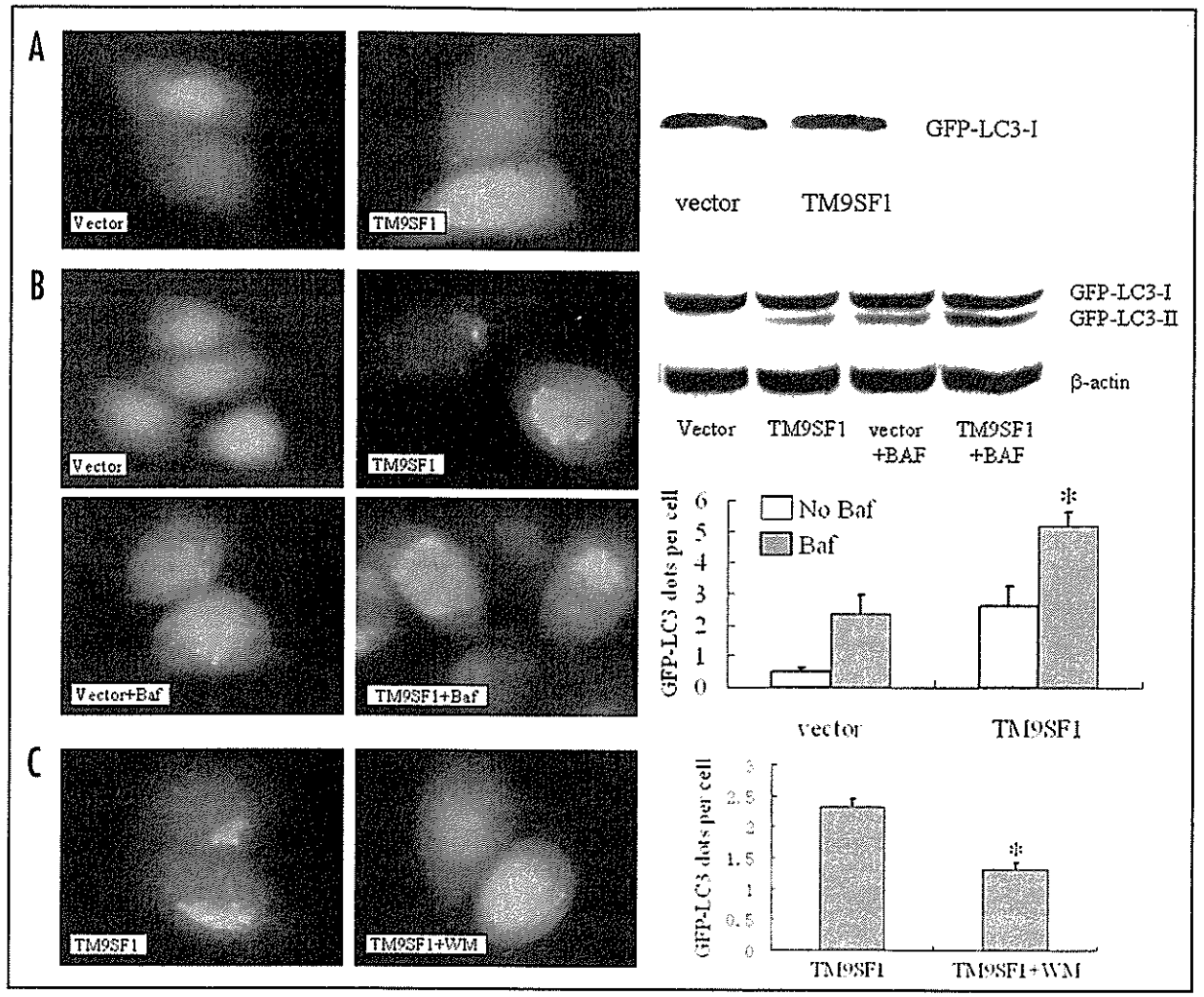

Figure 3. Validation for the autophagy-inducing role of *TM9SF1* by GFP-LC3(G120A), Bafilomycin A1 and wortmannin. (A) overexpression of *TM9SF1* could not induce the aggregate of GFP-LC3(G120A). HeLa cells were transfected GFP-LC3(G120A) and *TM9SF1* (or empty vector). 24 h after transfection, cells were examined under fluorescent microscope (left) or by western blotting using anti-LC3 antibodies (right). (B) After 48 h of transfection, Bafilomycin A1 (100 nM) was added or not added to the cell culture medium. The cells were examined under fluorescent microscope (left). The average number of GFP-LC3 dots per cell was quantified as the mean ± SD of at least three independent experiments (right). Western blot analysis was also given, which consistent with the GFP-LC3 punctate cells analysis. * Significantly different than control, $p < 0.05$. (C) Increment of GFP-LC3 punctate cells that induced by overexpression of *TM9SF1* could be inhibited by autophagy inhibitor wortmannin. HeLa cells treated with 100 nM wortmannin during co-transfection with GFP-LC3 and *TM9SF1* (or empty vector). After 24 h, the average number of GFP-LC3 dots per cell (left) was determined and expressed as the mean ± SD of three independent experiments (right). * Significantly different than control, $p < 0.05$.

in the MetaMorph 7.0 Imaging System (Molecular Devices, USA). In addition, we performed an automated image analysis to detect the number of "granules per cell" using the "granularity" application module of MetaMorph 7.0, which is designed to detect and count granules in cells and to measure the physical characteristics of granules. In this study, we developed an effective and convenient screening platform for identifying autophagy-related genes using an automated fluorescence imaging and analysis system, and we screened a library of 1,050 human genes of unknown function and identified three autophagy-related genes. This platform can be applied readily for high-throughput genomic screening using 384-well plates or cellular arrays.

GFP-LC3 was used as an autophagic marker in our screen for autophagy-inducing genes. However, it should be noted that most LC3-positive dots represent preautophagosomes and autophagosomes, rather than autolysosomes.[28] The appearance of GFP-LC3 dots or of increasing levels of LC3-II, strictly speaking, does not necessarily correspond to autophagic degradation. Therefore, the autophagy-related genes we identified based on the number of GFP-LC3 dots are considered autophagosome-inducing genes, but not necessarily autophagy-induced genes. Further experimentation, including assays to determine LC3-II turnover suggested by Isei Tanida, will be required to validate the exact roles of the "hits" with respect to autophagy.[28]

The three initial "hits" from our GFP-LC3-based screen, including *TM9SF1*, *TMEM166* and *TMEM74*, were validated using independent methods to detect autophagy, including TEM, LC3 immunoblot analysis, and MDC staining. *TMEM166* (transmembrane protein 166, also known as FLJ13391) was first identified as a novel gene associated with cell viability in our lab.[10] In our autophagy screen and apoptosis assays, *TMEM166* was identified as a novel regulator involved in both autophagy and apoptosis.[29]

TMEM74 (transmembrane protein 74) was first identified as a regulator of cell death in a previous study from our lab, and its role in autophagy has been further studied by Dr. Yu in our lab.[30]

TM9SF1 (transmembrane 9 superfamily member 1, NM_006405.5), also called MP70, is a nine-spanning transmembrane protein first cloned in 1997.[29] *TM9SF1* expression is ubiquitous in human tissues, and it is widely expressed and highly conserved among yeast, plants and mammals.[31] It was reported that *TM9SF1* could be induced by the neurotoxin 6-OHDA in a model of Parkinson's disease in PC12 cells.[32] In a recent study of identification tumor-associated antigens,[31] *TM9SF1* was overexpressed in tumors at levels 5.20 times higher than in normal breast tissue. The same study demonstrated that myc-tagged TM9SF1 localized to the cell surface of transfected COS-7L cells.[33] However, the function of *TM9SF1* remained unknown until now. Here, we found that *TM9SF1* may play an important role in autophagosome induction. We further found that TM9SF1-GFP localized to lysosomes, but not to other membrane compartments in transfected HeLa cells: TM9SF1-GFP colocalized extensively with LTR (lysosomal marker) and with DsRed-LC3 (autophagic vacuole marker). Furthermore, knockdown of *TM9SF1* by RNA interference could attenuate autophagosome formation. Collectively, we hypothesized that *TM9SF1* might play a modulating role in the process of autophagy, and then accelerate functional autophagy. But the molecular mechanism is still unclear.

In summary, we have established an effective and convenient cell-based screening platform, which can be applied for high-throughput genomic screening candidate autophagy-related genes using human cDNA and/or siRNA libraries. Three genes (*TMEM74*, *TMEM166* and *TM9SF1*) were identified as positive genes which overexpression can induce autophagosome formation. *TM9SF1* was further investigated as a novel autophagy-related gene. Further studies will be required to elucidate the specific role of *TM9SF1* in autophagy.

Materials and Methods

Materials. We generated rabbit anti-LC3 polyclonal antibodies using recombinant rat LC3 protein expressed in *E. coli* as the antigen, followed by affinity purification and validation by ELISA and immunoblot analysis. Monoclonal antibodies against β-actin and GFP were purchased from Santa Cruz Biotechnology Inc., (USA). HRP-conjugated secondary antibodies against mouse and rabbit IgG were purchased from Cell Signaling (USA). LysoTracker Red (LTR) and MitoTracker Red (MTR) were purchased from Molecular Probes (USA). Earle's Balanced Salt Solution (EBSS) and Bafilomycin A1 and wortmannin were purchased from Sigma (USA). Polyclonal antibody against TM9SF1 and Cathepsin D were purchased from Aviva Systems Biology (USA) and Santa Cruz (USA), respectively.

The vector for expression of GFP-LC3 was kindly provided by Dr. Zhenyu Yue (Mount Sinai School of Medicine, New York). The vector for expression of mutant GFP-LC3(G120A) was kindly provided by Dr. Tamotsu Yoshimori (Osaka University, Japan). The vector for expression of DsRed-LC3 was constructed by inserting the PCR-amplified LC3 coding sequence from GFP-LC3 into the in-frame restriction site of the DsRed vector. TM9SF1-GFP were constructed by inserting the coding sequence of *TM9SF1* into the

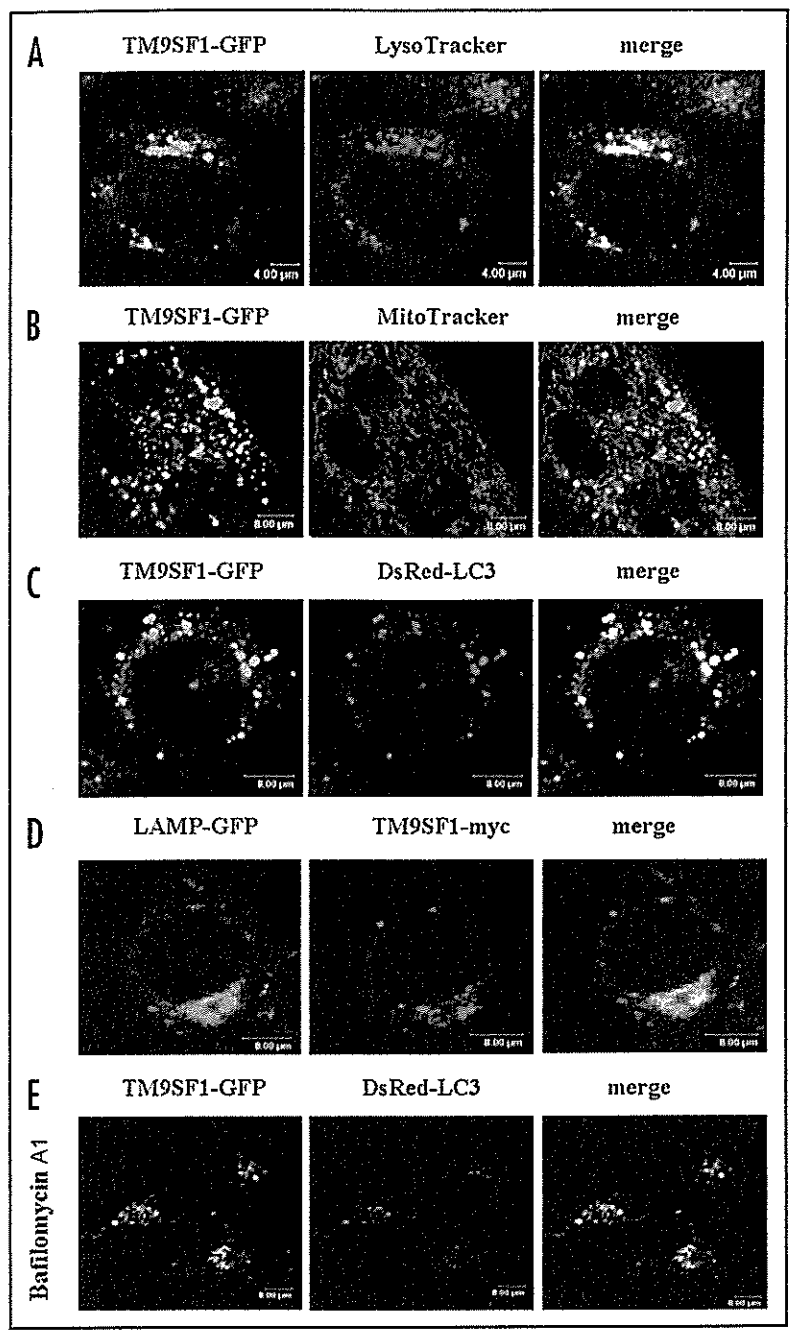

Figure 4. Subcellular localization of TM9SF1. For (A) LTR or (B) MTR labeling, HeLa cells were transfected with TM9SF1-GFP. At 24 h post-transfection, HeLa cells were probed with LTR or MTR and were analyzed using two-color confocal microscopy. (C) For DsRed-LC3 studies, HeLa cells were cotransfected with TM9SF1-GFP and DsRed-LC3. At 24 h posttransfection, HeLa cells were analyzed using two-color confocal microscopy. As shown in the merged image, TM9SF1 colocalized extensively with LTR and DsRed-LC3, but not with MTR. (D) Cells were transiently transfected with TM9SF1-myc and LAMP1-GFP. 24 h after transfection, cells were stained by indirect immunofluorescence with anti-myc antibody and analyzed by confocal microscopy. (E) TM9SF1-GFP and DsRed-LC3 were cotransfected into cells. Cells were incubated with 100 nM Bafilomycin A1 for 2 h and analyzed by confocal microscopy.