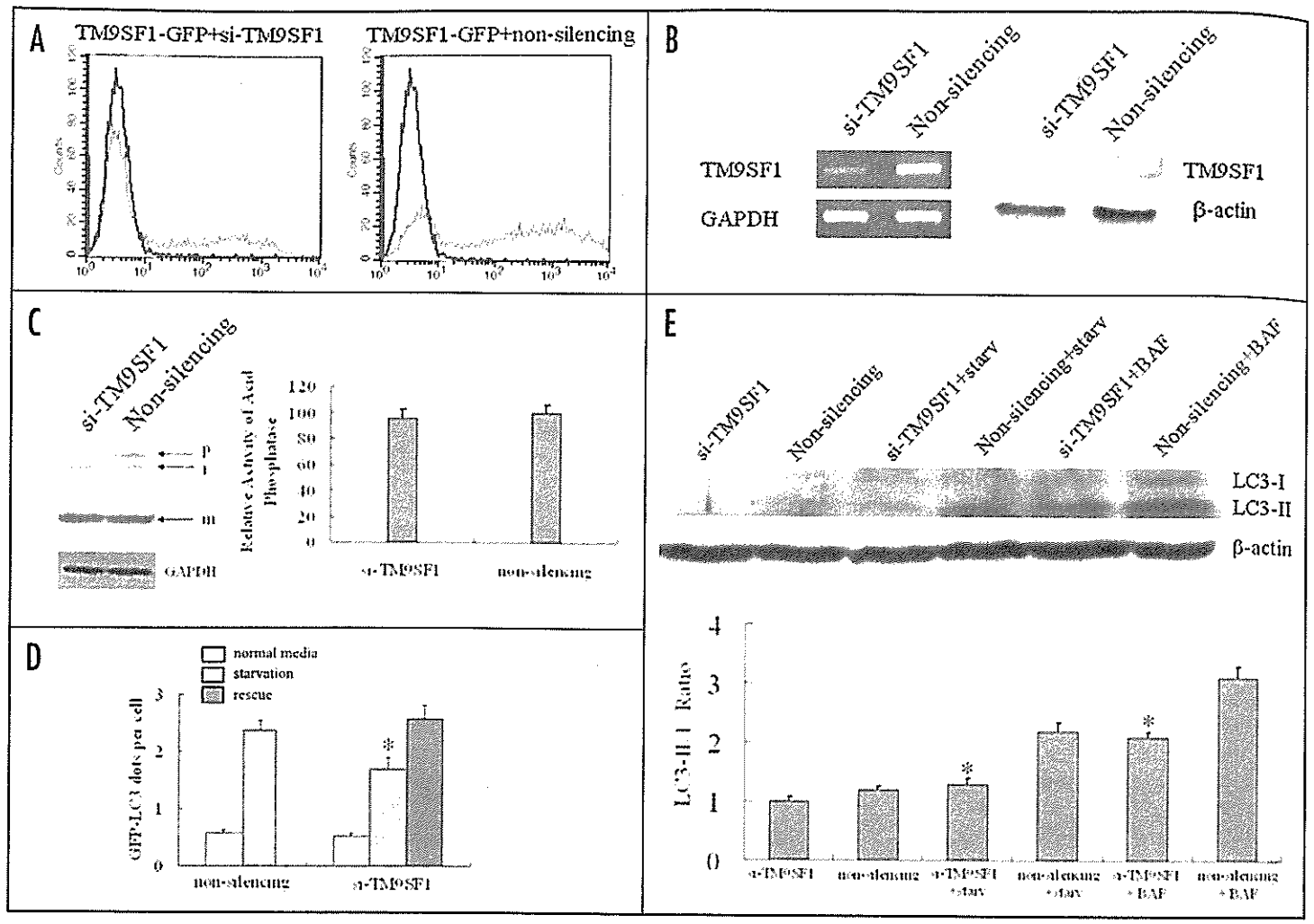

Figure 5. Knockdown of *TM9SF1* inhibits starvation-induced autophagy. (A) si-TM9SF1 inhibited the expression of TM9SF1-GFP fusion protein in HeLa cells. HeLa cells were cotransfected with si-TM9SF1 (or nonsilencing siRNA) and TM9SF1-GFP by electroporation. Fluorescent intensity was analysis by flow cytometry 48 h after transfection. Fluorescent intensity of cells transfected with si-TM9SF1 decreased over 80% compared with nonsilencing siRNA. Blue curve represent control cells (normal HeLa cells without GFP transfection). (B) Si-TM9SF1 inhibited TM9SF1 mRNA or protein expression. HeLa cells were transfected with si-TM9SF1 (or nonsilencing siRNA). 48 h later, mRNA or protein was prepared for RT-PCR or immunoblotting analysis respectively. (C) Western blotting of cathepsin-D from nonsilencing and si-TM9SF1 transfected HeLa cells. p, precursor; i, intermediate and m, mature form of the enzyme. Equal amounts of protein were loaded in each lane. Total proteins of nonsilencing and si-TM9SF1 transfected HeLa cells were extracted and their lysates were used to measure the lysosomal acid phosphatase enzymatic activity. Data represents the mean ± SD of three independent experiments. (D) Si-TM9SF1 attenuated the increment of GFP-LC3 punctate cells in starved HeLa cells. HeLa cells were transfected with GFP-LC3 and nonsilencing or si-TM9SF1 and cultured under the standard starvation protocol. After starvation, si-TM9SF1 transfected cells were rescued by transient transfection with knockdown-resistant TM9SF1-myc. The average number of GFP-LC3 dots per cell was determined and expressed as the mean ± SD of three independent experiments. * Significantly different than control, $p < 0.05$. (E) Si-TM9SF1 decreased the ratio of LC3-II/LC3-I in starved HeLa cells. HeLa cells were transfected with non-silencing siRNA or si-TM9SF1. 48 h after transfection, cells were starved for 2 h or treated with Bafilomycin A1 and then were lysed for immunoblot analysis. The ratio of cellular LC3-II/LC3-I are shown as the mean ± SD of three independent experiments. * Significantly different than control, $p < 0.05$.

in-frame restriction site of pcDNA.3.1/myc-His(-)B (Invitrogen, USA) and EGFP-N1 (Clontech, USA), respectively. To construct a plasmid expressing knockdown-resistant TM9SF1-myc, base substitutions were made by site-specific mutagenesis employing the overlap extension method and confirmed by DNA sequencing.

cDNA library construction. A human cDNA library was constructed as described previously.[10] A total of 1,050 human ORFs was generated for use in various cell-based functional screening approaches.[10,11] Our library consisted primarily of cDNAs encoding proteins of unknown function or with poorly defined functions; the role of most of these genes is still not known (Suppl. Table 1).

Cell culture and transfection. HeLa (ATCC CCL-2) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal calf serum (FCS) (Invitrogen, USA) at 37°C in a 5% CO_2 atmosphere. HeLa cells were cultured in 96-well plates at a density of 10,000 cells/well for the cell-based screening assays to examine GFP-LC3 localization. Twenty-four hours after plating, 90 ng of candidate plasmid DNA was cotransfected with 60 ng of GFP-LC3 plasmid DNA in each well using VigoFect (Vigorous, China), a nonliposomal cationic reagent, according to the manufacturer's instruction. For immunoblot analyses, confocal microscopy assays or electron microscopy assays, cells were electroporated for 20 ms at 120 V using 10 μg of plasmid DNA per 2 x 10^6 cells in 2 mm gap cuvettes using an ECM 830 Square Wave Electroporation System (BTX, USA). At 24 h post-transfection, HeLa cells were used in a biochemical and cell biological assays.

Quantitative analysis of GFP-LC3 dots. GFP-LC3-transfected cells cultured in 96-well plates were viewed using a 20x objective lens and autoscanned using an automated fluorescent microscope (Axiovert 200M, Zeiss, Germany) equipped with computer software-driven commands including "scan multiwell plate" and "auto focus" (MetaMorph 7.0 Imaging System, Molecular Devices, USA) (Fig. 1A). Exposure times were set at 300 ms, the number of punctate dots of GFP-LC3 signal per cell in GFP-LC3-positive cells was determined using the "granularity" application module of the MetaMorph 7.0 Imaging System (Fig. 1B). The "granularity" application module is designed to detect and count granules in cells and to measure the physical characteristics of granules (such as area and intensity), which can determine the average number of granules per cell directly. The number of GFP-LC3 dots per cell was counted in at least two independent visual fields from two independent wells. The results were expressed as the average number of GFP-LC3 dots in each cell (mean ± SD).

Confocal microscopy. HeLa cells transfected with *TM9SF1* or TM9SF1-GFP were grown on coverslips, and stained with 200 nM LTR for 15 min, 100 nM MTR for 15 min, or 0.05 mM Monodansylcadaverine (MDC) for 1 h at 37°C; cells were washed three times with PBS and observed using a Leica TCS SP2 Confocal System (Heidelberg, Germany) equipped with the appropriate filters. HeLa cells cotransfected with TM9SF1-GFP and DsRed-LC3 were observed directly using the same confocal system. For myc-tagged *TM9SF1*, indirect immunofluorescence assay was used.

Electron microscopy analysis. HeLa cells cultured on coverslips were prefixed in 4% glutaraldehyde in PBS at 4°C, treated with 1% OsO_4 for 2 h at 4°C, dehydrated in a graded series of ethanol, and flat embedded in epon. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEM-1230 transmission electron microscope (JEOL, Japan). The number of autophagosomes per cell profile was determined by an observer blinded to experimental conditions for a minimum of 50 cells for each specimen.

Immunoblot analysis. Immunoblotting was performed as described previously.[12] Total cell lysates were prepared from HeLa cells, and immunoblotting was performed using different antibodies.

Enzymatic assay. Lysosomal acid phosphatase activity was assayed using 4-methylumbelliferyl phosphate (4-MUP) substrates as described.[13,14] HeLa cells were lysed with enzyme lysis buffer (50 mM Tris-HCl pH 7.4, 1% Triton-X100, 300 mM NaCl) supplemented with a complete protease inhibitor cocktail (Pierce). The supernatants were incubated at 37°C for 30 min with 1 mM 4-MUP substrate in 0.1 M sodium acetate buffer (pH 4.5), the reaction was stopped by the addition of 0.15 M glycine buffer (pH 10.3), and the fluorescence was measured at Excitation 355 nm/Emission 460 nm by using a GENios Pro reader (Tecan, Switzerland).

TM9SF1 siRNAs synthesis and electroporation transfection. Specific siRNA against *TM9SF1* with targeting sequence 5'-GAA TGG CTG AGT CTT TGT A-3' (si-TM9SF1) was synthesized by RiboBio Corporation (Guangzhou, China). Nonsilencing siRNA that had no sequence homology to any known human genes was used as the control. Both siRNAs were dissolved at a concentration of 20 μM in buffer containing 20 mM KCl, 6 mM HEPES, pH 7.5, 0.2 mM $MgCl_2$. Cells were fed with fresh culture medium prior to experiments. HeLa cells were electroporated for 20 ms at 120 V, with 10 μg plasmid or 5 μl siRNA per 10^6 cells in 2 mm gap cuvettes using an ECM 830 Square Wave Electroporation System (BTX, USA).

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/HeAUTO5-1-Sup.xls

References

1. Debnath J, Baehrecke EH, Kroemer G. Does autophagy contribute to cell death? Autophagy 2005; 1:66-74.
2. Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. Oncogene 2004; 23:2891-906.
3. Levine B. Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. Cell 2005; 120:159-62.
4. Meijer AJ, Codogno P. Regulation and role of autophagy in mammalian cells. Int J Biochem Cell Biol 2004; 36:2445-62.
5. Klionsky DJ. The molecular machinery of autophagy: unanswered questions. J Cell Sci 2005; 118:7-18.
6. Reggiori F, Klionsky DJ. Autophagy in the eukaryotic cell. Eukaryot Cell 2002; 1:11-21.
7. Mizushima N. Methods for monitoring autophagy. Int J Biochem Cell Biol 2004; 36:2491-502.
8. Asanuma K, Tanida I, Shirato I, Ueno T, Takahara H, Nishitani T, Kominami E, Tomino Y. MAP-LC3, a promising autophagosomal marker, is processed during the differentiation and recovery of podocytes from PAN nephrosis. Faseb J 2003; 17:1165-7.
9. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. Embo J 2000; 19:5720-8.
10. Wang L, Gao X, Gao P, Deng W, Yu P, Ma J, Guo J, Wang X, Cheng H, Zhang C, Yu C, Ma X, Lv B, Lu Y, Shi T, Ma D. Cell-Based Screening and Validation of Human Novel Genes Associated with Cell Viability. J Biomol Screen 2006; 11:369-76.
11. Ma X, Wang X, Gao X, Wang L, Lu Y, Gao P, Deng W, Yu P, Ma J, Guo J, Cheng H, Zhang C, Shi T, Ma D. Identification of five human novel genes associated with cell proliferation by cell-based screening from an expressed cDNA ORF library. Life Sciences 2007; 81:1141-51.
12. Lv B, Shi T, Wang X, Song Q, Zhang Y, Shen Y, Ma D, Lou Y. Overexpression of the novel human gene, nuclear apoptosis-inducing factor 1, induces apoptosis. Int J Biochem Cell Biol 2006; 38:671-83.
13. Zhu M, Lovell KL, Patterson JS, Saunders TL, Hughes ED, Friderici KH. Beta-mannosidosis mice: a model for the human lysosomal storage disease. Hum Mol Genet 2006; 15:493-500.
14. Liang C, Feng P, Ku B, Dotan I, Canaani D, Oh BH, Jung JU. Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. Nat Cell Biol 2006; 8:688-99.
15. Biederbick A, Kern HF, Elsasser HP. Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. Eur J Cell Biol 1995; 66:3-14.
16. Bampton ET, Goemans CG, Niranjan D, Mizushima N, Tolkovsky AM. The dynamics of autophagy visualized in live cells: from autophagosome formation to fusion with endo/lysosomes. Autophagy 2005; 1:23-36.
17. Niemann A, Baltes J, Elsasser H-P. Fluorescence Properties and Staining Behavior of Monodansylpentane, a Structural Homologue of the Lysosomotropic Agent Monodansylcadaverine. J Histochem Cytochem 2001; 49:177-86.
18. Bagshaw RD, Mahuran DJ, Callahan JW. A proteomic analysis of lysosomal integral membrane proteins reveals the diverse composition of the organelle. Mol Cell Proteomics 2005; 4:133-43.