Eukaryotic cells maintain a sophisticated network of intracellular membranous system to ensure the proper distribution and compartmentalization of cellular proteins critical for diverse functions such as cell division or cell-cell communication. Yet, little is known about the mechanism that regulates the homeostasis of this system. While analyzing the impact of sorting nexins on the trafficking of membrane type matrix metalloproteinases, we unexpectedly discovered that the expression of SNX10 induced the formation of giant vacuoles in mammalian cells. This vacuolizing activity is sensitive to mutations at the putative phosphoinositide 3-phosphate binding residue Arg53. Domain-swap experiments with SNX3 demonstrate that the PX domain of SNX10 alone is insufficient to generate vacuoles and the downstream C-terminal domain is required for vacuolization. Brefeldin A, a chemical known to block the endoplasmic reticulum to Golgi transport, inhibited the vacuolization process. Together, these results suggest that SNX10 activity may be involved in the regulation of endosome homeostasis.

Eukaryotic cells differ from prokaryotic cells by the presence of membrane-enveloped organelles with distinct morphological features such as the nuclei, the Golgi complex, the endoplasmic reticulum, mitochondria, or chloroplasts in plants and those with less morphological characteristics such as secretory vesicles, endosomes, and lysosomes (1). These organelles help the cell establish an ordered structure within which its complex biochemical reactions and signaling processes are executed. Although highly compartmentalized, these organelles are interconnected through a network of vesicles shuffling between them to distribute important cargos among different compartments and deliver wastes for disposal in the cytosols or the lysosomes (2–5). Thus, each cell achieves a state of homeostasis within this intracellular organelle system such that the relevant cellular functions are carried out properly, and its disruption may lead to cellular malfunctions.

The organelles that constitute the secretory pathway are perhaps the most dynamic and best understood through both genetic and biochemical approaches (4). The secretory pathway achieves a state of homeostasis via rapid transfer of cargos through the organelles by the budding and fusion of vesicular intermediates from donor to acceptor compartments (4). Activated GTP-binding proteins such as Arfs and Rabs or a short lived lipid such as phosphoinositides (PIs)2 are known markers that confer identity to intracellular organelles (5–7). Distinct subcellular localization of individual PI species on different organelles is generated by a combination of organelle-specific PI kinases and phosphatases through cyclically phosphorylation/dephosphorylation of the PIs (6). The resulting PIs in turn recruit transport machinery and PI-binding proteins to a specific organelle (6). As such, a “PI-map” has been proposed to explain the compartmentalization of intracellular membrane systems (6, 7).

The PIs are recognized by PIBMs or PI-binding modules including the FYVE (Fab1, YOTB/ZK632.12, Vac1, and EEA1), PX (PHOX homology), PH (pleckstrin homology), ENTH (epsin N-terminal homology) and ANTH (AP180 N-terminal homology) domains (6, 8, 9). Each of the PIBMs can recognize one or several of the seven known PI species (PtdIns 3-phosphate or PI3P; PtdIns 4-phosphate or PI4P; PtdIns 5-phosphate or PI5P; PtdIns 3,4-bisphosphate or PI3,4P2; PtdIns 4,5-bisphosphate or PI4,5P2; PtdIns 3,5-bisphosphate or PI3,5P2; and PtdIns 3,4,5-trisphosphate or PI3,4,5P3), or each PI could be recognized by multiple PIBMs in mammalian cells, thus, generating a combinatorial binding specificities among intracellular vesicles and organelles (10). For example, the PH domain can recognize PI4P; PI3,4P2; PI4,5P2; PI3,5P2; and PI3,4,5P3, while PI3P can be recognized by both PX and FYVE domains (10). However, each PI species maps to specific subcellular organelles: the PI3P to the early endosomes, phagosomes, and multivesicular bodies (MVBs), the PI4P to secretory vesicles, PI4,5P2 to coated pits at the plasma membrane and Golgi complex, and PI3,5P2 to MVBs/late endosomes, and PI3,4,5P3 in plasma membrane during phagocytosis (10). This PI map suggests that the PI species play a critical role in generating and maintaining the compartmentalization of organelles through interactions with their binding partners.

The sorting nexins (SNXs) are a large diverse group of proteins unified by the presence of a PX domain (11). The first SNX, SNX1, was discovered by yeast two-hybrid screen as a regulator of EGFR trafficking and shares homology with a yeast protein called Mvp1 or multicopy suppressor of vacuolar sorting protein 1 (Vps1) (12). Subsequent studies revealed addi-
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A. HeLa cells were transfected with GFP alone (panels a and g), SNX1GFP (panels b and h), SNX2GFP (panels c and i), SNX3GFP (panels d and j), SNX6GFP (panels e and k), and SNX10GFP (panels f and l), and large vacuoles were found in cells transfected with SNX10GFP (panels f and l; red arrows). Note: the cells at the upper right corner also expressed SNX10GFP but were invisible at the setting in panel f. B, a time course observation of SNX10-mediated vacuolation. The same HeLa cells transfected with SNX10 were observed between 0 and 13.5 h and showed that vacuoles formed between 3.5 and 6 h and accumulated both in size and numbers from 6 to 13.5 h. The arrowheads indicated small vacuole formed around nuclei, and the arrows indicated the formation process of a large vacuole. C, electron microscopy of vacuoles. The red star indicated the large vacuoles in HeLa transfected with SNX10GFP (panel b), not in cell transfected with blank vector (panel a). D, the vacuoles stained by FM4-64. The red signal of FM4-64 was found primarily on the membranes of the vacuoles. E, SNX10GFP mediated vacuolation in different human cells including 293T (panels a and g), MCF-7 (panels b and h), GLC82 (panels c and i), h-LAMP1 (panels d and j), L78 (panels e and k), and SPC-A1 (panels f and l).

Induction of vacuoles in cells. A. HeLa cells were transfected with GFP alone (panels a and g), SNX1GFP (panels b and h), SNX2GFP (panels c and i), SNX3GFP (panels d and j), SNX6GFP (panels e and k), and SNX10GFP (panels f and l), and large vacuoles were found in cells transfected with SNX10GFP (panels f and l; red arrows). Note: the cells at the upper right corner also expressed SNX10GFP but were invisible at the setting in panel f. B, a time course observation of SNX10-mediated vacuolation. The same HeLa cells transfected with SNX10 were observed between 0 and 13.5 h and showed that vacuoles formed between 3.5 and 6 h and accumulated both in size and numbers from 6 to 13.5 h. The arrowheads indicated small vacuole formed around nuclei, and the arrows indicated the formation process of a large vacuole. C, electron microscopy of vacuoles. The red star indicated the large vacuoles in HeLa transfected with SNX10GFP (panel b), not in cell transfected with blank vector (panel a). D, the vacuoles stained by FM4-64. The red signal of FM4-64 was found primarily on the membranes of the vacuoles. E, SNX10GFP mediated vacuolation in different human cells including 293T (panels a and g), MCF-7 (panels b and h), GLC82 (panels c and i), h-LAMP1 (panels d and j), L78 (panels e and k), and SPC-A1 (panels f and l).
are 29 known or predicted members in the SNX family, with most of them remain uncharacterized (15). While the small group of SNXs that have been characterized so far appear to function in cargo-sorting in the endosomes, the function of most SNXs remains unknown (15).

We have been looking for strategies to identify regulators of membrane-type matrix metalloproteinases or MT-MMPs trafficking (16–18). Recently, we have undertaken a candidate gene family approach and screened all known members of the sorting nexin family as potential MT-MMP regulators (the results will be detailed elsewhere). We report here a surprising finding that SNX10 is capable of generating giant vacuoles in mammalian cells. Our results suggest that SNX10 is part of the regulatory machinery that governs endosome homeostasis.

**EXPERIMENTAL PROCEDURES**

*CDNA Constructions and Reagents—*Expressed sequence tag cDNA clones of human SNXs were either purchased from ATCC (Manassas, VA) or cloned by reverse transcription-PCR and confirmed by DNA sequencing with ABI3730 sequencer. Sequences were compared with those deposited at NCBI or GenBank™. The full-length coding fragments were cloned into eukaryotic expression vector pCR3.1-uni-tagged with FLAG or GFP-FLAG. The sequences were re-confirmed by DNA sequencing with ABI3730 sequencer. Point mutations of SNX10 were generated with QuikChange site-directed mutagenesis kit (Stratagene, CA) according to the manufacturer’s manual. Domain-swap mutants of SNX3 and SNX10 were generated by PCR and inserted into pCR3.1-uni-GFP-FLAG. Endosome marker protein expressing vectors FYVE-DsRed and Px-GFP were gifts from Dr. Jun Yuan (Harvard Medical School, Boston, MA). Rabbit anti-EGFR antibody was from Cell Signaling Technology. EGF was purchased from R&D and added to cell at 100 ng/ml. All the constructs were re-confirmed by sequencing.

**Cell Transfections, FM4–64 Staining, and Treatment with Inhibitors—**Cells were maintained at 37 °C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (for 293T) or 1640 (for HeLa, MCF-7, GLC82, h-LAPM1, L78, and SPC-A1) supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml ampicillin and 0.1% streptomycin. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. Briefly, 0.5 μg of plasmid DNAs were added per well in 24-well plate.

FM4–64 (Molecular Probes, Eugene, OR) was diluted with Me₂SO to 1 μg/μl, added to each well (1 μl) in 24-well plates 12 h post-transfection, and the cells were photographed 24 h later.

Brefeldin A (BFA) and LY290004 (Sigma) were prepared in Me₂SO (5 mg/ml) and added to cells at the same time when cells were transfected. Cells were photographed 48 h post-transfection.

![FIGURE 2. SNX10 co-localizes with endosome markers FYVE-DsRed and PX-GFP and the internalized EGFR. A, HeLa cells were co-transfected with SNX10 and marker genes. PX-GFP was transfected with SNX10-FLAG and immunostained with anti-FLAG antibody; FYVE-DsRed was co-transfected with SNX10GFP and analyzed by confocal microscopy. Note the significant co-localization signals in panels c and f. B, HeLa cells were transfected with SNX10GFP. After 24 h, cells were starved overnight and stimulated with 100 ng/ml EGF for 0 min (panels a–c) and 30 min (panels d–f). Cells were fixed and stained with rabbit anti-EGFR polyclonal antibody for the endogenous EGFR (red). Note the co-localization of EGFR and SNX10 in panels c and f. Bar represents 10 μm.](image-url)
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Western Blotting, Immunofluorescence, and Electron Microscopy—Immunoblotting and immunofluorescent staining were performed as described (19). Images were acquired with a digital camera attached to Olympus CKX41 fluorescent microscope and processed with Photoshop 7.0 (Adobe System, San Jose, CA). Confocal images were acquired using Leica TCS SP2 Spectral Confocal System. For electron microscopy, cells was transfected with pCR3.1 and SNX10GFP, then fixed with gluteraldehyde (Sigma) 48 h post-transfection. Cells were then dehydrated and embedded in epoxy resin. Ultrathin sections (60 nm) were cut, processed, and examined in the transmission electron microscope (at Southern China University of Agriculture, Guangzhou, China).

RESULTS

A Family-wide Screen for SNX Function Identified SNX10 as a Vacuolizing Factor in Mammalian Cells—We have previously shown that MT1-MMP is routed through early endosomes after internalization as a mechanism of down-regulation (18). As a major sorting complex at the early endosomes, members of the SNX family may serve as regulators of MT-MMP activity in both normal and tumor cells. To this end, we cloned all known SNXs and assessed their ability to regulate MT1-MMP through a gain-of-function screen. We observed, unexpectedly, the formation of large vacuole-type vesicles in HeLa cells transfected with SNX10 but not any other SNXs (Fig. 1A, panels f and l versus a–e and g–k). Given the close relatedness among the SNXs (11), this specific effect of SNX10 suggests that SNX10 may have a unique cellular function related to endosome homeostasis. While the number and size of vacuoles may not be directly correlative to the intensity of SNX10-GFP expression (see the upper right corner of panel f, Fig. 1A), SNX10-GFP expression and vacuolation are tightly coupled in individual cells. To further elucidate the vacuolation process, we performed a time course study on individual cells and observed the appearance of vacuoles ~4.5–6 h post-transfection (Fig. 1B, panels a–c). Vesicles of varying sizes appeared in the ensuing hours to reach a climax of one or a few giant vacuoles with concomitant disappearance of smaller vesicles (Fig. 1B, lanes c–f), suggesting that the large vesicles are formed by fusion of smaller ones continuously. Electron microscopy revealed that the vacuoles are mostly single membrane, and the large vacuole is formed through fusion of smaller ones at the periphery of the large vacuoles (Fig. 1C, panel b). The vacuolar membranes were stained with the lipophilic dye FM4-64 that were taken up through endocytosis, suggesting that these vacuoles are endosome-related (20) (Fig. 1D, panel b). We then screened multiple cell lines and found that SNX-10 is capable of mediating vacuolation in all cells tested (Fig. 1E, panels a–l). Thus, we concluded that the increased cellular activity of SNX10 perturbs the endosome homeostasis leading to the formation of giant vacuoles.

Co-localization of SNX10 with Endosome Markers and Internalized EGFR—To further demonstrate that SNX10-induced vacuoles are derived from endosomes, we characterized the localization of known endosome markers in SNX10 induced vacuoles. First we co-transfected SNX10 with a known endosome marker FYVE-DsRed that contains the FYVE domain of EEA1 fused to DsRed (8). As shown in panels a–c of Fig. 2A, FYVE-DsRed is co-localized with SNX10 in small and intermediate vesicles. Interestingly, the membranes of large vacuoles have much lower levels of SNX10 or FYVE-DsRed, suggesting that at least the small and intermediate vesicles are endosome-like organelles. We also analyzed the co-localization of SNX10 and the PX-GFP fusion that has been used as an endosome marker. It is clear that SNX10 co-localizes almost 100% with that of PX-GFP (Fig. 2A, panels d–f). Finally, we performed internalization experiment for EGFR upon stimulation with EGF (100 ng/ml) and observed the co-localization of EGFR with

FIGURE 3. SNX10 mediates vacuolization through its PI3P binding domain. A, a schematic diagram of three mutations in the PX domain of SNX10. B, wild-type and PX mutants of SNX10 expressed in HeLa cells as detected by Western blotting analysis probed with anti-FLAG antibody. C, vacuolizing activity of SNX10 and its three mutations. Note that R53A is negative, while K79A and R94A remain active. D, quantification of vacuoles per cell in C. Vacuoles in each of the transfected cell were counted and averaged. At least 100 cells were counted three times in each of the transfection and analyzed.
SNX10 in the small vesicles mostly (Fig. 2B, panels a–c versus d–f), suggesting again that SNX10 is associated with early endosomes.

The PI3P Binding Activity Is Required for SNX10-mediated Vacuolation—Members of the SNX family are proposed to mediate their physiological function via binding to PI3P on endosomes (15). To test if SNX10 functions through PI3P, we mutated the main PI3P binding residue Arg53 in SNX10 as predicted by homology modeling with yeast PX protein Grd19p (21). In addition, we also mutated a conserved Arg94, Arg99 in SNX10 (Fig. 3A). The mutants along with their wild type plasmids were transfected into HEK293 cells and analyzed by Western blotting analysis as shown in Fig. 3B. All mutants were expressed as stable proteins as judged by Western blotting analysis (Fig. 3B, lanes 3–5). We then analyzed the ability of these mutants to induce vacuole formation in HeLa cells. As shown in Fig. 3C, mutation at the main ligand binding residue Arg53 completely eliminated the ability of SNX10 to induce vacuole formation (Fig. 3C, panel c versus panel a). The secondary residue at Arg94 has a partial effect, while Lys79 has no effect on SNX10 (Fig. 3C, panels d and e versus b and Fig. 3D). These results suggest that SNX10 functions through its binding to the PI3P ligand on endosomes.

Both the PX and CD Domains of SNX10 Are Required for Vacuolation—To dissect the structure-function of SNX10, we performed domain swap and deletion experiments with SNX3 as illustrated in Fig. 4A. Their PX domains share strong homology, and both belong to the simple SNX group without any other recognizable motifs or domains. Their products were analyzed by Western blotting, and the expected proteins were obtained with the GFP fusions presented in Fig. 4B. More importantly, their abilities to induce vacuole formation were analyzed in transfected cells and presented in Fig. 4C. First, the PX domain of SNX10 alone is not sufficient to illicit vacuole formation (Fig. 4C, panels h/k versus g/j), suggesting that the C-terminal domain or CD is also required for vacuole formation. Second, swapping the CD of SNX10 into SNX3 did not generate a vacuolizing chimera (Fig. 4C, panels c/f), suggesting that a close collaboration between the PX and CD domains is needed to orchestrate the vacuolation of the endosome in mammalian cells by SNX10.

SNX10-induced Vacuolization Is Sensitive to BFA—SNX10 may induce the formation of these large vacuoles by either inhibiting the budding of vesicles from endosomes or accelerating the fusion of smaller vesicles to the growing endosomes. To gain insights into this process, we screened for small molecules that can either accelerate or inhibit the formation of vacuoles. Initially, we focused on compounds that known to impact the membrane trafficking process. Among the chemicals screened, brefeldin A specifically inhibited the formation of vacuoles in SNX10-GFP transfected cells in a dose-dependent fashion (Fig. 5, panels c/f versus b/e and a/d). The rest of the chemicals including the PI 3-kinase inhibitors, LY290004 or wortmannin, which are known to inhibit morphogenesis of MVBs (22), did not block the formation of vacuoles (data not shown), suggesting that the SNX10-mediated vacuolation is not dependent on phospholipids generated by the type I PI 3-kinases and the MVBs of the endosomal system (22, 23). Given the fact that BFA targets Golgi-associated ARF-GEFs to inhibit the budding of vesicles from the Golgi complex (24–26), we...
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conclude that SNX10-induced vacuolation is also a Golgi-dependent process, perhaps, through the fusion of Golgi-derived vesicles with the endosomes.

DISCUSSION

The sorting nexins have attracted considerable attention due to their purported role in mediating sorting of cargos in intracellular vesicles, mostly endosomes by binding to the signature PI3P localized on endosomes (5, 11, 12, 15, 23). Yet, the function of most the SNXs are unknown. Here we report a novel function for the SNX family by demonstrating the vacuolizing activity of SNX10. To our knowledge, this is the first reported function of this protein. As the mechanism that governs endosome homeostasis is poorly understood, the SNX10-mediated vacuolization may provide a model system to probe such mechanism.

The endosome morphogenesis or maintenance may be mediated by a process or complex participated by SNX10 or its interacting proteins. Our observation may be the result of the perturbation of this perhaps “dedicated” rheostat-type mechanism by the increased activity of SNX10. One may argue that the observed vacuolation is an artifact generated by the overexpressed SNX10. While SNX10 needs to be expressed over the endogenous level to generate vacuoles as presented in this report, we also observed that cells began to form vacuoles before the SNX10-GFP became detectable and many cells with high levels of SNX10-GFP did not form any vacuole or only small vacuoles. Yet, all cells that form vacuoles did express SNX10-GFP without any exception, suggesting that SNX10 is responsible for vacuolation. The precise mechanism for this discrepancy remains unknown. We speculate that SNX10 orchestrates vacuole formation by interacting with endogenous factors that regulate endosome size. One may argue that SNX10 may not be directly involved in regulating endosome formation. As such, we would predict that the targets of SNX10 are most likely involved in regulating endosome homeostasis in the mammalian cells.

The origin of the observed vacuoles can be traced at least to the Golgi complex because they are sensitive to brefeldin A, an antifungal drug that inhibits the ARF-GEFs in the Golgi complex and known to disrupt the function of Golgi (24, 25). On the other hand, FM4–64, a dye often used to track endocytosis and label endosomes (20), accumulated on the vacuole membranes (Fig. 1D), suggesting that the vacuoles are at least in part from endocytic vesicles, which is also corroborated by the internalized EGFR (Fig. 2). Consistently, BFA is also known to have similar effect on the endocytic pathway as it has on the Golgi complex (27). However, a dominant negative mutant of dynamin K44A failed to block the formation of these vacuoles (data not shown), suggesting that other forms of endocytosis other than clathrin-coated pits may be responsible to deliver the endocytosed FM4–64 to the vacuoles. Given their easily scored phenotype, the vacuoles generated by SNX10 transfection may be a model system for studying organelle biogenesis and morphogenesis in mammalian cells.

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Sorting Nexin 10 Induces Giant Vacuoles in Mammalian Cells
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