Supplementary information

TWEAK-Fn14 axis induces calcium-associated autophagy and cell death to control mycobacterial survival in macrophages

Yi-Ming Chen1-4, Po-Yu Liu2,3,5, Kuo-Tung Tang4, Hung-Jen Liu2,3,6,7, Tsai-Ling Liao1-3*

1Department of Medical Research, Taichung Veterans General Hospital, Taichung, Taiwan
2Rong Hsing Research Center for Translational Medicine, National Chung Hsing University, Taichung 402, Taiwan
3Ph.D. Program in Translational Medicine, National Chung Hsing University, Taichung 402, Taiwan
4Division of Allergy, Immunology and Rheumatology, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung, Taiwan
5Division of Infection, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung, Taiwan
6Institute of Molecular Biology, National Chung Hsing University, Taichung 402, Taiwan
7The iEGG and Animal Biotechnology Center, National Chung Hsing University, Taichung 402, Taiwan

Corresponding author and address reprint requests:

Dr. Tsai-Ling Liao, Department of Medical Research, Taichung Veterans General Hospital, Taichung, Taiwan

Address: No.1650, Sec.4, Taiwan Boulevard, Xitun Dist., Taichung City 40705, Taiwan
Tel.: 886-4-23592525, extension 4020; Fax: 886-4-23592705
Email: tlliao@vghte.gov.tw
## Supplementary Materials

| Reagent or Resource                          | Source                     | Identifier (Cat No.) |
|---------------------------------------------|----------------------------|----------------------|
| **Antibodies**                               |                            |                      |
| Mouse anti-β-actin antibodies                | Santa Cruz                 | sc-47778             |
| Mouse anti-IP3RII antibody                   | Santa Cruz                 | sc-398434            |
| Mouse anti-CHOP antibody                     | Cell Signaling Technology  | #2895                |
| Mouse anti-ORA11 antibody                    | Santa Cruz                 | sc-377281            |
| Mouse anti-STIM1 antibody                    | Santa Cruz                 | sc-166840            |
| Mouse anti-TRPM7 antibody                    | Santa Cruz                 | sc-271099            |
| Rabbit anti-AMPK antibody                    | Cell Signaling Technology  | #5832                |
| Rabbit anti-pAMPK(T172) antibody             | Cell Signaling Technology  | #2535                |
| Rabbit anti-Atg5 antibody                    | Cell Signaling Technology  | #12994               |
| Rabbit anti-Beclin-1 antibody                | Cell Signaling Technology  | #3738                |
| Rabbit anti-BiP antibody                     | Cell Signaling Technology  | #3177                |
| Rabbit anti-cleaved caspase-3 antibody       | Cell Signaling Technology  | #9661                |
| Rabbit anti-cleaved caspase-8 antibody       | Cell Signaling Technology  | #9496                |
| Rabbit anti-Fn14 antibody                    | Cell Signaling Technology  | #4403                |
| Rabbit anti-LC3B antibody                    | Cell Signaling Technology  | #2775                |
| Rabbit anti-pCaMKK2 (S511) antibody          | Cell Signaling Technology  | #12818               |
| Rabbit anti-pMLKL (S358) antibody            | Cell Signaling Technology  | #91689               |
| Rabbit anti-pRIPK1 (S166) antibody           | Cell Signaling Technology  | #65746               |
| Rabbit anti-pRIPK3 (S227) antibody           | Cell Signaling Technology  | #13526               |
| Rabbit anti-pULK1(S555) antibody             | Cell Signaling Technology  | #5869                |
| Antibody/Molecule | Technology | Catalog Number |
|-------------------|------------|---------------|
| Rabbit anti-ULK1 antibody | Cell Signaling Technology | #6439 |
| Rabbit anti-p62 antibody | Cell Signaling Technology | #8025 |
| Rabbit anti-TWEAK antibody | Abcam | ab37170 |
| Alexa Fluor 488 Goat anti-Rabbit IgG | Thermo Fisher Scientific | A11008 |
| Anti-mouse IgG, HRP-linked antibody | Cell Signaling Technology | #7076 |
| Anti-rabbit IgG, HRP-linked antibody | Cell Signaling Technology | #7074 |
| Chemicals, Peptides, and Recombinant Proteins | | |
| 3-methyladenine (3-MA) | Sigma-Aldrich | M9281 |
| A23187 calcium ionophore | Sigma-Aldrich | C7522 |
| BAPTA-AM | Sigma-Aldrich | A1076 |
| bepridil hydrochloride | Sigma-Aldrich | B5016 |
| BTP2 | Sigma-Aldrich | Y4895 |
| dihydrorhodamine 123 | Thermo Fisher Scientific | D23806 |
| dimethyl sulfoxide | Sigma-Aldrich | D8418 |
| diphenyleneiodonium | Sigma-Aldrich | D2926 |
| LysoTracker Green | Thermo Fisher Scientific | L7526 |
| Fluo 4-AM | Thermo Fisher Scientific | F14217 |
| JC-1 | Abcam | ab113850 |
| Mito-ID | Enzo Life Sciences | ENZ-51018 |
| MitoSOX Red | Thermo Fisher Scientific | M36008 |
| MitoTEMPO | Sigma-Aldrich | SML0737 |
| phorbol myristate acetate | Sigma-Aldrich | P1585 |
| Recombinant human TWEAK | PeproTech | 310-06 |
| Texas Red-X | Thermo Fisher Scientific | T20175 |
| Trizol reagent | Thermo Fisher | 15596018 |
|                            | Scientific          | Sigma-Aldrich       | V116   |
|-----------------------------|---------------------|---------------------|--------|
| Z-VAD-FMK                   |                     |                     |        |
| Cell lines                  |                     |                     |        |
| THP-1 Difluo™ hLC3 cells    | InvivoGen           |                     | THPDF-HLC3 |
| Others                      |                     |                     |        |
| Lipofectamine RNAiMAX       | Thermo Fisher       |                     |        |
| Transfection Reagent        | Scientific          |                     |        |
| Fn14 TaqMan Gene Expression Assays | Thermo Fisher  |                     | Hs00171993_m1 |
| Human TWEAK Instant ELISA kit | Thermo Fisher       |                     | BMS2006INST |
| On TARGETplus SMARTpool siFn14 | Dharmacoa         |                     | L-010661-00-0005 |
| Apoptosis/ Necrosis Assay kit | Abcam              |                     | ab176749 |
| CellTiter-Glo Luminescent Cell Viability Assay | Promega            |                     | G7570  |
Supplementary Figure S1 (A to D) Fn14 expression is increased after (A,B) BCG infection or (C,D) heat-killed *M. tuberculosis* (HKMT) treatment in a dose- and time- dependent manner. (E) The cell viability of THP-1 cell-derived macrophages with H37Rv or BCG infection at a MOI of 10. (F) The time kinetic of H37Rv proliferation in Fn14 knockdown cells. (G) The cell viability of vector-transfected or Fn14 over-expressing cells with or without H37Rv infection. Immunoblots were quantitated by densitometric analysis using ImageJ software and normalized to β-actin. Numbers below each lane are relative fold of the control level of a specific protein in mock-treated cells. All experiments were performed in triplicate and data are presented as the mean±SD. * P<0.05, ** P<0.01, *** P<0.005. NS, no significant.
Supplementary Figure S2 THP-1 cell-derived macrophages were transfected with pCMV-Fn14 to overexpress Fn14. After 24 h, (A) the levels of Fn14, LC3, Beclin-1, and β-actin were detected by immunoblotting. The bafilomycin A1 (BafA1) treatment was used as positive control. Immunoblots were quantitated by densitometric analysis using ImageJ software and normalized to β-actin. Numbers below each lane are relative fold of the control level of a specific protein in mock-treated cells. The relative levels of (B) Fn14, (C) LC3-II, and (D) Beclin-1 were calculated, respectively. All experiments were performed in triplicate and data are presented as the mean±SD. ** P<0.01, *** P<0.005. NS, no significant.
Supplementary Figure S3 THP-1 cells stably expressing RFP-GFP-LC3 fusion protein were transfected with Fn14 siRNA to knockdown Fn14. After 24 h, cells were infected with *M. bovis* BCG at a MOI of 10 for 24 h. The RFP-GFP-LC3 puncta were detected by confocal microscopy (upper panel) and quantified (lower panel). RPMI medium were used as solvent control (SC). Bafilomycin A1 (BafA1, 100nM) treatment were used as positive control. All experiments were performed in triplicate and data are presented as the mean±SD.**P<0.01, ***P<0.005.
**Supplementary Figure S4** THP-1 cell-derived macrophages were transfected with siFn14 or treated with anti-TWEAK monoclonal antibody for 6h. The cells were stained with Fluo-4 AM (5 µM, 1h at 37°C), then infected with *M. tuberculosis* H37Rv at MOI of 1 (20 min). The calcium mobilization in Fluo-4 AM loading cells was detected and quantified using flow cytometry. (A) H37Rv infection induced increased levels of cytosolic calcium in THP-1-derived macrophages. This effect was diminished when (B) intracellular Fn14 was knocked down or (C) in the presence of TWEAK blocker.
Supplementary File 1. Densitometric analysis of immunoblot results presented in this study. Immunoblots were quantitated by densitometric analysis using ImageJ software and normalized to β-actin. Numbers below each lane are relative fold of the control level of a specific protein in mock-treated cells. All results were obtained in three independent experiments, and the data is presented as the mean±SD. *$P<0.05$, ** $P<0.01$, *** $P<0.005$. 
Fig. 2A

- **Fn14**
  - siFn14
  - TWEAK
  - siCtrl
  - siCtrl

- **pAMPK**
  - (T172)
  - AMPK

- **pULK1**
  - (S555)
  - ULK1

- **LC3-I**
  - LC3-II

- **Beclin-1**

- **Atg5**

- **β-actin**

The images show bar graphs comparing the fold changes of various protein levels under different conditions. The graphs indicate significant changes in protein expression levels, with specific statistical significances denoted by asterisks.

- **Fn14/β-actin (fold)**
  - siFn14
  - TWEAK
  - siCtrl
  - siCtrl

- **pAMPK/AMPK (fold)**
  - LC3-II/β-actin (fold)

- **Beclin-1**

- **Atg5**

The bar graphs visually represent the fold changes in expression levels, with significant differences highlighted by asterisks.
Fig. 2F

![Diagram showing protein expression levels for Fn14, pAMPK, pULK1, LC3-I, LC3-II, Beclin-1, and β-actin after treatment with MOCK, SC, siCtrl, and siFn14 in H37Rv.](image-url)
Fig. 3C

|          | Mock | SC   | NAC  | DPI | MT  |
|----------|------|------|------|-----|-----|
| **pAMPK (T172)** | 1.00 | 2.80 | 1.04 | 1.09 | 1.07 |
| **AMPK**       | 1.00 | 2.80 | 1.04 | 1.09 | 1.07 |
| **pULK1 (S555)** | 1.00 | 2.50 | 1.32 | 1.42 | 1.23 |
| **ULK1**       | 1.00 | 2.61 | 1.03 | 0.99 | 0.90 |
| **LC3-I**      | 1.00 | 2.73 | 1.01 | 1.05 | 1.04 |
| **LC3-II**     | 1.00 | 2.73 | 1.01 | 1.05 | 1.04 |
| **Becnin-1**   | 1.00 | 2.61 | 1.03 | 0.99 | 0.90 |
| **Atg5**       | 1.00 | 2.56 | 0.98 | 0.95 | 0.90 |
| **β-actin**    | 1.00 | 0.98 | 0.97 | 1.02 | 1.03 |

**Histograms**

- **pAMPK/AMPK (fold)**
- **pULK1/ULK1 (fold)**
- **LC3-II/β-actin (fold)**
- **Becnin-1/β-actin (fold)**
- **Atg5/β-actin (fold)**
Fig. 4D

| Protein  | Condition | Mock | SC | siCtrl | siFn14 |
|----------|-----------|------|----|--------|--------|
| Fn14     | TWEAK     |      |    |        |        |
| STIM1    | TWEAK     |      |    |        |        |
| ORAI1    | TWEAK     |      |    |        |        |
| IP3R     | TWEAK     |      |    |        |        |
| TRPM7    | TWEAK     |      |    |        |        |
| pCaMKK2  | TWEAK     |      |    |        |        |
| LC3-I    | TWEAK     |      |    |        |        |
| LC3-II   | TWEAK     |      |    |        |        |
| β-actin  |           |      |    |        |        |

* Significant difference
** Highly significant difference
ns No significant difference
### Fig. 4E

|       | TWEAK |
|-------|-------|
|       | Mock  | SC   | DPI  | MT   | BTP2 |
|       | 1.00  | 2.77 | 1.05 | 1.01 | 1.29 |
| STIM1 |       |      |      |      |      |
| ORAI1 | 1.00  | 2.84 | 1.08 | 1.02 | 1.03 |
|       |       |      |      |      |      |
|       |       | 1.00 | 2.62 | 1.07 | 1.02 | 1.18 |
| pCaMKK2 | 1.00 | 2.75 | 1.13 | 1.05 | 1.32 |
|       |       | 1.00 | 2.47 | 0.98 | 0.99 | 1.04 |
| LC3-I | 1.00  | 2.69 | 0.99 | 0.91 | 1.11 |
| LC3-II| 1.00  | 2.59 | 1.17 | 1.09 | 1.25 |
| Beclin-1 | 1.00 | 2.59 | 1.17 | 1.09 | 1.25 |
|       | 1.00  | 0.98 | 0.97 | 1.02 | 1.03 |
| Atg5  | 1.00  | 2.59 | 1.17 | 1.09 | 1.25 |
| β-actin | 1.00 | 0.98 | 0.97 | 1.02 | 1.03 |

|       | 75 kDa |
|       |        |
|       |        |
|       |        |
|       |        |

**Notes:**
- **STIM1**
  - ** Mock: 1.00, SC: 2.77, DPI: 1.05, MT: 1.01, BTP2: 1.29
- **ORA11**
  - ** Mock: 1.00, SC: 2.84, DPI: 1.08, MT: 1.02, BTP2: 1.03
- **pCaMKK2**
  - ** Mock: 1.00, SC: 2.62, DPI: 1.07, MT: 1.02, BTP2: 1.18
- **pAMPK (T172)**
  - ** Mock: 1.00, SC: 2.75, DPI: 1.13, MT: 1.05, BTP2: 1.32
- **LC3-I**
  - ** Mock: 1.00, SC: 2.47, DPI: 0.98, MT: 0.99, BTP2: 1.04
- **LC3-II**
  - ** Mock: 1.00, SC: 2.69, DPI: 0.99, MT: 0.91, BTP2: 1.11
- **Beclin-1**
  - ** Mock: 1.00, SC: 2.59, DPI: 1.17, MT: 1.09, BTP2: 1.25
- **Atg5**
  - ** Mock: 1.00, SC: 2.59, DPI: 1.17, MT: 1.09, BTP2: 1.25

**Bar Graphs:**
- **STIM1**
- **ORA11**
- **pCaMKK2**
- **pAMPK**
- **LC3-II**
- **Beclin-1**
- **Atg5**
|        | Mock | SC   | siCtrl | siFn14 | anti-TWEAK | IgG  | kDa |
|--------|------|------|--------|--------|------------|------|-----|
| Fn14   | 1.00 | 3.23 | 3.38   | 1.11   | 1.10       | 2.85 | 17  |
| CHOP   | 1.00 | 3.08 | 3.11   | 1.28   | 1.16       | 3.09 | 25  |
| BiP    | 1.00 | 3.49 | 3.42   | 1.18   | 1.24       | 2.81 | 75  |
| STIM1  | 1.00 | 3.17 | 3.23   | 1.06   | 1.14       | 2.89 | 100 |
| ORAI1  | 1.00 | 3.42 | 3.39   | 1.42   | 1.40       | 2.61 | 35  |
| pCaMKK2| 1.00 | 3.09 | 3.04   | 1.02   | 1.04       | 2.70 | 75  |
| pAMPK1 | 1.00 | 3.13 | 3.01   | 1.03   | 0.95       | 2.94 | 63  |
| LC3-I  | 1.00 | 3.33 | 3.42   | 1.09   | 1.12       | 3.04 | 17  |
| LC3-II | 1.00 | 1.01 | 0.96   | 1.04   | 0.97       | 1.01 | 11  |
| β-actin| 1.00 | 0.97 | 0.98   | 0.96   | 1.01       | 0.97 | 48  |

**Fig. 5B**
Fig. 6A

**Table 1.**

|          | Mock | 24   | 48   | 72(h) |
|----------|------|------|------|-------|
| Fn14     | 1.00 | 2.24 | 2.89 | 3.06  |
| pRIPK1   | 1.00 | 2.11 | 2.89 | 2.76  |
| Casp-8   | 1.00 | 1.22 | 1.92 | 1.90  |
| cleaved Casp-3 | 1.00 | 2.44 | 2.50 | 2.48  |
| β-actin  | 1.00 | 0.97 | 1.03 | 1.05  |

**Figure Legends.**

- **Fn14**
- **pRIPK1**
- **Casp-8**
- **cleaved Casp-3**
Fig. 6B

![Graph showing changes in Fn14, pRIPK1, Casp-8, and cleaved Casp-3 expression levels with BCG treatment.](image)
Fig. 6G