Protocol: TBK1 knockdown and rescue in Hela-M cells

Olivia Harding, Erika L.F. Holzbaur
Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

Abstract

TANK-binding kinase 1 (TBK1) is a multifunctional kinase with roles in several crucial cell processes, including innate immune response, anti-viral response, and mitochondrial clearance, the last of which is of most interest to us. In order to further understand the role of TBK1 in mitophagy, we developed a protocol to transiently deplete TBK1 from a model system, HeLa cells, and re-introduce a tagged TBK1 along with other relevant components of mitophagy. Because of the many processes that rely on TBK1, cells depleted of the kinase exhibit poor health. Our protocol accomplishes ~70% depletion of endogenous TBK1 within 24 hours without causing excessive cell death. We employ this protocol to carry out biochemistry experiments such as Western blotting and organelle fractionation, and imaging experiments such as immunofluorescence, and live cell imaging.

Keywords

tissue culture | transfection | knockdown | TBK1

Guidelines

- This protocol was developed with the HeLa subtype, HeLa-M. HeLa-M cells are flatter than standard HeLa cells, making them easier to image. They also uptake siRNA better than standard HeLa. Regardless, the protocol would be easily adaptable to standard HeLa cells or other cell culture lines.
- HeLa cells fare poorly after TBK1 depletion. For this reason, I developed a 24-hour protocol with simultaneous knockdown and rescue. Even so, there will be a number of dead cells in the dish after transfection.
- This protocol was created in order to investigate Parkin-dependent mitophagy. Parkin and several other fluorescently-tagged mitophagy components are included in the protocol.

Before start

- Cells are best transfected before passage 30. Higher passage number could result in lower transfection efficiency. Use cells between P5 and P25 for best results.
- siRNA is easily degraded by RNAases that exist on everyday surfaces. Follow best practices for handling siRNA by suspending desiccated reagent in RNAase-free water, aliquoting into sterilized tubes, and using barrier pipet tips to handle aliquots.

Materials

- Countess slides (ThermoFisher Scientific, C10228)
- 10 mL conical tube (Corning CLS430055)
- 1.5 mL capped tubes (Millipore Sigma, EP022364120)
- 35 mm glass-bottomed dishes (MatTek, P35G-1.5-20-C)

Reagents

- Trypsin (ThermoFisher Scientific R001100)
- Trypan blue (ThermoFisher Scientific, T10282)
- DMEM (Corning, 10-017-CV)
- FBS (HyClone)
- GlutaMAX glucose supplement (Gibco, 35050061)
- Opti-MEM (ThermoFisher Scientific, 3198507)
- Plasmid DNA
    - untagged Parkin (subcloned from YFP-Parkin, a gift from R. Youle, NIH, Bethesda, MD)
    - Mito-DsRed2 (kindly provided by. T. Schwartz, Harvard Medical School, Boston)
    - Halo-TBK1 (subcloned from SNAP-TBK1, provided by T. Maniatis, see Ye et al, PNAS, 2019)
- siRNA
    - targeting 5' (UAACAAGAGGAUUGCCUGA) end of hTBK1 (Horizon Discovery)
    - targeting 3' (CCACUGUUAUACUGGGAUA) end of hTBK1 (Horizon Discovery)
- Lipofectamine 2000 (ThermoFisher Scientific, 11668027)

Equipment

- cell incubator to maintain 37 C and 5% CO2 atmosphere
- Countess automated cell counter (Invitrogen, AMQAX1000)
- mini centrifuge (Southern Labware, MLX-106)
- Compound microscope

Step-by-step

Day 1: Plating

- Trypsinize Hela-M cells by aspirating all media from a 10 cm dish of confluent cells, then dropping 0.75 mL Trypsin onto cells
- Incubate cells at 37 C, 5% CO2 for 5 min
- Resuspend detached cells and neutralize Trypsin with 1 mL DMEM with 10% FBS and 1% GlutaMAX for a final volume of 1.75 mL
- transfer this volume to 10 mL conical tube
- Combine 10 uL of suspension with 10 uL Trypan blue in a 1.5 mL tube.
- Drop 10 uL of this mixture onto a Countess slide and insert into the cell counter to calculate the concentration of cells in the resuspended solution
- Plate ~0.25 million HeLaM cells on 35 mm imaging dish in 2 mL DMEM/FBS/GlutaMAX

    - Note: The number of cells to plate should be calculated by a standard guide for confluence, in order to achieve ~80-90% confluence on Day 2.
    - Note: I prefer to pipet the volume of suspension needed (usually 20-50 uL) *for each dish* directly from that 10 mL tube and add it dropwise to 2 mL in the dish. I found that the cells uptake the exogenous nucleic acids best when they are in confluent patches instead of evenly distributed on the surface.

Day 2: Transfection

- Examine cells by compound microscope 18-24 hr after plating to confirm 80-90% confluence
    - Note: If cells are not at 80-90% confluence, do not transfect. Wait until they reach 80-90%.
- For each dish, prepare the following two solutions in 1.5 mL tubes
    - Tube 1 (nucleic acids): 200 uL Opti-mem
        - + 0.5 uL Halo-TBK1 (stock at 1 ug/uL)
        - + 0.25 uL mito-dsRed (" ")
        - + 0.5 uL Parkin (" ")
        - + 4.8 uL siTBK1-5'
        - + 4.8 uL siTBK1-3'
        - Note: invert stock solutions of each plasmid several times in order to ensure even distribution of plasmid.
    - Tube 2 (Lipofectamine 2000): 200 uL Opti-mem
        - +11.4 uL Lipofectamine 2000
        - Note: The Lipofectamine 2000 volume is calculated by this equation:
            - (X * 4) + (Y * 3.2), where X = ug plasmid DNA (in this protocol, X = 1.25) and Y = # of 4.8 uL aliquots of siRNA (in this protocol, Y = 2)
- Invert tubes 8 times to distribute the contents, then
- Incubate 5-10 min at room temperature
- spin 2 sec in a mini centrifuge
- Add Tube 2 to Tube 1 and invert 8 times to mix.
- Incubate 5-10 minutes at room temperature.
- Spin 2 sec in a mini centrifuge.
- Add entire volume (~ >400 uL) to the cells dropwise, distributing the drops mostly in the center of the dish (where the imaging window/coverslip is).

Day 3: Collection

- Cells are ready to collect for various assays 18-24 hr after transfection step.