocal microscopy. Finally, the viability of amphotericin B-treated *N. gruberi in combination* with Torin 1) will be evaluated.

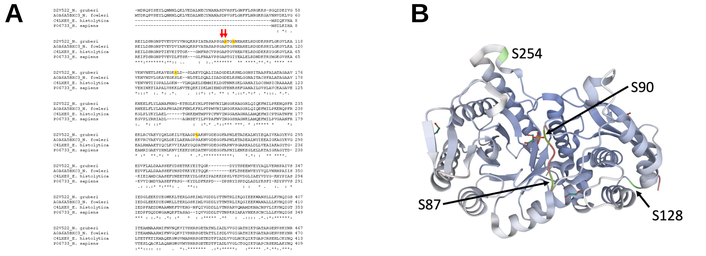

Figure 6. Phosphorylation sites of *N. gruberi* enolase
(A) Protein sequence alignment shows conserved regions of enolase among *N. gruberi*, *N. fowleri*, *E. histolytica*, and *H. sapiens* species. Phosphorylation sites of *N. gruberi* are highlighted in yellow. The red arrows point to residues for the substrate binding site. (B) Protein structure of *N. fowleri* enolase and corresponding four phosphorylation sites. The substrate of enolase, 2-phospho-D-glycerate, is in ball and stick form.

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