

# Ist1 Regulates Vps4 Localization and Assembly

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The ESCRT protein complexes are recruited from the cytoplasm and assemble on the endosomal membrane into a protein network that functions in sorting of ubiquitinated transmembrane proteins into the multivesicular body (MVB) pathway. This transport pathway packages cargo proteins into vesicles that bud from the MVB limiting membrane into the lumen of the compartment and delivers these vesicles to the lysosome/vacuole for degradation. The dissociation of ESCRT machinery by the AAA-type ATPase Vps4 is a necessary late step in the formation of MVB vesicles. This ATP-consuming step is regulated by several Vps4-interacting proteins, including the newly identified regulator Ist1. Our data suggest that Ist1 has a dual role in the regulation of Vps4 activity: it localizes to the ESCRT machinery via Did2 where it positively regulates recruitment of Vps4 and it negatively regulates Vps4 by forming an Ist1-Vps4 heterodimer, in which Vps4 cannot bind to the ESCRT machinery. The activity of the MVB pathway might be in part determined by outcome of these two competing activities.

## INTRODUCTION

Multivesicular bodies (MVBs) are endosomal structures that function in the transport of proteins between *trans*-Golgi, plasma membrane, and lysosomes/vacuoles. One route of traffic mediated by MVBs is the delivery of transmembrane proteins to the lumen of lysosomes/vacuoles for degradation (for review, see Gruenberg and Stenmark, 2004; Piper and Katzmann, 2007). This route requires the formation of cargo-containing vesicles that bud into the lumen of MVBs. On fusion with the lysosome/vacuole the MVB vesicles are exposed to hydrolytic enzymes that degrade both lipids and proteins. The MVB pathway represents the major protein degradation pathway for plasma membrane proteins and therefore plays an important role in regulating numerous cell surface-mediated processes, including cell signaling, nutrient uptake, and adaptation to environmental conditions.

Cargo sorting and vesicle formation in the MVB pathway are thought to be executed by a group of protein complexes called ESCRT-I, -II, and -III (endosomal sorting complex required for transport). These protein complexes are transiently recruited from the cytoplasm to the endosomal membrane where they bind transmembrane proteins previously marked for degradation by monoubiquitination. The current working model predicts that the ESCRTs concentrate the ubiquitinated proteins and package this cargo into forming vesicles that bud into the lumen of the compartment (for

review, see Babst, 2005; Hurley and Emr, 2006; Williams and Urbe, 2007). The mechanism of membrane deformation and vesicle budding remains unclear. One hypothesis suggests that MVB vesicle formation is intimately linked to the assembly of ESCRT-III, a complex composed of at least four subunits (Vps2, Vps24, Vps20, Snf7). The ESCRT-III subunits appear to assemble on the membrane into a large protein network that restricts cargo diffusion and might deform the membrane (Babst *et al.*, 2002; Muziol *et al.*, 2006; Shim *et al.*, 2007).

The disassembly of ESCRT-III is an essential step in the MVB vesicle formation and is executed by the AAA-type ATPase Vps4 (Babst *et al.*, 1998, 2002). Without Vps4 function the ESCRT machinery remains associated with the endosome and MVB formation is blocked. The ATPase activity of Vps4 is regulated by its oligomeric state (Babst *et al.*, 1998; Scott *et al.*, 2005a). As a dimer, Vps4 is inactive and localizes mainly to the cytoplasm. ESCRT-III recruits Vps4 dimers by binding to the N-terminal microtubule interacting and trafficking microtubule-interacting and transport (MIT) domain of Vps4. ESCRT-III associated Vps4 oligomerizes into a large ring structure of 12 subunits. This large Vps4 oligomer hydrolyzes the bound ATP, resulting in the disassembly of ESCRT-III and the dissociation of Vps4 into ADP-bound dimers that recycle back to the cytoplasm.

Two MVB-associated proteins, Vta1 and Did2 (also known as Fti1/Vps46), play distinct roles in regulating Vps4 activity. Vta1 interactions with Vps4 have been demonstrated both *in vitro* and *in vivo* (Yeo *et al.*, 2003; Shiflett *et al.*, 2004; Azmi *et al.*, 2006; Lottridge *et al.*, 2006). Biochemical studies indicate a positive regulatory role for Vta1 in promoting the oligomeric assembly of Vps4 and stimulating its ATPase activity (Azmi *et al.*, 2006). Did2 associates with ESCRT-III and binds to both Vps4 and Vta1 (Howard *et al.*, 2001; Lottridge *et al.*, 2006; Nickerson *et al.*, 2006). Loss of Did2 results in the accumulation of ESCRT-III components on the endosomal membrane (Nickerson *et al.*, 2006). These data point toward a role for Did2 in coordinating the disassembly of ESCRT-III from endosomal membranes via Vps4.

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Abbreviations used: MVB, multivesicular body; ESCRT, endosomal sorting complex required for transport.

**Table 1.** *S. cerevisiae* strains and plasmids used in this study

Strain or plasmid	Descriptive name	Genotype or description	Reference or source
<b>Strain</b>			
MBY41	<i>vps4Δvps2Δ</i>	MBY3, <i>VPS2::HIS3</i>	Babst <i>et al.</i> (2002)
EEY26-1	<i>vps4Δdid2Δ</i>	MBY3, <i>DID2::HIS3</i>	This study
MBY12	<i>vps4Δvps24Δ</i>	MBY3, <i>VPS24::HIS3</i>	Babst <i>et al.</i> (2002)
EEY17	<i>did2Δ</i>	SEY6210, <i>DID2::HIS3</i>	This study
JPY47	<i>vta1Δ</i>	SEY6210.1, <i>VTA1::HIS3</i>	Azmi <i>et al.</i> (2006)
EEY12	<i>vps4Δdid2Δ</i>	MBY3, <i>SNF7::HIS3</i>	Babst <i>et al.</i> (2002)
MBY63	<i>ist1Δ</i>	SEY6210, <i>IST1::HIS3</i>	This study
MCY2	<i>vta1Δist1Δ</i>	SEY6210, <i>VTA1::HIS3, IST1::HIS3</i>	This study
MCY3	<i>vps4Δist1Δ</i>	MBY3, <i>IST1::HIS3</i>	This study
CAY2	<i>did2Δist1Δ</i>	EEY17, <i>IST1:URA3</i>	This study
MBY3	<i>vps4Δ</i>	SEY6210, <i>VPS4::TRP1</i>	Babst <i>et al.</i> (1997)
MCY31	<i>did2Δ</i>	W303, <i>DID2::HIS3</i>	This study
W303	WT	<i>MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15</i>	Thomas and Rothstein (1989)
SEY6210	WT	<i>MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	Robinson <i>et al.</i> (1988)
<b>Plasmids</b>			
pGO45	GFP-CPS	<i>URA3 Ap<sup>R</sup> (pRS426) GFP-CPS1</i>	Odorizzi <i>et al.</i> (1998)
pCS24	Ste2-GFP	<i>URA3 Ap<sup>R</sup> (pRS426) STE2-GFP</i>	Odorizzi <i>et al.</i> (1998)
pMB241	IST1-HA	<i>URA3 Ap<sup>R</sup> (pRS416) IST1-HA</i>	This study
pMB243	<i>IST1-GFP</i>	<i>URA3 Ap<sup>R</sup> (pRS416) IST1-GFP</i>	This study
pMB287	<i>GST-IST1(CC)</i>	<i>Ap<sup>R</sup> (pGEX-KG) GST-IST1(CC)</i>	This study
pCD2	<i>GST-IST1</i>	<i>Ap<sup>R</sup> (pGEX-KG) GST-IST1</i>	This study
pCJ7	<i>IST1(ΔN)-GFP</i>	<i>URA3 Ap<sup>R</sup> (pRS416) IST1(ΔN)-GFP</i>	This study
pAH26	<i>2 μ DID2-HA</i>	<i>URA3 Ap<sup>R</sup> (pRS425) DID2-HA</i>	This study
pMB149	<i>vps4(E233Q)-HA</i>	<i>URA3 Ap<sup>R</sup> (pRS416) vps4(E233Q)-HA</i>	Babst <i>et al.</i> (2002)
pMB283	<i>VPS4-HA</i>	<i>URA3 Ap<sup>R</sup> (pRS416) VPS4-HA</i>	This study
pAH27	<i>DID2(ΔC58)-HA</i>	<i>LEU2 Ap<sup>R</sup> (pRS425) DID2(ΔC58)-HA</i>	This study
pAH25	<i>DID2(ΔC29)-HA</i>	<i>LEU2 Ap<sup>R</sup> (pRS415) DID2(ΔC29)-HA</i>	This study
pMB65	<i>vps4-ts</i>	<i>LEU2 Ap<sup>R</sup> (pRS415) vps4-ts-29</i>	This study
pMB286	<i>P(GAL1)-IST1</i>	<i>TRP1 Ap<sup>R</sup> (pRS414) P(GAL1)-IST1</i>	This study
pMB277	<i>P(GAL1)-IST1-HA</i>	<i>URA3 Ap<sup>R</sup> (pRS416) P(GAL1)-IST1-HA</i>	This study

In this study we provide evidence for an additional regulator of Vps4 activity, Ist1. By interacting with Did2 and Vps4, Ist1 appears to regulate the recruitment and oligomerization of Vps4. We propose that Ist1, Did2, and Vta1 form a network of interconnected regulatory proteins that modulate Vps4 activity, thereby regulating the flow of cargo through the MVB pathway.

## MATERIALS AND METHODS

### Materials

HA-tag (hemagglutinin-tag) specific antibody was purchased from Covance (Princeton, NJ). The antisera against Snf7 and Vps24 have been characterized previously (Babst *et al.*, 1998).

### Strains and Media

Table 1 lists the *Saccharomyces cerevisiae* strains used in this work. Yeast strains were grown in standard yeast extract-peptone-dextrose (YPD) or synthetic medium supplemented with essential amino acids as required for maintenance of plasmids (YNB; Sherman *et al.*, 1979). To construct deletion strains, yeast cells were transformed with DNA fragments containing the *HIS3* or *URA3* gene flanked by 50 base pairs specific for the 5' and 3' region of the corresponding gene. The cells were selected for the presence of the marker gene and the deletions were confirmed by PCR analysis of the chromosomal DNA.

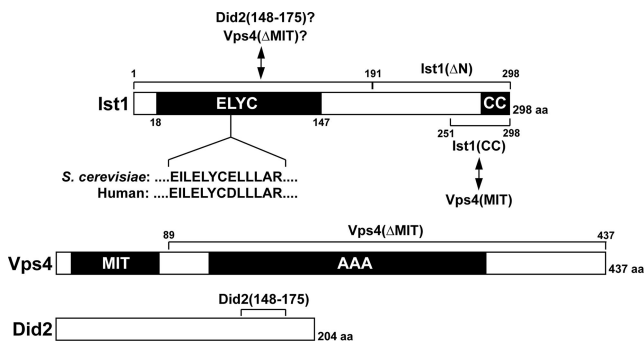
### DNA Manipulations

Plasmids used in this study are listed in Table 1. The pRS shuttle vectors used in this study have been described previously (Christianson *et al.*, 1992). The *IST1* gene including the promoter region was amplified by PCR from SEY6210 chromosomal DNA and fused either to a 3-HA tag or to the green fluorescent protein (GFP) containing NheI/SalI fragment of pEGFP-C1 (Clontech Laboratories, Palo Alto, CA), resulting in the gene fusions *IST1-HA* (pMB241) and

*IST1-GFP* (pMB243). The *IST1* gene was amplified by PCR and inserted into the BamHI/SalI digested *Escherichia coli* expression vector pGEX-KG, resulting in the plasmid pCD2. A SacI/HindIII fragment of pMB241 encoding the C-terminal coiled-coil domain of Ist1 fused to 3-HA was inserted into the SacI/HindIII sites of pGEX-KG, resulting in the plasmid pMB287 [*GST-IST1(CC)-HA*]. pCJ7 was constructed by fusing a PCR product containing the 3' region of *IST1* with NheI/SalI fragment of pEGFP-C1 [*IST1(ΔN)-GFP*, see Figure 1]. The *DID2* gene was amplified by PCR from genomic DNA, fused to the 3-HA tag and inserted into SacI/SalI of pRS426, resulting in the plasmid pAH26 (*2 μ DID2-HA*). A SacI/SalI fragment of pMB76 (Babst *et al.*, 1998) was inserted into pRS416 to obtain plasmid pMB283 (*VPS4-HA*). PCR products encoding the N-terminal 146 or 175 amino acids of Did2 were fused to 3-HA tags, resulting in pAH27 [*DID2(ΔC58)-HA*] and pAH25 [*DID2(ΔC29)-HA*], respectively. pMB65 was obtained by inserting the NotI/SalI fragment of pMB59 (Babst *et al.*, 1997) into pRS415. PCR products containing either *IST1* or *IST1-HA* were fused to the *GAL1* promoter and inserted into pRS414 or pRS416 to obtain pMB286 [*P(GAL1)-IST1*] and pMB277 [*P(GAL1)-IST1-HA*].

### Experimental Procedures

Immunofluorescence microscopy was performed on fixed spheroplasted cells as described (Babst *et al.*, 1998). Fluorescence microscopy was performed on a deconvolution microscope (DeltaVision, Applied Precision, Issaquah, WA). Sephacryl S300 (16/60 column, GE Healthcare, Waukesha, WI) gel filtration analysis of yeast cell extract was performed as described in Curtiss *et al.* (2007). Superose 6 (3.2/30 column, GE Healthcare) gel filtration analysis of purified proteins was performed in presence of 1 mM ATP, 150 mM KAc, 20 mM HEPES, pH 7.4, and 5 mM MgAc<sub>2</sub> at a flow rate of 40 μl/min. The method used for the ATPase activity tests was described previously (Babst *et al.*, 1998). For subcellular fractionation experiments yeast was grown in 8 ml minimal medium to a density of OD<sub>600</sub> = 0.6. The cells were harvested, washed with water, treated with 100 mM Tris, pH 9.4, 10 mM dithiothreitol for 10 min, and spheroplasted with oxalyticase in minimal medium containing 1 M sorbitol for 45 min. The spheroplasts were lysed by osmotic stress in 0.5 ml of 100 mM KCl, 50 mM KAc, 20 mM PIPES, pH 6.8, 5 mM MgAc<sub>2</sub>, 100 mM sorbitol, 0.1 mM AEBSF, and protease inhibitor cocktail (Complete, Roche Molecular Biochemicals, Indianapolis, IN). The cell extract was sepa-



**Figure 1.** Domain structure of Ist1, Vps4, and Did2. The borders of the ELYC domain were determined based on domain prediction by Swissprot (DUF292). Arrows indicate interactions between Ist1 and Vps4 and between Ist1 and Did2. Interactions with the N-terminal region containing the ELYC domain are predicted based on the observation that deletion of this domain results in loss of Ist1 binding to Did2 and Vps4( $\Delta$ MIT), which are labeled with question marks.

rated by centrifugation at  $5000 \times g$  for 10 min into a soluble and a pellet fraction. The proteins of the fractions were precipitated by the addition of 10% trichloroacetic acid (TCA). The resulting pellet was washed with acetone and resuspended in 0.25 ml SDS-PAGE sample buffer (2% SDS, 0.1 M Tris, pH 6.8, 10% glycerol, 0.01% bromophenol blue, 5%  $\beta$ -mercaptoethanol). Five microliters of each sample were used for Western blot analysis.

## RESULTS

### Identification of IST1

We identified the Ist1 gene product in a two-hybrid screen as a potential Vps4-interacting protein. Three different *IST1* clones were isolated in this screen encoding either the full-length Ist1 protein or N-terminal truncated derivatives of it (data not shown). The shortest clone encoded the final 96 amino acids of Ist1, suggesting that this C-terminal region was responsible for the interaction between Vps4 and Ist1. *IST1* (SGD: YNL265c) was previously identified in a study where increased sodium tolerance was observed in strains deleted for this gene (*IST*: Increased Sodium Tolerance; Entian *et al.*, 1999).

The *IST1* gene encodes a 298-amino acid soluble protein that is predicted to contain a C-terminal coiled-coil motif (predicted by Coiles, Lupas *et al.*, 1991; Figure 1). Amino acid sequence comparison with other genomes indicated that Ist1 is conserved among all eukaryotes with the most conserved region within the first  $\sim 150$  amino acids of the protein. This region contains a highly conserved amino acid sequence, ELYC, which we used to name this putative domain (Figure 1).

### Ist1 Binds to Vps4 and Prevents Vps4 Assembly

To characterize the potential interaction between Ist1 and Vps4 *in vitro*, we expressed *IST1* in *E. coli* and purified the protein. The native molecular weight of recombinant Ist1 was determined by gel filtration analysis. Relative to protein standards Ist1 eluted from the column at  $\sim 90$  kDa, more than twice its predicted molecular weight of 35 kDa which suggested that Ist1 may dimerize (Figure 2A). To compare this result with endogenously expressed Ist1, we constructed a functional C-terminal HA-tagged version of Ist1 (Ist1-HA, Supplementary Figure 1), expressed the *IST1*-HA fusion in wild-type yeast, and analyzed its molecular weight by gel filtration. The result was consistent with the size observed with recombinant Ist1 (Figure 2A). Molecular weight determination by gel filtration is sensitive to the

shape of the protein and thus the Ist1 data could be misleading. Therefore, analytical centrifugation experiments were performed using purified Ist1 at concentrations of 1, 0.5, and 0.25 mg/ml. These experiments indicated that at all three concentrations the majority of Ist1 is present as a monomer (measured MW/theoretical MW = 1.2). However, the analysis was complicated by the presence of higher molecular weight particles, suggesting that Ist1 has the tendency to oligomerize/aggregate. Together, the data from gel filtration and analytical centrifugation experiments suggested that Ist1 is a monomeric protein with a nonglobular, elongated structure.

We studied the predicted Ist1-Vps4 interaction *in vitro* using recombinant Ist1 and ATPase-deficient Vps4(E233Q) protein (Figure 2B). Gel filtration analysis has shown that the ATPase-deficient mutant protein Vps4(E233Q) assembles in presence of ATP into a stable  $\sim 500$ -kDa complex, representing the higher-order ATP-bound complex (no. 1 in Figure 2B; Babst *et al.*, 1998). Interestingly, we found that the addition of equimolar amounts of Ist1 abolished Vps4 assembly and instead resulted in the formation of a Vps4-Ist1 complex that was  $<150$  kDa (no. 2 in Figure 2, B and C). High-resolution gel filtration analysis of this complex indicated a native molecular weight of 100 kDa, suggesting that Ist1 (35 kDa) and Vps4 (48 kDa) may assemble into a hetero-dimer (Figure 2D).

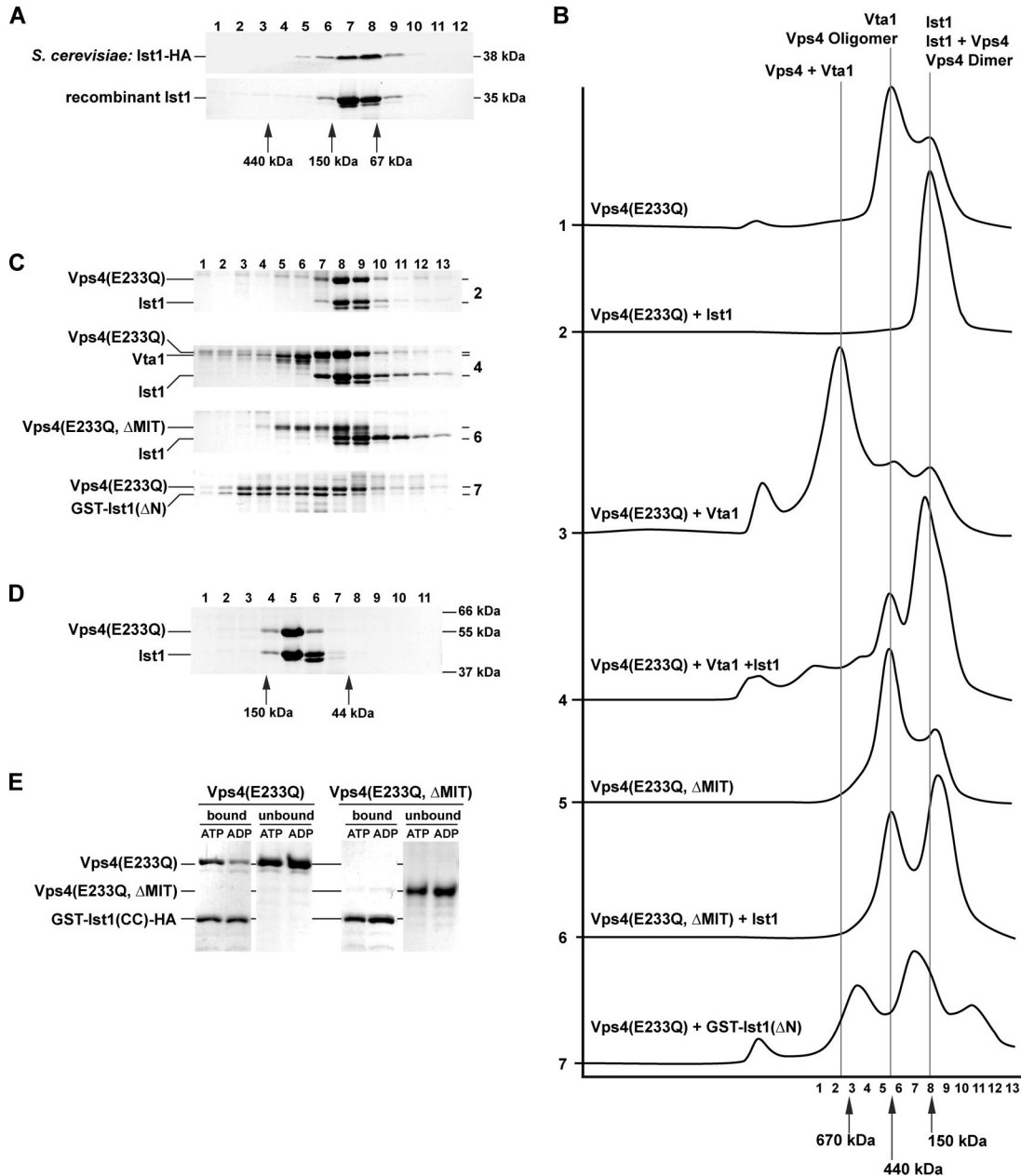
Vta1 binds Vps4 as a positive regulator, promoting Vps4 assembly and ATPase activity. As previously shown, the addition of Vta1 to ATP-bound Vps4(E233Q) resulted in the formation of a large  $\sim 1$ -MDa complex, (no. 3 in Figure 2B; Azmi *et al.*, 2006). The void volume peak present in this experiment is an artifact of Vta1 aggregation. The presence of Ist1 not only inhibited the formation of the Vps4-Vta1 complex, but it also successfully competed with Vta1 for binding to Vps4. As a consequence, the gel filtration analysis showed the presence of free Vta1 in addition to the Vps4-Ist1 complex (no. 4 in Figure 2, B and C).

The N-terminal MIT domain of Vps4 interacts with ESCRT-III subunits and thus functions as the substrate-binding domain of Vps4 (Scott *et al.*, 2005b; Vajjhala *et al.*, 2007). Deletion of this domain does not interfere with the homotypic oligomerization or the ATPase activity of Vps4 (no. 5 in Figure 2B; Babst *et al.*, 1998). Gel filtration analysis showed that Ist1 did not stably associate with Vps4(E233Q,  $\Delta$ MIT) and thus did not block the oligomerization of the mutant ATPase (no. 6 in Figure 2, B and C). This result indicated that the MIT domain of Vps4 is required for the formation of a stable Ist1-Vps4 complex.

The two-hybrid data suggested that the C-terminal 96 amino acids of Ist1 were able to bind to Vps4. Therefore a glutathione *S*-transferase (GST)-fusion protein containing the C-terminal 107 amino acids of Ist1 was purified [GST-Ist1( $\Delta$ N)], mixed with Vps4(E233Q) and analyzed by gel filtration. The resulting chromatogram and SDS-PAGE data confirmed that the C-terminal region of Ist1 could bind Vps4 (no. 7 in Figure 2, B and C). However, in contrast to full-length Ist1, the binding of GST-Ist1( $\Delta$ N) to Vps4 did not inhibit oligomerization of Vps4. As a consequence, two protein peaks were observed: one at  $\sim 250$  kDa likely representing Vps4(E233Q) dimers bound to GST-Ist1( $\Delta$ N) and one at  $\sim 600$  kDa likely formed by the binding of GST-Ist1( $\Delta$ N) to oligomeric Vps4(E233Q). This result indicated that the C-terminal region of Ist1 binds to Vps4 but this interaction alone is not sufficient to inhibit Vps4 assembly.

Thus far our experiments had identified two interaction sites important for the formation of the Vps4-Ist1 complex: the MIT domain of Vps4 and the C-terminal 107 amino acids





**Figure 2.** Ist1 binds to Vps4 and inhibits oligomerization of the ATPase. (A) Gel filtration analysis of cell extracts and purified recombinant protein using an S300-Sephacryl column. The resulting fractions were analyzed for the presence of Ist1-HA by Western blot and for the presence of the recombinant Ist1 by SDS-PAGE followed by Coomassie staining (top panel, extract from MBY63 pMB241; bottom panel, purified Ist1). (B) Superose S6 gel filtration analysis of purified recombinant proteins in presence of 1 mM ATP (X-axis, time; Y-axis,  $UV_{280\text{nm}}$  absorption). All proteins analyzed were present at 1 mg/ml in the loading sample. (C) SDS-PAGE analysis of the gel filtration fractions from the experiments in B. The sample number corresponds to the fraction number in B. The gel number indicates from which experiment in B the samples originate. (D) Gel filtration analysis using an S200-Sephacryl column of purified Ist1 and Vps4(E233Q) in presence of 1 mM ATP (1 mg/ml protein each). The fractions were analyzed by SDS-PAGE. (E) GST-Ist1(CC)-HA was immobilized on GSH-Sepharose and incubated with either Vps4(E233Q) or Vps4(E233Q, ΔMIT) in presence of 1 mM ATP or ADP. The bound and unbound fractions were analyzed by SDS-PAGE.

of Ist1. The MIT domain of Vps4 has been shown to bind to the C-terminal coiled-coil domains of ESCRT-III subunits (Scott *et al.*, 2005b). Therefore we tested if the MIT domain might also bind to the C-terminal coiled-coil motif of Ist1 (see Figure 1). A GST fusion protein containing the coiled-coil domain of Ist1 [GST-Ist1(CC)-HA] was purified and immobilized on GSH (reduced glutathione)-Sepharose beads. An excess of ATPase-deficient proteins Vps4(E233Q)

and Vps4(E233Q, ΔMIT) were added either in presence of ATP or ADP. The beads were washed and the bound and unbound fractions were analyzed by SDS-PAGE. The resulting gel illustrated that in presence of ATP Vps4(E233Q) bound to GST-Ist1(CC)-HA in an ~1:1 ratio (Figure 2E). A control experiment under the same conditions demonstrated that Vps4(E233Q) did not interact with immobilized GST (data not shown). In the presence of ADP Vps4(E233Q) still

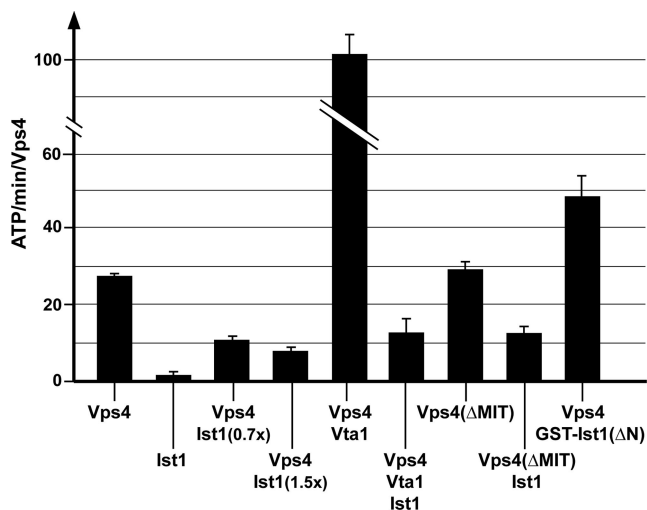
interacted with GST-Ist1(CC)-HA but the affinity between the two proteins was diminished. In contrast the MIT-deleted Vps4 mutant protein did not bind to the C-terminus of Ist1 independent of the nucleotide binding state (Figure 2E). Together, the GST pulldown experiments indicated a direct interaction between the coiled-coil motif of Ist1 and the MIT domain of Vps4. This interaction was in part dependent on the nucleotide-binding state of Vps4.

In summary, the *in vitro* data demonstrated a direct interaction between Ist1 and Vps4, resulting in the formation of a heterodimeric Vps4-Ist1 complex. This complex was able to compete with both the oligomerization of Vps4 and the binding of the regulator Vta1. The interaction between the Vps4 MIT domain and the coiled-coil motif of Ist1 was required for the formation of a stable Vps4-Ist1 complex. However, this interaction alone was not sufficient to prevent Vps4 oligomerization, suggesting the presence of at least one more Vps4-Ist1 interaction site.

### Ist1 Inhibits ATPase Activity of Vps4

The assembly of Vps4 into the large oligomeric form is necessary for the hydrolysis of the bound ATP (Babst *et al.*, 1998). As such, we predicted that interfering with Vps4 oligomerization by adding Ist1 would inhibit Vps4 ATP hydrolysis activity. We tested this prediction by measuring ATPase activity of Vps4 *in vitro* in the presence or absence of Ist1 (Figure 3). As expected, the addition of even substoichiometric amounts of Ist1 (0.7 times the amount of Vps4) strongly reduced the ATP turnover rate of Vps4. This effect on Vps4 activity only increased slightly when the concentration of Ist1 was doubled, suggesting that Ist1 reached maximal inhibition (about threefold) at approximately equimolar amounts relative to Vps4 (Figure 3). This result was consistent with the gel filtration analysis that predicted a one-to-one protein ratio of the Ist1-Vps4 complex. It is interesting to note that Ist1 is not able to completely block the ATPase activity of Vps4, suggesting that the interaction between Ist1 and Vps4 that is responsible for blocking ATPase activity might be dynamic.

As previously demonstrated, the presence of Vta1 increased the ATPase activity of Vps4 substantially (Figure 3;



**Figure 3.** Ist1 inhibits ATPase activity of Vps4. Proteins were present at 1  $\mu$ M concentration, except where specifically labeled (0.7 $\times$  and 1.5 $\times$  Ist1). The results shown represent the average activity and SEM obtained from four individual time points.

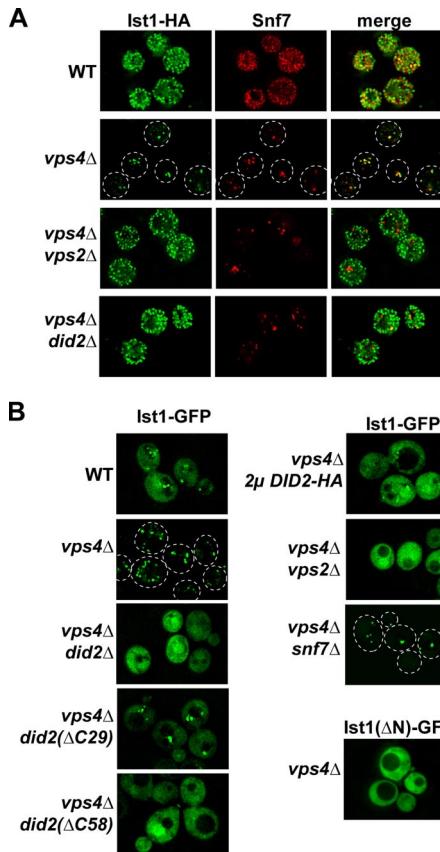
Azmi *et al.*, 2006). Consistent with the gel filtration analysis we found that Ist1 is able to compete with Vta1 and can reduce the activity of Vps4 to similar levels as observed for the Ist1-Vps4 complex alone (Figure 3). To our surprise, Ist1 reduced the ATPase activity of the MIT-deleted Vps4 protein to a similar extent as observed for wild-type Vps4 (Figure 3). The gel filtration data together with the GST pulldown experiments indicated that the MIT domain plays an important role in interactions between Ist1 and Vps4. Therefore we expected that the addition of Ist1 to Vps4( $\Delta$ MIT) would have only minor effects on the ATPase activity. The discrepancy between the two experiments could be explained by the predicted presence of a second interaction site between Ist1 and Vps4 that is dynamic and plays a key role in blocking both oligomerization and ATPase activity of Vps4. The dynamic nature of this second interaction would fit to the observation that Ist1 is not able to completely block Vps4 ATPase activity (Figure 3) and would explain why the second site alone is unable to maintain a stable Ist1-Vps4 complex during the gel filtration analysis. In support of this model we found that the presence of GST-Ist1( $\Delta$ N), which lacks the second interaction site did not inhibit but instead increased ATPase activity of Vps4 (Figure 3). This increase of ATPase activity is likely do in part to GST dimerization promoting the assembly of Vps4 bound to GST- Ist1( $\Delta$ N). However, we cannot exclude the possibility that the interaction between the C-terminus of Ist1 with the Vps4 MIT domain is responsible for the observed increase in enzymatic activity.

In summary, the ATPase activity tests and the gel filtration data supported a model of two distinct interaction sites between Ist1 and Vps4. A stable interaction between the two proteins requires the binding of the Ist1 coiled-coil motif to the Vps4 MIT domain. This interaction is nucleotide-dependent and thus resembles the binding of Vps4 to its substrate ESCRT-III. The second interaction formed between the N-terminal region of Ist1 and Vps4 is weaker but is crucial to inhibiting Vps4 assembly and ATPase activity.

### Ist1 Localizes to MVBs by Binding to Did2

A genome-wide localization study in yeast suggested that Ist1 associates with endosomes (Huh *et al.*, 2003). We confirmed this observation by immunofluorescence microscopy of yeast strains expressing *IST1-HA*. Ist1-HA localized predominantly to the cytoplasm of wild-type cells but colocalized with the ESCRT-III subunit Snf7 on enlarged endosomal structures in cells deleted for *VPS4* (Figure 4A). This Vps4-dependent endosomal localization is typical for proteins of the ESCRT machinery, which have been shown to cycle on and off MVBs. We confirmed the immunofluorescence data by live cell imaging of cells expressing a C-terminal GFP-tagged version of Ist1, Ist1-GFP. This fusion protein was found to be nonfunctional (Supplementary Figure 1), but fluorescence microscopy of cells expressing Ist1-GFP showed the same Vps4-dependent endosomal localization as observed with functional Ist1-HA (Figure 4B), indicating that localization was not affected by the C-terminal GFP fusion. Live cell imaging of Ist1-GFP was necessary because fixation procedures result in a punctate distribution of soluble proteins when visualized by immunofluorescence microscopy, preventing a clear distinction between localization to the cytosol or to small compartments.

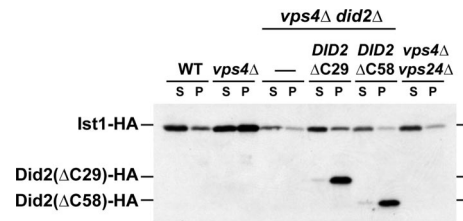
We combined mutations in different ESCRTs with *vps4 $\Delta$*  and tested which double mutant blocked endosomal localization of Ist1. These tests revealed that mutations in the Vps2-Vps24 subcomplex of ESCRT-III disrupted association of Ist1-HA and Ist1-GFP with the MVB (*vps4 $\Delta$ vps2 $\Delta$* : Figure



**Figure 4.** Ist1 localization to MVBs requires Did2. (A) Immunofluorescence microscopy of yeast strains expressing *IST1-HA* (pMB241). Fixed cells were stained using anti-HA and anti-Snf7 antibodies (WT, SEY6210; *vps4Δ*, MBY3; *vps4Δvps2Δ*, MBY41; and *vps4Δdid2Δ*, EY26-1). (B) Fluorescence microscopy of cells expressing *IST1-GFP* (pMB243) or *IST1(ΔN)-GFP* (pCJ7). The following strains have been used for the experiments: MBY3 (*vps4Δ*), EY26-1 (*vps4Δdid2Δ*), MBY3 pAH26 (*vps4Δ 2μ DID2-HA*), EY26-1 pAH27 [*vps4Δ did2(ΔC58)*], EY26-1 pAH25 [*vps4Δ did2(ΔC29)*], and EY12 (*vps4Δsnf7Δ*).

4, A and B; *vps4Δvps24Δ*: data not shown). In contrast, deletions of subunits of the ESCRT-III Snf7-Vps20 subcomplex did not affect endosomal accumulation of Ist1-GFP (*vps4Δsnf7Δ*: Figure 4B; *vps4Δvps20Δ*: data not shown). This result is consistent with the observation that localization of Vps2-Vps24 to endosomal membranes does not require the presence of Snf7 or Vps20 (Babst *et al.*, 2002; Muziol *et al.*, 2006; Shim *et al.*, 2007). A recent study demonstrated that localization of the Vps4-interacting protein Did2 to the endosome was dependent on the presence of Vps2 and Vps24 (Nickerson *et al.*, 2006). Interestingly, the *vps4Δdid2Δ* cells exhibited cytosolic localization of Ist1, similar to that observed in ESCRT-III mutants (Figure 4, A and B), suggesting that Ist1 associates with ESCRT-III by binding to Did2. In addition, overexpression of *DID2* resulted in the redistribution of Ist1-GFP to the cytoplasm in *vps4Δ*, suggesting that cytoplasmic Did2 is able to interact with Ist1-GFP, thereby competing with ESCRT-III-associated Did2 for Ist1-GFP (Figure 4B). Furthermore, the mutant protein Ist1(ΔN)-GFP did not localize to endosomal structures, indicating that the N-terminal region containing the ELYC domain is required for the interaction with Did2 (Figure 4B).

Using fluorescent microscopy, we determined which region of Did2 was required for proper localization of Ist1-



**Figure 5.** Subcellular fractionation of Ist1 in different yeast strains. Yeast cell extracts were fractionated by centrifugation into soluble, cytoplasmic fractions (S) and membrane-associated pellet fractions (P). The resulting samples were analyzed for the presence of Ist1-HA (pMB241), Did2(ΔC29)-HA (pAH12), and Did2(ΔC58)-HA (pAH27) by Western blot using anti-HA antibodies (WT, SEY6210; *vps4Δ*, MBY3; *vps4Δdid2Δ*, EY26-1; and *vps4Δvps24Δ*, MBY12).

GFP. A previous study suggested that the N-terminal half of Did2 is involved in the binding to the Vps2-Vps24 subcomplex of ESCRT-III, whereas the C-terminus might interact with Vps4 (Nickerson *et al.*, 2006). Our fluorescence microscopy experiments showed a partial loss of endosome-associated Ist1-GFP in cells expressing Did2(ΔC29)-HA (Did2 lacking the final 29 amino acids) and a complete redistribution of Ist1-GFP to the cytosol in cells expressing Did2(ΔC58)-HA (Figure 4B).

Additional Ist1 localization studies were performed by subcellular fractionation experiments that have been previously shown to separate cytoplasmic ESCRTs from MVB-associated ESCRT machinery (Babst *et al.*, 1998). Yeast cells expressing Ist1-HA were spheroplasted, lysed, and separated by centrifugation at  $13,000 \times g$  into the membrane-bound, pellet fractions (P) and the cytoplasmic, soluble fractions (S). The fractions were then analyzed by Western blot for the presence of Ist1-HA. Consistent with the microscopy data Ist1-HA was found mostly in the cytoplasmic fraction of wild-type cells, accumulated in the pellet fraction in *vps4Δ*, and was almost completely lost from the pellet in *vps4Δdid2Δ* and *vps4Δvps24Δ* double mutants (Figure 5). The accumulation of Ist1-HA in the pellet fraction of *vps4Δ* was less pronounced than expected based on the microscopy data. This discrepancy between the two methods might be the result of partial dissociation of Ist1-HA from the endosomes during the fractionation procedure. As predicted from the microscopy studies, the fractionation experiments showed a partial loss of membrane-associated Ist1 in cells expressing Did2(ΔC29)-HA and a complete loss of membrane association in cells expressing Did2(ΔC58)-HA (Figure 5). However, both C-terminal deleted Did2 proteins associated with the membrane fraction, indicating that these mutant proteins are able to bind to ESCRT-III (Figure 5). In addition to changes in subcellular localization, we also observed variations in the overall amounts of Ist1 in the different yeast strains. In particular loss of Did2 resulted in reduced levels of Ist1, suggesting that Ist1 might be less stable in this mutant strain (Figure 5).

In summary, the subcellular localization data indicated that Ist1 is a cytosolic protein that transiently associated with ESCRT-III by binding to Did2. This interaction was mediated by the N-terminal region of Ist1 and the C-terminal amino acids 148–175 of Did2.

#### Both Deletion and Overexpression of *IST1* Impair MVB Sorting

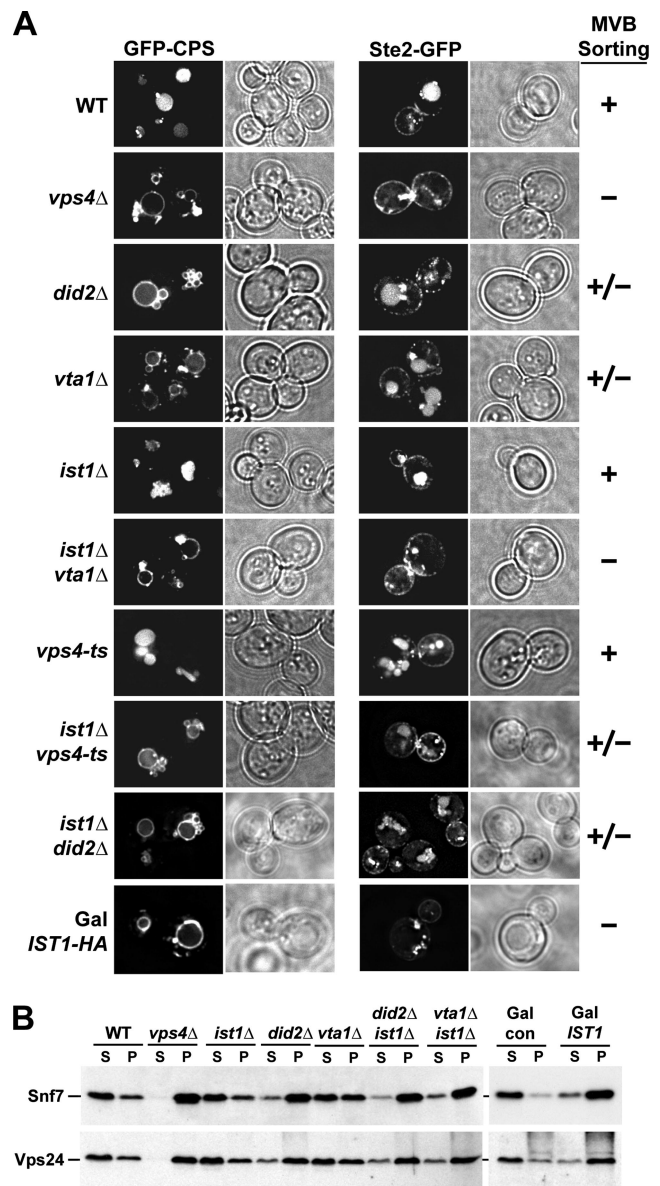
The interaction between Ist1 and Vps4 suggested that Ist1 might function in the MVB pathway. Therefore we tested the



effect of an *IST1* deletion on the trafficking of the MVB cargoes GFP-CPS and Ste2-GFP using fluorescence microscopy. The vacuolar peptidase CPS is synthesized as a transmembrane precursor and is delivered via the MVB sorting pathway into the lumen of the vacuole for its proper function. Ste2 is a cell-surface signaling receptor that is internalized and sorted by the MVB pathway to the vacuole for degradation (Odorizzi *et al.*, 1998). In wild-type cells the fluorescence of both GFP-tagged cargoes are observed predominantly in the vacuole, indicating that these proteins have been efficiently sorted by the MVB machinery (Figure 6A). In contrast, mutations in the ESCRT machinery block delivery of GFP-CPS and Ste2-GFP to the vacuolar lumen causing them to accumulate in an aberrant endosomal structure called the class E compartment. Additionally, ESCRT mutants mislocalize GFP-CPS, and to a lesser extent Ste2-GFP, to the limiting membrane of the vacuole (e.g., *vps4Δ* in Figure 6A; Odorizzi *et al.*, 1998).

A strain deleted for *IST1* exhibited no obvious MVB trafficking defects and efficiently transported GFP-CPS and Ste2-GFP to the vacuolar lumen (Figure 6A). In comparison, mutations in two other known Vps4-interacting proteins, Vta1 and Did2, resulted in trafficking phenotypes in which sorting of GFP-CPS and Ste2-GFP was partially affected (Figure 6A; Azmi *et al.*, 2006). This result is consistent with the model that Vta1 and Did2 are not essential factors of the ESCRT machinery but rather are regulators of Vps4 activity. Similarly, Ist1 might regulate some aspect of Vps4 activity and loss of this regulatory function might not be easily detected under optimal growth conditions present in the laboratory. Therefore we decided to test for a functional connection between *IST1* and the three genes *VPS4*, *VTA1*, and *DID2* by analyzing potential synthetic genetic interactions. We combined *ist1Δ* with a temperature-sensitive allele of *VPS4*, *vps4-ts*. This mutant allele of *VPS4* encodes a protein that is active at 30°C but loses function at 37°C. Analyzing the trafficking of GFP-CPS and Ste2-GFP in the *ist1Δvps4-ts* strain at permissive temperature (30°C) revealed a partial MVB sorting defect demonstrating a genetic interaction between *IST1* and *VPS4* (Figure 6A). Similarly, deleting *IST1* in *vta1Δ* strongly enhanced the trafficking defects, resulting in a block in vacuolar delivery of GFP-CPS and Ste2-GFP as severe as observed in *vps4Δ* (Figure 6A, see also Supplementary Figure 1). These results are consistent with the idea that Ist1 functions side-by-side with Vta1 in regulating Vps4 activity. In contrast, the deletion of *IST1* did not change the severity of the MVB trafficking phenotype of a *did2Δ* strain (Figure 6A), which is consistent with the role of Did2 in localizing Ist1 to ESCRT-III. Deletion of *DID2* blocks proper Ist1 function and therefore the additional deletion of *IST1* cannot worsen the *did2Δ* phenotype. This result further suggested that the positive function of Ist1 in regulating Vps4 is dependent on Did2 and most likely occurs in association with ESCRT-III.

Vps4 functions in the disassembly of the ESCRT-III complex, resulting in the dissociation of the ESCRT-III subunits from the endosomal membrane. As a consequence, in subcellular fractionations of wild-type cells the majority of ESCRT-III subunits were found in the soluble, cytoplasmic fraction, whereas deletion of *VPS4* resulted in the accumulation of ESCRT-III in the membrane-bound, pellet fraction (Figure 6B; Babst *et al.*, 2002). Consistent with the partial MVB trafficking phenotype, mutations in *VTA1* caused partial accumulation of ESCRT-III subunits Vps24 and Snf7 on endosomal membranes (Figure 6B; Azmi *et al.*, 2006). Deleting *DID2* caused a more pronounced ESCRT-III accumulation than observed in *vta1Δ* but in contrast to a previous



**Figure 6.** Phenotypic analysis of strains deleted for *IST1* or overexpressing *IST1*. (A) Fluorescence microscopy of GFP-CPS (pGO45) or Ste2-GFP (pCS24) in different yeast strains (*vps4-ts*, MBY3 pMB65; *ist1Δvps4-ts*, MCY3 pMB65; other strains are listed in Table 1; for Ste2-GFP experiments the isogenic a-strains were used). *Ist1-HA* was overexpressed from a *GAL1* promoter in wild-type cells (W303) growing in presence of galactose (Gal *IST1-HA*). The severity of the observed MVB trafficking phenotypes is indicated [plus (+): no phenotype; plus/minus (+/-): intermediate phenotype; minus (-): severe MVB sorting phenotype]. (B) Separation of yeast cell extracts by centrifugation into soluble (S) and membrane-associated pellet (P) fractions (see Table 1 for yeast strains). *IST1* fused to a *GAL1* promoter was overexpressed in wild-type cells grown in presence of galactose (Gal *IST1*: W303 pMB286). W303 containing an empty vector was used as a control (Gal con). The fractions were analyzed by Western blot using anti-Snf7 and anti-Vps24 antibodies.

publication (Nickerson *et al.*, 2006), we reproducibly observed a less severe accumulation phenotype than in *vps4Δ* (Figure 6B). As expected from the lack of a trafficking phenotype, *ist1Δ* showed a wild-type distribution of the ESCRT-III (Figure 6B). However, deleting *IST1* in a *vta1Δ* mutant strain resulted in a synthetic phenotype that was as severe as

that observed in *did2Δ* (Figure 6B). In contrast, combining *ist1Δ* with *did2Δ* did not increase the ESCRT-III accumulation of the *DID2* deletion strain, consistent with *Did2* being necessary for *Ist1* function in MVB sorting (Figure 6B).

The analysis of the MVB trafficking and ESCRT-III localization phenotypes supported the model that *Ist1* functions together with *Vps4* in the dissociation of ESCRT-III. However, loss of *Ist1* function displayed only minor phenotypes, consistent with the idea that *Ist1* functions as a regulator rather than a cofactor of *Vps4*.

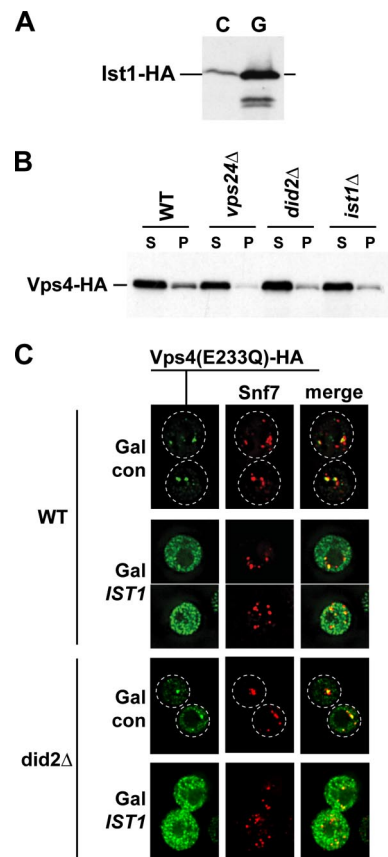
Because the *in vitro* studies suggested that *Ist1* functions as an inhibitor of *Vps4* activity, we tested if overexpression of *IST1* would have a more severe effect on *Vps4* function than the deletion of *IST1*. For these overexpression studies we fused the *IST1* gene to the *GAL1* promoter, a strong, galactose-regulated promoter. Cells containing either the *P(GAL1)-IST1-HA* construct on a CEN plasmid or the empty vector (con) were grown in galactose medium and tested for a potential MVB trafficking phenotype by determining the localization of GFP-CPS and *Ste2-GFP* in these cells. Western blot analysis showed that the *GAL1* promoter fusion resulted in at least 10-fold higher *Ist1-HA* levels than normal (Figure 7A). These high levels of *Ist1* caused a strong MVB sorting defect that was as severe as observed in a *VPS4* deletion strain (Figure 6A). Similarly, subcellular fractionation revealed that overexpression of *IST1* caused an accumulation of ESCRT-III subunits in the membrane fraction, further indicating that high levels of *Ist1* interfere with proper *Vps4* function (Figure 6B).

#### *Ist1* Regulates the Recruitment of *Vps4* to ESCRT-III

The observation that *Ist1* interacted both with *Vps4* directly and with ESCRT-III via *Did2* suggested that *Ist1* might function in regulating the recruitment of *Vps4* to ESCRT-III. Therefore we tested the effects of deletion or overexpression of *Ist1* on the localization of *Vps4* to the endosomal membrane. Subcellular fractionation demonstrated that compared with wild type, *ist1Δ* cells showed reduced amounts of *Vps4* in the membrane-bound pellet fraction (Figure 7B). The reduction was similar to that of a *DID2* deleted strain but less severe than observed in cells deleted for *VPS24* (Figure 7B). These results indicated that *Ist1* indeed plays a role in the recruitment of *Vps4* to MVBs. The fact that *Did2* is essential for the localization of *Ist1* to MVBs explains why a deletion of *DID2* resulted in the same *Vps4* recruitment defect as found in *ist1Δ*. Finally, unlike *Vps24* that is crucial for localizing *Vps4* to MVBs (Babst *et al.*, 2002), *Did2* and *Ist1* play a regulatory role in *Vps4* recruitment, but are not required for the recruitment itself (Nickerson *et al.*, 2006).

A potential effect of high levels of *Ist1* on *Vps4* localization was studied using immunofluorescence microscopy of cells expressing the *Vps4(E233Q)-HA* mutant protein. This point mutant of *Vps4* is trapped in the ATP-bound state and therefore accumulates on MVBs (Babst *et al.*, 1998). However, in cells overexpressing *IST1* from a strong *GAL1* promoter, *Vps4(E233Q)-HA* was mislocalized to the cytoplasm (Figure 7C), indicating that high cellular levels of *Ist1* were able to block *Vps4* recruitment to MVBs. This *Vps4* mislocalization phenotype in *IST1*-overexpressing cells was not dependent on the presence of *Did2* (Figure 7C).

Together, the phenotypic analysis suggested a role of *Ist1* in regulating the recruitment of *Vps4* to the MVB-localized ESCRT-III complex. Lack of *Ist1* as well as high levels of *Ist1* interfered with proper localization of *Vps4*, suggesting that cellular levels of *Ist1* could play a role in regulating *Vps4* function.



**Figure 7.** *Ist1* regulates the recruitment of *Vps4* to MVBs. (A) Western blot analysis of cell extracts from yeast strains grown in galactose-containing medium expressing *IST1-HA* either from its own promoter (C, control) or the *GAL1* promoter (G). (B) Subcellular fractionation of yeast strains expressing *Vps4-HA* into soluble (S) and membrane-bound pellet (P) fractions (WT, MBY3 pMB283; *vps24Δ*, MBY12 pMB283; *did2Δ*, EY26-1 pMB283; and *ist1Δ*, MCY3 pMB283). The fractions were analyzed by Western blot using anti-HA antibodies. (C) Localization of *Vps4(E233Q)-HA* (expressed from pMB149) and *Snf7* in cells overexpressing *IST1* from a *GAL1* promoter (Gal *IST1*: pMB286) was determined by immunofluorescence microscopy. The strains were grown in presence of galactose (WT, W303; *did2Δ*, MCY31). Strains containing the empty expression vector were used as controls (Gal con).

## DISCUSSION

In the MVB pathway, the AAA-type ATPase *Vps4* functions in the disassembly of the ESCRT-III complex and the subsequent recycling of all ESCRT complexes from the endosomal membrane (Babst *et al.*, 1998, 2002). Without *Vps4* function, the ESCRT machinery remains on the endosomal membrane and MVB vesicle formation is inhibited. *Vps4* is the only known essential enzyme within the ESCRT machinery and therefore is ideally positioned to play a key role in regulating the MVB pathway according to cellular needs.

Previous studies suggested a model in which inactive *Vps4* dimers are recruited from the cytosol to the ESCRT-III complex where they assemble into a large double-ring structure of 12 subunits (Babst *et al.*, 1998; Scott *et al.*, 2005a). This oligomeric form of *Vps4* is the active ATPase, which disassembles the bound ESCRT-III complex using the energy from ATP hydrolysis. *Vps4* activity seems to be regulated through oligomerization of the protein. One *Vps4* regulator is *Vta1*, a dimeric protein that binds to the ATPase domain



of Vps4 and promotes both Vps4 assembly as well as ATP hydrolysis (Azmi *et al.*, 2006). In this study we identified Ist1 as a regulator of Vps4 recruitment that may function together with Vta1 in modulating Vps4 activity.

*IST1* encodes a 35-kDa soluble protein that contains a conserved N-terminal domain of ~150 amino acids, which we named the ELYC (pronounced “ellis”) domain, and a putative C-terminal coiled-coil domain of ~30 amino acids. Size determination experiments under native conditions indicated that Ist1 is a monomeric protein with a nonglobular, elongated structure.

Yeast two-hybrid data and published proteomics studies (Krogan *et al.*, 2006) indicated that Ist1 interacts with Vps4 *in vivo*. Our *in vitro* studies using recombinant Ist1 and Vps4 protein revealed that Ist1 binds directly to Vps4, resulting in the formation of a heterodimeric Vps4-Ist1 complex. Because Vps4 is predicted to be either a dimer or a dodecamer, the data suggested that Vps4 complexes disassemble in order to form the Vps4-Ist1 heterodimer. Moreover, this interaction requires the MIT domain of Vps4 and the coiled-coil motif of Ist1. The MIT domain has been shown to function as the substrate-binding domain Vps4 by interacting with the coiled-coil rich ESCRT-III subunits. Therefore it seems that the Ist1-Vps4 interaction mimics the binding of Vps4 to its substrate ESCRT-III.

The formation of the Vps4-Ist1 complex efficiently blocks the oligomerization of Vps4 into the dodecameric complex, resulting in a strong reduction of Vps4 ATPase activity. This inhibition of Vps4 oligomerization was observed even in presence of the assembly-promoting factor Vta1, suggesting that Ist1 is able to compete with Vta1 for interaction with Vps4. The interaction between the Vps4 MIT domain and the coiled-coil region of Ist1 is sufficient to obtain a stable Vps4-Ist1 complex, but cannot block Vps4 oligomerization, indicating the existence of at least one more interaction site, possibly via the N-terminal Ist1 ELYC domain (Figure 1). This second interaction site alone is sufficient to impair hydrolysis activity of Vps4 in ATPase assays, but is not stable enough to form an Ist1-Vps4 complex that can be observed by gel filtration analysis.

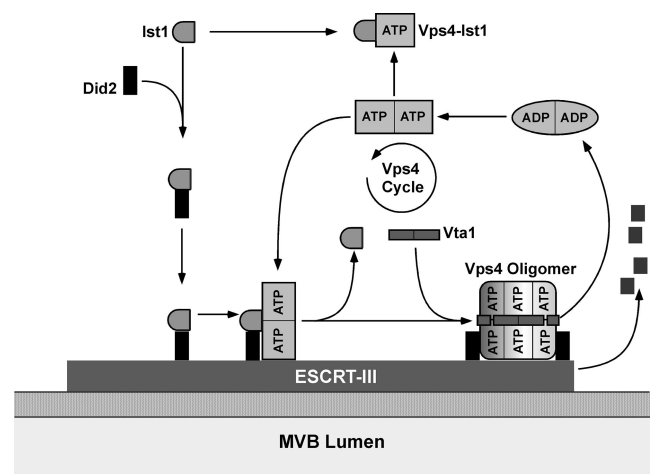
In wild-type cells Ist1 localizes to both the cytoplasm and MVBs. In contrast, in cells deleted for *VPS4* Ist1 accumulates on the aberrant endosomal structures formed in these cells. Therefore Ist1 localization mimics the localization of the ESCRT complexes, suggesting that, like the ESCRTs, Ist1 cycles on and off MVBs in a Vps4-dependent manner. The recruitment of Ist1 to MVBs requires the presence of Did2, a protein homologous to the ESCRT-III subunits. Previous studies have shown that Did2 localizes to ESCRT-III by binding to the Vps24 subunit, where it functions in Vps4-dependent disassembly of the protein complex (Nickerson *et al.*, 2006). This activity requires the binding of the Did2 C-terminal region to the Vps4 MIT domain. However, our data indicate that the same region of Did2 recruits Ist1 to ESCRT-III and that the MIT domain of Vps4 interacts with the Ist1 coiled-coil region. Further studies will be necessary to determine if Ist1 competes with the Did2-Vps4 interactions or if all three proteins are able to bind to each other simultaneously.

Deletion of *IST1* caused no obvious MVB trafficking phenotypes. However, genetic studies revealed synthetic interactions between *ist1Δ* and mutations in *VTA1* and *VPS4*, suggesting that Ist1 plays a positive role in Vps4 function. No synthetic genetic interactions were observed between *ist1Δ* and *did2Δ*, consistent with the idea that Ist1 requires Did2 for its positive effect on Vps4 activity. Cells deleted for *IST1* or *DID2* exhibited a similar reduction in the amount of

membrane associated Vps4, suggesting that the Ist1-Did2 complex might function to regulate the proper recruitment of Vps4 to ESCRT-III. In contrast to the modest defects observed in *ist1Δ*, overexpression of *IST1* resulted in a severe MVB trafficking phenotype. This phenotype was caused by a block in Vps4 recruitment to MVBs, resulting in endosomal accumulation of ESCRT-III. The overexpression data suggested that high levels of Ist1 in the cytoplasm might result in the formation of a Vps4-Ist1 complex that competes with the recruitment of Vps4 by ESCRT-III localized Did2-Ist1.

The phenotypic analysis suggests two distinct functions for Ist1 in regulating Vps4 activity. Associated with Did2, Ist1 localizes to ESCRT-III, where it seems to have a positive effect in the recruitment of Vps4. In contrast the free, cytoplasmic pool of Ist1 binds to Vps4, resulting in the formation of an Ist1-Vps4 complex that is not recruited to ESCRT-III. This latter complex seems to represent an inactive pool of Vps4. Both regulatory functions of Ist1 have mild effects on Vps4 activity. As a result of this, MVB trafficking defects are only observed either when *ist1Δ* is combined with other mutations in the ESCRT machinery or when Ist1 is overexpressed to artificially high levels. These observations are consistent with the idea that at normal cellular levels Ist1 acts as a modulator of the MVB pathway, affecting the kinetics of sorting but not blocking the pathway. One possible mechanism by which the cell could modulate Vps4 activity is by regulating the concentration of Ist1 in the cytoplasm.

In summary, we propose the following model for the function of Ist1 in regulating Vps4 activity (Figure 8). In the cytoplasm Ist1 interacts with Did2, resulting in the formation of Ist1-Did2 complex that is recruited to ESCRT-III. On ESCRT-III Ist-Did2 seems to function in regulating the recruitment of dimeric Vps4. The Ist1-Did2 complex is not required for binding of Vps4 to ESCRT-III but the complex might affect the kinetics and the proper timing of Vps4 recruitment. Furthermore, when bound to ESCRT-III the interaction of Vps4 with Ist1-Did2 might inhibit premature oligomerization of the ATPase, ensuring that ESCRT-III has completed its function in the MVB-sorting pathway before dissociation is initiated. At the proper time Ist1 dissociates from Vps4 and Did2 and is exchanged for the assembly factor Vta1, which initiates the oligomerization of Vps4 into the active complex. Previous studies showed that Vta1 in-



**Figure 8.** Model for the regulation of Vps4 by Ist1 (see Discussion for details).

teracts with Did2, suggesting that Did2 might also function in the Vps4 assembly step (Lottridge *et al.*, 2006). This would explain why loss of Did2 results in more severe MVB trafficking defects than observed in *ist1Δ*. The oligomeric form of Vps4 is the active ATPase that disassembles ESCRT-III in an ATP-dependent manner. In addition to the interaction with Did2, Ist1 can also form a heterodimeric complex with Vps4 in the cytoplasm. This Ist1-Vps4 complex represents an inactive form of Vps4 that competes with the function of Vps4 in ESCRT-III disassembly. By regulating the level of Ist1 in the cytoplasm the cell would be able to shift the equilibrium either in favor of the Ist1-Did2 or the Ist1-Vps4 complex, thereby regulating the recruitment of Vps4 and thus the efficiency of MVB sorting.

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