Drosophila Sec16 mediates the biogenesis of tER sites upstream of Sar1 through an arginine-rich motif

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ABSTRACT

tER sites are specialised cup-shaped ER subdomains characterized by the focused budding of COPII vesicles. Sec16 has been proposed to be involved in the biogenesis of tER sites by binding to COPII coat components and clustering nascent-coated vesicles. Here, we show that Drosophila Sec16 (dSec16) acts instead as a tER scaffold upstream of the COPII machinery, including Sar1. We show that dSec16 is required for Sar1-GTP concentration to the tER sites where it recruits in turn the components of the COPII machinery to initiate coat assembly. Last, we show that the dSec16 domain required for its localisation maps to an arginine-rich motif located in a non-conserved region. We propose a model in which dSec16 binds ER cups via its arginine-rich domain, interacts with Sar1-GTP that is generated on ER membrane by Sec12 and concentrates it in the ER cups where it initiates the formation of COPII vesicles, thus acting as a tER scaffold.
INTRODUCTION

A crucial step in the anterograde membrane transport is the packaging and exit of newly synthesized proteins from the ER. This takes place at specialized ribosome-free ER subdomains, the ER exit sites, (also called tER sites), that are characterized by a cup-shaped ER membrane from which COPII vesicles bud. The COPII machinery comprises the small GTPase Sar1 that is recruited to the ER membrane by its guanine nucleotide exchange factor (GEF) Sec12, and the proteins forming the coat itself, the two complexes Sec23/24 and Sec13/31 (Bonifacino and Glick, 2004).

Throughout the eukaryotic kingdom, tER sites are characterized by the presence of COPII components. Except for Sacharomyces cerevisiae (Rossanese et al., 1999), the tER sites in animal species studied so far are focused and form a limited number of sites: 1 in trypanosome (He et al., 2005), 2-5 in Pichia pastoris (Bevis et al., 2002), more than 200 in HeLa cells (Hammond and Glick, 2000) and 17±8 in non synchronized Drosophila S2 cells (Kondylis et al., 2007). What controls the focused organisation and the number of tER sites remains largely unknown.

A genetic screen searching for factors influencing the organisation of tER sites in P. pastoris has identified Sec16. A temperature-sensitive sec16 mutant causes fragmentation of tER sites at non-permissive temperature (Connerly et al., 2005). Similarly, depletion of Sec16 by RNAi in mammalian cells leads to disruption of tER sites and inhibition of anterograde transport, accompanied by the accumulation of cargo proteins in the ER (Bhattacharyya and Glick, 2007; Watson et al., 2006).

Sec16 is a large ~240-280 kDa peripheral membrane protein that was originally characterized in S. cerevisiae as a factor involved in the formation of COPII vesicles (Kaiser and Schekman, 1990). In yeast, it is co-purified with ER-derived COPII transport vesicles formed in vitro (Espenshade et al., 1995), but also plays an active role in their formation (Supek et al., 2002). Consistently, Sec16 displays biochemical interactions with the COPII subunits Sec23 (Bhattacharyya and Glick, 2007; Espenshade et al., 1995), Sec24 (Gimeno et al., 1996), Sec31 (Shaywitz et al., 1997) and possibly Sar1 (Supek et al., 2002). A genetic (Kaiser and Schekman, 1990) but not biochemical (Gimeno et al., 1995) interaction with Sec12 has also been shown.
As largely debated for the Golgi complex, tER sites have been proposed to be generated on a tER matrix (Mogelsvang et al., 2003; Shaywitz et al., 1997; Soderholm et al., 2004) in which Sec16 functions as a molecular platform upstream of COPII assembly, dictating where the focused budding of the COPII vesicles takes place. Alternatively, Sec16 could physically interact with several COPII coat components of the nascent COPII vesicles after the initial coat assembly on ER membrane (Espenshade et al., 1995; Gimeno et al., 1996; Shaywitz et al., 1997). Sec16 oligomerisation properties (Bhattacharyya and Glick, 2007) would result in the clustering of COPII vesicles, thus forming distinct and focused tER sites. Supporting this Sec16 role downstream of COPII assembly, Sar1-GTP has been recently shown to recruit Sec16 to tER sites in human cells (Iinuma et al., 2007; Watson et al., 2006).

Here, using Drosophila S2 cells as a model system (Kondylis and Rabouille, 2003; Kondylis et al., 2005), we show that dSec16 acts a tER scaffold dictating where the tER sites are formed. We first characterised the Drosophila Sec16 (dSec16), whose properties strictly conform to its orthologues in other species. Second, using a combination of light and immuno-electron microscopy, we show that dSec16 can associate to discrete ER domains in the absence of assembled tER sites, COPII vesicles and even Sar1, suggesting that it acts upstream of the COPII machinery. We find that Sar1 concentration to tER sites is Sec16-dependent. In dSec16 depleted cells, Sar1 is no longer concentrated on tER sites and is dispersed to the ER. Conversely, a form of dSec16 artificially localized to endosomes is able to recruit Sar1 but also other COPII subunits, indicating that Sec16 is part of the molecular machinery regulating the positioning and building of tER sites. Third, we identified the dSec16 domain involved in its localisation to tER sites to a stretch of 65 aminoacids arginine-rich domain in the N-terminal non-conserved region of the protein.

We propose a model for tER site biogenesis in which dSec16 acts as a tER matrix by binding ER cups through its arginine-rich domain and adjacent sequences followed by its oligomerisation. Concomitantly, Sar1-GTP is recruited to ER membrane by Sec12 GEF activity and gets concentrated to ER cups by interacting with dSec16, initiating the assembly of the COPII coat.
MATERIALS AND METHODS

Antibodies

The polyclonal anti-dSec16 antibody #764 was generated by immunizing rabbits with a GST-fusion protein comprising amino acids 655-817 of dSec16. The other rabbit polyclonal antibodies used were: anti-Sec23 (clone PA-069, Affinity Bioreagents, Goden, CC, (Kondylis and Rabouille, 2003), anti-GFP (Abcam, Cambridge, UK), anti-Sec31 (gift from F. Gorelick, Yale University), anti-Sar1 (Fromme et al., 2007) from B. Kleizin, VU Amsterdam, The Netherlands) and the anti-dGMAP (Friggi-Grelin et al., 2006). The following monoclonal antibodies were from the indicated sources: anti-Delta (Hydridoma from DSHB), anti-d120kd (Calbiochem, (Kondylis and Rabouille, 2003), anti-GFP (Roche, Woerden, The Netherlands), anti α-tubulin (Sigma), anti-V5 (Invitrogen) and anti-VSVG (clone P5D4, Roche).

Double-stranded (ds)RNA

dSec16 cDNA RE70141 was used to PCR a 820-bp fragment with flanking T7 RNA polymerase binding sites (TTAATACGACTCACTATAGGGAGA) using the primers 5’ T7-CGTCTGGGACAAGGAGC and 3’ T7-CCTGCTGGAAAGTTGAC corresponding to the C-terminus of dSec16. Sec23 cDNA was used as a template to amplify by PCR a 793-bp fragment with the primers 5’ T7-GTGCAGGATATGCTCGGAAT and 3’ T7-GTGGAGCTGGGATTCAATGT. Full length Sar1 (nucleotides 1-465) was amplified using 5’ T7-GTTTTCCTCGCTTCCAGATG and 3’T7-CTTGCCGGGTGTGTTAGCTGAT as primers.

These fragments were subjected to in vitro transcription for generating dsRNA using MEGASCRIPT T7 transcription kit (Ambion, Austin, TX), according to manufacturer's protocol and as described (Kondylis and Rabouille, 2003).

Plasmids

The cDNA clone LP14866 was obtained from Flybase (http://flybase.org) and was used to generate reporter constructs. This clone encodes the short isoform of dSec16, except for the last 68 amino acids. All constructs were amplified by PCR and cloned in pMT/V5-HisA or HisB (Invitrogen). The restriction sites and the corresponding size of the resulting constructs are described in Table S1. The
pEGFP-2xFYVE containing the tandem FYVE domain of mouse Hrs was a gift from Dr. Harald Stenmark (Oslo, Norway). This construct was used to PCR the 2xFYVE domain, followed by subsequent ligation into the XbaI site of pMT/V5-dSec16 to generate dSec16-2xFYVE-V5. To obtain GST-dSec16, the dSec16 region comprising amino acids 655-817 was cloned into XhoI-NotI sites of pGEX-4T1. Sec23 was PCR amplified from the *Drosophila* cDNA clone RE35250 (containing the full-length open reading frame of the gene) and ligated in pRmeGFP vector to generate an in frame GFP-dSec23 construct. Sar1 was amplified by PCR from a reverse transcriptase reaction of a total RNA extract from *Drosophila* S2 cells and cloned into the pRmeGFP vector as a C-terminally GFP fusion. Quick Change Mutagenesis kit (Stratagene) was used to create the NC23-CCD 807/861AAA, as well as the Sar1[H74G] and [Sar1T34N] mutants. Sec16-VSVG was made by replacement of the V5 epitope with VSVG (YTDIEMNRLGK) in the dSec16-containing pMT/V5-His plasmid. All constructs were checked by sequencing.

**Cell culture, RNAi, transfection and drug treatment.**

Wild type S2 cells or stably transfected with Delta-WTNdeMYC, Fringe-GFP, dSar1[H74G]-GFP or GFP-Sec23 (from Drosophila) were grown and depleted by RNAi as previously described (Kondylis and Rabouille, 2003; Kondylis et al., 2007). Cells were analyzed after 5 days of RNAi treatment in case of Sec16 depletion or 4 days in case of Sar1.

For transient transfections, approximately $1 \times 10^6$ S2 cells were plated on glass coverslips in 3.5-cm dishes and transfected the next day with 0.5-1µg DNA using the Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After 48-72h, the protein synthesis was induced by adding 1mM CuSO$_4$ in the cell medium for 2-3h at 27°C. The CuSO$_4$ was washed out and the cells were further incubated for 1-2h in fresh medium before being fixed for immunofluorescence (IF) or immuno-electron microscopy (IEM). On average, 50% transfection efficiency was achieved. For biochemical experiments, cells were processed 2-3h after induction.

For the Brefeldin A (BFA) experiment, stably transfected Sar1[H74G]-GFP S2 cells were induced for expression by adding 1mM CuSO$_4$ for 1.5h. The medium was changed and the cells were mock treated or treated with BFA
(20µM) for 2h. Control S2 cells transiently transfected with Fringe-GFP were treated with BFA for 30 min prior to Fringe-GFP expression induced by addition of 1mM CuSO4 for 1.5h in the presence of BFA.

**Immunofluorescence (IF) and Immuno electron microscopy (IEM)**

S2 cells grown on coverslips were fixed in 4% PFA in PBS and processed for IF as described (Kondylis and Rabouille, 2003). Cells were viewed under a Leica TCS-NT (Jena, Germany) or a Zeiss LSM-510 confocal microscope (Wetzlar, Germany). When a whole cell projection is presented, it is indicated as “proj”. When nothing is indicated, a confocal section is presented. Immuno-electron microscopy was performed as published (Kondylis and Rabouille, 2003).

**Delta/Fringe transport assay**

Delta and Fringe-GFP transport assays were done as described (Kondylis and Rabouille, 2003; Kondylis et al., 2007). A Fringe-GFP stable cell line was generated according to the manufacturer’s protocol.

**dSec16-FYVE and Sar1 recruitment to endosomes**

To examine whether dSec16-2xFYVE-V5 localised to endosomes a BSA-gold uptake was included after the 2h pulse with CuSO4 in the 1h or 2h chase period of transfected S2 cells for the IEM or IF analysis, respectively. The effect of delocalising dSec16 on the localisation of dSar1 was determined by double transfection of dSec16-2xFYVE-V5 and Sar1[T34N]-GFP or Sar1[H74G]-GFP. The quantification of the co-localisation between dSec16-FYVE and Sar1-GTP or Sar1-GDP is based on analyzing 113 and 104 endosomes, respectively from 40 cells in 2 independent experiments.

**Subcellular fractionation**

S2 cells (~80 millions) were harvested by centrifugation, resuspended in 1.2 ml homogenization buffer (20mM Hepes, pH7.5, 250mM sucrose and protease inhibitors) and broken by three freeze/thaw cycles. The nuclei and non-broken cells were pelleted by centrifugation 15 min at 800xg and the resulting post-nuclear supernatant (PNS) was applied to ultracentrifugation at 100000xg for 1h using a TLA 100.2 rotor to generate the cytosol (C) and the membrane fraction. To analyze the partition of dSec16 between the membrane soluble (S) and insoluble
fraction (P), the total membranes were further resuspended and incubated for 1h at 4°C in a PNS-equivalent volume of homogenization buffer to which NaCl and/or TritonX-100 were added to a final concentration of 0.5M and 1%, respectively. The suspension was ultracentrifuged as above to separate the soluble from the insoluble material. The insoluble fraction was finally dissolved in Laemmli sample buffer. Equal volumes of PNS, C, S and P were loaded on an SDS-PAA gel and analyzed by western blot with anti-dSec16 antibodies.

**In vivo interaction between dSec16 and Sar1**

S2 cells co-transfected with Sar1[H74G]-GFP or Sar1[T34N]-GFP and dSec16-V5 were resuspended in lysis buffer (20mM Hepes, pH 7.5, 150mM NaCl, 5mM MgCl₂, 0.2% NP-40 and protease inhibitors) for 30 min on ice. The lysates were centrifuged for 15 min and the resulting supernatants were subjected to immunoprecipitation with a polyclonal anti-GFP antibody. The precipitated proteins were resolved by SDS-PAGE and analyzed by immunoblotting with monoclonal anti-GFP and anti-V5 antibodies.

**dSec16 oligomerisation**

S2 cells transfected with the indicated plasmids were lysed in 50mM Tris, pH 7.5, 150mM NaCl 1% TritonX-100 (immunoprecipitation buffer) supplemented with protease inhibitor cocktail (Roche) for 30 min on ice. The lysate was centrifuged for 15 min and the resulting supernatant was subjected to immunoprecipitation using anti-V5 antibodies bound to protein A-agarose beads (Repligen). To improve the coupling of the V5 antibody to protein A-agarose beads, a bridging Affinity purified rabbit anti-mouse antibody (Jackson Immunoresearch) was used. The incubation was carried out for 2h in cold. The beads were then washed for three times with immunoprecipitation buffer. Immunoprecipitated proteins were eluted from the beads by heating in Laemmli reducing sample buffer (RSB) for 7 min at 95°C and analysed by SDS-PAGE and western blot using anti VSVG- and anti-V5 antibodies.
RESULTS

Drosophila Sec16 is the orthologue of yeast and human Sec16

The Drosophila melanogaster homologue of human and yeast Sec16 is encoded by the gene CG32654 (flybase) that exhibits at least two predicted splice variants. CG32654-RD encodes a 2221-aminoacid form that lacks the conserved C-terminus reported in other species, whereas CG32654-RE encodes a 2021-aminoacid form that contains the C-terminus but has a shorter second non-conserved region (Figure 1A). Using an antibody we raised against the 162 aminoacid sequence upstream of the central conserved domain (Figure 1A, see Materials and Methods) to blot a S2 cell lysate fractionated on SDS-PAGE, a single band was detected at the molecular weight of ~280-300 kDa (Figure 1B, lane 3), similar to that of the human and yeast proteins (Bhattacharyya and Glick, 2007; Espenshade et al., 1995; Watson et al., 2006). This band had an identical migration as the transfected shorter form of V5-tagged Sec16 (Figure 1B, lane 4), and was very efficiently knocked down (up to 90-95%) using a double-stranded RNA corresponding to the C-terminus (+ds dsec16) (Figure 1B, lane 7 and 8). As previously reported for the yeast homologue (Espenshade 1995, Supek 2002), overexpressed dSec16 was partially degraded, giving rise to several breakdown products (Figure 1B, lane 4, bracket). Blotting with the preimmune serum (pi) did not yield any band (Figure 1B, lane 5 and 6), confirming the specificity of our antibody. This shows that S2 cells only express the short form of Sec16, and we will refer to it as dSec16.

dSec16 is overall 23% identical and 35% similar to the long form of the human Sec16 protein, hSec16L (Bhattacharyya and Glick, 2007), with the highest conservation in the Central Conserved Domain (CCD, Figure 1A) (33% identity and 50% similarity) and the C-terminus (28% identity and 39% similarity) (using EMBOSS align program, http://emboss.sourceforge.net/).

Human and Pichia Sec16 localise to tER sites (Bhattacharyya and Glick, 2007; Connerly et al., 2005; Watson et al., 2006). Immunofluorescence (IF) localisation using the antiserum we generated showed that, as expected, endogenous dSec16 localises to the 17±8 tER sites in S2 cells (Figure 1C) (Kondylis et al., 2007), co-localises with GFP-Sec23 (Figure 1C’) and Sar1-GFP (Supplementary Figure S2D).
As previously described (Kondylis and Rabouille, 2003), the *Drosophila* tER sites are in close proximity to the Golgi complex. Accordingly, dSec16 localization is juxtaposed to that of the integral Golgi membrane proteins Fringe (Kondylis et al., 2007) (Figure 2D) and d120kd, the *Drosophila* homologue of the vertebrate 160kD medial Golgi sialoglycoprotein MG160 (Yano et al., 2005) (Figure 3A). By immuno-electron microscopy (IEM), dSec16 was found to decorate the typical pleomorphic tubular/vesicular membrane marked by Sec23 and located near a Golgi stack, the characteristic morphology of tER sites (Figure 1D), as well as the adjacent ER cup albeit at much lower level (Figure 1D, arrow).

Finally, both human and *S. cerevisiae* Sec16 peripherally associate to the membrane fraction from which they can be extracted by high salt concentrations and pH, but not by Triton X-100 or urea (Espenshade et al., 1995; Watson et al., 2006). The subcellular fractionation of dSec16 shows that it is mostly associated to the membrane fraction (Figure 1E, control) in a salt sensitive manner, consistent with the properties of yeast and human proteins. However, its solubility in detergents was significantly higher than in the above species, perhaps reflecting differences in the lipid membrane composition (Figure 1E).

Taken together, these data suggest that, as human and yeast Sec16, dSec16 localises to tER sites and exhibit similar membrane association properties.

**Depletion of dSec16 leads to disruption of tER sites**

Human cells depleted of Sec16, and *P. pastoris* harboring a non-functional Sec16 show a severe disruption in the organisation of tER sites (Bhattacharyya and Glick, 2007; Connerly et al., 2005; Watson et al., 2006). In agreement, depletion of dSec16 from S2 cells by RNAi (as above, Figure 1B) caused a significant fragmentation of tER sites. The typical Sec23 IF pattern (Figure 2A) was converted into a haze of numerous small fluorescence punctae (Figure 2B). By EM, the tER sites and the COPII vesicles were no longer visible (Figure 2B", S1C"), and Sec23 was mostly cytoplasmic (Figure 2C").

Few larger Sec23 fluorescent spots sometimes remained (Figure 2B) that correspond to Sec23 aggregates associated to clustered membrane remnants close to an ER cisterna, likely representing non-functional tER sites (Figure 2C") (compare to the tER sites in a non depleted cell, Figure 2C).
In dSec16-depleted S2 cells, the Golgi stacks were absent, as observed by the re-distribution of the peripheral cis-Golgi marker dGMAP throughout the cytoplasm (Figure 2B’). By EM, Golgi cisternae were not visible (compare Figure 2B” to Figure 2A”). Furthermore, dSec16 depletion led to the proliferation of ER membrane, as exemplified by long aligned ER sheets that were only rarely seen in mock depleted cells (not shown), a very reproducible and significant feature that resembles the S. cerevisiae sec16 mutant phenotype (Kaiser and Schekman, 1990).

In the absence of functional Sec16, the anterograde transport both in yeast and human cells is largely inhibited (Bhattacharyya and Glick, 2007; Espenshade et al., 1995; Watson et al., 2006). To test this in dSec16-depleted cells, we performed an ER to plasma membrane transport assay making use of the inducible expression of the plasma membrane marker Delta (Klueg et al., 1998; Kondylis and Rabouille, 2003) as well as the Golgi marker Fringe-GFP (Kondylis et al., 2007; Munro and Freeman, 2000). We found that both Delta (Figure 2E) and Fringe-GFP (Figure 2D) were retained in the ER in more than 80% of dSec16-depleted cells, indicating that ER export is largely inhibited. This was confirmed by the severe cell lethality in Drosophila tissues depleted of dSec16 by RNAi (not shown).

Taken all together, dSec16 has similar sequence homology, size, localisation, biochemical properties and role in tER site organisation and anterograde transport as the other Sec16 proteins already described, demonstrating that it functions as an orthologue.

dSec16 localisation to tER sites is independent of COPII-coated vesicle assembly
A wealth of data has proven the essential role of Sec16 in the organisation of ER exit sites (Hughes and Stephens, 2008). However, the mechanism behind it is largely unknown. As outlined in the introduction, one model proposes that Sec16 is recruited by the COPII coat components to the nascent vesicles and clusters them into a typical tER site due to its oligomerisation properties (Connerly et al., 2005).

If this is true, dSec16 localisation should be clearly affected if COPII coat formation is prevented. To test this, we first depleted Sec23 from S2 cells, as observed by Western blot (Supplementary Figure S1A) and by the large
downregulation of GFP-Sec23 (Supplementary Figure S1C). As for dSec16 depletion, the IF pattern of the Golgi marker dGMAP (not shown) and d120kd (Figure 3B) was dispersed in the cytoplasm, and the ER was also significantly proliferated and dilated (Supplementary Figure S1D-D’), in agreement with the ER patterns in fibroblasts of patients carrying a mutation in the sec23a gene (Boyadjiev et al., 2006; Fromme et al., 2007). By IEM, typical tER sites and Golgi stacks were no longer visible (Figure 3C).

Remarkably, in Sec23-depleted cells, the IF pattern of endogenous dSec16 was indistinguishable from mock-treated cells (Figure 3A,B). When investigated by IEM, dSec16 was found on distinct ER cups and dense material underneath that contains almost no vesicular profiles (Figure 3C). This suggests that COPII coat assembly is not necessary for dSec16 recruitment, and therefore its localisation occurs upstream of the Sec23/Sec24 complex.

In human cells, Sec16 has been reported to be recruited and stabilized to tER sites by Sar1-GTP (Iinuma et al., 2007; Watson et al., 2006). To investigate whether this is also the case in Drosophila, we first tested their interaction by immunoprecipitation using the Drosophila inactive GDP-locked Sar1[T34N] and the active GTP-locked Sar1[H74G] mutants. dSec16 was found to interact only with the active form of Sar1 (Figure 4A).

To investigate the functional relevance of this interaction, we tested whether Sar1 recruits dSec16. We first displaced endogenous Sar1 from the tER sites by overexpressing the GDP-locked Sar1[T34N] mutant. This has been shown to potently inhibit the formation of COPII vesicles (Aridor et al., 1995; Aridor et al., 2001; Kuge et al., 1994; Rowe et al., 1996) by binding and inhibiting the Sec12-mediated exchange activity on wild type Sar1 (Weissman et al., 2001), thus blocking anterograde transport (Prescott et al., 2001). Accordingly, the Golgi complex was fragmented and dispersed as assessed by dGMAP and d120kd distribution (Supplementary Figure S2A,B), the latter also partially found in the ER (not shown). In cells overexpressing Sar1[T34N], endogenous dSec16 was still localised in spots reminiscent of tER sites (Supplementary Figure S2C), though they were less numerous and larger than in cells transfected with wild type Sar1 and Sar1[H74G] (Supplementary Figure 2D,E, see discussion). Surprisingly, although dSec16 and wild type Sar1 co-localise perfectly at tER sites, dSec16 and Sar1[H74G]-GFP did not. Instead, their labeling seem to juxtapose, perhaps
because, as COPII vesicles that are formed in the presence of the active Sar1 mutant do not uncoat, dSec16 is not accessible to its antibody by immunofluorescence. By IEM, however, dSec16 and Sar1[H74G]-GFP were shown localize on the same tER site (Supplementary Figure S2F).

To confirm that dSec16 is not released from the tER sites in the absence of functional Sar1, we depleted Sar1 from S2 cells for 4 days (Figure 4B) and assessed the localisation of endogenous dSec16. Again, dSec16 was localised in spots (Figure 4D). As in cells overexpressing Sar1[T34N], their number was reduced and their size larger when compared to non-depleted cells (Figure 4C, see discussion). Using IEM, the IF spots were shown to correspond to discreet ER cups decorated by endogenous dSec16. The rest of the ER was not labeled. As expected, most of the vesicle budding was inhibited (Figure 4E). Despite the tER site morphometric changes (see discussion), these results suggest that dSec16 localisation is independent of Sar1.

Taken together, these results indicate that dSec16 is able to localise to tER sites in the absence of COPII coated membrane (achieved either by Sec23 or Sar1 depletion). This shows that its localisation is not mediated by binding to Sec23, the pre-budding complex (Sar1-Sec23/24) (Kuehn et al., 1998) or Sar1-GTP. Although dSec16 could associate to the tER sites by interacting with the Sec13/31 complex, this is very unlikely as recent data have shown that the tER localisation of Sec13/31 critically depends on the Sar1-Sec23/24 complex with the Sec23 Phe380 residue central for this association (Bi et al, 2007; Fromme et al, 2007). Therefore, our results fit with the hypothesis that Sec16 functions upstream of the COPII coat formation.

dSec16 mediates the recruitment of Sar1 to tER sites

We next investigated whether Sar1 localisation depends on dSec16. To do so, we localised Sar1[H74G] in dSec16 depleted S2 cells. As expected, in mock-treated cells, Sar1[H74G] localised to tER sites (Figure 5A), but depletion of dSec16 led to the complete re-localisation of the GTPase in a dispersed pattern (Figure 5B), probably corresponding to the ER (Figure 5C), in agreement with the membrane association of GTP locked form of the small GTPases (Novick and Zerial, 1997). This results shows that dSec16 recruits Sar1-GTP and concentrates it to tER sites.
However, Sar1[H74G] dispersion could be an indirect effect of the transport inhibition caused by Sec16 depletion. To test this, we analyzed the distribution of Sar1[H74G] when ER-to-Golgi transport was inhibited by Brefeldin A (BFA), as shown by the localization of the Golgi protein Fringe-GFP in the ER (Supplementary Figure S3B). Under BFA treatment, the tER localization of Sar1[H74G]-GFP was largely unchanged when compared to mock-treated cells (Supplementary Figure S3A), though a faint nuclear envelope staining was visible. This shows that its dispersion upon dSec16 depletion is not due to anterograde transport inhibition, but to the loss of dSec16 protein. This suggests that dSec16 functions upstream of Sar1-GTP, concentrating it from the ER to ER cups, where it can drive the local budding of COPII-coated vesicles, thus inducing tER biogenesis.

If this hypothesis is correct, dSec16 should be able recruit COPII components, including Sar1-GTP, to a membrane to which it is ectopically localised. To test this, dSec16-V5 was fused to a tandem FYVE domain, known to mediate localisation to endosomes (Gillooly et al., 2000). Accordingly, dSec16-FYVE-V5 was found significantly localised on large cytoplasmic ring-like structures (2-5 per cell confocal section, Figure 6B), shown by BSA-gold uptake and IEM to correspond to endosomes (Figure 6B,C). As predicted, dSec16-V5 did not co-localise with BSA-gold (Figure 6A).

Sar1[H74G]-GFP was then co-transfected with dSec16-FYVE-V5. We chose this form of dSar1 to bypass the requirement for the GEF activity of Sec12. Sar1[H74G] was found to significantly co-localise to dSec16-FYVE/BSA-gold positive endosomes (Figure 6D, arrows). 44% of the endosomes labeled by ectopic dSec16 were also positive for Sar1[H74G]-GFP versus 2.8% for dSar1[T34N]-GFP (Figure 6E). Furthermore, Sar1[H74G] transfected to wild-type cells (Supplementary Figure 2E) or co-transfected with dSec16-V5 (not shown) was not localised to endosomes. Importantly, we also found that other COPII components, such as Sec23 (not shown) and Sec31 (Figure 5F) co-localised with Sar1[H74G] and ectopic dSec16 on endosomes. These results show that dSec16 is able to recruit COPII components, including activated Sar1 at the site of its localisation.

Together with the loss of Sar1 localisation upon dSec16 depletion and the fact that, conversely, dSec16 localisation does not change upon Sar1 or Sec23
depletion, this experiment suggests that dSec16 recruits the COPII machinery, starting with the concentration of the GTP loaded Sar1 to ER cups (alone or as a pre-budding complex) where it drives the biogenesis of tER sites.

**A tER specificity motif is mapped to a 65-aminoacid arginine-rich domain**

As dSec16 is not recruited to the tER through binding to COPII components, we set out to identify the domain mediating its localisation. To do so, we generated a series of V5 tagged C-terminal truncations of dSec16 (Figure 7A) and analyzed their intracellular localisation by IF and IEM.

dSec16-V5 localised very efficiently to tER sites in a pattern indistinguishable from the endogenous dSec16. By IF, it overlapped with the Golgi marker dGMAP (Figure 7B), and by IEM, it labeled tER sites (Figure 7B'). It appeared, though, that dSec16-V5 is more restricted to the ER cup outlining the tER sites when compared to the endogenous dSec16 in control cells that was found more prominently on the vesicular-tubular pleiomorphic membrane (compare Figure 7B' and Figure 1D, see discussion). Removing the 400 C-terminal aminoacids (∆C-dSec16-V5), did not change the localisation characteristics (Supplementary Figure S4A,A' [2]), showing that the C-terminus is dispensable. Accordingly, the C-terminal conserved domain alone localised to membrane in a non-specific manner (ER, Plasma membrane, Golgi), (not shown).

We then generated further truncations of ∆C-dSec16-V5 (Figure 7A). Alone, the central conserved domain (CCD) behaved as the C-terminus (Supplementary Figure S4B, [3]), indicating perhaps the presence of a general membrane-binding domain lacking specificity to confer tER localisation. However, it is necessary for the localisation to tER sites, as truncations lacking it were mislocalised (Figure 7A, [7,8,9,10]). We found that the CCD domain extended at its N-terminus by 376 amino acids (NC2.3.4-CCD) is sufficient to mediate localisation to tER sites (Supplementary Figure S4C, [4]). An extension of 176 amino acids only (NC3.4-CCD) was less efficient (Supplementary Figure S4D, [5]), suggesting that NC2 is necessary.

To test this further, we designed a chimera encompassing the CCD extended at its N-terminus by NC2 (NC2-CCD) that was poorly localised to tER sites (Supplementary Figure S4E, [11]). When the CCD was extended by NC2.3 (NC2.3-CCD), we recovered full tER site localisation and decoration of the ER cup
(Figure 7C, C', [12]). To confirm the role of NC2.3 in the localisation of dSec16, we deleted this region (aa 690-989) from dSec16-V5. This chimera ($\Delta$NC2.3) did not properly localise to tER sites and became largely cytoplasmic (Figure 7D, [13], Figure 8C). Taken together, these results show that the domain mediating the localisation of dSec16 to tER sites (NC2.3) is located in the non-conserved region. On its own, however, it does not confer the specificity for tER membrane binding that requires the presence of the CCD.

hSec16 has been shown to oligomerise (Bhattacharyya and Glick, 2007), see below). To rule out that the observed localisation of our truncated proteins is due to oligomerisation with the endogenous dSec16, we monitored the localisation of $\Delta$C-dSec16-V5 (Supplementary Figure S4F, [2]) and NC2.3-CCD (Supplementary Figure S4G, [12]) in dSec16-depleted cells. Both localised in puncta that by IEM were shown to correspond to cupped ER membrane (Supplementary Figure S4F'), indicating that their tER site localisation is not due to oligomerisation with the endogenous protein.

The tER localisation domain in hSec16L is also found in the non-conserved 300 amino acid sequence upstream of the CCD (924-1227, (Bhattacharyya and Glick, 2007). When NC2.3 was aligned to this region, several residues appeared conserved, of which two consensus sequences for PKC sites at positions 807 and 861, and a 65-aminoacid arginine-rich domain (aa 860-925) present in many species (Figure 8A). A role of a putative PKC-mediated phosphorylation in the dSec16 localisation was proven negative. Although dSec16-V5 is phosphorylated (unpublished results), the mutation of the two putative PKC sites to alanine did not abrogate its localisation to tER sites (unpublished results), even in dSec16 depleted cells to avoid oligomerisation with the endogenous protein (see below).

The arginine-rich domain, however, has an important role. Expression of a dSec16-V5 mutant lacking the 65 aminoacids covering this domain (860-925, $\Delta$Arg-V5, Figure 7A [14]) resulted in a strong cytoplasmic distribution of the protein (compare Figure 8B to Figure 7B), though the tER sites localisation was not completely abrogated. The residual localization of $\Delta$Arg-V5 to the tER sites can be explained by its binding to the localized coat components and their oligomerization to the endogenous protein (see below). The fractionation experiment confirmed a 30-40% increase the cytosolic pool of $\Delta$Arg-V5 and $\Delta$NC2.3-V5 relative to the full-length protein (Figure 8C).
Taken together, these results show that the arginine-rich domain is involved in conferring the specificity for dSec16 localisation to tER sites.

**The localisation of dSec16 to tER site does not require its oligomerisation**

The function of Sec16 in the organisation of ER exit sites has been proposed to depend on its self-oligomeric properties (Bhattacharyya and Glick, 2007; Connerly et al., 2005). We first tested whether dSec16 also exhibits oligomeric properties *in vivo*. Transfected dSec16-V5 and dSec16-VSVG were efficiently co-immunoprecipitated from detergent extract of S2 cells, showing that dSec16 oligomerises (Figure 8D, lane 2). However, dSec16-missing NC2.3 (ΔNC2.3-V5, [13]) did not (Figure 8D, lane 3), suggesting that NC2.3 is required for oligomerisation.

We next asked whether dSec16 oligomerisation and localisation were linked. As dSec16 missing the arginine-rich domain (ΔArg-V5, [14]) does not localise to tER sites very efficiently (Figure 8B), we tested whether ΔArg-V5 oligomerises with dSec16-VSVG. They did as strongly as dSec16 with itself (compare Figure 8D, lane 4 with lane 2). This suggests that oligomerisation is not sufficient to mediate localisation. Conversely, NC2.3-CCD-V5 alone that localises very efficiently to tER sites (Figure 7A, C) oligomerised very poorly with dSec16-VSVG (Figure 8D, lane 5). A similar result was obtained with NC3.4-CCD and importantly, the CCD alone did not oligomerise.

Taken together, these results show that NC2.3 is required but not sufficient for oligomerisation and suggest that the tER site localisation and oligomerisation are two independent properties.

**DISCUSSION**

Here, using a combination of biochemical methods, RNAi depletion and re-transfection followed by immuno-fluorescence and immuno-electron microscopy analyses, we present evidence that dSec16, the *Drosophila* orthologue of human and yeast Sec16, is part of a tER scaffold regulating where tER sites assemble on the ER. dSec16 binds to ER cups through an arginine rich domain and concentrate the active form of Sar1, either alone or as a pre-budding complex together with Sec23/24 (Kuehn et al., 1998), thus initiating COPII coat formation.
dSec16 shares at least four critical features with human and *P. pastoris* Sec16. They all are large hydrophilic proteins (above 250kD) that exhibit a conserved central domain (CCD) and a conserved C-terminus of ~200aa. They localise to tER sites and their inactivation by depletion or mutation leads to disruption of the functional organisation of tER sites, causing in turn a severe inhibition in anterograde transport. Last, they display an increased solubility in ionic over detergent based buffers (this study, and Supek et al., 2002; Watson et al., 2006).

**dSec16 localisation to tER sites is specified by an arginine-rich domain**

As for the long form of human Sec16, hSec16L, the domain mediating dSec16 localisation has been mapped to 300 amino acids present in a non-conserved region upstream of the CCD (this study and Bhattacharyya and Glick, 2007). For hSec16, this domain is both necessary and sufficient to mediate its tER site localisation, but not for dSec16. In *Drosophila*, this domain has to be expanded by the conserved central domain that on its own localises to all intracellular membrane. This indicates that the 300 aminoacid domain confers tER specificity and that the two domains cooperate to bring about the binding of dSec16 to tER site membrane.

The dSec16 tER specificity motif was further narrowed to a 65-amino acid arginine-rich domain that is also present in hSec16L and other species. Arginine-rich domains have been shown to interact with ionic lipids (Hitz et al., 2006), to remodel membrane (Shaw et al., 2007) and even cross membrane (Futaki, 2005), as it is the case for the twin arginine transport (TAT) pathway (Sargent, 2007). It remains to be tested whether the arginine-rich domain of dSec16 has such properties.

As hSec16, dSec16 also oligomerises. However, oligomerisation is not necessarily followed by efficient tER site localisation, and conversely, localisation can occur without oligomerisation. This suggests that these two properties are independent from one another. However *in vivo*, they both are likely to be used in parallel or in a sequential fashion. We propose that the recruitment of dSec16 to the ER cups could be followed by its oligomerisation that in turn strengthens its localization (see below). Alternatively, Sec16 could oligomerise in the cytoplasm.
and be recruited as a performed oligomer to the ER cups. What drives the initial recruitment needs to be further investigated (see below).

**dSec16 acts as a tER matrix upstream of COPII**

We provide here several lines of evidence that dSec16 acts upstream of the COPII machinery in defining the ER subdomain at which tER sites are built. First, we show that dSec16 can still associate to distinct ER cups in the absence of COPII coat formation, either upon Sec23 depletion or in the absence of (functional) Sar1 (depletion and expression of the inactive Sar1[T34N] mutant).

Second, as its yeast homologue (Supek et al., 2002), dSec16 interacts with Sar1-GTP and dSec16 is required to concentrate it to tER sites. Depletion of dSec16 led to a significant loss of Sar1-GTP from tER sites, and the ectopic localisation of a chimeric form of dSec16 to endosomes results in the quantitative recruitment of Sar1 to the same compartment. These findings are not in strict agreement with the conclusions drawn from studies in human cells, suggesting that Sar1 recruits/maintains Sec16 to tER sites. The first study shows that overexpression of GTP-locked Sar1 leads to an apparent dispersal of endogenous hSec16, whereas transfected Venus-hSec16 appears enriched in crescent-like Sar1-positive structures reminiscent of tER sites (Watson et al., 2006). In a second study, the membrane association of hSec16 (measured in vitro) is shown to increase upon addition of GTP-loaded Sar1 (Iinuma et al., 2007). However, hSec16 was not dissociated from membrane in the presence of excess of GDP-loaded form of Sar1, suggesting that the initial binding of Sec16 to membrane does not depend on Sar1.

Taken together and in agreement with data showing that Sec16 stabilises Sar1-GTP (Supek et al., 2002), our results demonstrate that Sec16 not only acts upstream of the structural COPII coat subunits, but also upstream of Sar1, suggesting that Sec16 is part of an initial molecular platform (or tER matrix) that regulates the biogenesis of the tER sites by nucleating COPII coat assembly and vesicle budding.

Our results, however, do not exclude a role for Sar1 in the tER site organisation in influencing directly or indirectly dSec16 functions. Indeed, the number of tER sites labeled by dSec16 under conditions where Sar1 is inactivated or depleted is reduced and their size is larger when compared to control cells.
Several explanations are possible: First, Sar1 has been shown to activate a PI4 kinase (Blumental-Perry et al., 2006), and as such, it indirectly contributes to the generation of phospholipids, such as PI4P on the ER membrane, that are required for tER site maintenance. Second, Sar1 depletion could impede on the dynamics of tER site fusion/fission (Bevis et al., 2002; Stephens, 2003), thus leading to larger and less numerous sites. Last, the binding of Sar1-GTP to dSec16 could change the properties of dSec16, for instance modulating its oligomeric state. On its own, Sec16 would form very large oligomers in a reduced number of spots, but the binding of Sar1-GTP would drive the dissociation of monomeric Sec16 from the oligomer, thus allowing its incorporation in the forming COPII vesicles (see below). This will have to be further investigated.

**Localisation versus function**

Given the large size and multi-domain structure of the protein, the role of Sec16 as a tER matrix does not exclude an additional role in COPII vesicle formation and budding (Kaiser and Schekman, 1990). In yeast and human, Sec16 C-terminus has been shown to bind Sec23 (Bhattacharyya and Glick, 2007; Espenshade et al., 1995). As this domain is also found and conserved in dSec16, it is likely that its interaction with Sec23 will also be conserved in *Drosophila*.

We propose that these two independent functions of Sec16 are reflected in its localisation. dSec16 is located to the ER cup where COPII components are initially recruited to form nascent COPII vesicles that bud, uncoat, and form pleiomorphic membrane clusters, that also contain dSec16. In control cells, endogenous dSec16 is mostly found on this membrane, but under conditions where COPII budding is inhibited (in Sar1 depletion), the localisation shifts towards the ER cup. dSec16 is never seen diffused in the entire ER. Whether the ER cups pre-exist as such or are formed upon dSec16 binding remains to be established.

Strikingly, transfected dSec16 constructs localise also preferentially to ER cups, suggesting that they recapitulate the initial association of dSec16 to ER membrane, but not its incorporation in COPII vesicles, resulting in the inhibition anterograde transport. This could be explained by the fact that the function of Sec16 in transport depends drastically on its stoechiometry. Sec16 overexpression causes a lethal secretion defect in yeast (Espenshade et al., 1995), and inhibition of ER export in mammalian cells (Watson et al., 2006). The mapping of the
domains required for transport by using the usual transfection techniques will therefore be difficult. Genome tagging under endogenous promoters, so far only feasible in yeast, will prevent this overexpression.

**dSec16 acts as a tER scaffold involved in the biogenesis of functional tER sites**

We propose a model for tER site biogenesis in which dSec16 dictates the site of COPII coat components recruitment and vesicle budding, before being incorporated in the vesicles themselves. First, dSec16 binds to ER cups upstream of the COPII coat and Sar1, through the dual activity of its central conserved domain and its arginine-rich domain. Second, localised dSec16 assembles into oligomeric complexes, possibly increasing its stability in the ER cup. Third, concomitant to dSec16 localising in ER cups, Sar1-GTP is generated on ER membrane due to the GEF activity of Sec12, itself exhibiting an ER localisation in S2 cells (not shown) and other organisms (Weissman et al., 2001). Fourth, Sar1-GTP diffuses in the plane of the ER and reaches ER cups enriched in dSec16 oligomers to which it binds, leading to its concentration and the initiation of the COPII coat assembly. Alternatively, before binding to Sec16, Sar1 is perhaps already part of the pre-budding complex with Sec23/24 (Kuehn et al., 1998). As dSec16 does not interact with the GDP loaded form of Sar1, we propose that Sec16 acts in parallel with Sec12, though Sec16 itself might also be capable of recruiting first Sec12 from the ER to the ER cup. In Pichia, Sec12 associates to the tER sites by binding to a partner protein (Soderholm et al., 2004) and it would be tempting to speculate that this protein is Sec16. Once the coat is formed, Sec16 associates to it, leading to the stabilisation of the COPII coat and its incorporation into functional COPII vesicles. The Sec16 molecule that associates to the coat can either be recruited from the cytosol or dissociates from the oligomers.

Whether Sec16 is the most upstream factor driving the building of tER sites remains to be established. Although its putative membrane deformation properties and oligomerisation properties (see above) could lead to its self-assembly to tER sites, other factors are likely to be involved. Phosphatidylinositol 4-phosphate has been recently shown to be locally regulated at tER sites (Blumental-Perry et al., 2006), and depletion of the phospholipase A1-like protein p125, specifically
disrupts the organization of tER sites, but not their function (Shimoi et al., 2005). In addition, we cannot rule out the existence of a receptor. The first half of the dSec16 localisation domain encompasses a short coiled-coil known to mediate protein-protein interactions. Furthermore, dSec16 overexpression does not cause tER site enlargement (this study and (Connerly et al., 2005), suggesting a saturable binding. Identification of this potential receptor and other binding partners will shed light onto the mechanism of dSec16-membrane interaction.

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REFERENCES


LEGEND FOR FIGURES

Figure 1: dSec16 is the Drosophila orthologue of human and yeast Sec16.

(A) Schematic representation of dSec16. The conserved domains are in light grey, whereas the non-conserved ones in dark grey. The region used to raise antibodies against dSec16 is indicated.

(B) Western blot of a lysate from S2 cells transfected (+) or not (-) with dSec16-V5 or depleted for dSec16 was probed with the α-dSec16 antibody. One band of ~280-300 kDa was specifically detected in both non-transfected and transfected cells, which was not detected by the pre-immune serum (pi). The V5 antibody was used as a positive control for the transfected protein. dSec16 was depleted to 90-95% using dsRNA against the C-terminus. α-tubulin served as a loading control. Note the degradation products in lane 4 corresponding to proteolysis from the C-terminus, therefore not detected by the V5 epitope but by the anti-dSec16 antibody.

(C, C') Localisation of endogenous dSec16 was assessed by IF in one wild type S2 cells (C, projection), and in one GFP-Sec23 expressing S2 cells (C', confocal section). Note that both proteins co-localise significantly.

(D) Double labeling of endogenous dSec16 (10nm) and Sec23 (15nm) on S2 cells frozen sections shows that they both decorate tER sites next to a Golgi stack (G) and an ER cisterna. Arrows indicate a small pool of dSec16 on ER membrane.

(E) S2 cell post nuclear supernatant (PNS) was fractionated in cytosol (C) and membranes (M). The membrane fraction was further separated into pellet (P) and supernatant (S) after treatment with 1% Triton X-100, 0.5mM NaCl or combination of the two. dSec16 is tightly associated to the membrane fraction and is highly soluble in NaCl. Detergent lysate was loaded as control.

Scale bars: 5µm (C) and 200 nm (D).

Figure 2: dSec16 depletion affects the functional organisation of the early exocytic pathway.

(A-C'') S2 cells were mock treated (-ds RNA, A-A'', C) and dSec16 depleted for (+ds dsec16, B-B'', C', C''), and processed for IF (A, A’, B, B”), conventional EM (A”, B””) or cryo-IEM (C, C’, C”). Both the tER marker Sec23 (B) and the Golgi marker dGMAP (B’) are dispersed. The typical tER-Golgi organisation (A”) is absent in Sec16 depleted cells (B’”; brackets indicate a cytoplasmic area where a
tER-Golgi unit would typically be present). IEM of Sec23 (10nm gold) shows that a large majority of Sec23 is displaced from tER sites (C) and is found in the cytoplasm (C’, arrows), whereas a pool aggregates in abortive tER sites (C’’).

(D, E) Anterograde transport of Fringe-GFP (green, D) and Delta (green, E) in mock (-ds RNA) and dSec16 depleted cells (+ds dsec16). Endogenous dSec16 is labeled in red (D, E). The panels presented are confocal sections. Note that upon depletion, both Fringe-GFP and Delta are retained in the ER, exemplified by the nuclear envelope labeling (arrow in D and E). The level of Delta expression is reduced when dSec16 is depleted, suggesting that it is perhaps partially degraded. Scale bars: 5µm (A, B, E and F) and 200nm (C-D).

**Figure 3: dSec16 localises to tER sites in Sec23-depleted cells.**
(A-B) IF localisation of endogenous dSec16 (red) and the Golgi marker d120kd (green) in mock-treated (-ds RNA, A) and Sec23-depleted (+ds sec23, B) S2 cells (C) IEM localisation of dSec16 (10nm gold) in Sec23 depleted cells. The panels presented are projections. Note that dSec16 localises to tER sites in the absence of Sec23. Scale bars: 5µm (A, B) and 200nm (C).

**Figure 4: Sar1 does not mediate dSec16 localisation to tER sites.**
(A) Western blot of immunoprecipitates using an anti GFP antibody on lysates of S2 cells co-transfected with dSec16-V5 and Sar1[H74G]-GFP and dSec16-V5 and Sar1[T34N]-GFP. Transfected dSec16 co-immunoprecipitates with the active form of Sar1. (B) Western blot of Sar1 in mock (-ds RNA) or Sar1 depleted (+ds sar1) S2 cells. (C-D) IF localisation of endogenous dSec16 (red) and the Golgi marker d120kd (green) in mock-treated (-ds RNA, C) and Sar1-depleted (+ds sar1, D) S2 cells. (E) IEM localisation of dSec16 (10nm gold) in Sar1 depleted cells. Note that dSec16 localises to ER cup in the absence of Sar1. Scale bars: 5µm (C, D) and 200nm (E).
**Figure 5: Sar1 localisation to tER sites depends on dSec16**

(A-C) IF localisation of Sar1[H74G]-GFP (green) and the Golgi marker dGMAP (red) in mock- (-dsRNA, A) and dSec16 depleted S2 cells (+ds dsec16, B). Note that depletion of dSec16 completely delocalizes Sar1[H74G]-GFP from the tER sites to the ER (C). In A and B, the panels are projections. In C, the panels are confocal sections.

Scale bars: 5µm

**Figure 6: Ectopically localised Sec16 recruits Sar1-GTP on endosomes**

(A, B) IF localisation of dSec16-V5 (A) or dSec16-FYVE-V5 (B) in S2 cells that have internalized BSA-gold (10nm) using an anti-V5 antibody (red). BSA-gold is visualised by reflective light. Note that dSec16-FYVE-V5 localises in ring-like compartments positive for BSA-gold.

(C) IEM localisation of dSec16-FYVE-V5 in S2 cells that have internalized 5nm BSA-gold using an anti V5 antibody (10nm). dSec16-FYVE-V5 localises to endosomes (E), one of them marked by BSA-gold (E').

(D-E) Gallery of IF localisation of Sar1[H74G]-GFP (green, D-D') and Sar1[T34N]-GFP (E) in cells expressing dSec16-FYVE-V5 (red) fed with BSA-gold (blue, D, D'). D' shows a single endosome at high magnification. The Sar1[H74G]/dSec16/BSA gold positive endosomes are marked by arrows. Note that there are no Sar1[T34N] positive Sec16 rings.

(F) Gallery of IF co-localisation of dSec16-FYVE-V5 (red), Sar1[H74G]-GFP (green) and endogenous Sec31(blue) on endosomes (arrows).

All the panels presented in this figures are confocal sections.

Scale bars: 5µm (A,B), 200nm (C), 2µm (D, E, F).

**Figure 7: Mapping of the dSec16 domain that mediates its localisation to tER sites**

(A) Schematic representation of the truncation and deletion mutants of dSec16. The numbers these constructs are referred to in the text are indicated in between brackets. The degree of colocalisation of these mutants with dGMAP was evaluated. Strong colocalisation was given ++, moderate colocalisation is +, whereas - means hardly or no colocalisation.
(B-D) IF localisation of the indicated constructs tagged with V5 (green) and the Golgi marker dGMAP (red) (B, C, D) and by IEM using the anti-V5 (10nm) (B’, C’). In B and C, the merge of both confocal sections and projections are presented, whereas in D only single channels are shown. NC2.3-CCD-V5 [12] and dGMAP co-localise extensively. Note the fragmented and dispersed pattern of \(\Delta\)NC2.3-dSec16-V5 [13] and its very poor colocalisation with dGMAP (D).

Scale bars: 5µm (B, C, D), 200nm (B’, C’).

Figure 8: The Arginine-rich domain is involved in the dSec16 localisation to tER sites

(A) Alignment of the arginine-rich domain of Sec16 in Drosophila (CG 32654E), in human (hSec16L, KIAA0310), in fowl (Gallus Gallus, XP_415419), in wasp (Nasonia vitripennis, XP_001604818) and in bee (Apis, XP_393378). The number in brackets indicates the start of the motif in each sequence. The conserved arginine (R) are outlined in gray, the conserved acidic aminoacids (D and E), in green, and the conserved S, T and Y in yellow. In blue, aminoacids conserved between Human and Gallus (and xenopus, not shown) are indicated. The conserved aminoacids in all 5 species are marked by a star.

(B) IF localisation of \(\Delta\)Arg-dSec16-V5 [14] (green) and the Golgi marker dGMAP (red). Their co-localisation is very reduced and the \(\Delta\)Arg-dSec16-V5 labeling is mostly dispersed. The left hand panel is a projection whereas the two other panels are confocal sections.

(C) Subcellular fractionation of dSec16-V5, \(\Delta\)NC2.3-dSec16-V5 and \(\Delta\)Arg-dSec16-V5 expressing S2 cells as described in Materials and Methods. Both \(\Delta\)NC2.3-dSec16-V5 and \(\Delta\)Arg-dSec16-V5 show an increase in the cytosolic pool compared with dSec16-V5.

(D) Western blotting of the immunoprecipitated dSec16 oligomeric complex. dSec16-VSVG was co-transfected in S2 cells with the indicated V5 tagged dSec16 constructs. Cells were lysed in 1% TritonX-100 containing buffer and subjected to immunoprecipitation (ip) with an anti-V5 antibody. The ip was immunoblotted with the anti-VSVG and the anti-V5 antibodies.

Scale bars: 5µm (B).
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**Figure D**
- **dSec16-V5**
- **proj**
- **dGMAP**

**Figure B**
- **dSec16-V5**
- **proj**
- **dGMAP**

**Figure C**
- **[12]**
- **proj**
- **dGMAP**

**Figure B'**
- **[12] NC2.3.CCD-V5**
- **proj**
- **dSec16-V5**

**Figure C'**
- **[12] NC2.3.CCD-V5**
- **proj**
- **dSec16-V5**
Drosophila (868) RRSA AEE DDDY ERER NRS--- RRSTKPQSSA EKRSSG G---RRNY ENSGRSARA DDG----RRY
Human long (1111) RRYWCDAF YDAY REHSAFG RRPKRDNNWR YDPRTGS FDDDP DP PH RDPYG BEV E----RRS
Gallus (524) RMY DGP HNY PQKREAPYGN RHQRY DDRWRYDPRTGS FDDPAE PH RDPYG DEE E----RRS
Nasonia (767) RREEID E----- RGRRLD RRGRRR RLDDRRH DRS ADPR RL DREPREF DSRYPD
Apis (991) RRRPDDRKR DRVPREIRDY ORYSDRDYLD RRRRDR --RRRYD YDIRPYR--REYDDP-- YR-- Y

B

[14] proj

[14]
dGMAP

C

PNS  C  M

WB α-V5
dSec16
dSec16ΔNC2.3
dSec16ΔArg

D

empty pMT-V5
dSec16-V5
ΔNC23-V5
ΔArg-V5
NC2.3-CCD-V5
NC2-CCD-V5
NC3.4-CCD-V5
NC4-CCD-V5
CCD-V5

WB α-V5

E

Oligomerisation

dSec16 ++
[3] CCD -
[4] NC3.4-CCD -
[5] NC4-CCD -
[11] NC2-CCD -
[12] NC2.3-CCD -/+ 
[13] ΔNC2.3 -
[14] ΔArg ++