Ubiquitination and dynactin regulate TMEPAI lysosomal trafficking

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The transmembrane prostate androgen-induced protein (TMEPAI) has been reported to be elevated in various tumor cells, is localized to the lysosome and promotes lysosome stability. The molecular mechanism of TMEPAI trafficking however to the lysosome is unknown. Here we report that clathrin and CI-M6PR mediate TMEPAI transport from the Golgi directly into the endo-lysosomal pathway. TMEPAI is ubiquitinated at its C-terminal region and ubiquitination modification of TMEPAI is a signal for its lysosomal trafficking. Moreover, TMEPAI binds the ubiquitin binding proteins Hrs and STAM which is required for its lysosomal transport. In addition, TMEPAI interacts with the dynactin pointed-end complex subunits dynactin 5 and dynactin 6. The aa 132–155 domain is essential for specific TMEPAI binding and deletion of this binding site leads to mis-trafficking of TMEPAI to the plasma membrane. These results reveal the pathway and mechanism regulating transport of TMEPAI to the lysosome, which helps to further understand the role of TMEPAI in tumorigenesis.

Protein trafficking in the secretory and endocytic pathways is a multistep process involving the transport of proteins from a particular intracellular or extracellular compartment to another. This is regulated by an array of pathways including membrane trafficking, protein translocation, and endocytosis or exocytosis

Ubiquitination was originally described as a protein degradation signal to the 26 S proteasome. More attention has been attracted however to the discovery that ubiquitination is also found to modulate numerous biological processes in yeast and mammalian cells, including vesicular trafficking, signaling transduction, endocytosis, cell-cycle modification, DNA damage repair and gene transcription. The process of ubiquitinylination involves the sequential transfer of the evolutionarily conserved 76 amino acid protein ubiquitin, between ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin–protein ligase (E3), to the specific lysine residues of the target proteins. Proteins can either be mono, multi, or poly-ubiquitinated according to the degree of ubiquitin linkage to the lysine residues of the substrate or of ubiquitin itself. The different forms of ubiquitin modification on a protein dictate its distinct functions. Poly-ubiquitination provides the main targeting signals for degradation to the proteasome, whereas the mono-ubiquitination and multi-ubiquitination, act as a sorting

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signal that regulate the intracellular protein trafficking from TGN to endosomes or lysosomes and endocytosis of plasma membrane proteins. The function of the Nedd4 family of E3 ubiquitin ligases in regulating endocytosis and of the sorting of transmembrane proteins has been demonstrated. In mammalian cells, ubiquitination of GGA3 by Nedd4 regulates the sorting of LAPTM5 from the Golgi to endosomes/lysosomes.

The endosomal sorting complex required for transport (ESCRT) system is critical for the degradation of ubiquitinated proteins and comprises a major pathway for multivesicular body (MVB) formation. The ubiquitinated proteins can be recognized by intracellular proteins that contain one or more ubiquitin-binding domains, such as Hrs and STAM which are two components of the ESCRT subunit ESCRT-0, and provide an additional targeting module that promote their binding to cargo-enriched endosomes. Thus, ESCRT-0 is the detection module for initiating the ESCRT pathway at endosomes, and ubiquitination is a vital modification for sorting ubiquitinated cargoes into ESCRT-mediated MVB vesicles and their subsequent transfer to lysosomes.

In addition to sorting signal of ubiquitination, microtubules provide the tracks for protein trafficking, along which cargo is carried to its destination. Microtubule-based transport is of critical importance for the localization and motility of endomembranes. Motor proteins consisting of dynein, kinesin and myosin co-ordinate to physically move cargo along the microtubule network. Dynactin is a multisubunit protein complex that is required for dynein activity through the direct binding of dynein with p150glued and allows the motor to traverse the microtubule lattice over long intracellular distances. It has been suggested that dynactin contributes to microtubule anchoring since impairment of dynactin structure or its function commonly leads to the redistribution of endomembrane compartments from the cell center to the periphery. Loss of p25 (dynactin 5) significantly weakened the physical interaction between dynein and early endosomes, leading subsequent redistribution of early endosome.

Transmembrane prostate androgen-induced protein (TMEPAI), also known as PMEPA1 (prostate transmembrane protein androgen induced 1) or STAG1 (solid tumor-associated 1 protein), has been reported to be overexpressed in various tumor cells. TMEPAI is a type Ib transmembrane protein containing 287 amino acids containing a transmembrane domain at the N-terminus and two PY motifs (PPxY) which interact with E3 ubiquitin ligase Nedd4. Previous studies have shown that TMEPAI localizes to the lysosome and downregulates TGF-β signaling by competing with SARA (Smad anchor for receptor activation) for R-Smad binding to sequester R-Smad phosphorylation and promoting lysosomal degradation of TGF-β receptor (TβR). TMEPAI promotes lysosome stability and autophagy. Moreover, it has been reported that the E3 ubiquitin ligase Nedd4-binding defective mutant of TMEPAI was translocated to the plasma membrane. The molecular mechanism of TMEPAI trafficking to the lysosome and of E3-binding defective mutant TMEPAI (2YA) translocation to the plasma membrane is unknown. Here, we further investigated and demonstrated that clathrin and CI-M6PR mediated TMEPAI transport from the Golgi directly into the endo-lysosomal pathway, and ubiquitination modification of early endosome.

Results

Clathrin and CI-M6PR modulate TMEPAI transport from the Golgi to the lysosome. It has been reported that TMEPAI was mainly localized to the lysosome, while the Nedd4-binding defective mutant of TMEPAI was translocated to the plasma membrane. We further verified that endogenous TMEPAI was mainly localized to the lysosome, partially overlapped with TIR and not colocalized with the Golgi (Fig. 1A), whilst the TMEPAI (2YA) was localized to the plasma membrane (Fig. 1B). Lysosomal proteins can reach the lysosome through either direct or indirect pathways which are distinguishable in whether the transport process passes through the plasma membrane or not. To determine if the intracellular transport of TMEPAI is via a plasma membrane route, the endocytosis inhibitors dynasore and genistein were used to define the trafficking pathway of TMEPAI to the lysosome. Dynasore acts as an inhibitor of endocytic pathways and depends on dynamin 2 by rapidly blocking coated vesicle formation. Genistein is a tyrosine-kinase inhibitor that causes local disruption of the actin network at the site of endocytosis and inhibits the recruitment of dynamin 2. Our results showed that TMEPAI remained in the lysosome with no apparent alternation after treatment with endocytosis inhibitor (Fig. 1C), and the function of dynasore and genistein in inhibiting endocytosis was verified (Fig. 1D). Furthermore, depletion of dynamin 2 by RNA interference and expression of dynamin 2 GDP mutant (K44A) also did not affect the lysosomal localization of TMEPAI (data not shown). These results indicate that TMEPAI transports to the lysosome without passing through the plasma membrane.

Dynasore and genistein both act on dynamin 2, an essential protein engaged in clathrin-mediated endocytosis. Clathrin serves to mediate vesicle-coating on TGN exits. To determine the potential involvement of clathrin in both TMEPAI and its mutant form in sorting from TGN, clathrin-shRNA targeting the clathrin heavy chain was performed in order to deplete clathrin and block clathrin coated vesicles (CCVs) formation. Depletion of clathrin showed an enrichment of TMEPAI and its mutant TMEPAI (2YA) was observed in the Golgi labelling with the Golgi marker Golgin 84 (Fig. 2A,B), together with the redistribution to the Golgi and plasma membrane of transferrin receptor (TIR), a receptor coated by clathrin during its exit from TGN and internalization on plasma membrane, and depletion of clathrin also resulted in the redistribution of Lamp2 to the perinuclear region. Western blot analysis was used to confirm the efficient depletion of clathrin (Fig. 2C). These results indicate that TMEPAI lysosomal transport is clathrin-dependent.

Mannose-6-phosphate receptors (M6PRs), as well as clathrin are involved in lysosomal protein trafficking. There are two types of M6PRs, cation-independent mannose-6-phosphate receptors (CI-M6PR) and cation-dependent mannose-6-phosphate receptors (CD-M6PR), both of which deliver newly synthesized acid hydrolases to the endo-lysosomal pathway and then return them to the TGN. To investigate whether M6PRs regulate TMEPAI transport, M6PR levels were reduced by M6PR-shRNA lentivirus infection. Depletion of CI-M6PR resulted in TMEPAI retention to the Golgi (Fig. 3A), whereas no effect was observed on TMEPAI...
Ubiquitination regulates TMEPAI lysosomal transport. Since TMEPAI sorting to the lysosome requires interaction with Nedd4, the lysosomal sorting defect of the TMEPAI mutant lacking the Nedd4 binding sites is due to either blocking the TMEPAI-Nedd4 interaction or to ubiquitination modification of TMEPAI.
We next tested whether TMEPAI ubiquitination is involved in its sorting to the lysosome. To determine if the ubiquitination of TMEPAI is mono- or poly-ubiquitination, His-tagged ubiquitin protein and methylated ubiquitin protein defective in mediating poly-ubiquitination were used in an in vitro ubiquitination assay. Both the wide-type ubiquitin and ubiquitin mutant resulted in a similar pattern of Nedd4-mediated ubiquitin ligation, which suggested that Nedd4 catalyzes mono-ubiquitination of TMEPAI (Fig. 4A). Further, we analyzed the potential ubiquitination sites on the TMEPAI protein sequence using the online ubiquitination sites prediction tool of UbiPred (http://iclab.life.nctu.edu.tw/ubipred/index.php), and found that potential ubiquitination sites presented at the C-terminal tail of TMEPAI (277-KEKDKQKGHPL-287). We then generated TMEPAI mutants TMEPAI-KR with four Lysine (K) residues mutated to Arginine (R) and produced the TMEPAI cytoplasmic recombinant protein His-HA-TMEPAI-WT, the mutants His-HA-TMEPAI-2YA and His-HA-TMEPAI-KR for additional in vitro ubiquitination experiments. Ubiquitination of TMEPAI-2YA and TMEPAI-KR were inhibited compared to TMEPAI-WT (Fig. 4B). Moreover, in vivo ubiquitination performed by immunoprecipitation of A549 cells stably expressing Flag-tagged TMEPAI-WT, TMEPAI-2YA and His-HA-TMEPAI-KR for additional in vitro ubiquitination experiments. Ubiquitination of TMEPAI-2YA and TMEPAI-KR were inhibited compared to TMEPAI-WT (Fig. 4B). Moreover, in vivo ubiquitination performed by immunoprecipitation of A549 cells stably expressing Flag-tagged TMEPAI-WT, TMEPAI-2YA and TMEPAI-KR showed that ubiquitination of both TMEPAI-2YA and TMEPAI-KR was disrupted (Fig. 4C). To examine whether the ubiquitination site is required for TMEPAI sorting to the lysosome, A549 stably expressing TMEPAI-KR-Flag was double labeled with anti-Flag antibody and WGA-FITC, indicating that TMEPAI-KR-Flag was present on the plasma membrane (Fig. 4D). These results indicated that ubiquitination of TMEPAI at its C-terminal is responsible for transport to the lysosome.

Ubiquitin binding proteins (UBPs) play an important role in the recognition and recruitment of ubiquitinated protein, and ubiquitinated proteins require binding to specific UBPs for targeting to relevant endomembrane compartment. To investigate whether UBPs serve as an adaptor to supervise TMEPAI trafficking, a series of UBPs including Hrs, STAM, GGA3, Tsg101 and Eap45 were selected to test the TMEPAI interaction by yeast two-hybrid analysis. The yeast two-hybrid results showed that TMEPAI interacted with Hrs and STAM (Fig. 5A). Interaction of TMEPAI between Hrs and STAM was further identified using an immunoprecipitation assay by co-expression of pEF-TMEPAI-WT-Flag and pcDNA3.1+ -HA-Hrs or pcDNA3.1+ -HA-STAM constructs respectively in HeLa cells (Fig. 5B). Furthermore, since ubiquitinated protein links with ubiquitin binding proteins through ubiquitin (Henne et al.16), GST-TMEPAI was first in vitro ubiquitinated to produce ubiquitinated GST-TMEPAI (Ub) for the GST pull-down assay, and the results indicated that ubiquitination is critical for TMEPAI interaction with Hrs.
and STAM (Fig. 5C). We next investigated the roles of Hrs and STAM in regulating TMEPAI intracellular transport. RNA interference of Hrs and STAM was performed in A549 cells and showed that TMEPAI was significantly distributed to the plasma membrane in either Hrs or STAM depleted cells, although with little colocalization with lysosomal marker Lamp2 and early endosome marker EEA1 (Fig. 5D and E). These results together demonstrate that ubiquitination is a sorting signal for TMEPAI lysosomal trafficking and that the ubiquitin binding proteins Hrs and STAM are involved in its lysosomal transport.

**Dynactin involvement in TMEPAI intracellular trafficking.** Dynactin acts as an important adaptor in combination with dynein and maintains dynein activity to drive membrane vesicles along microtubules for long-distance movement. The dynactin pointed-end complex consists of dynactin 4/p62, dynactin 5/p25 and dynactin 6/p27 and is proposed to be a cargo-targeting module. We initially found that TMEPAI interacted with dynactin 6 by yeast two-hybrid screening, thus we next set out to investigate whether TMEPAI interacts with the other two subunits (dynactin 4 and dynactin 5). Yeast two-hybrid experiments showed that TMEPAI interacted with both dynactin 5 and dynactin 6, but not with dynactin 4, or with another dynactin complex subunit Arp1, which forms the Arp1 rod for binding to membrane vesicles, indicating that TMEPAI specifically combined with the point-end complex through dynactin 5 and dynactin 6 (Fig. 6A). Interaction of TMEPAI between dynactin 5 and dynactin 6 was further verified by GST pull-down assay using recombinant proteins (Fig. 6B). Since the integrity of dynactin point-end complex is essential for membrane binding, we addressed the question of whether the interaction between TMEPAI and dynactin 5, dynactin 6 might be reinforced when the point-end complex formed. We performed a GST pull-down assay to test the binding of GST-TMEPAI with different combinations of point-end complex subunits recombinant proteins. Interestingly, the interaction of TMEPAI with dynactin 5 and dynactin 6 was not strengthened with a combination of different subunits. In contrast, binding of TMEPAI to dynactin 5 and dynactin 6 was inhibited in the presence of dynactin 4 (Fig. 6C).

To further identify the binding domain of TMEPAI with dynactin, we analyzed the homology sequence of TMEPAI protein and generated truncated versions of TMEPAI to test their interaction with dynactin 5 and dynactin 6 using the yeast two-hybrid system. The yeast two-hybrid experiments indicated that the N-terminal region of TMEPAI bound to both dynactin 5 and dynactin 6 (Fig. 7A), and further deletion mutation screening showed the aa132–155 domain was required for this interaction, as mutants lacking this region failed to bind dynactin.
and dynactin 6 (Fig. 7B), but without affecting its binding to Nedd4 (data not shown). Furthermore, GST pull-down assay tests confirmed that GST-TMEPAI bound dynactin 5 and dynactin 6 but not the deletion mutant GST-TMEPAI (Δ132–155) (Fig. 7C). Moreover, we investigated whether the binding of TMEPAI to dynactin 5 and dynactin 6 contributed to its lysosomal localization. We generated TMEPAI mutants TMEPAI (Δ132–155) and TMEPAI (Δ63–86) with deletion of regions aa 132–155 and aa 63–86 respectively, and expressed these in A549 cells before double labelling with anti-Flag antibody and other membrane compartment markers. We observed that the mutant TMEPAI (Δ132–155) was significantly distributed to the plasma membrane, though with little colocalization with lysosomal marker Lamp2 and early endosome marker EEA1. The TMEPAI-WT and deletion mutant TMEPAI (Δ63–86) were localized to the lysosome normally (Fig. 7D). These results suggest that the aa132–155 domain of TMEPAI is required for its interaction with dynactin and lysosomal trafficking.

Discussion

Each protein that enters the endo-lysosomal pathway passes a number of decision stations that determine the rest of its journey. Alternative pathways for the delivery of newly lysosomal proteins to the endo-lysosomal system demand multiple TGN exits with two lysosomal trafficking pathways options. Apart from the clathrin- and M6PR-mediated pathway, the biosynthetic pathways to the lysosome are poorly understood. Identification of distinct sorting mechanisms for direct targeting of lysosome membrane proteins (LMPs) or lysosomal hydrolases to the lysosome raises challenges in lysosomal trafficking research. Even a cross-link between lysosomal hydrolases and LMPs such as LIMP2 has been shown to interact with β-glucocerebrosidase (β-GC) and mediate lysosome transport38. Blocking the interaction of TMEPAI with Nedd4 leads to translocation of TMEPAI to the plasma membrane, which raises the question of whether TMEPAI is delivered initially to plasma membrane before being internalized into the endo-lysosomal pathway. We found that endogenous TMEPAI remained in the lysosome location in cells treated with the endocytic inhibitor dynasore and genistein to inhibit the clathrin-dependent endocytosis. This suggests that TMEPAI is directly delivered to the endo-lysosome system from the Golgi, and it seems that TMEPAI is ubiquitinated before travelling into the endosome. A clathrin-dependent or -independent TGN exit pathway could be utilized by LMPs, Lamp139 and LAPTM515 separate from TGN via clathrin-coated vesicles (CCVs). Our study indicated that TMEPAI and its mutant targeted to their respective destination in a CCV-dependent pathway.

LMPs such as Lamp1 and Lamp2 are not modified with mannose 6 phosphate (M6P) groups and therefore are in the M6PR-independent sorting pathway. Instead, they undergo sorting as directed by their cytosolic tails that mediate both endo-lysosomal targeting and rapid endocytosis after travelling to the cell surface. Similar to other TGN sorting and endocytic signals, most lysosomal targeting signals belong to the YXXO or [DE][XXX][LI] types with certain features that make them functional for lysosomal trafficking60. In order to define whether TMEPAI targeting to the lysosome is M6PR-mediated, we found that transport of TMEPAI was blocked in the Golgi under conditions of CI-M6PR depletion but not with CD-M6PR. However, the transport of TMEPAI
Figure 5. Ubiquitin binding proteins Hrs and STAM regulate TMEPAI lysosomal trafficking. (A) Human Nedd4, Hrs, STAM, GGA3, Tsg101 and Eap45 were fused to the GAL4 activation domain (AD fusions) and tested for interaction with the TMEPAI cytoplasmic domain (BD fusion) in the yeast two-hybrid system. Yeast diploids co-expressing GAL4 AD fusions and GAL4 BD fusions were grown in liquid selective media, diploids were titrated (5⁻¹, 5⁻², 5⁻³; total 3 μl) and patched on the DDO, QDO and QDO/X/A plates. Interaction between the indicated proteins results in growth on the QDO and...
(2YA) mutant to the plasma membrane was M6PR-independent. Although TMEPAI does not contain the typical residues for lysosome sorting signals described above, TMEPAI contains potential glycosylation sites according to the online N-glycosylation prediction software analysis (http://www.cbs.dtu.dk/services/NetNGlyc/), which suggests that glycosylation may serve as a sorting signal for TMEPAI lysosomal trafficking mediated by M6PR.

Ubiquitin, particularly monoubiquitin, has several proteasome independent functions in regulating membrane protein trafficking, including serving as a sorting signal on plasma membrane proteins for internalization and sorting into the endosomal vesicles for delivery into the lysosome. It also regulates biosynthetic membrane proteins trafficking from the Golgi to the endosomes or lysosomes. Ubiquitination-defective mutants. In contrast, ubiquitinated proteins are sorted into intraluminal vesicles (ILVs) and develop into multivesicular bodies (MVBs)11,42. At the endosome, a series of proteins form ESCRT (endosomal sorting complex required for transport) to recruit ubiquitinated cargo to the endosome and sort into the interior of MVBs. These proteins are referred to as the ubiquitin binding proteins (UBPs) including Eps15, which operate at the plasma membrane to early endosome45. GGA3, mediates transport from the Golgi to the endosomes; Hrs/STAM, functions on interface between endosomes46,47; Tsg101, primary recruitment to late endosome48 and other ESCRT subunit complexes, all of which together are presumed to be involved in MVB formation. We found that TMEPAI directly interacts with both Hrs and STAM both by yeast two-hybrid and biochemical essays, and that either depletion of Hrs/STAM leads to redistribution of TMEPAI to the plasma membrane. A possible trafficking pathway of TMEPAI to the plasma membrane following UBp depletion involves TMEPAI being transported to the early endosome without recruitment by Hrs/STAM to ILVs, then recycling back to the Golgi and reaching the plasma membrane through the constitutive pathway.

Dynactin is responsible for dynein activity and drives cargo along microtubules for long distance movement. Our study showed that TMEPAI bound to both dynactin 5 and dynactin 6 simultaneously, but not dynactin 4 and the cargo-binding unit Arp1, indicating a specific association of TMEPAI with dynactin 5 and dynactin 6. Interestingly, dynactin 4 inhibited the binding of TMEPAI to dynactin 5 and dynactin 6, which suggested that dynactin 4 might function as a switch in modulating the binding and regulating the release of TMEPAI from the complex during its transport along the microtubule. Apart from dynactin, dynactin also contributes to the activity of another microtubule-based motor protein, kinesin II46. Dynactin concentrates at the microtubule plus end and presents on various endo-membranes such as the early endosome and cell periphery47. Although dynactin is reported to conduct minus direction transport of microtubules, a recent study implied that dynactin could facilitate the mobility of adenovirus both towards and away from the nucleus48. In addition to binding dynein and maintaining its activity, dynactin functions as a cargo-targeting module by the point end complex, as dynactin 5 was discovered to be involved in endosome movement49 and dynactin 6 was found to assist polo-like kinase 1 target kinetochores during mitosis50. To investigate the role of dynactin in regulating TMEPAI intracellular trafficking, we identified the aa132–155 domain of TMEPAI as the dynactin binding region, and the dynactin-binding defective mutant TMEPAI (Δaa132–155) was found localized to plasma membrane. Although it has been reported that dynactin 5 and dynactin 6 are required for binding of dynactin and recruitment of dynein to the vesicles that recycle TIR to the cell surface, but not to late endosomes51, how this interaction with the dynactin complex regulates TMEPAI trafficking from the Golgi to lysosomes is still unclear and needs further investigation.

It has been reported that TMEPAI inhibits TGF-β signaling by blocking both R-Smad phosphorylation and by promoting degradation of TβR. This is not observed in the ubiquitination loss mutant TMEPAI (2YA)50,51, which suggests that ubiquitination contributes to TMEPAI lysosomal localization and the regulation of TGF-β signaling. A recent study has shown that TMEPAI promotes lysosome stability and autophagy and thus enhances
cancer cells drug resistance to chemotherapeutics. Protein expression, protein trafficking and subcellular localization, signaling transduction all co-ordinate to regulate normal cellular function. Our studies demonstrate that modification by ubiquitination and interaction with dynactin regulates TMEPAI intracellular trafficking to the lysosome. These findings extend the known roles of TMEPAI in tumorigenesis and might provide a novel strategy to modulate the transport and intracellular function of TMEPAI as a potential target for cancer treatment.

Methods
Materials and antibodies. All materials were from Sigma unless otherwise stated. Yeast two hybrid system kit and dropout solution screening medium were purchased from Clontech. Rabbit polyclonal antibody to TMEPAI was generated using the His-tagged cytoplasmic domain of TMEPAI as immunogen, antibody was affinity-purified against the GST-TMEPAI covalently coupled to Glutathione Sepharose (GE Healthcare). The specificity of the TMEPAI antibody was assessed by Western blot using the purified recombinant TMEPAI proteins and cell lysates. Mouse anti-HA, Mouse anti-Flag and WGA-FITC were all from Sigma. Mouse anti-β-actin was from Tianjin Sungene Biotech. Sheep anti-Golgin84 and mouse anti-Lamp2 was from Abcam. Goat anti-EEA1 (N-19), mouse anti-TF/CD71 (3B82A1), mouse anti-Rab7 (B-3), mouse anti-Clathrin HC (TD.1), rabbit anti-CI-M6PR (H-300), mouse anti-Hrs (D-3) and mouse anti-STAM (B-2) were from Santa Cruz Biotechnology. Fluorophore secondary antibodies were purchased from Invitrogen.

Cell culture, transfection and stable cell lines. A549 cells were cultured in F-12K medium, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. HeLa cells
were maintained in DMEM medium containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Transfection was performed with Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's instructions. A549 cell lines stably expressing TMEPAI-2YA-Flag or TMEPAI-KR-Flag were obtained by transfection with pEF-IREs-puro-TMEPAI-2YA-Flag or pEF-IREs-puro-TMEPAI-KR-Flag recombinant plasmid and selected in 1 μg/ml puromycin for 2 weeks.

**Immunofluorescence microscopy.** The cells were grown on coverslips and fixed in 4% PFA in PBS at room temperature (RT) for 20 min. Cells were quenched with 10 mM glycine, pH 8.5 (in PBS), and permeabilized with 0.1% TritonX-100 (in PBS) for 5 min at RT. Coverslips were incubated for 2 h at RT with primary antibodies diluted into PBS containing 3% BSA, washed three times with DPBS, incubated for a further 30 min at RT with fluorophore-conjugated secondary antibodies then DNA dye Hoechst 33342 (200 ng/ml) was mixed with the secondary antibodies. Coverslips were mounted in Mowiol and allowed to dry. Immunofluorescent images were viewed and analyzed using an Olympus FV1000 Confocal Microscope with the FV10-ASW 3.0 Viewer software.

**RNA interference.** Lentiviral vectors expressing clathrin-shRNAs (clathrin-1: 5′-CCGGTA ATCCAAATCGAGAAGCAATTAGGTTCTGGATTGATTTTGT-3′; clathrin-2: 5′-CCGGCGGTGCCATCTTCATGGTGATTTTGT-3′; clathrin-3: 5′-CCGGCCGATCCCTTCATGGTGATTTTGT-3′; CI-M6PR-shRNAs (CI-M6PR-1: 5′-CCGGCCGAGGAATACATCTGAATCTCGAGATTGGTCTTGC-3′; CI-M6PR-2: 5′-CCGGCCGATCCCTTCATGGTGATTTTGT-3′), Hrs-shRNAs (Hrs-1: 5′-CCGGCCGATCCCTTCATGGTGATTTTGT-3′; Hrs-2: 5′-CCGGCCGATCCCTTCATGGTGATTTTGT-3′), STAM-shRNAs (STAM-1: 5′-CCGGTTAAATCCGATTTGACCTCACTGACTGGGTAG-3′; STAM-2: 5′-CCGGTAAAAATGCATTTGACCTCACTGACTGGGTAG-3′), Tsg101-shRNAs (Tsg101-1: 5′-CCGGCCGCAGACTTTTGGATTTTGT-3′; Tsg101-2: 5′-CCGGCCGCAGACTTTTGGATTTTGT-3′), Esp45-shRNAs (Esp45-1: 5′-CCGGCTGGATTTTGT-3′; Esp45-2: 5′-CCGGCTGGATTTTGT-3′), Hrs and Tsg101-shRNAs (Hrs-1: 5′-CCGGCCGATCCCTTCATGGTGATTTTGT-3′; Hrs-2: 5′-CCGGCCGATCCCTTCATGGTGATTTTGT-3′), TMEPAI-KR, dynactin 4, dynactin 5, and dynactin 6 were transformed into BL21-CodonPlus (DE3)-RIL cells. Protein expression and purification. Plasmids encoding His-HA-tagged TMEPAI-WT, TMEPAI-2YA or TMEPAI-KR were washed twice with PBS and lysed in the ice-cold lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM NaVO₄, 10 mM NaF, and protease inhibitors). Lysates were left on ice for 40 min and centrifuged for 10 min at 12000 g at 4 °C. The supernatants were adjusted to equal protein concentration and volume, and subjected to SDS-PAGE. Separated proteins were transferred to PVDF membranes followed by blocking with 5% non-fat milk in PBS. The membranes were incubated with primary antibody, and then subjected to SDS-PAGE followed by Western blot analysis.

**In vitro ubiquitination assay.** Purified His-HA-TMEPAI or His-HA-TMEPAI-2YA/KR proteins were incubated with recombinant E1 (Boston Biochem), recombinant UbCH5C (Boston Biochem), GST-Nedd4 and methylated ubiquitin or His-tagged ubiquitin (Boston Biochem) in reaction buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.3 mM DTT, 2 mM ATP) for 60 min at 30 °C. The reaction products were analyzed by SDS-PAGE and Western blot.

**In vivo ubiquitination assay.** A549 cells stably expressing Flag-tagged TMEPAI-WT, TMEPAI-2YA or TMEPAI-KR were washed twice with PBS and lysed in the ice-cold lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM NaVO₄, 10 mM NaF, and protease inhibitors). Lysates were left on ice for 40 min and centrifuged for 10 min at 12000 g at 4 °C. The supernatants were incubated with anti-Flag antibody for 2h at 4 °C with rotation and further incubated for 2 h at 4 °C after addition of protein A/G Sepharose (Thermo Scientific). Immunoprecipitates were washed 3 times with lysis buffer and once with PBS, and then subjected to SDS-PAGE followed by Western blot analysis.

**Western blot.** The cells were collected in RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM NaVO₄, 10 mM NaF, and protease inhibitors) for 40 min. Homogenates were clarified by centrifugation at 12000 g for 10 min at 4 °C and the supernatants were mixed with SDS-loading buffer and heated for 5 min at 95 °C. Samples were adjusted to equal protein concentration and volume, and subjected to SDS-PAGE. Separated proteins were transferred to PVDF membranes followed by blocking with 5% non-fat milk in PBS. The membranes were incubated with primary antibody, and then subjected to SDS-PAGE followed by Western blot analysis.

**Molecular biology and yeast two-hybrid analysis.** Standard molecular biology techniques were used for all constructs. For the yeast two-hybrid assay, the full-length and truncated versions of TMEPAI cDNA lacking the trans-membrane domain were inserted into the NdeI and EcoRI sites of the pGBKT7 vector. The full-length of human Nedd4, Hrs, STAM, GGA3, Tsg101, Esp45, dynactin 4 (DCTN4), dynactin 5 (DCTN5), dynactin 6 (DCTN6) and Arp1 cDNA were inserted into the pGADT7 vector (CLONTECH Laboratories, Inc.). The pGBKTT7/TMEPAI and pGADT7 related plasmids were co-transformed into the yeast reporter strain Y187 on synthetic medium lacking leucine and tryptophan (double dropout, DDO) (low selection) and then patched onto synthetic medium lacking leucine, tryptophan, histidine, and adenine (quadruple dropout, QDO) (medium selection), as well as onto synthetic medium lacking leucine, tryptophan, histidine, and adenine with 40 μg/ml X-α-gal, 125 ng/mL Aureobasidin A (Aba A) (quadruple dropout with X-α-gal and Aba A, QDO/X/A) (high selection) according to the CLONTECH Laboratories, Inc. yeast protocol handbook.

**Protein expression and purification.** Plasmids encoding His-HA-tagged TMEPAI-WT, TMEPAI-2YA, TMEPAI-KR, dynactin 4, dynactin 5, and dynactin 6 were transformed into BL21-CodonPlus (DE3)-RIL cells. Cells were induced with 0.5 mM IPTG overnight at 16 °C. Cells were lysed in ice cold lysis buffer (50 mM phosphate sodium buffer, pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, protease inhibitors) and the recombinant proteins were purified with Ni Sepharose beads (GE Healthcare).
Figure 7. Interaction between TMEPAI and dynactin is required for TMEPAI lysosomal localization. (A,B) DCTN5 and DCTN6 fused to the GAL4 activation domain (AD fusions) were tested respectively for interaction in the yeast two-hybrid system with full-length, truncated and deleted versions of TMEPAI cytoplasmic domain fused to the GAL4 DNA-binding domain (BD fusion). Yeast diploids co-expressing GAL4 AD fusions and GAL4 BD fusions were grown in liquid selective media. Diploids were titrated (5^{−1}, 5^{−2}, 5^{−3}; total 3 μl) and patched on DDO, QDO and QDO/X/A plates. (C) GST, GST-tagged cytoplasmic domains of TMEPAI (GST-TMEPAI) and GST-tagged cytoplasmic domains of TMEPAI with deletion of amino acids 132–155 (GST-TMEPAI (Δ132–155)) were coupled to beads and incubated with either His-HA-tagged recombinant proteins DCTN5 and DCTN6. Bound proteins were determined by Western blot with antibody to HA (left). GST, GST-TMEPAI and GST-TMEPAI (Δ132–155) proteins used for pull-down assay was analyzed by Western blot with antibody against GST (right). (D) Immunofluorescence microscopy of A549 cell lines transfected with Flag-tagged TMEPAI-WT, TMEPAI (Δ63–86) and (TMEPAI (Δ132–155) labelled with antibodies to Flag (red), Golgin84, Lamp2, EEA1 and WGA-FITC (green). Scale bar, 10 μM.
GST pull-down assay. Glutathione Sepharose beads coupled with 5 μg GST-fusion protein were incubated with the clarified cell lysates in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) with complete protease inhibitors at 4°C for 3 h with rotation. The beads were washed four times with RIPA buffer and resuspended in SDS-PAGE sample buffer, bound and input proteins were subjected to SDS-PAGE and Western blot using indicated antibodies.

Immunoprecipitation. A549 cell lines stably expressing Flag-tagged TMEPAI-WT, TMEPAI-2YA and TMEPAI-KR were extracted in IP buffer (20 mM Tris-Cl pH 7.4, 0.1 M KCl, 0.1 M NaF, 1 mM DTT, 1% TritonX-100) with complete protease inhibitors and clarified by centrifugation at 12000g for 10 min, then incubated with anti-Flag M2 Magnetic Beads (Sigma M8823) for 4 h at 4°C with gentle rotation. The beads were washed with IP buffer four times and eluted by boiling in SDS-PAGE sample buffer and analyzed by Western blot with appropriate antibodies.

Statistical analysis. Statistical analysis was performed using the Student t-test with GraphPad Prism software, and P < 0.05 represented statistically significant difference.

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**Author Contributions**

S.L., L.J., A.D. designed the study. S.L., L.J., T.Z., Y.L., Z.L. carried out research experiments. All authors analyzed data. S.L., L.J., A.D. wrote the paper. All the authors have reviewed the final version of the manuscript.

**Additional Information**

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