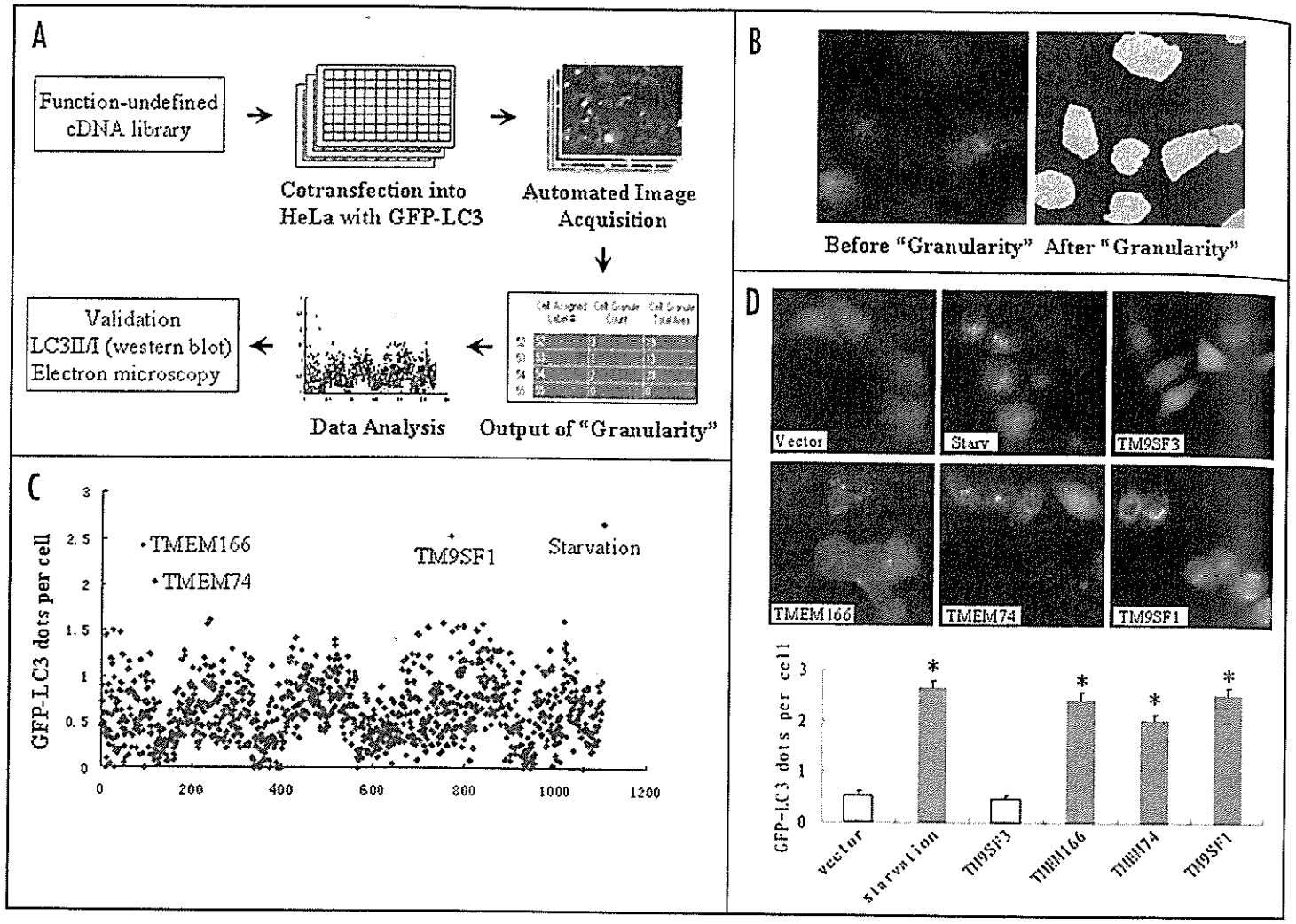

Figure 1. Screening for autophagy-related genes in HeLa cells using GFP-LC3. (A) Work flow of functional screening for autophagy-related genes based on automated fluorescence imaging. (B) "Granularity" module of the MetaMorph 7.0 Imaging System (Molecular Devices, USA). The "granularity" application module is designed to detect and count granules in cells and to measure the physical characteristics of granules. (C) Quantitative analysis of GFP-LC3 dots in HeLa cells overexpressing the gene of unknown function from our cDNA library. The number of GFP-LC3 dots per cell in GFP-LC3-positive cells was determined using the "granularity" application module of the MetaMorph 7.0 Imaging System (Molecular Devices, USA) at 24 h post-transfection. The number of GFP-LC3 dots 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 per cell. Starvation (autophagy induced by replacing DMEM medium to Earle's balanced salts solution (EBSS) for HeLa for 12 h) and three "hits" from the cDNA library (*TMEM166*, *TMEM74* and *TM9SF1*) were marked with a red spot. (D) The punctate distribution of GFP-LC3 induced by *TMEM166*, *TMEM74* or *TM9SF1* overexpressed in HeLa cells for 24 h. Negative controls corresponding to the vector alone or to cells expressing an irrelevant membrane protein control (TM9SF3: transmembrane 9 superfamily member 3, a homology of TM9SF1) did not induce a punctate distribution of GFP-LC3 in HeLa cells. HeLa cells that were cotransfected pcDNA.3.1/myc-His(-)B and GFP-LC3 grown in EBSS for 12 h were set as positive control. Pictures were taken using a 20× objective lens. The number of dots per cell of different genes was quantified. Results are the mean ± SD of three independent experiments. * Significantly different than control, $p < 0.05$.

we detected two major lysosomal proteinases. The result showed no difference in the cathepsin D and acid phosphatase enzymatic activity between nonsilencing and si-TM9SF1 transfected HeLa cells (Fig. 5C). Further, we evaluated whether si-TM9SF1 treatment could inhibit starvation-induced autophagy. HeLa cells transfected with nonsilencing siRNA or si-TM9SF1 were induced by starvation for 2 h to promote autophagy. As illustrated in Figure 5D and E, si-TM9SF1 could inhibit both overexpressed GFP-LC3-II and endogenous LC3-II levels in starved HeLa cells. Furthermore, rescue assay showed knockdown-resistant gene can suppress the defect of the si-TM9SF1 and induce autophagy. While treated with Bafilomycin A1, the LC3- II/LC3-I ratio of non-silencing siRNA transfected HeLa cells was also significantly increased compared with si-TM9SF1 transfected cells. These data suggest that *TM9SF1* plays a key role in the regulation of cell autophagy.

Discussion

Cellular based large-scale screens have played important roles in elucidating the functions of human genes in a variety of cell signal pathways and in diverse cellular processes.[19-21] Autophagy is associated with many forms of human diseases.[4] However, many of the mammalian genes involved in autophagy remain unidentified and studies to monitor autophagy in mammalian cells have only been performed on a small scale.[7-9,22,23] Therefore, it is necessary

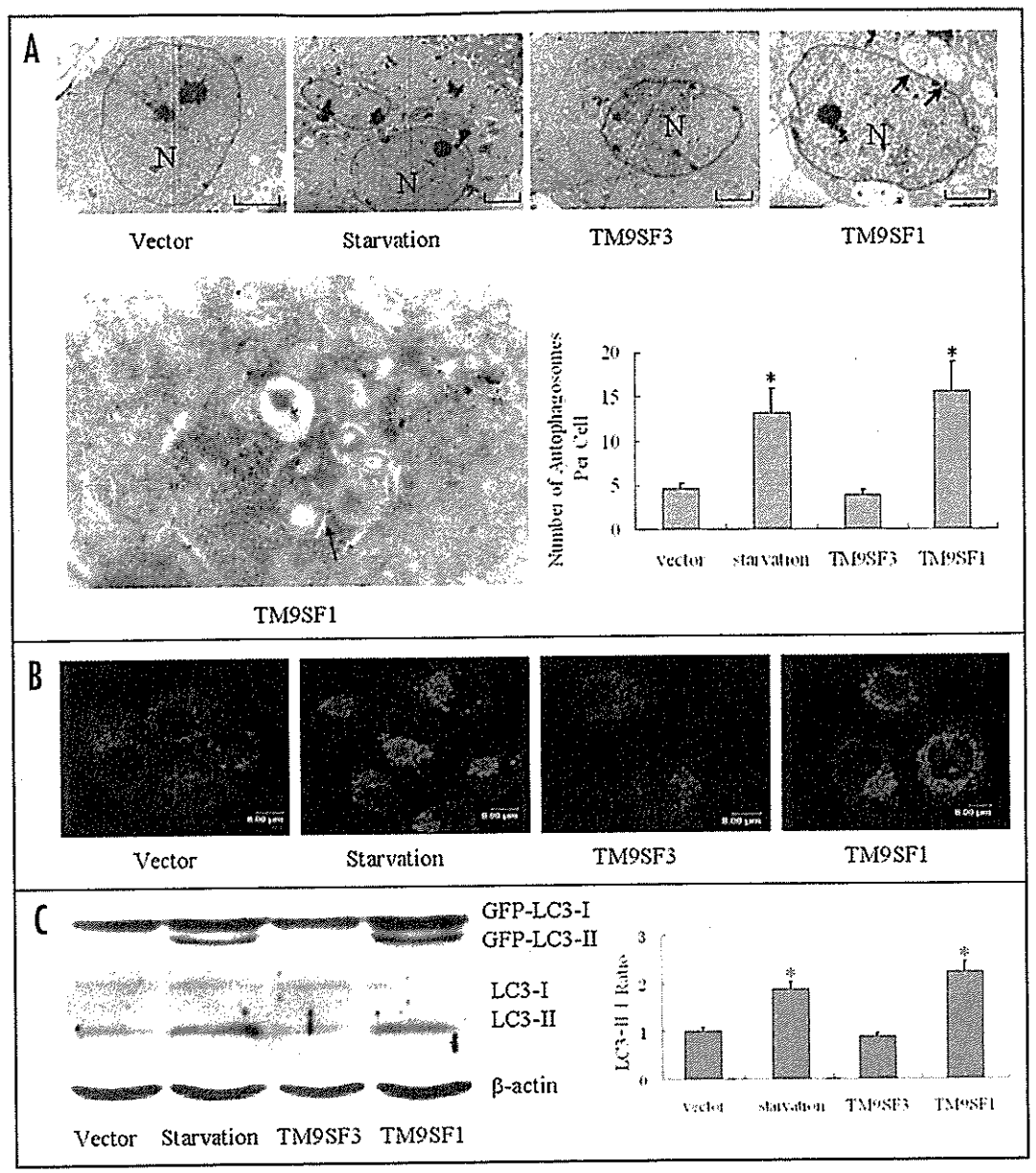

Figure 2. Autophagosomes are accumulated in cells overexpressed *TM9SF1*. (A) Electron microscopic images obtained from transfected HeLa cells. Extensive cytoplasmic vacuolization was observed in starved (starvation) and *TM9SF1*-transfected HeLa cells. A higher magnification (x16,000) image of *TM9SF1* transfected cell was also shown. The number of autophagosomes per cell was quantified by electron microscopy. N, nucleus; arrows indicate autophagosome vacuoles. Scale bars, 2 μM. (B) *TM9SF1* overexpression led to increased MDC staining in HeLa cells. MDC is used as a marker for acidic cellular vacuoles. The pictures were taken using a 20x microscope objective lens. (C) Increased ratio of GFP-LC3-II/GFP-LC3-I and endogenous LC3-II/LC3-I induced by *TM9SF1* overexpression but not *TM9SF3*. The ratio of cellular LC3-II/LC3-I are shown as the mean ± SD of three independent experiments. * Significantly different than control, $p < 0.05$.

to establish a platform suitable for large-scale functional screening mammalian genes involved in autophagy.

There are varieties of autophagy detection methods being used in higher eukaryotes, such as quantitative electron microscopy, LC3 and TOR and p62 western blotting, GFP-LC3 and tandem RFP-GFP fluorescence microscopy, and turnover of long-lived proteins, etc.[24,25] However, many of them are time-consuming and labored. LC3 was identified as a specific and convenient autophagic marker in mammalian cells, and GFP-LC3 was recommended for use in high-throughput functional screens to target autophagy.[8,9] Examination of GFP-LC3 localization is relatively simple, and requires only a high-resolution fluorescence microscope. In order to use GFP-LC3 in a high-throughput fluorescence microscopy-based screen, a platform must be established for automated image capture and analysis. Automated fluorescence microscopy imaging and analysis technologies have developed rapidly and have been widely applied in several high-throughput cell image-based screens in recent years.[19,26] A number of commercial instruments and software are available from well-known companies including Cellomics, Molecular Devices, Axon, Q3DM and GE Health Care.[27] Here, we developed an assay to measure autophagy using automated fluorescence microscopy imaging with 96-well plates and an automated fluorescent microscope (Axiovert 200M, Zeiss, Germany) controlled by "scan multiwell plate" and "auto focus" commands