Lysosomal targeting of the ABC transporter TAPL is determined by membrane localized charged residues

Running Title
Lysosomal trafficking of TAPL

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Abstract
The human lysosomal polypeptide ABC transporter TAPL (ABC subfamily B member 9, ABCB9) transports 6–59 amino-acids-long polypeptides from the cytosol into lysosomes. The subcellular localization of TAPL depends solely on its N-terminal transmembrane domain TMD0, which lacks conventional targeting sequences. However, the intracellular route and the molecular mechanisms that control TAPL localization remain unclear. Here, we delineated the route of TAPL to lysosomes and investigated the determinants of single trafficking steps. By synchronizing trafficking events by retention using selective hooks (RUSH) assay and visualizing individual intermediate steps through immunostaining and confocal microscopy, we demonstrate that TAPL takes the direct route to lysosomes. We further identified conserved charged residues within TMD0 transmembrane helices that are essential for individual steps of lysosomal targeting. Substitutions of these residues retained TAPL in the endoplasmic reticulum (ER) or Golgi. We also observed that for release from the ER, a salt bridge between Asp-17 and Arg-57 is essential. An interactome analysis revealed that Yip1-interacting factor homolog B, membrane-trafficking protein (YIF1B) interacts with TAPL. We also found that YIF1B is involved in ER-to-Golgi trafficking and interacts with TMD0 of TAPL via its transmembrane domain and that this interaction strongly depends on the newly identified salt bridge within TMD0. These results expand our knowledge about lysosomal trafficking of TAPL and the general function of extra transmembrane domains of ABC transporters.

Introduction
ATP-binding cassette (ABC) transporters belong to one of the largest protein families in all organisms (1). In eukaryotes, they are almost exclusively exporters, transporting a diverse set of solutes across membranes while hydrolyzing ATP (2). The cytosolic nucleotide binding domains (NBDs) translate their ATP-binding induced dimerization and subsequent ATP-hydrolysis in conformational changes of the transmembrane domains (TMDs), which leads to translocation of the solute across the membrane. The common core architecture of human ABC exporters comprises 2 x 6 transmembrane helices (TMHs) and two NBDs, arranged either as a single polypeptide chain (full transporter) or as dimers (half-transporters).

In human, 48 ABC proteins are found. A small subgroup of them forms the transporter associated with antigen processing (TAP)-family, consisting of TAP1 (ABCB2) and TAP2 (ABCB3) forming a heterodimer, and the homodimeric TAP-Like (TAPL / ABCB9). TAPL is able to transport polypeptides varying between 6 and 59 amino acids in length (3). In contrast to ER-resident TAP, which is part of the peptide loading complex (4, 5), TAPL is localized to lysosomes (6). To date, the physiological role of TAPL remains ill-defined. It was shown that TAPL expression is strongly upregulated after maturation of monocytes to professional antigen presenting cells like macrophages and dendritic cells (7). However, no evidence was found for TAPL being involved in antigen cross-presentation (8). In contrast, it is associated with phagosomal maturation, consistent with earlier studies on the TAPL orthologous HAF-4 and HAF-9 of Caenorhabditis elegans, which are essential for gut granule biogenesis (9).

TAPL can be dissected into two functional parts (10). CoreTAPL, comprising the six C-terminal TMHs and the cytosolic NBD, forms homodimers that are fully active in ATP-dependent peptide transport. The four additional N-terminal TMHs, named TMD0, are negligible for peptide transport, but essential for lysosomal localization (11) and for interaction with the lysosomal membrane proteins LAMP-1 and LAMP-2B (12). CoreTAPL, lacking the TMD0, is mislocalized to the plasma membrane (PM), while the TMD0 itself is trafficked to lysosomes. If both parts are co-expressed, coreTAPL interacts non-covalently with TMD0, which leads to lysosomal localization (11).

For lysosomal membrane proteins, two intracellular biosynthetic routes are described (13, 14): the direct route leads from the trans-Golgi network (TGN) via early endosomes and late endosomes to lysosomes. In contrast, the
indirect route includes an intermediate trafficking step at the PM, a subsequent endocytosis, and transport to early endosomes. Short linear targeting motifs determine individual trafficking steps. Most common are tyrosine (YXXΦ) or di-leucine ([D/E]XXX[L/I])-based motifs in a cytosolic region of the lysosomal membrane protein. These sequences are recognized by cytosolic adaptor proteins such as the five adaptor protein complexes (AP-1 to -5) or the monomeric GGAs (Golgi-localizing, gamma-adaptin ear domain homology, ARF-binding proteins). None of these consensus sequences associated with protein trafficking can be found in the TMD0 of TAPL. However, an increasing body of evidence clearly demonstrates that lysosomal localization can also be mediated by atypical targeting determinants ranging from anomalous leucine or tyrosine motifs to post-translational modifications (15).

In this study, we investigated the trafficking of TAPL to elucidate the intracellular route to lysosomes, especially with regard to a possible PM intermediate step. In mutational studies, we were able to determine conserved, charged residues within TMD0 that are essential for individual trafficking steps and thus form the targeting determinants of TAPL. Immunoprecipitation-mass spectrometry (IP-MS) allowed us to identify YIF1B, a factor in ER to Golgi trafficking, as an interaction partner of TAPL involved in the targeting process. The interaction between the two proteins is mediated by the TMD of YIF1B and TMD0 of TAPL, with significantly reduced interaction if the salt bridge between two conserved, charged residues in TMD0 is disrupted.

Results

Intracellular route of TAPL

To decipher the intracellular route of TAPL to lysosomes, we applied the retention using selective hooks (RUSH) assay (16), which enables synchronization of protein trafficking and thus visualization of intermediate steps. HeLa Kyoto cells were transiently transfected with a bicistronic plasmid. One gene coded for the invariant chain (Ii, CD74) containing an N-terminal, cytosolic ER-retention signal and core streptavidin as hook, while the other coded for TAPL, fused C-terminally with a streptavidin binding peptide (SBP) followed by eGFP (Fig. 1A). In the absence of biotin, TAPL colocalized with the hook protein, demonstrating efficient ER retention, and did not overlap with GM130 (cis-Golgi), EEA1 (early endosomes), and LAMP-1 (lysosomes) (Fig. S1A). By adding biotin, the interaction between TAPL and the hook protein was outcompeted, TAPL was released and trafficked to lysosomes in a time dependent manner. Within 15 min after the addition of biotin, TAPL accumulated in a perinuclear area partially overlapping with the cis-Golgi marker GM130, which represents the first trafficking step from ER to the Golgi (Fig. 1B and Fig. S1B). After 45-60 min, TAPL colocalization with GM130 was reduced and a fraction of TAPL was found in distinct vesicular structures stained with the early endosomal marker EEA1. Finally, a first overlap with the late endosomal and lysosomal marker LAMP-1 was observed after 60-90 min. In this context, it should be noted that trafficking periods vary between cells dependent on cellular TAPL levels, with faster kinetics for low expressing cells. According to the observed localizations, TAPL takes an intracellular route from the ER, via Golgi and early endosomes to late endosomal and lysosomal compartments.

To exclude that we missed a short-lived PM trafficking step that reflects the indirect route, we inhibited endocytosis by the dynamin inhibitor Dyngo-4a (17). HeLa Flp-In T-REx cells were used to allow stable, inducible expression of tapl under the control of the tetracycline-regulated promoter. To inhibit endocytosis during TAPL synthesis and trafficking, Dyngo-4a was added together with the inducer doxycycline. 2 h after induction, TAPL was detected in lysosomes but no PM localization was observed (Fig. 1C). To verify the inhibitory function of Dyngo-4a, we analyzed the clathrin- and dynamin-dependent endocytosis of the transferrin receptor, which can be triggered by addition of transferrin (18, 19). In the presence of Dyngo-4a, transferrin was not taken up by endocytosis, in contrast to the inactive control compound Dyngo-Ø, which did not interfere with endocytosis as proven by immunofluorescence microscopy as well as by
flow cytometry (Fig. S2). Thus, Dyngo-4a is capable to fully block dynamin-dependent endocytosis. Collectively, these data demonstrate that TAPL solely takes the direct route from the ER via Golgi and early endosomes to lysosomes.

Residues involved in intracellular trafficking

Intracellular trafficking steps are mediated by targeting motifs. However, none of the tyrosine or dileucine-based motifs classically associated with lysosomal targeting are found in the cytosolic loops of TMD0, the targeting domain of TAPL. To elucidate how TMD0 mediates individual targeting steps along the intracellular route, we performed multiple sequence alignments of TAPL orthologues of phylogenetically distant species in order to identify conserved residues (Fig. S3).

As derived from a recently published secondary structure model of TMD0 (Fig. 2) (20), all eight conserved residues, three leucines and five charged residues found in species from human to C. elegans, are localized in the transmembrane helices 1, 2 and 3. Since charged residues in the hydrophobic core of the membrane often accompany special functions, we focused our targeting studies on the charged residues D17 in TMH1, D49 and R57 in TMH2, and K100 in TMH3. The following studies were performed with HeLa and HEK cells, in which endogenous TAPL expression was detected at mRNA level (21). However, at protein level, endogenous TAPL could not be detected by either immunoblot or immunofluorescence microscopy, most likely due to low abundance of TAPL in these cells (7).

Therefore, analysis of intracellular localization of TAPL mutants was carried out using stably transfected HeLa Flp-In T-REx cells. This system enabled mild overexpression of TAPL by means of low inducer concentrations and thus led to a more robust localization analysis across the cell population than would be possible in transiently transfected cells.

First, we replaced the conserved, positively charged residues with alanine and the conserved, negatively charged residues with asparagine. D49 was always substituted in combination with D45, which is one helical turn upstream of D49 in TMH2 and could compensate for charge deletion of D49. Subcellular localization was examined by confocal immunofluorescence microscopy (Fig. 3A), and lysosomal localization was quantified by Pearson correlation of 50 single cells (Fig. 3B). The substitution of any of the four conserved, charged residues interfered with trafficking of TAPL. TAPL<sub>R57A</sub> still displayed a substantial overlap with LAMP-1 (PCC = 0.54 ± 0.02) but was significantly reduced compared to wildtype TAPL (PCC = 0.8 ± 0.1). Further reduction in lysosomal localization was detected for TAPL<sub>D45N,D49N</sub> (PCC = 0.24 ± 0.03) and TAPL<sub>R57A</sub> (PCC = 0.20 ± 0.02). Most strikingly, TAPL<sub>D17N</sub> was not detected in LAMP-1 positive compartments at all (PCC = -0.23 ± 0.03). TAPL<sub>D17N</sub> also showed no overlap with the cis-Golgi marker GM130 but strong colocalization with the ER marker PDI (Fig. S4).

Because of this result, we replaced both conserved positive charged residues and observed that TAPL<sub>R57A,K100A</sub> (PCC = -0.19 ± 0.02) behaved like TAPL<sub>D17N</sub>, showing no lysosomal localization. TAPL mutants, which are unable to reach lysosomes due to these substitutions, are localized in the ER. A pronounced ER localization could mask a weak lysosomal population in case of TAPL<sub>D17N</sub>. Therefore, we treated the cells with cycloheximide (CHX) and chased them for 5 h to inhibit protein synthesis and allow trafficking (Fig. S5). Lysosomal localization of TAPL<sub>D45N,D49N</sub> (PCC = 0.27 ± 0.02) and TAPL<sub>K100A</sub> (PCC = 0.59 ± 0.02) was unaltered (Fig. S6). In contrast, lysosomal localization of TAPL<sub>R57A</sub> was significantly increased (PCC = 0.45 ± 0.02) by CHX treatment implying a more challenging folding of TAPL<sub>R57A</sub>. Importantly, TAPL<sub>D17N</sub> and TAPL<sub>R57A,K100A</sub> did not show colocalization with LAMP-1, confirming that these TAPL variants were unable to reach lysosomes and were stuck in the ER. In summary, substitution of the conserved, charged residues within TMD0 impacts lysosomal targeting of TAPL, and therefore these residues determine its subcellular localization. Moreover, TAPL was trapped in the ER, if the positive charges at R57 and K100, or the negative charge at D17 were deleted. Since the deletion of these charged residues had the same effect, we hypothesize that D17 forms an intramolecular salt bridge with one of these two positively charged residues to yield a trafficking competent conformation. It should
be noted that the deletion of the positive charge at position 57 had a stronger effect than the charge removal at position 100. If the ER localization of TAPL<sub>D17N</sub> was caused by disruption of a salt bridge, the simultaneous inversion of the charges of D17 and R57 or K100 should yield a trafficking competent TAPL variant. To prove any lysosomal localization, we again applied CHX for 5 h (Fig. 4). TAPL<sub>D17R</sub> (PCC = -0.29 ± 0.02), like TAPL<sub>D17N</sub>, showed no overlap with LAMP-1. TAPL<sub>R57D</sub> (PCC = -0.32 ± 0.02), in stark contrast to its neutral substitution TAPL<sub>R57A</sub>, also fully lacked colocalization with LAMP-1 whereas TAPL<sub>K100D</sub> (PCC = 0.51 ± 0.02) was trafficked as well as TAPL<sub>K100A</sub> to lysosomes. These findings indicated that R57 is more relevant for yielding a trafficking competent conformation than K100 and also possibly in closer proximity to D17 for forming a salt bridge. This hypothesis was supported by the fact that TAPL<sub>D17R,K57D</sub> was indeed trafficking competent and showed colocalization with lysosomes (PCC = 0.49 ± 0.02) strongly supposing a salt bridge between D17 and R57. In contrast, TAPL<sub>D17R,K100D</sub> (PCC = -0.27 ± 0.02) was not found in lysosomes pointing to a minor role of K100 in context of D17.

Since TAPL<sub>K100D</sub> (PCC = 0.51 ± 0.02) was trafficked as well as TAPL<sub>K100A</sub> to lysosomes but TAPL<sub>D17R,K100D</sub> (PCC = -0.27 ± 0.02) was not found in lysosomes, a salt bridge between D17 and K100 could be excluded. Remarkably, TAPL<sub>R57D</sub> (PCC = -0.32 ± 0.02), in stark contrast to its neutral substitution TAPL<sub>R57A</sub>, also fully lacked colocalization with LAMP-1. However, TAPL<sub>D17R,R57D</sub> was indeed trafficking competent and showed strong colocalization with lysosomes (PCC = 0.49 ± 0.02). Since only simultaneous inversion of the charges at residue 17 together with residue 57 and not residue 100 restored lysosomal localization of TAPL, we propose an intramolecular salt bridge between D17 and R57.

During the analysis of the conserved, charged residues in TMD0 of TAPL, we noticed that TAPL<sub>D45N,D49N</sub> showed a significantly reduced lysosomal localization in comparison to TAPL<sub>wt</sub> (Fig. 3). Inversion of the charges (TAPL<sub>D45K,D49K</sub>) abolished lysosomal localization (Fig. 5A and B). This effect could not be compensated by the additional substitution of K100D (TAPL<sub>D45K,D49K,K100D</sub> PCC = -0.16 ± 0.02). While lysosomal localization was clearly absent, TAPL<sub>D45K,D49K</sub> and TAPL<sub>D45K,D49K,K100D</sub> showed strong colocalization with the cis-Golgi marker GM130 (TAPL<sub>D45K,D49K</sub> PCC = 0.50 ± 0.02 and TAPL<sub>D45K,D49K,K100D</sub> PCC = 0.56 ± 0.01) as detected in the presence (Fig. 5) as well as in the absence (Fig. S7) of CHX. Correct folding of the triple mutant TAPL<sub>D45K,D49K,K100D</sub> was demonstrated by similar peptide transport activity as TAPL<sub>wt</sub> (Fig. 5C).

In conclusion, D17 in TMH1 of TAPL and its salt bridge to R57 are essential for the release of the transporter from the ER, whereas D45 together with D49 are important for the second trafficking step from the Golgi to endosomal and lysosomal compartments.

**TAPL<sub>D17N</sub> is correctly folded**

Defects in trafficking and especially ER retention can be caused not only by missing trafficking determinants but also by misfolding. Therefore, we tested correct folding of TMD0<sub>wt</sub> and its mutants in vivo by the interaction with coreTAPL. We co-expressed transiently TMD0 variants containing a C-terminal FLAG-tag and coreTAPL in HEK293T cells and performed co-immunoprecipitation with an α-FLAG antibody. CoreTAPL was precipitated together with all TMD0 variants but not in the absence of TMD0, demonstrating correct folding of all TMD0 mutants (Fig. 6A). Since substituting D17 showed the strongest effects in subcellular trafficking and ER retention, we addressed the folding state of TMD0<sub>D17N</sub> and TAPL<sub>D17N</sub> more in detail. First, we evaluated folding of TMD0<sub>D17N</sub> by solution NMR. <sup>15</sup>N labelled variants of TMD0<sub>wt</sub> (cf-TMD0) and TMD0<sub>D17N</sub> (cf-TMD0<sub>D17N</sub>) were synthesized by cell-free (cf) expression, and [<sup>15</sup>N,<sup>1</sup>H]-BEST-TROSY spectra were recorded (Fig. 6B). The well-resolved peak distribution and peak overlaps between both spectra indicated that cf-TMD0<sub>D17N</sub> is a folded protein with a conformation similar to cf-TMD0. Next, the localization of TMD0 in stably transfected HeLa Flp-In T-REx cells was analyzed (Fig. 6C). TMD0<sub>wt</sub> significantly overlapped with the lysosomal marker LAMP-1, whereas TMD0<sub>D17N</sub> was not localized in lysosomes. Adding the cytosolic, tyrosine based lysosomal targeting sequence of LAMP-2C at the C-terminus of TMD0<sub>D17N</sub> partially
restored lysosomal localization (Fig. 6D). Therefore, ER retention must be due to the absence of a lysosomal targeting determinant in TMD0D17N, whereas misfolding can be excluded. Lysosomal localization of full-length TAPLD17N was also recovered if co-expressed with TAPLwt in HeLa Kyoto cells, demonstrating that TAPLD17N dimerizes with TAPLwt and is not retained in the ER (Fig. 7A and B). Finally, correct folding and dimerization of TAPLD17N was demonstrated by the comparable ATP-dependent peptide transport activity of TAPLwt and TAPLD17N in crude membranes derived from HeLa Flp-In T-REx cells (Fig. 7C). Taking together these results, we conclude that altered lysosomal trafficking and ER or Golgi retention of TAPL mutants are not caused by misfolding but are due to the absence of a targeting determinant.

**Trafficking chaperones**

Since the impaired ER to Golgi trafficking of TAPLD17N and TAPLD17R is not due to misfolding, we hypothesized that the identified salt bridge stabilizes a specific conformation of TMD0 that allows transient interactions with trafficking chaperones. The only known interaction partners for TAPL are LAMP-1 and -2B, identified by tandem affinity purification of digitonin solubilized membranes. However, these proteins are neglectable for TAPL trafficking since in double LAMP-1/2 knock-out cells TAPL is correctly targeted to lysosomes (12). Therefore, we aimed to identify components of the trafficking interactome, which are assumed to interact with TAPL only transiently and are consequently associated with TAPL in low abundance. Thus, we performed a fast, single step purification followed by mass spectrometry and applied Comparative Proteomic Analysis Suite (CompPASS) to enable identification of high-confidence candidate interacting proteins (HCIPs) (22). In short, CompPASS ranks identified proteins based on peptide abundance and occurrence in pre-determined proteomic datasets. A protein frequently found across several samples in the datasets is ranked lower than a unique one in a given sample. HeLa Flp-In T-REx cells non-transfected or stably expressing TAPL-HA or coreTAPL-HA were solubilized by the detergent Nonidet P40 (NP40), purified in a single step with an α-HA antibody and analyzed by tandem mass spectrometry. CompPASS analysis revealed 14 HCIPs of TAPL that were absent in coreTAPL samples or samples derived from untransfected cells. Among these HCIPs (Fig. 8A), the Yip1 interacting factor homologue B (YIF1B) is the only one that is a transmembrane protein and also involved in trafficking of a transmembrane protein. YIF1B is a member of the FinGER protein family and, together with YIF1A, the human homologues of yeast YPT-interacting protein 1 (YIP1). In prior studies, both proteins were implicated in ER to Golgi protein trafficking (23, 24). YIF1B is composed of an N-terminal cytosolic domain, followed by a C-terminal TMD comprised of five predicted TMHs (Fig. S11A). First, we verified the interaction of TAPL with YIF1B by co-immunoprecipitation. Endogenous YIF1B was co-immunoprecipitated with C-terminally FLAG-tagged TAPL and TMD0 stably expressed in HeLa Flp-In T-REx cells, but not with coreTAPL (Fig. 8B). Importantly, TMD0 was pulled-down in a reverse immunoprecipitation via YIF1B if transiently co-expressed in HEK293T cells (Fig. 8C), demonstrating that YIF1B interacts with the TMD0 of TAPL. The TMD of YIF1B, devoid of its cytosolic tail, was still able to interact with TAPL in transiently co-transfected HEK293T cells (Fig. 8D), validating that the interaction occurs via the TMDs of both proteins.

Next, we investigated if YIF1B is essential for TAPL targeting. HeLa Flp-In T-REx YIF1B knock-out clones were created by CRISPR/Cas9 using two separate sgRNAs targeting exon 2 or exon 3. The knock-out was confirmed by immunoblot and sequencing of the targeted exons (Fig. S8A). In the YIF1B knock-out cell lines, TAPL showed no alteration in localization between 2 h and 24 h after induction of expression compared to the original cell line (Fig. S8B, Fig. S9A). Next, YIF1B KO cells were transfected with the aforementioned vector for the RUSH assay, yielding no differences in the early trafficking process of TAPL (Figure S9B). This demonstrates that YIF1B is not essential for TAPL targeting and its absence does not impact trafficking kinetics. Redundancy in subcellular trafficking is common, therefore we investigated whether...
YIF1A, with a sequence identity of 53% to YIF1B, can compensate for YIF1B deficiency (Fig. S10). Nevertheless, YIF1B, but not YIF1A, was co-immunoprecipitated with TMD0 if transiently co-expressed in HEK293T cells, consistent with the mass spectrometry results. To assess whether YIF1B plays a role in TAPL targeting, even if it is not an essential factor, we aimed to outcompete alternative binding partners as well as endogenous YIF1B by overexpression of truncated YIF1B (TMD\textsubscript{YIF1B}). If we assume that YIF1B is involved in ER to Golgi targeting of TAPL, and harbors its targeting determinant in its cytosolic domain, the overexpression of TMD\textsubscript{YIF1B} should influence TAPL targeting. In contrast to YIF1B, which is found dominantly in the ER, TMD\textsubscript{YIF1B} is strongly enriched in the cis-Golgi (Fig. S11B and C), implying that a targeting or retrieval signal is indeed localized in the cytosolic domain of YIF1B. Upon transient co-expression in HeLa Kyoto cells, TAPL showed a reduction in lysosomal localization dependent on the TMD\textsubscript{YIF1B} level (Fig. 9A, B and E), with more pronounced effects in cells with higher TMD\textsubscript{YIF1B} levels. TAPL was strongly enriched in lysosomes of cells not transfected by YIF1B or TMD\textsubscript{YIF1B}. Overexpression of TMD\textsubscript{YIF1B} significantly decreased colocalization of TAPL with LAMP-1, compared to cells overexpressing full-length YIF1B (Fig. 9D and E). Interestingly, TAPL colocalized strongly with TMD\textsubscript{YIF1B} in cis-Golgi (Fig. 9C).

Finally, we tested our initial hypothesis whether D17N substitution in TMD0 affects the interaction with YIF1B. HEK293T cells were transiently transfected with TMD0-FLAG or TMD0\textsubscript{D17N}-FLAG and immunoprecipitation was carried out using α-FLAG antibody. The amount of endogenous YIF1B pulled down by TMD0\textsubscript{D17N} was reduced by a factor of four in comparison to TMD0 (Fig. 9F and G), based on the four individual immunoprecipitations we performed (Fig. S12). This indicates a conformational change of the TMD0 interaction interface by disruption of the salt bridge between D17 and R57.

In summary, the newly identified transient interaction partner YIF1B interacts via its TMD\textsubscript{YIF1B} directly or indirectly with TMD0. Although YIF1B is not essential for lysosomal targeting of TAPL, overexpression of TMD\textsubscript{YIF1B} strongly interferes with correct localization of TAPL. Interaction of both proteins is strongly dependent on the conserved D17 within TMD0.

**Discussion**

In this study, we demonstrated that TAPL takes the direct route from the Golgi to lysosomes. Further, we found four conserved, charged amino acids in the transmembrane helices of TMD0, which affect the subcellular localization of TAPL and, therefore, represent atypical targeting determinants. Additionally, we identified YIF1B as a new interaction partner of TAPL, being the first piece of the, presumably more complex, trafficking interactome.

By synchronizing intracellular trafficking using the RUSH assay, we proved that TAPL chooses the direct route to lysosomes via the Golgi and early endosomes but not the indirect route via the PM. The lack of PM localization is supported by the use of the dynamin inhibitor Dyngo-4a and missing surface biotinylation (11). Furthermore, TAPL was not detected on the PM in bone marrow derived dendritic cells (8). This is in contrast to lysosomal-localized ABCB6, which takes the indirect route and can be accumulated at the PM by adding Dyngo-4a (25). While the trafficking kinetics should be analyzed with caution due to their dependence on cells with high TAPL-SBP-eGFP levels for detection, rough estimates can be made based on our results. ER to Golgi trafficking occurs within 15-30 min after biotin addition. After 30-60 min, Golgi localization decreases and early endosome localization is detectable. To reach lysosomes, TAPL needs up to 90 min. This timeframe is in line with data from inducible HeLa Flp-In T-REx cells used in this study, where TAPL co-localized with LAMP-1 two hours after induction of expression. Moreover, the trafficking time of TAPL is in good agreement with that of other proteins in the secretory pathway and lysosomal membrane proteins (26–28).

Atypical cytosolic targeting determinants, which differ from the consensus tyrosine or dileucine motifs, can be found, for example, in the Longin domain of VAMP7 (29), as PY-motifs (L/PPXY) in LAPTM5 (30), or as “extended acidic dileucine signal” in TMEM106B (31). For some proteins, these motifs are, by themselves, not
sufficient to mediate lysosomal localization but are dependent on additional motifs. One example is the lysosomal targeting of TMEM106B, which additionally depends on N-glycosylation of its luminal loops (32). Another type of atypical sorting determinants are TMH parameters like their length, amount and positioning of hydrophobic or charged amino acids, or interplay with lipids (33). In case of TAPL, charged amino acids within the TMHs of TMD0 determine its subcellular localization. Charged residues within TMHs are often essential for protein function and complex assembly. For instance, correct complex assembly and therefore PM localization of the T-cell receptor (TCR) depends on charged residues in the TMHs (34, 35). In case of TAPL<sub>D17N</sub>, full ER retention is not due to an incomplete assembly of TAPL homodimers since TAPL<sub>D17N</sub> is fully active in peptide transport. By means of immunoprecipitations, NMR analysis of cf-TMD0<sub>D17N</sub> and restoration of lysosomal localization of TMD0<sub>D17N</sub> by supplying the targeting motif of LAMP-2C, we demonstrated that ER retention was not caused by misfolding neither. In conclusion, while D17 is indispensable for targeting, it is not essential for the structural integrity of TAPL or TMD0.

YIF1B shuttles between ER, ERGIC and Golgi (23). Furthermore, it is involved in lysosomal trafficking of TAPL, since overexpression of TMD<sub>YIF1B</sub>, which accumulates in the Golgi, has a strong impact on TAPL targeting. Moreover, TMD0 of TAPL interacts transiently with TMD<sub>YIF1B</sub>, and this interaction is strongly weakened by mutating D17 of TAPL. Therefore, we postulate that subcellular targeting of TAPL depends on its interactions with other transmembrane proteins that link it to components of the trafficking machinery. Since YIF1B is not implicated in trafficking steps beyond the Golgi, other transmembrane proteins probably interact with TAPL at the TGN and endosomes to mediate further trafficking steps. This would coincide with the observed strong Golgi retention of TAPL upon D45, D49 and K100 substitution. Such a piggyback mechanism is described for the ABC transporter ABCD4, which interacts with the classical tyrosine-based motif exhibiting LMBD1 (36). Similarly, the endosomal and lysosomal localization of MHC class II is the result of its interaction with the invariant chain (37, 38), which blocks premature peptide binding (39) and also exhibits a dileucine motif (40).

YIF1B, implicated in anterograde transport (23), plays a role in 5-HT<sub>1A</sub> receptor trafficking (41). However, this cell surface-localized protein interacts with YIF1B via its cytosolic tail and not its TMD. In contrast, the closely related YIF1A, which shares high sequence identity in its TMD with YIF1B, interacts with VAPB via its transmembrane domain (24), indicating that these proteins may have multiple binding sites for different proteins. Despite their similarities in the TMD, YIF1A is unable to interact with TMD0, as demonstrated by immunoprecipitation.

CRISPR/Cas9-mediated knock-out of YIF1B in our stable cell line system did not alter TAPL localization and is therefore apparently not an essential factor for targeting. Thus, other transmembrane proteins also have to be considered for TAPL ER to Golgi trafficking. Redundant trafficking pathways and mechanisms are observed for several proteins. For instance, three different trafficking routes were described for LAMP-1/2. Usually, these proteins are trafficked in a clathrin dependent manner. They can either take the direct route (42), mediated by AP-1 (43) and AP-3 (44), or the indirect route, mediated by AP-2 (45). Additionally, trafficking from the TGN to late endosomes by non-clathrin coated “LAMP-carriers” was described (46, 47).

Of the four conserved charged residues, D17 substitutions revealed the strongest impact on TAPL localization. Mutation of D17 retained TAPL completely in the ER, while single substitutions of the other conserved amino acids allowed partial lysosomal localization. Intriguingly, the other two members of the TAP-family, TAP1 and TAP2, which form heterodimers, also have a conserved aspartate in their first and a conserved arginine in their second TMH (48). It was shown that this aspartate is essential for an intermolecular salt bridge with tapasin. This interaction is placing TAP in the peptide loading complex, which is responsible for MHC class I loading. Based on molecular dynamics simulations, this aspartate forms an intramolecular salt bridge with the
arginine, which is replaced by an intermolecular salt bridge with lysine 428 in the transmembrane helix of tapasin, while the arginine side chain snorkels to the polar headgroups of the lipid bilayer to avoid an unfavorable uncompensated charge in the TMD. Based on the strong impact of charge inversion substitutions of D17 and R57 on TAPL trafficking, as well as the partial rescue of lysosomal localization by simultaneous charge reversal of both, we demonstrated that D17 in TAPL does not form an intermolecular salt bridge but an intramolecular one with R57. However, deletion of the charge at R57 was partially compensated by K100 since R57A substitution, unlike D17N, did not abolish lysosomal localization. This indicates that D17 and K100 are able to form a salt bridge but yield a conformation that is less trafficking competent than the wildtype conformation. Charge inversion of D17 and K100 (TAPL_D17R,K100D) does not produce a trafficking competent conformation possibly due to the positive charge of R57, which would be in close proximity to D17R, causing destabilizing effects. In line with the proposed salt bridges, deletion of both conserved positive charges again completely prohibited lysosomal targeting. Summing up our data, we propose that disruption of the salt bridge between D17 and R57 induces subtle conformational changes in the binding interface. These changes weaken the interaction with YIF1B, and possibly other trafficking chaperones, resulting in increased ER localization of TAPL. Interestingly, substitution and charge reversals of D45, D49, and K100 strongly increased Golgi localization and also abolished lysosomal localization, further highlighting the importance of the conserved, charged residues. Whether a salt bridge is formed between K100 and the negative cluster of D45/D49 cannot be derived from these experiments. These substitutions seem to impact the second trafficking step of TAPL from the Golgi to early endosomes, indicating an involvement in a second protein interaction interface, separate from that affected by D17 and R57 substitutions.

In summary, TAPL targeting is determined by conserved, charged residues in its TMD0, forming intramolecular salt bridges. Disruption of these salt bridges arrests TAPL either in the ER or Golgi by rendering TMD0 trafficking incompetent. This disability to travel along the direct route to lysosomes is due to a diminished capability to interact with interaction partners involved in trafficking as exemplified with YIF1B.

Experimental procedures

Peptides and antibodies

Primary and secondary antibodies used in this study are listed in Table S2. α-YIF1B was a kind gift from Michèle Darmon (Centre de Psychiatrie et Neurosciences, Paris, France) (41). α-C8 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Chessie 8 from Dr. George Lewis (49). HA peptide was purchased from Sigma-Aldrich/Merck and Charité (Berlin, Germany). RRYQNSTCL peptide (Charité) was labelled with 5-iodoactamidofluorescein (I9271, Sigma-Aldrich/Merck) as published elsewhere (50).

Cloning

Cloning primers, corresponding templates, plasmids and cloning techniques are listed in Table S3. Q5 Site-Directed Mutagenesis Kit (New England BioLabs) was used according to manufacturer’s instructions. Restriction enzyme cloning was performed by digesting backbone and PCR product with the listed restriction enzymes (ThermoFisher Scientific or New England BioLabs). Str-il_VSVGwt-SBP-EGFP (16) was a gift from Franck Perez (Institute Curie, Paris, France) and pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang (Addgene plasmid # 62988) (51). HA-YIF1A and HA-YIF1B genes were synthesized by Eurofins Genomics. All constructs were verified by DNA sequencing.

DNA extraction

To extract DNA from mammalian cells for PCR, 0.1 x 10^6 cells were transferred in a tube, washed 3 times with PBS (4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and frozen at -20 °C. Pellet was resuspended in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), incubated at 80 °C for 10
min and then incubated at -20 °C for 5 min. Proteinase K (0.5 µg/µl, ThermoFisher Scientific) was added and incubated at 50 °C for 30 min and at 80 °C for 10 min. Samples were centrifuged at 10,000x g for 2 min, and 1 µl of supernatant was used as template for PCR.

**Cell culture**

HeLa Kyoto, HEK293T and HeLa Flp-In T-REx cells were cultured at 37 °C, 5% CO₂ and 95% humidity. HeLa Kyoto and HEK293T were cultured in DMEM/Dulbecco’s Modified Eagle Medium (Gibco/ThermoFisher Scientific) with 10% fetal bovine serum (FCS, Capricorn Scientific). For culturing stable cell lines of the HeLa Flp-In T-REx system, DMEM with 10% tetracycline-free FCS (Bio&Sell) was used. Selection of stable HeLa Flp-In T-REx cells was performed with 200 µg/ml hygromycin B (ThermoFisher Scientific) in combination with 2 µg/ml blasticidin S HCl (ThermoFisher Scientific). Selection of transiently transfected HeLa Flp-In T-REx cells was performed with 1 µg /ml puromycin (ThermoFisher Scientific). Induction of expression in stable HeLa Flp-In T-REx cells was performed with 1 ng/ml - 5 µg/ml of doxycycline (D9891, Sigma-Aldrich/Merck), depending on the gene of interest and application. For cycloheximide (CHX) (2112, Cell Signaling Technology) treatment, cells were induced for 19 h and then treated with 25 µg /ml CHX for additional 5 h. All cells were tested regularly for mycoplasma contamination.

**Transfection**

Transfections of HeLa Kyoto, HeLa Flp-In T-REx and HEK293T Flp-In T-REx cells were performed with Lipofectamin2000 (ThermoFisher Scientific) in a 1:2.5 ratio (µg DNA per µl transfection reagent). HEK293T cells were transfected using 18 mM polyethylenimine (PEI) stock solution in a 1:5 ratio (µg DNA per µl transfection reagent). WE used 0.8 µg DNA per well in a 24-well plate, 2.5 µg per 6-well and 15 µg for 10 cm dishes. DNA and Lipofectamin2000 or PEI were diluted in Opti-MEM I Medium (ThermoFisher Scientific), incubated for 5 min, mixed and incubated for 15 min prior to transfection. Cells were seeded 6 to 20 h prior to transfection to ensure complete adhesion. Lipofectamin2000 and PEI transfections were performed at 80 to 95% and 40% confluency, respectively.

**CRISPR/Cas9**

Exon and intron sequences were obtained from Ensembl Genome Browser (ensembl.org). Exon 2 or 3 were used as input sequences for sgRNA design (crispr.mit.edu). sgRNAs were picked according to their score and potential off-target effects (Table S3). Corresponding DNA was ordered from Eurofins Genomics. SpCas9-2A-Puro V2.0 was used for transfection of HeLa-Flp-In T-REx cells. Selection was performed using Puromycin. Post-selection limited dilution cloning was performed to obtain monoclonal cells. DNA was extracted and used for PCR. PCR products were separated by 2% agarose gel, extracted, purified and sequenced. Knock-out was further confirmed by immunoblot (52, 53).

**Sequence alignments**

All protein sequences were obtained from UniProt (uniprot.org) (54). Sequence alignments were performed as Multiple Sequence Comparison by the Log-Expectation (MUSCLE) online tool (ebi.ac.uk/Tools/msa/muscle) (55, 56).

**Immunostaining**

Cells were seeded on sterile coverslips in 24-well plates, washed three times with PBS and fixed using 4% formaldehyde (Carl Roth) in PBS for 10 min. Permeabilization and blocking were performed by addition of 0.1% saponin (S4521 Sigma-Aldrich/Merck) in PBS for 20 min and 5% BSA (Carl Roth) in 0.1% saponin/PBS, respectively. Cells were stained by primary and secondary antibody, diluted in 0.1% saponin/PBS containing 1% BSA, at room temperature for 2 h and 1 h, respectively. DAPI (4’,6-Diamidino-2-phenylindole dihydrochloride, D8417 Sigma-Aldrich/Merck) was added during incubation with secondary antibody. After washing and drying, coverslips were mounted in Mowiol 4-88 and DABCO (Carl Roth).

**Confocal imaging**

The intracellular route of TAPL was imaged using a confocal laser-scanning TCS SP5 microscope (Leica) with a Plan-Appochromat
63x/1.4 oil differential interference contrast (DIC) objective. Sequential settings for scans were used. For excitation the following laser lines were used: 405 nm (diode laser) for 4,6-diamidino-2-phenylindole (DAPI), 488 nm (argon laser) for eGFP, 561 nm (diode-pumped solid-state laser) for Cy3 and 633 nm (helium-neon laser) for Alexa Fluor 647. Intensities of channels were adjusted over the whole image for better visualization of overlap and exported by Leica Application Suite X.

All other experiments were performed using a confocal laser-scanning LSM880 microscope (Zeiss) with a Plan-Appochromat 63x/1.4 oil DIC M27 objective. Sequential settings for scans were used. For excitation the following laser lines were used: 488 nm (argon laser) for Alexa Fluor 488 and eGFP, 543 nm (helium-neon laser) for Cy3, 594 nm (helium-neon laser) for Alexa Fluor 568, 633 nm (helium-neon laser) for Alexa Fluor 647. Intensities of channels were adjusted over the whole image for better visualization of overlap and exported by Zen blue (Version 2.3 lite, Zeiss).

**Live-cell imaging**

Cells were cultured and transfected in 8 well x-well slides (Sarstedt). 18-24 h after transfection cells were washed once with PBS and then shortly incubated in 200 µl live cell imaging solution (Invitrogen/ThermoFisher Scientific) in the incubation chamber of microscope at 37 °C. 200 µl of biotin (80 µM) in live cell imaging solution was added to release TAPL-SBP-eGFP. Z-stacks were recorded every 60 - 90 s. Maximum intensity z-projections were generated using Zen black (Version 2.3, Zeiss).

**Quantification of colocalization**

Unprocessed micrographs were exported by ZEN blue as “tiff” files without compression. Images were loaded into Fiji/ImageJ (57, 58). Then, 50 to 90 individual cells were outlined as regions of interest (ROI) and Pearson correlation coefficient (PCC) was determined by Coloc 2. Automated threshold selection (Costes) was performed. PCC values above threshold were plotted by GraphPad Prism 5 (GraphPad Software) as vertical scatter plot with means and SEM. P < 0.05 were considered significantly different. For intensity plots, intensity along the line was measured by Zen blue, normalized and plotted by GraphPad Prism 5.

**Statistical analysis**

Statistical significance analyses were performed using GraphPad Prism 5 (GraphPad Software). For pairwise comparison statistics unpaired two-tailed Student’s t-tests were applied. For multiple comparison of flow cytometry data one-way ANOVA with post hoc Tukey’s test was used. For multiple comparison of PCC values obtained from colocalization analysis the non-parametric Kruskal-Wallis test with post hoc Dunn’s test was used. Test results are listed in Table S1.

**Deconvolution of Images**

All microscopy images, except maximum intensity z-projections, used for Figures were deconvoluted. Point-spread functions (PSF) were generated by PSF Generator (59) for Fiji/ImageJ, using the Born-Wolf algorithm. Images were deconvoluted using DeconvolutionLab2 (60) for Fiji/Image2, using the Richardson-Lucy algorithm with 50 iterations.

**Synthesis of Dyngo compounds**

Dyngo-4a and Dyngo-Ø were synthesized as recently described (61) using the reflux method. Products were dissolved as 30 mM stock solutions in 100% DMSO (D2650, Sigma-Aldrich/Merck) and stored at -20 °C. Compounds were diluted in DMEM without FCS to 30 µM directly before usage.

**Transferrin uptake assay**

Transferrin conjugated with Alexa Fluor 647 (T23366, ThermoFisher) was reconstituted in H2O at 5 mg/ml. Cells were washed with OptiMEM I (without FCS) twice and pre-incubated with 30 µM Dyngo-4a, Dyngo-Ø or DMSO in Opti-MEM I for 30 min at 37°C. Then cells were incubated with 25 µg/ml transferrin and 30 µM Dyngo-4a or Dyngo-Ø in Opti-MEM I at 37 °C for 30 min. Afterwards cells were trypsinized, mixed with DMEM / 10% FCS, centrifuged at 600x g at 4°C, washed once with cold PBS and twice with cold FACS buffer (1% FCS in PBS). To assess background signal, a sample was trypsinized after the pre-incubation, mixed with DMEM / 10% FCS,
centrifuged at 600x g at 4 °C. Subsequently, cells were washed twice with cold PBS, incubated with transferrin\textsuperscript{AF647} and Dyngo-4a in Opti-MEM I for 30 min on ice and then washed once with cold PBS and twice with cold FACS buffer. Cells were fixed in 1% PFA in FACS buffer prior to analysis. All samples were analyzed by FACS Celesta (BD Biosciences) with λ\textsubscript{ex/em} 633/670 nm. Data analysis was performed with FlowJo V10 to determine the mean fluorescence intensity. The statistics are based on three separately prepared samples.

**Immunoprecipitation**

50 µl of Dynabeads M-280 sheep anti-mouse or sheep anti-rabbit (Life Technologies) were washed with 3 ml IP buffer (20 mM Tris, 150 mM NaCl, 5 mM MgCl\textsubscript{2}, pH 7.4) supplemented with 0.1% BSA. Washed beads were incubated in 800 µl IP buffer with either 2.5 µl anti-FLAG M2, 2.5 µl anti-HA or 5 µl mouse monoclonal IgG1-κ as isotype control overnight at 4 °C and washed with 3 ml IP buffer. 8.8 x 10\textsuperscript{6} cells were used for each individual IP. Harvested cells were stored at -80 °C, thawed on ice and solubilized using IP buffer containing 0.5% Nonidet P40 (NP40, Fluka now Sigma-Aldrich/Merck) or 1% digitonin (Millipore/Merck) and 1x HP protease inhibitor mix (Serva) for 1 h at 4 °C. Lysate was centrifuged at 20,000x g for 20 min at 4 °C. Supernatant was incubated with antibody coated beads for 2 h at 4 °C and washed with 3 ml IP buffer containing 0.05% NP40 or 0.1% digitonin. Proteins were eluted by incubation at 90 °C for 20 min in elution buffer containing 2x SDS sample buffer (125 mM Tris, 4% (w/v) SDS, 4 mM EDTA, 0.02% (w/v) bromophenol blue, 20% (v/v) glycerol, pH 6.8) without reducing agents in 25 mM NaOAc, pH 5.0. Samples were analyzed by 10% SDS-PAGE or 10% Tricine-SDS-PAGE followed by immunoblotting.

**Cell free expression and NMR spectroscopy**

Synthesis of TMD0 variants, purification and NMR spectroscopy was performed as published before (62). In short, \textsuperscript{15}N uniformly labelled cf-TMD0 and cf-TMD0\textsubscript{D17N} were synthesized in a continuous exchange \textit{E. coli} based cell-free expression system. Proteins were solubilized in 1-myristoyl-2-hydroxy-sn-glycerol-3-[phospho-rac-(1-glycerol)] (LMPG) (Avanti Polar Lipids). During immobilized metal affinity chromatography (IMAC), detergent was exchanged to 1,2-dihexanoyl-sn-glycero-3-phosphocholine (c6-DHPC). After elution, buffer was exchanged to NMR sample buffer (25 mM NaOAc, 100 mM NaCl, 0.75% c6-DHPC, 1x HP protease inhibitor mix, pH 5.0). \textsuperscript{[15N, \textsuperscript{1}H]-BEST-TROSY NMR spectra of 160 µM of cf-TMD0 and 220 µM of cf-TMD0\textsubscript{D17N} were recorded at sample temperatures of 313 K on a 700 MHz Bruker AvIII HD spectrometer equipped with a cryogenic \textsuperscript{1}H/\textsuperscript{13}P/\textsuperscript{13}C/\textsuperscript{15}N quadruple resonance probe.

**Peptide transport**

40 x 10\textsuperscript{6} HeLa Flp-In T-Rex cells were washed with 60 ml PBS, harvested by centrifugation for 10 min at 1000x g and 4 °C and stored at -80 °C. For membrane preparation, cells were thawed on ice, resuspended in Tris buffer (20 mM Tris, 1 mM DTT, 2.5 mM benzamidine, 1 mM PMSF, pH 7.4) and disrupted by dounce homogenization (40 times) with a tissue grinder (Wheaton). Sucrose was added to a final concentration of 250 mM and cells were dounced again (10 times). Cell lysate was sequentially centrifuged for 4 min at 200x g and 8 min at 700x g at 4 °C. Subsequently, supernatant was pelleted for 45 min at 100,000x g and 4 °C. Crude membranes were resuspended in PBS and aliquots were snap-frozen in liquid nitrogen and stored at -80 °C. Protein concentration in membranes was determined by Bradford assay (ThermoFisher Scientific). For peptide transport, crude membranes (120 µg protein) were incubated in 50 µl PBS supplemented with 3 mM MgCl\textsubscript{2} and 3 µM NST-F (RRYQNST\textsubscript{Fluorescein}L) peptide. Peptide transport was started by addition of 3 mM ATP for 12 min at 37 °C. Transport was stopped with 1 ml ice-cold stop buffer (PBS supplemented with 10 mM EDTA). Membranes were collected on microfilter plates (MultiScreen plates, Durapore Membrane, 1.2 µm pore size; Millipore now Merck) preincubated with 0.3% (w/v) polyethylenimine. Filters were washed three times with 250 µl of ice-cold stop buffer and incubated at RT for 10 min in lysis buffer (PBS, 1% SDS, pH 7.5). The amount of transported peptides was analyzed on a fluorescence plate.
reader (CLARIOstar, BMG LABTECH) at λex/em = 485/520 nm. ATP-dependent peptide accumulation in crude membranes from non-transfected cells was subtracted and transport was normalized on TAPL amount determined by immunoblot.

**Mass spectrometry (MS) based proteomics**

Anti-HA-immunoprecipitation was performed as previously described (63–65, 22). Summarily, expression of TAPL-HA and coreTAPL-HA was induced by addition of 4 µg/ml doxycycline for 24 h in HeLa Flip-In T-REx cells. Parental non-transfected HeLa Flip-In T-REx cells were used as negative control. For each sample, 6.4 x 10⁷ cells were harvested, frozen in liquid nitrogen and stored at -80 °C. Cells were lysed in 3 ml MCLB buffer (50 mM Tris, 150 mM NaCl, 0.5% NP40, pH 7.4) supplemented with cOmplete EDTA-free protease inhibitor tablets (Roche, 2 tablets for 50 ml MCLB buffer) on ice for 30 min. Lysate was cleared by centrifugation (18,000x g, 10 min, 4 °C) followed by filtering through a 0.45 µm spin filter (Millipore). For isolation of HA-tagged proteins, cell lysate was incubated overnight at 4 °C with 60 µl of equilibrated α-HA agarose beads (Sigma-Aldrich/Merck). Subsequently, beads were washed four times with 1 ml MCLB and 1 ml PBS, respectively. 50 µl of 250 µg/ml HA peptide was added to dry beads and incubated for 30 min at room temperature. Elution was repeated twice obtaining a final volume of 150 µl. Proteins were precipitated with 20% tri-chloroacetic acid (TCA), resuspended in 20 µl 50 mM ammonium bicarbonate pH 8.0 containing 10% acetonitrile and 750 ng tryspin (Promega) and incubated for 4 h at 37 °C. Desalting was performed using stage tips. Samples were analyzed as technical duplicates on an LTQ Velos (ThermoScientific) and spectra were identified by Sequest searches as previously described (66). For CompPASS analysis, the identified peptides were compared to IP-MS data of 99 unrelated bait proteins, which were previously processed using the same experimental conditions (22), to obtain weighted and normalized D-scores (WDN-score)(Table S4). Proteins with WDN ≥ 1.0 and APSM (average peptide spectral matches) ≥ 4 were considered as high-confident candidate interacting proteins (HCPs). To account for co-purifying (background) proteins in HeLa cells and coreTAPL-binding proteins, proteins found in these two IP conditions were subtracted from the list of TAPL HCIPs. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (67) partner repository (ebi.ac.uk/pride/archive/) with the dataset identifier PXD010989.

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**Conflict of interest**

The authors declare that they have no conflicts of interest with the contents of this article.

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**Figures**

**Figure 1: Intracellular route of TAPL**

**A** Constructs for the RUSH assay. Invariant chain of MHC class II (luminal domain is not depicted), containing a cytosolic HA-tag, streptavidin, and an ER retention signal, served as ER “hook” protein. TAPL was C-terminally fused to SBP and eGFP. To guarantee simultaneous expression, both genes were cloned in a bicistronic IRES vector under the control of the cytomegalovirus promotor (pCMV) and separated by a synthetic intervening sequence (IVS) and an internal ribosome entry site (IRES).

**B** Time dependent trafficking of TAPL. HeLa Kyoto cells were transiently transfected with the bicistronic IRES vector coding for both RUSH constructs and incubated with biotin for the indicated time-points. Subsequently, cells were fixed and immunostained by α-HA (hook), α-GM130 (cis-Golgi), α-EEA1 (early endosomes) and α-LAMP-1 (late endosomes/lysosomes). White arrowheads at zoom-in point to an overlap of TAPL and subcellular marker. Scale bar: 10 μm; inset 5 μm.

**C** TAPL is not found on the plasma membrane. TAPL-FLAG expression was induced by doxycycline in stably transfected HeLa Flp-In T-REx cells in the presence of 30 μM Dynago-4a for 2 h. Cells were fixed and immunostained by α-FLAG (TAPL) and α-LAMP-1 (lysosomes) or α-MHC I (plasma membrane). Scale bar: 10 μm; inset 5 μm.
Figure 2: Conserved, charged residues within TMD0
Conserved residues of human, mouse, chicken, zebrafish and sea lamprey TAPL, as well as *C. elegans* HAF-4 and HAF-9 (see Fig. S3) are depicted in a TMD0 secondary structure model (20). Charged residues within TMH1-3 investigated in this study are highlighted by a bold border.
Figure 3: Conserved, charged residues within TMD0 determine TAPL localization

A Subcellular localization of TAPL mutants. 24 h after induction of TAPL variants expression, HeLa Flp-In T-REx cells were fixed and immunostained by α-FLAG (TAPL) and α-LAMP-1 (lysosomes). Scale bar: 10 µm; inset 5 µm.

B Quantification of lysosomal localization. For each TAPL construct, the Pearson correlation coefficient of TAPL and LAMP-1 colocalization was determined for 50 individual cells. Individual values are depicted as rectangles, mean values and corresponding SEMs are shown in red. ***, p < 0.001; *, p < 0.05; ns, nonsignificant by Kruskal-Wallis test with post hoc Dunn’s test. Mean values, corresponding SEMs and test results are listed in Table S1.
Figure 4: Intramolecular salt bridge between D17 and R57

A Subcellular localization of TAPL variants. 19 h after induction of TAPL variants expression, HeLa Flp-In T-REx cells were treated with 25 µg/ml CHX for 5 h. Cells were fixed and immunostained by α-FLAG (TAPL) and α-LAMP-1 (lysosomes). Scale bar: 10 µm; inset 5 µm.

B Quantification of lysosomal localization. For each TAPL construct, the Pearson correlation coefficient of TAPL and LAMP-1 colocalization was determined for 50 individual cells. Individual values are depicted as rectangles, mean values and corresponding SEMs are shown in red. ***, p < 0.001; ns, nonsignificant by Kruskal-Wallis test with post hoc Dunn’s test. Mean values, corresponding SEMs and test results are listed in Table S1.
Figure 5: Golgi retention of TAPL substitutions

A: Subcellular localization of TAPL variants. 19 h after induction of TAPL variants expression, cells were treated with 25 µg/ml CHX for 5 h. Cells were fixed and immunostained by α-FLAG (TAPL), α-LAMP-1 (lysosomes) or α-GM130 (cis-Golgi). Scale bar: 10 µm, inset 5 µm.

B: Quantification of subcellular localization. For each TAPL construct, the Pearson correlation coefficient of TAPL and LAMP-1 or GM130 colocalization was determined for 50 individual cells. Individual values are depicted as rectangles, mean values and corresponding SEMs are shown in red.

C: TAPL substitutions are correctly folded. Peptide transport of crude membranes derived from HeLa Flp-In T-REx cells not transfected (n.t.) or containing TAPL constructs was performed with NST-F peptide (3 µM) in the presence of ATP (3 mM) for 12 min at 37 °C. Peptide accumulation in crude membranes from not transfected cells was subtracted. Transport, performed in triplicates, was normalized on TAPL amount determined by immunoblot of crude membranes derived from HeLa Flp-In T-REx cells not transfected (5 µg) or expressing wt TAPL (2.5 µg) or TAPL D45K,D49K,K100D (5 µg). Error bars indicate SD.
Figure 6: TMD0 is correctly folded

A TMD0 variants interact with coreTAPL. HEK293T cells transiently co-transfected with TMD0-Flag constructs and coreTAPL were solubilized by 1% digitonin (cell lysate). Co-immunoprecipitation was performed using α-FLAG antibody (FLAG) or an IgG isotype control antibody (IC). CoreTAPL was detected by α-TAPL.

B Structural integrity of TMD0

15N, 1H]-BEST-TROSY NMR spectra were recorded of 15N uniformly labeled cf-TMD0 (red) and cf-TMD0 D17N (green) at 313 K.

C Restoration of lysosomal localization of TMD0 D17N. 24 to 48 h after induction of TMD0-FLAG, FLAG-TMD0 D17N and FLAG-TMD0 D17N-LAMP-2C expression, HeLa Flp-In T-REx cells were fixed and immunostained by α-FLAG for TMD0 constructs and α-LAMP-1 (lysosomes). Scale bar: 10 µm; inset 5 µm.

D Quantification of lysosomal localization. For each TMD0 construct, the Pearson correlation coefficient of TMD0 and LAMP-1 colocalization was determined for 50 individual cells. Individual values are depicted as rectangles, mean values and corresponding SEMs are shown in red.***, p < 0.001 by Kruskal-Wallis test with post hoc Dunn’s test. Mean values, corresponding SEMs and additional test results are listed in Table S1.
Figure 7: TAP\textsubscript{L\textsubscript{D17N}} is correctly folded

A TAP\textsubscript{L\textsubscript{D17N}} is accompanied to lysosomes by TAP\textsubscript{L\textsubscript{wt}}. HeLa Kyoto cells, transiently co-transfected with TAPL\textsubscript{FLAG} and TAPL\textsubscript{D17N\textendash}C8, were fixed and stained by α-C8, α-FLAG and α-LAMP-1 (lysosomes). Scale bar: 5 µm; inset 2.5 µm.

B TAP\textsubscript{L\textsubscript{D17N}}, TAP\textsubscript{L\textsubscript{wt}} and LAMP-1 colocalize. Intensity of all three channels from micrograph depicted in Fig. 7A is shown along the white arrow for better visualization. Scale bar: 10 µm.

C TAPL-dependent peptide transport. Peptide transport of crude membranes derived from HeLa Flp-In T-REx cells not transfected (n.t.) or containing TAPL\textsubscript{wt} or TAPL\textsubscript{D17N} was performed with NST-F peptide (3 µM) in the presence of ATP (3 mM) for 12 min at 37 °C. Peptide accumulation in crude membranes from untransfected cells was subtracted. Transport, performed in triplicates, was normalized on TAPL amount determined by immunoblot of crude membranes derived from HeLa cells not induced (5 µg) or expressing wt TAPL (2.5 µg) or TAPL\textsubscript{D17N} (10 µg). TAPL wt data are taken from Figure 5C since the experiments were performed for all mutants simultaneously. Error bars indicate SD.
Figure 8: YIF1B interacts with TAPL

A TMD0 dependent TAPL interactome. HeLa Flp-In T-REx cells stably expressing HA-tagged TAPLwt or coreTAPL and their parental empty counterparts were lysed by Nonidet P40 (NP40) and subjected to IP-MS. HCIPs were identified by CompPASS based on a WD<sup>N</sup>-score ≥ 1.0 and APSM value ≥ 4. Proteins found in coreTAPL or parental non-transfected HeLa Flp-In T-Rex were subtracted from the TAPL HCIPs. Full CompPASS results are listed in Table S4.

B YIF1B interacts with TAPL via TMD0. HeLa Flp-In T-REx cells stably expressing FLAG-tagged variants of TAPL<sup>wt</sup>, coreTAPL or TMD0<sup>wt</sup> were solubilized by NP40, immunoprecipitated by α-FLAG antibody (FLAG) and immunoblotted. Specificity of immunoprecipitation was verified by an IgG isotype control (IC) and using non-transfected cells. The contrast in the right part of α-YIF1B immunoblot was enhanced due to low signal intensity.

C Reverse co-immunoprecipitation of YIF1B and TMD0. HEK293T cells transiently transfected with TMD0<sup>wt</sup>-FLAG, HA-YIF1B or a combination of both were lysed by NP40. YIF1B was immunoprecipitated by α-HA antibody (HA) and immunoblotted. Specificity of immunoprecipitation was verified by an IgG isotype control (IC) and using cells transfected with a plasmid devoid of a gene of interest (-/-).

D TMD<sub>YIF1B</sub> interacts with TAPL. HEK293T cells transiently transfected with TAPL<sup>wt</sup>-FLAG alone or in combination with HA-YIF1B (fl.), HA-cyt<sub>YIF1B</sub> (cyt.) or HA-TMD<sub>YIF1B</sub> (TMD) were solubilized by NP40, and immunoprecipitation was performed by α-Flag antibody (FLAG). Specificity of immunoprecipitation was verified by an IgG isotype control (IC) and using cells transfected with a plasmid devoid of a gene of interest (-/-).
Figure 9: YIF1B is involved in TAPL targeting and its interaction is weakened by D17N substitution

A TMD of YIF1B interferes with TAPL targeting. HeLa Kyoto cells were transiently co-transfected with TAPL<sub>wt</sub>-FLAG and HA-TMD<sub>YIF1B</sub>. After 24 h, cells were fixed and stained by α-HA (TMD<sub>YIF1B</sub>), α-FLAG (TAPL) and α-LAMP-1 (lysosomes / LY). Scale bar: 5 µm; inset 5 µm.

B Intensity of all three channels of micrograph depicted in Fig. 9A is shown along the white arrow for better visualization of colocalization. Scale bar: 10 µm.

C TMD<sub>YIF1B</sub> accumulates TAPL in the Golgi. HeLa Kyoto cells were transiently co-transfected with TAPL<sub>wt</sub>-FLAG and HA-TMD<sub>YIF1B</sub>. After 24 h, cells were fixed and stained by α-HA, α-FLAG and α-GM130 (cis-Golgi). Scale bar: 5 µm; inset 5 µm.

D TAPL is localized in lysosomes if full length YIF1B is coexpressed. HeLa Kyoto cells were transiently co-transfected with TAPL<sub>wt</sub>-FLAG and HA-YIF1B. After 24 h, cells were fixed and stained by α-HA, α-FLAG and α-LAMP-1 (lysosomes / LY). Scale bar: 5 µm; inset 5 µm.

E For quantification of YIF1B dependent lysosomal localization of TAPL by Pearson correlation coefficient, 90 individual cells were quantified for each transfection. Individual values are depicted as rectangles, mean values and corresponding SEMs are shown in red. ***, p < 0.001 by Kruskal-Wallis test with post hoc Dunn’s test. Mean values, corresponding SEMs and additional test results are listed in Table S1.
F D17 of TAPL is important for YIF1B interaction. HEK293T cells transiently transfected with TMD0<sub>wt</sub>-FLAG or TMD0<sub>D17N</sub>-FLAG were lysed by NP40. TMD0 variants were immunoprecipitated by α-FLAG antibody (FLAG) and immunoblotted using α-FLAG and α-YIF1B. Specificity of immunoprecipitation was verified by an IgG isotype control (IC) and using cells transfected with a plasmid devoid of a gene of interest (Vector).

G YIF1B and TMD0-FLAG immunoblot signals from four independent experiments were quantified and the TMD0<sub>wt</sub>-FLAG normalized ratio of YIF1B to TMD0 is plotted. Individual immunoblots used for quantification are shown in Fig. S12. Individual data points are shown as squares and mean value and corresponding SD are depicted in red.
Lysosomal targeting of the ABC transporter TAPL is determined by membrane localized charged residues
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