Vesicles versus Tubes: is ER-Golgi Transport in Plants Fundamentally Different to other Eukaryotes ?

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One sentence summary:

This article contains the opinions of four secretory pathway experts on the dynamics of ER membrane export as well as on the ongoing controversy about how ER-Golgi trafficking in plants occurs.

Introduction

Cell biology textbooks usually give a description of the Golgi apparatus as it is seen and functions in a mammalian cell. This is understandable considering the huge body of literature on this organelle in animal cells, especially in relation to disease. It also reflects and is designed to accomodate the large readership from the medical sciences. Indeed, and with few exceptions (e.g. Ito et al., 2014) there are few reviews on the Golgi apparatus which give a balanced account of its structure and function across the whole eukaryotic kingdom.

The plant Golgi apparatus is nevertheless a fascinating organelle that has unique features, especially when compared with its mammalian counterpart. It is polydisperse rather than being present as a continuous perinuclear ribbon, it does not disassemble during mitosis, and it is motile. These characteristics are of great importance, especially since Golgi stacks in plants, along with their protein processing functions, have been termed “polysaccharide factories“ and their activity is essential for formation of a cell wall during cytokinesis and growth. Nevertheless, it is Golgi stack motility that makes bidirectional protein transport between the ER and the Golgi apparatus in plants conceptually more difficult to grasp and therefore even more intriguing than other in other eukaryotic kingdoms.

There is general consensus that in all eukaryotes anterograde protein transport out of the ER is largely dependent upon COPII proteins, and that retrograde transport both within the Golgi stack and between the Golgi and the ER requires COPI proteins (Sztul and Sztul, 2011; Barlowe and Miller, 2014). Since recruitment of the COP proteins in yeast and mammals culminates in the formation of a transport vesicle, it is logical to assume that this may also be the case for plants. However, whereas there appears to be no doubt about the formation and release of COPI vesicles at the periphery of plant Golgi cisternae (Pimpl et al., 2000; Donohoe et al., 2007) the existence of COPII-coated vesicles and their operation as anterograde transport vectors in the narrow (< 500 nm) interface between the ER and Golgi apparatus of land plants is a matter of considerable controversy (Hawes et al., 2008; Hawes, 2012). In contrast, there are numerous examples amongst lower eukaryotes where electron micrographs reveal a Golgi stack immediately adjacent (✝ 500 nm) to transitional ER – per definition a domain of the ER (or NE) showing vesicle budding

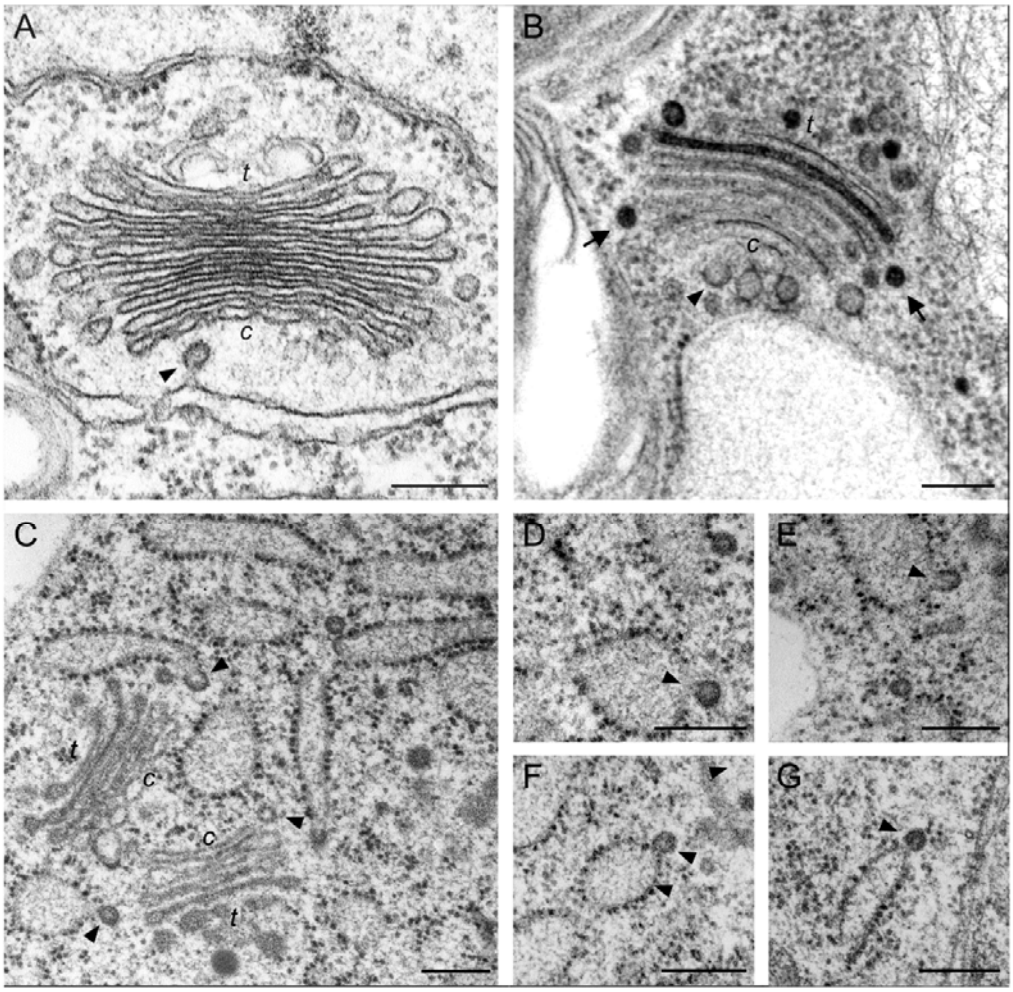

Figure 1

Figure 1: Electron microscopy of COPII-budding. A, B: transitional ER plus adjacent Golgi stacks in the green alga *Chlamydomonas noctigama* as seen in (A) chemically fixed and (B) high pressure frozen samples. The *cis-trans (c,t)* polarity of the Golgi stacks are clearly visible, so too are budding and released COPII vesicles (arrowheads). Putative COPI vesicles are marked with arrows. C: High pressure frozen endosperm cell of *Arabidopsis thaliana*. Budding COPII vesicles are marked with arrowheads, free putative COPII vesicles are marked with arrows. D – G: Collage of COPII budding profiles. Note that many of the buds are at the termini of ER cisternae. Note that the ER in high pressure frozen samples is in general much more dilated than in chemically fixed samples; in *Chlamydomonas* it is extremely dilated (the ER in B, can be recognized by the ribosomes at the left of the vacuole-like structure). Bars are 200 nm. (HPF specimen preparations of *Chlamydomonas* and *Arabidopsis* are courtesy of Stefan Hillmer, Heidelberg and York-Dieter Stierhof, Tübingen).

profiles and lacking ribosomes. The best examples for this are probably the yeast *Pichia pastoris* (Mogelsvang et al., 2003), and the algae *Chlamydomonas* (Fig. 1 A, B; see also Hummel et al., 2007), *Tribonema* and *Melosira* (see Getty Images # 169272449, 128618249). ER vesiculation profiles have often been recorded for

mammalian cells going right back to the early papers of George Palade. Interestingly in all of these cases, as with the algae just mentioned, classical chemical fixation was sufficient to obtain the images. Therefore one would expect that higher plants would be no different in this regard. Unexpectedly, this is not the case. So far, only in rapidly frozen samples has it been possible to visualize ER vesiculation profiles. Even then such images are rare (see Fig. 1 C, D; also Robinson et al., 2007; Kang and Staehelin, 2008; Langhans et al., 2012).

Golgi stacks are invariably associated with tubular ER, and only rarely with the edges of cisternae (Sparkes et al., 2009b). Moreover, In highly vacuolated plant cells such as in the leaf epidermis, Golgi stacks move (several um/sec) in a stop-and-go fashion along the surface of the ER (Boevink et al., 1998; Nebenführ et al., 1999). This contrasts with the situation in mammalian cells and in the aforementioned algae, where the ER and the Golgi are more or less stationary. So is perhaps Golgi motility the clue to the controversy surrounding COPII vesicle identification in higher plants?

The only alternative to vesicle-mediated transport is through some form of interconnecting tubules, either permanent or more probably temporal in nature. If so, the early secretory pathway of plants would appear to be fundamentally different to that of other eukaryotes. The purpose of this article is to examine whether this conclusion is warranted and indeed valid.

Four scientists who have made major contributions in this area have come together to give their views on the matter. However, their divergent opinions have precluded a joint review. It was therefore decided that their opinions should appear separately. Our paper starts with a contribution from Federica Brandizzi who sets the scene at the molecular level, followed by two articles: one summarizing the data pro tubules (Chris Hawes) and the other arguing in favour of vesicles (David Robinson). The final article is from Aki Nakano whose recent successful application of super high resolution microscopy on yeast, allows for new insights into ER-Golgi trafficking in higher plants. We believe that the plant sciences community cannot fail to benefit from witnessing how four experienced cell biologists percieve the current state of play in this controversy. Although being unable to come to a final agreement on this issue, we dwell in the conclusions section on further possible courses of action.

Federica Brandizzi: The "Secretory Units" model for ER protein transport in highly vacuolated cells

In live cell imaging analyses, the Arabidopsis COPII coat components (Sec13, Sec23, Sec24 and Sec31) when expressed in highly vacuolated leaf epidermal cells in tobacco and Arabidopsis have been found in punctate structures that are associated with the ER and move with the Golgi stacks (Stefano et al., 2006; Hanton et al., 2007; Sieben et al., 2008; Wei and Wang, 2008; Faso et al., 2009; Hanton et al., 2009; Takagi et al., 2013; Tanaka et al., 2013), which in fully-expanded plant cells are highly dispersed and motile (Boevink et al., 1998; Stefano et al., 2014). The punctae labelled by the COPII coat proteins are commonly indicated as ER exit sites (ERES). With the exception of Sec13 which has also been found at the nuclear envelope (Yang et al., 2005), Sec23, Sec24 and Sec31 are predominantly localized at such areas. Three-dimensional projection reconstruction of confocal images followed by rendering analyses have shown that Sec16 is localized in cup-like structures where the ER assumes a high-degree curvature (Takagi et al., 2013; see also Fig. 2), supporting the intriguing possibility for specific requirements of ER membrane curvature for the ERES establishment and maintenance. In a Sec24A partial loss-of-function mutant, a functional fluorescent protein fusion to Sec24A has been identified also in bright structures of unknown identity (Faso et al., 2009). It has been hypothesized that such structures may represent ERES in formation or protein aggregates (Faso et al., 2009). The COPII recruiting GTPase, Sar1, has been found at the ERES but also over the ER network to a variable degree that may depend on the specific Sar1 isoform (Hanton et al., 2008). The distribution of Sec16, Sar1 and the COPII coat proteins drastically differs from that of the Sar1-GEF, Sec12, which has been found distributed largely at the ER (Bar-Peled and Raikhel, 1997; daSilva et al., 2004; Yang et al., 2005). Similar to other eukaryotic cells transport of proteins in plant cells may occur by bulk flow, as demonstrated for soluble proteins (Crofts et al., 1999; Phillipson et al., 2001), as well as in dependence of signals that can be present in the transmembrane domain (Brandizzi et al., 2002; Schoberer et al., 2014), or through the presence of specific motifs that are recognized by COPII proteins.

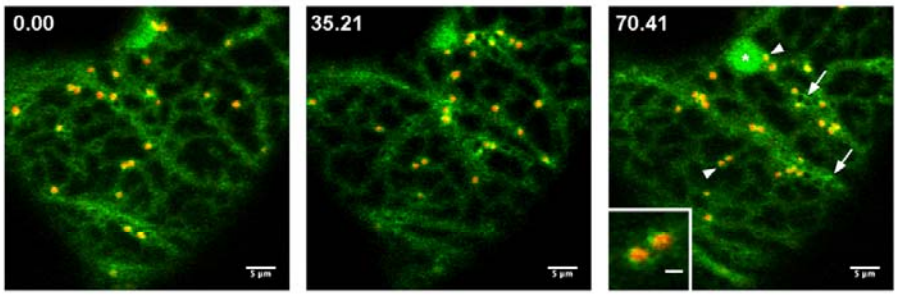

Figure 2

Figure 2: Golgi cisternae (ST-YFP, red) and the ER exit site marker (SEC16-GFP, green) visualized in tobacco leaf epidermal cells. Images from time-lapse sequence acquired at the cortical region of tobacco leaf epidermal cell with a Zeiss LSM510 confocal microscope. The Sec16 marker distributes at the peri-Golgi area (arrowheads) as well as to structures of unknown identity that are not associated with the Golgi marker (Arrows) (see also Takagi et al., 2013). The structures labelled by Sec16 can assume a ring-like shape (Takagi et al., 2013). Bars, 5 µm (1 µm, in inset). Time of frames in the sequence is indicated at the left-hand corner of images (secs). Asterisk indicates a chloroplast, which is visible through chlorophyll autofluorescence. Images by Dr. Luciana Renna, DOE-MSU Plant Research Lab, Michigan State University, East Lansing, USA.

As it would be expected for specialized ER export domains in which cargo is packaged for export and release to the Golgi, ERES are dynamic entities. Indeed photobleaching experiments on fluorescent protein fusions to Sec13 and Sec24 have

shown a high degree on turnover of these proteins on and off ERES. Functional analyses on the effect of Sec16A loss-of-function mutation on COPII assembly in live cells have also demonstrated that Sec16A is involved in the dynamic association of coat components onto the ER because in its absence Sec24 and Sec13 were found to cycle on and off the ERES to a much faster rate than in wild-type cells (Takagi et al., 2013). These findings support the possibility that Sec16 has a regulatory role on the COPII coat assembly likely by influencing the GTPase activity of Sar1, which recruits the outer COPII coat components (Takagi et al., 2013). Increase in ERES size and number was verified when ER-export competent membrane cargo was expressed transiently in tobacco leaf epidermal cells compared to ER-export incompetent membrane cargo and bulk flow cargo (Hanton et al., 2008), supporting that the establishment and maintenance of ERES is responsive to the cell's necessity to export membrane and cargo from the ER. It will be interesting to test in the future whether modulation of ERES number and size depends on Sec16 and its functional interactions with COPII proteins.

A striking feature of the ERES is their movement. The subcellular distribution of ERES with respect to Golgi stacks has been debated for quite some time (Brandizzi and Barlowe, 2013). Although a transient association of the COPII machinery with the Golgi apparatus is plausible if partially coated COPII carriers are linked with the Golgi membrane before the COPII coat is completely shed (Langhans et al., 2012), a recent study on the subcellular localization and function of a plant Sec16 homolog has revealed new insights that further support an association of ERES and motile Golgi (daSilva et al., 2004; Takagi et al., 2013). In particular, through fluorescence recovery after photobleaching analyses using a functional fluorescent protein to Sec16A, Sec16A was found to undergo dynamic binding and release from the membranes to slower rates compared to those of the outer COPII coat components such as Sec13, which was found to interact with Sec16 together with Sec31, and Sec24 (Takagi et al., 2013). If the punctate distribution of fluorescent protein fusion to COPII components observed in live cell imaging studies were the result of association of these proteins with Golgi membranes then Sec 16 should show dynamics on and off membranes similar to the outer COPII components. However, the evidence is that Sec16 cycles on and off the membranes at slower rate compared to Sec24 and Sec13 and that the pool of membrane-associated protein significantly

differs between Sec16 and Sec13 or Sec24 (Takagi et al., 2013) supports that a subpopulation of Sec16 is excluded from the coat of formed COPII carriers and labels ERES that face the Golgi apparatus. The discrete steady-state localization of Sec16A at ERES that are associated with the Golgi stacks supports that the plant ER/Golgi interface is uniquely organized such that ERES move together with associated Golgi stacks. In this model, known as the “secretory units“ model (daSilva et al., 2004), ERES would facilitate ER export to the Golgi at a Golgi-facing surface that is relatively static. Such organization does not exclude the possibility that non-Golgi associated ERES may also exist. In this light, it is possible that ERES/Golgi unit may encounter ERES that are not associated with a Golgi stack and eventually associate with it. It is similarly possible that non-Golgi associated ERES may assemble to form new Golgi stacks. The association of ERES with the Golgi could also favor efficient retrograde transport mediated by cargo carriers such as COPI vesicles that have been clearly visualized in plant cells (Pimpl et al., 2000; Donahue et al., 2007). Arf1, the GTPase involved in COPI coat assembly and dissociation, as well as coatomer, have been localized at the plant Golgi (Stefano et al., 2006; Matheson et al., 2007). It is possible to hypothesize that COPI vesicles might fuse proximally to COPII-enriched ERES. Retrograde transport of membrane from the Golgi at the ER-Golgi interface close to the ERES region could facilitate fast retrieval and concentration of SNAREs and other components of the machinery necessary for anterograde transport from the ER towards the Golgi.

The close spatial relationship between Golgi and ER and the evidence for a continuous exchange of Golgi enzymes with the ER (Brandizzi et al., 2002) raise the question on whether the close association of ERES with Golgi could have a role in holding the ER and the Golgi in close association. Such organization would facilitate ER protein export to a motile organelle. That the plant ER and the Golgi are attached has been demonstrated through the application of optical laser tweezers through which induced movement of tweezers-pulled Golgi stacks caused movement of the ER (Sparkes et al., 2009). It is possible that ERES and Golgi may be tethered by a proteinaceous mesh, which could not only facilitate the ER-Golgi directionality of COPII carriers but also hold the two organelles together. It may also be that ERES have a more direct role in the ER-Golgi interaction. It is also possible that the transient state between formation and dissipation of COPII carriers could enable the

formation of a dynamic bridge that is sufficient to hold the Golgi in place on ERES. Similar to the Golgi biogenesis model proposed earlier (Donohoe et al., 2013), the dynamic attachment of the ER and the Golgi would be facilitated during cisternae biogenesis whereby the most ER-proximal cisterna of the Golgi would be produced de novo by partial or complete fusion of COPII carriers and then mature into a distal cisterna via retrograde recycling of membranes and proteins.

Chris Hawes: Let it be tubes

What can EM tell us?

Back in the 1970's and 1980's prior to the discovery of COPI and COII vesicles, various EM studies of the plant endomembrane system suggested that there may be direct membrane connections between the ER and Golgi bodies which were often termed dicytosomes in those days (e.g. Harris 1979, Mollenhauer at al. 1975). To enable selective enhancement of the membranes of these organelles and to permit studies in three-dimensions using thick sections and high voltage electron microscopy, osmium impregnation techniques combined with either zinc iodide or potassium ferricyanide, to enhance the deposition of osmium on membranes and within the lumen of EM tubules and ER/Golgi cisternae, were often applied (Harris 1979, Hepler 1981). This resulted in a number of reports suggesting that the ER was directly connected to Golgi bodies via membrane bounded tubules and that Golgi bodies themselves presented many tubules at the margins of their cisternae.

In our laboratory we undertook a limited survey of ER-Golgi interactions in a number of tissues using the ZIO osmium impregnation technique (Juniper et al. 1982) and concluded that in maize root caps, bean root tips and leaves (probably erroneously) that the ER and Golgi were not connected. However, in the heavily protein secreting glands of the Venus's Fly Trap, after stimulation of secretion, we observed numerous tubular extensions from cisternal rims, including *cis*-cisternae, that appeared to interconnect with the ER. At the same time a study of the Golgi apparatus in developing wheat endosperm Parker and Hawes (1982), using high voltage electron microscopy of thick sections of osmium impregnated tissue, reported fine peripheral cisternal tubules connecting the *cis*-Golgi as well as other cisternae to the ER. This

was also reported in in developing bean and mung bean seeds (Harris 1979, Harris & Oparka 1983). In wheat these fine tubules were often much thinner than an ER tubule and unless heavily osmium impregnated, under normal contrasting conditions in the EM would unlikely scatter sufficient electrons to be readily visible. More recently we reported ER-Golgi connections in tobacco leaves using osmium impregnation (Brandizzi et al. 2002). Of course the presence of tubules in electron micrographs does not prove they are involved in transport, retrograde or anterograde, but does show perhaps a more intimate relationship between the two organelles than is often assumed. Likewise, it has been shown that the ER surface itself is highly motile (Runions et al. 2006), therefore conceptually there should be no problem in envisaging membrane flow from the ER through to the Golgi

In contrast to these results there are relatively few reports of vesicles budding from the ER or existing at the interface between the ER and Golgi, although as far back as 1980, Schnepf and Christ suggested that a vesicle flow exits in the epithelial cells of the nectaries of *Asclepias curassavica.* Only in a few publications using ultra rapid high pressure freeze-fixation followed by freeze-substitution and electron tomography have shown COPII vesicles budding from the ER (Kang & Staehelin 2008, Donohoe et al. 2013, Hawes 2012), mainly in root meristem cells. An unpublished example in developing arabidopsis endosperm tissue can be seen in Figure 1C-G of this paper. Unfortunately, three-dimensional analysis and reconstruction by electron tomography of data such as these still relies on manual tracing of images as auto-segmentation algorithms cannot as yet differentiate the subtle differences in contrast in such electron micrographs to permit totally unbiased autosegmented reconstructions. However, it is possible to observe tubular cnnections between ER and *cis*-Golgi in tomograms of osmium impregnated root material (Hawes unpublished, Fig. 3A).). At this stage it should be noted that lack of COPII vesicles is not restricted to plants, but has been reported in several micro-organisms. Likewise depletion of COPII components does not always inhibit cargo transport from the ER in yeasts and animals (see Mironov 2014 for references).

An argument has been made on the lack of COPII EM images based around the calculation that in reality there are very few COPII vesicles at any one time in the ER-Golgi interface and that in any one thin section at most only one vesicle would be

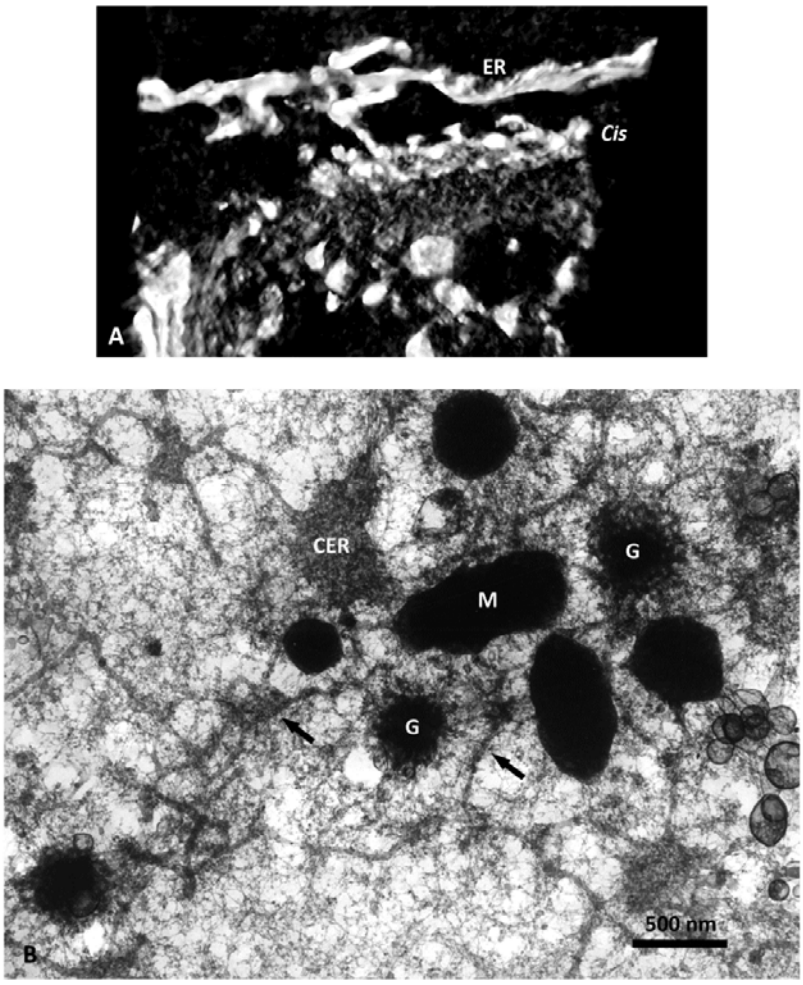

Figure 3

Figure 3: A: Maximum intenstity projection in negative contrast of a stack of thin sections from a tomogram of a pea root tip Golgi body and associated ER impregnated by the osmium zinc iodide (ZIO) technique. The reconstruction is presented at an angle to show a clear tubular connection between the ER and *cis*-Golgi. (Reconstruction courtesy of Louise Hughes). B: Inside face view of a dry-cleaved carrot suspension culture cell. The cell had been fixed on a coated EM grid, dehydrated and critical-point dried prior to dry-cleaving on double sided tape. The view onto the plasma membrane shows dark mitochondria (M), complete Golgi stacks in face view (G), plus cisternal (CER) and tubular ER (arrows). Not the huge difference between the diameter of a Golgi body and ER tubules.

seen (Langhans et al 2012, this article). This argument of course only holds true if such vesicles do exist, if they don't then they obviously they would not be seen. Likewise if tubules are transient in nature they would rarely be seen in conventional electron micrographs and when caught in cross section would appear to be vesicular!

An obvious experiment where COPII vesicles should be seen is in the reformation of Golgi after Brefeldin A induced reabsorption into the ER. One such study on tobacco BY2 cells reported buds on the ER surfaces, which were infrequent, and tubular vesicular clusters representing the earliest observable stage of stack regeneration cells (Langhans et al. 2007). These clusters immunolabelled for COPI coat components but not for COPII proteins. Considering the number of Golgi stacks in such cells which is in the hundreds, if COPII vesicles exist then it is surprising that none were seen in these experiments.

Of course the “get out of jail” card and perhaps a lazy answer to all of these discrepancies in the ultrastructural literature is simply to state that data from chemically fixed material is artefactual in nature due to slow fixation rates and only data from ultra-rapidly frozen freeze-substituted material is acceptable. This of course ignores the fact that in chemically fixed material it is easy to visualise both COPI and clathrin coats, so why not COPII coats, especially as they are relatively easy to visualise in chemically fixed cells of algae such as chlamydomonas (Hummel et al. 2007)? Two other golden rules of thin section transmission electron microscopy also have to be remembered: 1. A thin section presents a two dimensional image, thus a tubule in cross-section can easily be mis-interpreted as a vesicle, 2: Any biological material has to scatter sufficient electrons to form an image. Thus a membrane in transverse section, spanning 70nm of resin, scatters sufficient electrons to form a classic unit-membrane image, whereas the same stained membrane in face view may not present sufficient heavy metal stain molecules and thus be electron lucent and not form an image, thus fine tubules and membranes in face view can be missed. Selective membrane staining techniques overcome this latter limitation. Of course other EM techniques exist such as freeze-fracture or freeze-fracture deep-etch, which should reveal structured exit sites on ER and COPII coats, but as far as I am aware, apart from the occasional image showing clathrin coated vesicles and COPI vesicles (Coleman et al. 1987, Andreeva et al. 1998, no such images of COPII structures have been published in plants.

Has live cell imaging helped?

Our initial observations on Golgi and ER in living leaf epidermal cells let us observe, for the first time, the dynamic nature of the organelles and the fact that Golgi bodies in leaves appeared to move over the surface of the ER (Boevink et al 1998). This led us to propose the "hoover model" of Golgi bodies travelling over the ER surface sucking up vesicles produced by the ER, thus making the serious, but all too common, mistake of assuming that the plant ER-Golgi interface would function exactly the same as the mammalian ER in the production of COPII vesicles. However, over the past decade or so we have refined our ideas and developed the "secretory unit" concept of ER exit sites and Golgi bodies travelling as single units around the cell with the motile surface of the ER (daSilva et al 2004, Langhans et al. 2012). Such advances were made possible by the revolution in live cell imaging offered by fluorescent protein technology and direct organelle labelling combined with techniques such as photobleaching and photoactivation of fluorescent probes. This enabled a range of experiments to be undertaken on the ER Golgi interface, and contrary to what is often stated it was shown there is no real evidence that transport between the ER and individual Golgi bodies only takes places when stacks are stationary (the stop and go model, Brandizzi et al. 2002). Indeed we demonstrated that in a FRAP experiment, recovery of fluorescence of a Golgi membrane protein could be demonstrated in moving Golgi indicating a continual transfer of protein from ER to Golgi (da Silva et al. 2004). Subsequently laser manipulation of Golgi has demonstrated that when captured and translated through the cytoplasm by an infrared laser beam, Golgi bodies almost always drag a tubule of ER behind them (Sparkes et al. 2009a). One of the conclusions from this work was that the ER and Golgi are closely associated and tethered together not via the cytoskeleton but most likely by a number of structural proteins such as the Golgins/Golgi matrix proteins (Hawes et al. 2008). In studies of Golgi destruction and reformation in tobacco leaf cells we showed that with either a genetic block of secretion or inhibition by Brefeldin A, reabsorption of the Golgi back into the ER was an ordered process, starting with the release of *trans*-located Golgins into the cytoplasm followed by sequential reabsorption of *trans,* medial and *cis*-membranes into the ER. However, a *cis*-located matrix component, CASP remained associated with ER exit sites and we suggest that this protein may be part of the tethering complex holding the Golgi to the ER (Osterrieder et al. 2010, Schoberer et al. 2010). How, this ordered membrane

transport back to the ER is mediated is not known perhaps some of the cisternal associated Golgi-ER tubules are involved in this pathway.

Interestingly, Golgi bodies in vacuolated tissue such as leaf epidermal cells are only ever associated with curved membranes of the ER such as tubules or more rarely the edges of cisternae (Sparkes et al. 2009b) and are never seen on the flat faces of ER cisternae. This situation has also been reported for yeast, where ERES were associated with high curvature domains containing the membrane curving ER proteins reticulon 1 (Okamoto et al. 2012), and we have preliminary evidence that in arabidopsis reticulons may interact with SEC12 the Sar1-GEF which recruits the GTPase to the ER membrane as part of the COPII coat building process (Kriechbaumer & Hawes unpublished). Thus, we can conclude that plant ERES probably requires a curved ER surface on which to form.

Due to the nature of fluorescence and the diffraction limit of the microscopies used, an artificial impression is given in typical confocal micrographs of the diameter of ER tubules, , in comparison to the diameter of a Golgi body (around 1 micron), giving a ratio of approximately 1:2. However when observed by EM techniques such as dry cleave to show the cortical ER, whose tubes are in reality are around 30 to 90 nm, and associated Golgi, it is obvious that this ratio is more like 1:8 even taking into account the fact that *cis*-Golgi cisternae tend to be smaller in diameter than the rest of the stack (see Fig. 3b). Thus, ER exit sites have to form on a relatively restricted area of highly curved membrane and not a flat surface, and perhaps the requirement for membrane bending proteins to produce a bud is lessened. Also, an ER exit site would need to be linear in structure along roughly 200nm of ER tubule. Could such a structure produce sufficient 70nm vesicles to transfer the required protein and membrane to a Golgi body? Such calculations have not yet been made.

Of course, it is generally accepted that COPII components are required in plants for some if not all transport between the ER and Golgi. Indeed as described above it is easily demonstrated that inhibition of the formation of a COPII coat by expressing a non-hydrolysable form of the coat initiating GTPase SAR1p results in the disruption of Golgi stack homeostasis and resorption of Golgi membrane back into the ER (Osterrieder et al 2010). However, coating of a membrane patch to promote curvature and to concentrate cargo at the tip of a transiently produced tubule is a

distinct possibility. Indeed it has been shown *in vitro* that COPII components can tubulate liposome membrane (Bacia et al. 2011) and it has even been suggested that COII coat components have sufficient flexibility to form 300nm tubular structures that can accommodate large filamentous cargo such as procollagen fibrils (Miller & Scheckman 2013). Such tubes could easily span the narrow >300nm interface between ER and *cis*-Golgi in plants.

David G. Robinson: The odds are stacked in favour of vesicles

The mobile secretory unit: what are the consequences for bidirectional ER-Golgi traffic?

In the frequently used leaf epidermal system it is well-established that COPII-fluorescence labelling always colocalizes with fluorescent Golgi markers, whether the Golgi stacks are mobile or not (daSilva et al., 2004; Hanton et al., 2009). For most researchers punctate fluorescent COPII-signals on the surface of the ER are synonymous with ER exit sites (ERES), but this may not necessarily be so since the visualization of COPII-binding does not actually reveal the actual exit event. Langhans et al. (2012) have also questioned the fidelity of COPII-fluorescence labelling in recording ERES on the surface of the ER, and have suggested instead that the signals may instead represent pre-fusion COPII vesicles lying in the interface between ERES and the *cis*-Golgi. Despite these caveats it seems that anterograde traffic from the ER is restricted to a domain of the ER immediately adjacent to a Golgi stack, and is embodied in the concept of the “secretory unit“ (da Silva et al., 2004; Hanton et al., 2009). It now appears that retrograde traffic between the Golgi and the ER is also spatially restricted, since it has been demonstrated that the t-SNAREs required for COPI vesicle fusion with the ER localize to ER domains immediately underneath Golgi stacks (Lerich et al. 2012). This unique feature of the secretory pathway in plants probably serves the purpose of control and regulation, since the problem of stochastic release of COPII vesicles into the cytosol and their subsequent capture by Golgi stacks is avoided.

The concept of the *secretory unit* received great support from laser-trapping technology, which demonstrated that ER tubules moved with individual Golgi stacks when the latter were displaced (Sparkes et al., 2009). This key observation could be interpreted as proof of direct membrane continuities between the ER and the *cis*-Golgi, but in the opinion of the majority it is a consequence of the existence of tethering/matrix proteins which not only anchor the Golgi stack to the ER surface (Latijnhouwers et al., 2005; Osterrieder et al., 2012) but also maintain the integrity of the Golgi stack (Ito et al., 2014). An important feature of these experiments is that the Golgi stacks were immobilized by actin inhibitors, obviously a prerequisite for capturing otherwise mobile Golgi stacks. In this situation interlocking matrix protein interactions between the ER and the Golgi are probably comparable to the real-life situation of a “stop“ period in Golgi travel. However, it remains unclear as to whether the actual exit of anterograde cargo from the ER i.e. vesicle budding (or COPII tube formation) is restricted to the stationary phase or occurs continuously during Golgi movement. We also do not know whether antero- and retrograde transport are separate or coordinated, synchronized events. Lerich et al. (2012) have suggested that pre-fusion clusters of COPII- and COPI-vesicles might accompany mobile Golgi stacks, but the colocalization of Golgi stacks with the Qc-SNARE SYP72 of the t-SNARE fusion complex only in the immobilized condition suggests that entry of COPI retrograde cargo into the ER is restricted to stationary Golgi stacks.

Why are COPII vesicles difficult to visualize in higher plants?

COPI vesicles have been detected at the periphery of Golgi cisternae in higher plants (Pimpl et al., 2000; Donohoe et al., 2007). Although their positive identification as retrograde carriers to the ER in plants awaits confirmation, it is very likely that they do fulfill this function. This being so, there seems to be no *a priori* reason for questioning the participation of COPII vesicles in anterograde ER-Golgi traffic, yet their visualization in higher plant cells has proved difficult. In mammalian cells, there is a transport intermediate between the ER and the perinuclear immobile Golgi complex known as the ER-Golgi intermediate compartment (ERGIC; Appenzeller-Herzog and Hauri, 2006). It is this structure rather than the Golgi apparatus which is engaged in bidirectional COPII/COPI-mediated trafficking. During its formation (apparently through homotypic COPII vesicle fusion) and before it begins moving in the direction

of the Golgi complex, it lies close to the surface of the ER (200-500 nm distant) in the immediate vicinity of ER export sites. Nevertheless, free COPII-coated carriers/vesicles have been reported on several occasions in the ER/ERGIC interface (Zeuschner et al., 2006; Hughes et al., 2009; Witte et al., 2011). In support of these observations, it has been recently demonstrated that loss of function of a cytosolic protein complex (TFG, Trk-fused gene) which forms aggregates that temporarily trap COPII carriers in the ER/ERGIC interface, causes free COPII-carriers to accumulate throughout the cytoplasm (Johnson et al., 2015).

A similarly narrow interface exists between ER and the *cis*-Golgi (the probable ERGIC equivalent in plants) so why the problem in seeing COPII vesicles in thin sections of higher plant cells? Langhans et al. (2012) have attributed this to the relatively small numbers of transport vesicles (at the very most 20) in the interface at any one time which extrapolates to only a single vesicle in a thin section. There may be other contributing factors. For example the speed of vesicle transport, and obviously if bidirectional transport is restricted to the stationary phase of Golgi movement, the timing of transport. The chances of visualizing transport vesicles in the ER-Golgi interface being seriously affected by the mobile status of the Golgi stack at the moment of fixation.

Have "gap-spanning" tubules been observed in the interface between the ER and cis-Golgi?

This can be answered with a categorical no, despite opposing claims made in some recent reviews (Sparkes et al., 2009; Stefano et al., 2014). A careful scrutiny of the electron micrographs in the papers cited in these reviews (see section Hawes for details) in support of direct membrane continuity at the ER/Golgi interface, reveals that the connections are not at the interface but are in fact lateral connections between undefined, but probably median Golgi cisternae and the ER. Moreover, the frequency of such continuities is enhanced under non-physiological conditions, e.g. cold (Mollenhauer et al., 1975) or brefeldin A (BFA) (Ritzenthaler et al., 2002) treatments. The physiological significance of such lateral connections remains obscure, especially since it is not in harmony with the glycoprotein processing reactions which are supposed to occur in a sequential manner through the Golgi

stack (from *cis* to *trans*). Also, lateral connections of this type are difficult to reconcile with Golgi stacks gliding over the surface of the ER. Finally, if tubular connections between the ER/NE and the *cis*-face of a Golgi stack do exist, one would expect that the chances of visualizing them would be greater in those cases where the Golgi is immobile. However, such structures have never been seen in algal cells with naturally stationary Golgi stacks, nor in higher plant cells where the Golgi has been immobilized through actin inhibitors.

What are the advantages of vesicles?

Vesicles allow organelles to communicate among themselves and with the cell exterior (via the plasma membrane). With such transport vectors, cargo molecules can be transferred between organelles without disturbing their integrity. By excluding certain molecules and including others, vesicles also allow for a high degree of selectivity in intracellular transport. The efficient sorting of proteins into vesicles is to a great extent related to coat proteins at their surface which interact with several different types of integral transmembrane proteins: cargo receptors, helper proteins such as the p24 proteins and SNAREs. All of the coat proteins (COPI, COPII and clathrin) can polymerize to form spherical structures (cages) and thus have the inherent ability to form vesicles, although it is true that COPII does it differently to COPI and clathrin (Hughson 2010). However, the special properties of the COPII coat allow the incorporation of cargos of different sizes, something which cannot be achieved with clathrin or COPI vesicles. Therefore, COPII vesicles of different sizes can accommodate export of different cargo from the ER (Miller and Schekman 2013) without the need for direct tubular connections. Finally, by concentrating SNAREs into a small amount of membrane the efficiency of vesicle fusion with a specific target compartment is increased. Indeed, the inhibition of vesicle formation can lead to uncontrolled fusion of organelles mediated by v- and t-SNAREs as seen for the Golgi-ER hybrid structures formed in the presence of BFA (Elazar et al., 1994; Ritzenthaler et al., 2002).

In contrast, tubular contacts (irrespective of their longevity), or direct contacts between organelles culminating in fusion, then fission (as for example in “hug and kiss“ models, Kurokawa et al., 2014), have the inherent caveat that an unspecific

516 mixing of organelle contents may occur. Another problem is that if there is direct
517 contact between the lumina of two adjacent compartments how can pH differences
518 can be maintained? This is particularly important in the case of the ERD2 (KDEL)
519 receptor which in mammalian cells is thought to bind to its KDEL-ligands at an acid
520 pH (pH 6.7) in the *cis*-Golgi and to release them at the higher pH (pH 7.2) of the ER
521 lumen (Wilson et al., 1993; Majoul et al., 1998; Paroutis et al., 2004). As is the case
522 with the mannosyl-6 phosphate receptor in mammalian endosomes, apparently only
523 a relatively small shift in pH is sufficient to cause ligand release. A similar pH gradient
524 of about 0.5 pH units between the ER and the Golgi also exists in plants (Martiniere
525 et al., 2013) and it has recently been shown that the binding of COPII proteins to a
526 plant ERD2 homolog is favored at neutral pH conditions whereas the recruitment of
527 COPI proteins to ERD2 is more optimal at an acidic pH (Montesinos et al., 2014).
528
529 Bidirectional protein trafficking between the ER and the Golgi apparatus also entails
530 the movement of membrane. In the anterograde direction this serves the purpose of
531 replenishing membrane lost at the *trans*-face of the Golgi stack through release of
532 the TGN (Viotti et al., 2010) and continually drives the process termed cisternal
533 maturation. In the retrograde direction it is a consequence of receptor recycling.
534 While vesicles clearly fulfill this requirement, it is less easy to see how tubes can
535 achieve it. If ERES and *cis*-Golgi cisternae briefly touch each other, fuse and rapidly
536 separate there can be no net movement of membrane at all. This is a stringent
537 interpretation of the “hug and kiss“ model of Kurokawa et al. (2014). However, if there
538 is an active uptake of membrane (a patch of previously COPII coated ERES)
539 together with soluble cargo as the *cis*-Golgi docks onto ERES, the model should
540 perhaps be renamed “hug and bite“.
541
542 As new data becomes available researchers are often compelled to revise their
543 standpoints on particular issues. My initial interpretation of ER-Golgi traffic, based on
544 immunofluorescent studies in tobacco BY-2 cells (Yang et al., 2005) led me to
545 believe that ER exit sites were greatly in excess of Golgi stacks. Switching to leaf
546 epidermal cells and perhaps more stringent localization criteria via transient
547 expression of (X)FP-tagged, convinced me of the validity of the secretory unit
548 concept. Nevertheless, I was never in doubt about vesicles, and I maintain that the
549 unique features of the secretory unit, i.e. mobile Golgi stacks with a narrow interface

to the ER, are not necessarily an impediment to COP-vesicles as mediators for bidirectional protein traffic between the ER and the Golgi apparatus in higher plants. In contrast, direct membrane continuities between the ER and the Golgi have physiological drawbacks, and there is no convincing evidence for their existence, even in organisms where Golgi stacks remain permanently stationary.

Akihiko Nakano: A common mechanism for ER-to-Golgi traffic -- a view from super-resolution live imaging microscopy

How proteins traffic between the ER and the Golgi apparatus is an interesting and controversial issue. Problems have arisen in part through limitations of time and space resolution in observing the two organelles. My opinions section will focus on how we have tackled this problem by super-resolution live imaging.

The budding yeast *Saccharomyces cerevisiae* has been used as an ideal model system to study molecular mechanisms of membrane trafficking, because it is amenable to both genetics and biochemistry. In addition, its simplicity in organellar organization offers advantages in live imaging. We have applied high-speed confocal microscopy (Nakano, 2002) to *S. cerevisiae* and have made many discoveries that would have never been possible without live imaging at high spatio-temporal resolutions. Here, I will describe first what we learned from this yeast, and then move on to compare similarly obtained data from plant and animal cells.

Golgi cisternae do not stack in *S. cerevisiae* (Glick and Nakano, 2009; Suda and Nakano, 2012), but not all budding yeasts have this feature. *Pichia pastoris* has for example stacked Golgi. This peculiar property of *S. cerevisiae* provides a wonderful opportunity to observe individual Golgi cisternae in living cells. Our and Ben Glick's groups demonstrated that the yeast Golgi cisternae change properties from *cis* to medial and then to *trans* over time (Losev et al., 2006; Matsuura-Tokita et al., 2006). This gave strong support for the cisternal maturation model of intra-Golgi protein transport (Glick and Nakano, 2009; Nakano and Luini, 2010).

583 During the course of our studies on *S.cerevisiae*, we found that the microscopic
584 method we were using (combination of a high-speed spinning-disc confocal scanner
585 and a high-sensitivity camera system) had a great potential in improving resolution
586 not only in time but also in space (Matsuura-Tokita et al., 2006). I will skip the details
587 here, but briefly, accurate image acquisition and minute data processing by
588 deconvolution allow for amazing super-resolution beyond the diffraction limit
589 (Kurokawa et al., 2013). Indeed, we have achieved 50-60-nm resolution in 3-
590 dimensional space with the time resolution of a few seconds per volume (the spec is
591 further rising now). This method has been given the acronym SCLIM, standing for
592 super-resolution confocal live imaging microscopy (Kurokawa et al., 2013).
593
594 With such a high spatiotemporal resolution, we next tried to understand how *cis*-Golgi
595 cisternae form. According to a classic cisternal maturation model, newly formed
596 COPII vesicles containing cargo fuse with each other and *cis*-Golgi proteins would
597 join via COPI vesicles. First, we observed that the fluorescence of COPII coats,
598 indicators of ERES, shows a very dynamic behavior. The ERES enlarge and shrink,
599 and are often very mobile. They appear to be stabilized when approaching high-
600 curvature ER domains, such as the saddle-shaped surface of the ER sheet edge and
601 along the ER tubules (Fig. 4). On the Golgi side, *cis* cisternae show a significantly
602 high probability of staying in the vicinity of the ERES, whereas *trans* cisternae do not
603 (Okamoto et al., 2012).
604
605 A more detailed analysis of their behavior unveiled that the *cis*-Golgi frequently
606 approaches the ERES, keeps contact for a few seconds, and then leaves there. In
607 addition, the fluorescence intensity of COPII often goes down upon this contact,
608 suggesting an uncoating event during this process. *trans*-Golgi cisternae also
609 approach the ERES, but do not share the same tendency for a collapse of the COPII
610 coat (Kurokawa et al., 2014). We reasoned that what we later termed the “hug-and-
611 kiss” behavior of the *cis*-Golgi towards the ERES indicates the capture of newly
612 forming COPII vesicles followed by their uncoating and subsequent fusion with the
613 *cis*-Golgi. To confirm this, we set up a system to pulse-chase the cargo by live
614 imaging. Fluorescent cargo that is synthesized and accumulated in the ER at a high
615 temperature (39°C) proceeds to the secretory process upon shift down to a low
616 temperature (25°C). Cargo first relocates to the ERES and then gradually moves to

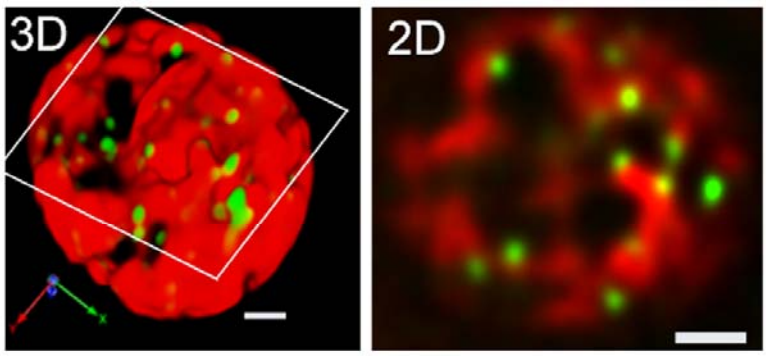

Figure 4

FIgure 4: Organization of the ER exit sites in the budding yeast *Saccharomyces cerevisiae.* (left) Dual-color three-dimensional (3D) image of Sec13–GFP (ERES marker, green) and mRFP–Sec12 (bulk ER marker, red) obtained by SCLIM. (right) Two-dimentional (2D) slice image taken from the 3D data. ERES localize at the high-curvature domains of the ER such as along tubules and at the edge of the sheet. Bar: 1 μm. Photograph by Kazuo Kurokawa, Live Cell Super-Resolution Imaging Research Team, RIKEN Center for Advanced Photonics (see also Okamoto et al., 2012).

the Golgi apparatus. When *cis*-Golgi approaches the ER, the cargo signal overlaps with the *cis*-Golgi signal, and then the *cis*-Golgi leaves the ER together with the cargo (Kurokawa et al., 2014). Thus we conclude that cargo is delivered from the ERES to the *cis*-Golgi through such a hug-and-kiss event. This seems to be a safer and more

efficient way to send cargo to the destination than by releasing free COPII vesicles into the cytosol. It also explains why it has been so difficult to observe COPII vesicles by electron microscopy. Now, this hug-and-kiss delivery of cargo from the ER to the Golgi raises many new questions:

1) Do the COPII signals seen in our experiments represent clusters of COPII vesicles or patches of COPII coat?

-- We do not know at the moment. Their sizes and shapes vary dynamically, suggesting that they do not correspond to individual single COPII buds. Considering the flexible nature of COPII coat (Miller and Schekman, 2013), they could be either clusters of COPII buds or large patches of COPII coat as has been proposed for clathrin coats.

2) Is the COPII vesicle formation completed before or during the hug and kiss? In other words, when does the fission of COPII vesicles occur?

-- This is a good question. As the sorting of cargo from ER resident proteins must occur during the budding event, discontinuity would be desired. But as has been discussed for the presence of tubular connections in the Golgi stacks (Glick and Nakano, 2009; Nakano and Luini, 2010), a physical discontinuity of membrane may not be necessarily required for sorting of proteins.

3) If the hug-and-kiss mechanism ensures efficient and safe transfer of cargo, why are COPII vesicles necessary?

-- We are not proposing that COPII vesicles are not released at all. At least in certain i*n vitro* or cell-free reconstructed systems, COPII vesicles do form in a Sar1 GTPase-dependent manner (Oka and Nakano, 1994; Barlowe et al., 1994; Matsuoka et al., 1998; Sato and Nakano, 2004; Tabata et al., 2009). In *sec17* (SNAP) and *sec18* (NSF) mutants, which are defective in vesicle fusion, numerous vesicles including COPII vesicles accumulate in the cytosol (Novick et al., 1980). However, releasing free vesicles in a large amount would be wasteful and even dangerous. Under normal conditions, we believe that complete release of COPII vesicles is maintained to a minimum.

4) If the *cis*-Golgi acts as a preexisting compartment to capture cargo from the ER, is there a stable pool? How does this model reconcile with the cisternal maturation from *cis* to *trans*?

-- In our previous work (Matsuura-Tokita et al., 2006), we stated that the *cis*-Golgi appears to form *de novo*. However, with improved resolution it looks more likely that small structures containing a *cis*-Golgi marker move around and grow over time before entering the maturation phase. We usually use Sed5 (the counterpart of SYP31/32 of plants and syntaxin-5 of mammals) as a *cis*-Golgi marker, but considering the findings we have made in tobacco BY-2 cells (Ito et al., 2012), the yeast Sed5 compartment may represent both a sorting platform like the ERGIC as well as the first enzymatic station of the glycosylation factory (see also below).

5) Is a hug-and-kiss mechanism specific to yeast?

-- This is also a frequently asked question. We believe that the answer is no. In the remaining part of my essay, I would like to proceed to a comparison between yeast and other eukaryotes.

Plant cells have beautiful stacks of the Golgi apparatus, which are largely associated with the ER. To try to address how Golgi stacks are assembled and maintained in tobacco BY-2 cells, we set up a system for live imaging, in which Golgi stacks are disassembled by brefeldin A (BFA) treatment and reformed after BFA removal (Ito et al., 2012). While most of Golgi markers were absorbed into the ER upon BFA treatment as previously reported (Takeuchi et al., 2000, 2002; Ritzenthaler et al., 2002), we realized that some of the *cis*-Golgi markers (SYP31 and RER1B) remained in the cytosol as small punctate structures. Another *cis*-Golgi marker, ERD2, diffuses into the ER together with medial and *trans* markers, suggesting that there are two different classes of *cis*-Golgi proteins. Upon BFA wash, the small punctate structures of SYP31 nucleate to build new cisternae, to which other Golgi proteins then join in a *cis*-to-*trans* sequence. The small punctate structures of SYP31 are in the close vicinity of the ERES but do not completely overlap with them (Ito et al., 2012).

All these observations suggest that the SYP31 compartment represents a scaffold to build the Golgi stack around like the ERGIC (ER-to-Golgi intermediate compartment)

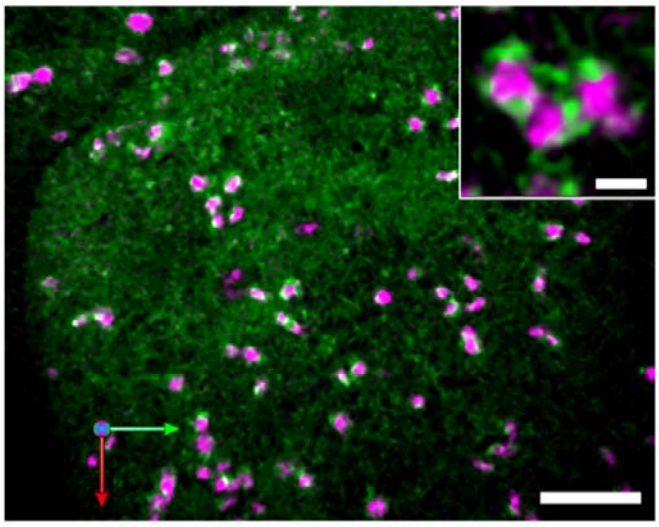

Figure 5

Figure 5: *cis*-Golgi cisternae (mRFP-SYP31, magenta) and the ER exit sites (SEC13-YFP, green) visualized in tobacco BY-2 cells. Confocal images were captured by SCLIM and reconstructed into 3D with deconvolution. A typical trajectory image from a 3D time-lapse movie is shown. Almost all of the Golgi stacks were associated with bright spots of ER exit sites. From the magnified image (inset), we could observe the ER exit sites surrounding the *cis*-Golgi making ring-shaped fluorescent patterns. Bar, 5 μm (1 μm in inset). Photograph by Yoko Ito, Live Cell Super-Resolution Imaging Research Team, RIKEN Center for Advanced Photonics. (see also Ito et al., 2012).

688 of mammalian cells. The other type of *cis*-Golgi, in which ERD2 resides, may be a
689 little distal to the SYP31 compartment. Indeed simultaneous imaging of SYP31 and
690 ERD2 shows their slightly different localizations in the stack (Yoko Ito, unpublished
691 results). Distinction of the *cis*-most cisterna of the Golgi from the later compartments

692 in terms of function has also been reported by electron tomography. The *cis*-most
693 cisterna is proposed to function as a protein-sorting platform, whereas the later
694 compartments are involved in the biosynthetic activities of the Golgi (Donohoe et al.,
695 2013). Regarding the relationship with the ERES, it should also be mentioned that, in
696 our study of BY-2 cells, the number of ERES is larger than that of *cis*-Golgi cisternae
697 (see Fig. 5). The ERES without associated Golgi stacks are extremely mobile and
698 may become stable when they encounter the Golgi (Ito et al., 2012).
699
700 Consideration of the role ERGIC plays for a subset of *cis*-Golgi compartments leads
701 to the notion that it may be a very general feature of ER-to-Golgi trafficking (Ito et al.,
702 2014; Kurokawa et al., 2014). It is widely accepted that ERGIC is a typical structure
703 of vertebrate cells, which have radial organization of microtubules, and receives
704 cargo from the ERES in the cell periphery before transporting it to the Golgi ribbon in
705 the centrosomal region. Other organisms that do not have such astral patterns of
706 microtubules, e.g. invertebrates, plants and fungi, are believed to lack ERGIC.
707 However, as discussed above, plant cells appear to have a structure functionally
708 related to ERGIC. Drosophila cells, which have dispersed Golgi, have also been
709 reported to have an ERGIC-like structure (Witte et al., 2011). Furthermore, even in
710 the yeast *S. cerevisiae*, we now propose that the mobile Sed5 structures that show a
711 hug-and-kiss action towards ERES may be regarded as ERGIC-like (Kurokawa et al.,
712 2014). ERGIC of mammalian cells is closely associated with the ERES (Budnik and
713 Stephens, 2009), and so is the Drosophila ERGIC (Witte et al., 2011). A SCLIM
714 image of ERES and *cis*-Golgi in a BY-2 cell is presented in Fig. 5. Altogether, the
715 currently available data envisage a common mechanism of cargo delivery in the
716 spatially close relationship of ERES and ERGIC (or its counterpart). Such an
717 association of ERES and ERGIC is relatively stable in the cases of mammals,
718 Drosophila, plants and the yeast *P. pastoris*, but is transient in *S. cerevisiae*.
719 Interaction of these two compartments must require special molecular machinery,
720 and we have proposed a role for the tethering factor Uso1 (yeast counterpart of
721 p115) in the association of ERES and the *cis*-Golgi. Interestingly, when the Uso1
722 function is compromised by a temperature-sensitive mutation, the action of *cis*-Golgi
723 is frozen during the hug and kiss event (Kurokawa et al., 2014).
724

There are still many problems remaining. Obviously, the observation of COPII vesicles in the spatiotemporal resolution good enough to distinguish individual dynamics has urgent priority for us. There is biochemical evidence that cargoes are selected during COPII vesicle budding (Sato and Nakano, 2005) and ER-Golgi shuttling proteins are retrieved back from the Golgi to the ER via COPI vesicles (Cosson et al., 1998; Sato et al., 2001). These events also await a revisit by live imaging. We are now in the process of developing the 2nd generation SCLIM, which will provide us with far better images of what is going on in the narrow interface between the ER and the Golgi apparatus.

Conclusions

After having gone through the information and arguments presented above, it will not come as a surprise to the reader that the authors found themselves unable to reach a consensus about the modality of membrane traffic between the ER and the Golgi apparatus in higher plants. Whilst there is no question that COPII proteins are essential for this process, doubt continues as to whether vesicles are the vectors of bidirectional traffic between the two organelles. It is clear that fluorescence microscopy, immunolabelling and live cell imaging in combination have revolutionised our conceptual understanding of the structure and functioning of the eukaryotic Golgi apparatus and its relationship with the ER. However, the present super resolution fluorescence microscopic techniques such as SIM, STED and PALM are not sufficient to unequivocally identify discrete individual COPII vesicles. On the other hand, new techniques such as SCLIM are emerging, that have the potential for even higher super-resolution not only in space but also in time, may well bring about a breakthrough in understanding what really is going on in living cells. Of course, electron microscopy is still the most powerful technique to solve the finer structural details of the interface between the ER and the Golgi whether in chemically-fixed or frozen samples. Thus, perhaps a combination of ultra-rapid freezing and freeze-substitution combined with selective membrane staining and one of the new high resolution 3-D SEM technologies such as FIB-SEM or serial block face imaging might solve some of the mysteries of the plant ER-Golgi interface and help settle the controversy explored in this article. Unfortunately, irrespective of the method of preparation and the type of imaging, the downside with electron microscopy is that

only single Golgi stacks can visualized with the caveat that the one being visualized might temporarily not be engaged in trafficking with the ER.

There is also an issue with the experimental systems being used. Beautiful live cell imaging data has been obtained with the leaf epidermis system, but obviously it will be interesting to explore other cell types. While it seems that the situation in tobacco BY-2 cells is not too different from that of leaf epidermal cells, there are other cell types such as in the meristem or in the endosperm during cellularization whose secretion status is unclear and where it is difficult, especially by conventional live cell imaging, to recognize a close spatial relationship between the ER and Golgi stacks, let alone being able to say anything about Golgi motility due to the sheer density of the cytoplasm. On the other hand, such cell types because of their relatively high cytoplasm/cell volume ratio freeze well and are therefore most suitable for electron microscopy studies. The reverse is true for leaf epidermal cells which are excellent objects for live cell imaging, but present an almost unsurmountable obstacle for ultra-rapid freezing. It remains to say that, even with the ideal plant cell, in order to capture the complexity of bidirectional ER-Golgi traffic in plant cells correlative light and electron microscopy is necessary. Establishing this technique for studies on plants is going to be a major challenge for the future.

Acknowledgements

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Legends to Figures

Figure 1: Electron microscopy of COPII-budding. A, B: transitional ER plus adjacent Golgi stacks in the green alga *Chlamydomonas noctigama* as seen in (A) chemically fixed and (B) high pressure frozen samples. The *cis-trans (c,t)* polarity of the Golgi stacks are clearly visible, so too are budding and released COPII vesicles

(arrowheads). Putative COPI vesicles are marked with arrows. C: High pressure frozen endosperm cell of *Arabidopsis thaliana*. Budding COPII vesicles are marked with arrowheads, free putative COPII vesicles are marked with arrows. D – G: Collage of COPII budding profiles. Note that many of the buds are at the termini of ER cisternae. Note that the ER in high pressure frozen samples is in general much more dilated than in chemically fixed samples; in *Chlamydomonas* it is extremely dilated (the ER in B, can be recognized by the ribosomes at the left of the vacuole-like structure). Bars are 200 nm. (HPF specimen preparations of *Chlamydomonas* and *Arabidopsis* are courtesy of Stefan Hillmer, Heidelberg and York-Dieter Stierhof, Tübingen).

Figure 2: Golgi cisternae (ST-YFP, red) and the ER exit site marker (SEC16-GFP, green) visualized in tobacco leaf epidermal cells. Images from time-lapse sequence acquired at the cortical region of tobacco leaf epidermal cell with a Zeiss LSM510 confocal microscope. The Sec16 marker distributes at the peri-Golgi area (arrowheads) as well as to structures of unknown identity that are not associated with the Golgi marker (Arrows) (see also Takagi et al., 2013). The structures labelled by Sec16 can assume a ring-like shape (Takagi et al., 2013). Bars, 5 µm (1 µm, in inset). Time of frames in the sequence is indicated at the left-hand corner of images (secs). Asterisk indicates a chloroplast, which is visible through chlorophyll autofluorescence. Images by Dr. Luciana Renna, DOE-MSU Plant Research Lab, Michigan State University, East Lansing, USA.

Figure 3: A**:** Maximum intenstity projection in negative contrast of a stack of thin sections from a tomogram of a pea root tip Golgi body and associated ER impregnated by the osmium zinc iodide (ZIO) technique. The reconstruction is presented at an angle to show a clear tubular connection between the ER and *cis*-Golgi. (Reconstruction courtesy of Louise Hughes). B: Inside face view of a dry-cleaved carrot suspension culture cell. The cell had been fixed on a coated EM grid, dehydrated and critical-point dried prior to dry-cleaving on double sided tape. The view onto the plasma membrane shows dark mitochondria (M), complete Golgi stacks in face view (G), plus cisternal (CER) and tubular ER (arrows). Not the huge difference between the diameter of a Golgi body and ER tubules.

FIgure 4: Organization of the ER exit sites in the budding yeast *Saccharomyces cerevisiae.* (left) Dual-color three-dimensional (3D) image of Sec13–GFP (ERES marker, green) and mRFP–Sec12 (bulk ER marker, red) obtained by SCLIM. (right) Two-dimentional (2D) slice image taken from the 3D data. ERES localize at the high-curvature domains of the ER such as along tubules and at the edge of the sheet. Bar: 1 μm. Photograph by Kazuo Kurokawa, Live Cell Super-Resolution Imaging Research Team, RIKEN Center for Advanced Photonics (see also Okamoto et al., 2012).

Figure 5: *cis*-Golgi cisternae (mRFP-SYP31, magenta) and the ER exit sites (SEC13-YFP, green) visualized in tobacco BY-2 cells. Confocal images were captured by SCLIM and reconstructed into 3D with deconvolution. A typical trajectory image from a 3D time-lapse movie is shown. Almost all of the Golgi stacks were associated with bright spots of ER exit sites. From the magnified image (inset), we could observe the ER exit sites surrounding the *cis*-Golgi making ring-shaped fluorescent patterns. Bar, 5 μm (1 μm in inset). Photograph by Yoko Ito, Live Cell Super-Resolution Imaging Research Team, RIKEN Center for Advanced Photonics. (see also Ito et al., 2012).

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