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Vesicles versus tubes: is endoplasmic reticulum-Golgi transport in plants fundamentally different from other eukaryotes?.

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

3

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23

24

25 **One sentence summary:**

26

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

Introduction

30

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

38

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

49

50 There is general consensus that in all eukaryotes anterograde protein transport out of
51 the ER is largely dependent upon COPII proteins, and that retrograde transport both
52 within the Golgi stack and between the Golgi and the ER requires COPI proteins
53 (Sztul and Sztul, 2011; Barlowe and Miller, 2014). Since recruitment of the COP
54 proteins in yeast and mammals culminates in the formation of a transport vesicle, it is
55 logical to assume that this may also be the case for plants. However, whereas there
56 appears to be no doubt about the formation and release of COPI vesicles at the
57 periphery of plant Golgi cisternae (Pimpl et al., 2000; Donohoe et al., 2007) the
58 existence of COPII-coated vesicles and their operation as anterograde transport
59 vectors in the narrow (< 500 nm) interface between the ER and Golgi apparatus of
60 land plants is a matter of considerable controversy (Hawes et al., 2008; Hawes,
61 2012). In contrast, [there are numerous examples amongst lower eukaryotes where
62 electron micrographs reveal a Golgi stack immediately adjacent \(† 500 nm\) to
63 transitional ER – per definition a domain of the ER \(or NE\) showing vesicle budding](#)

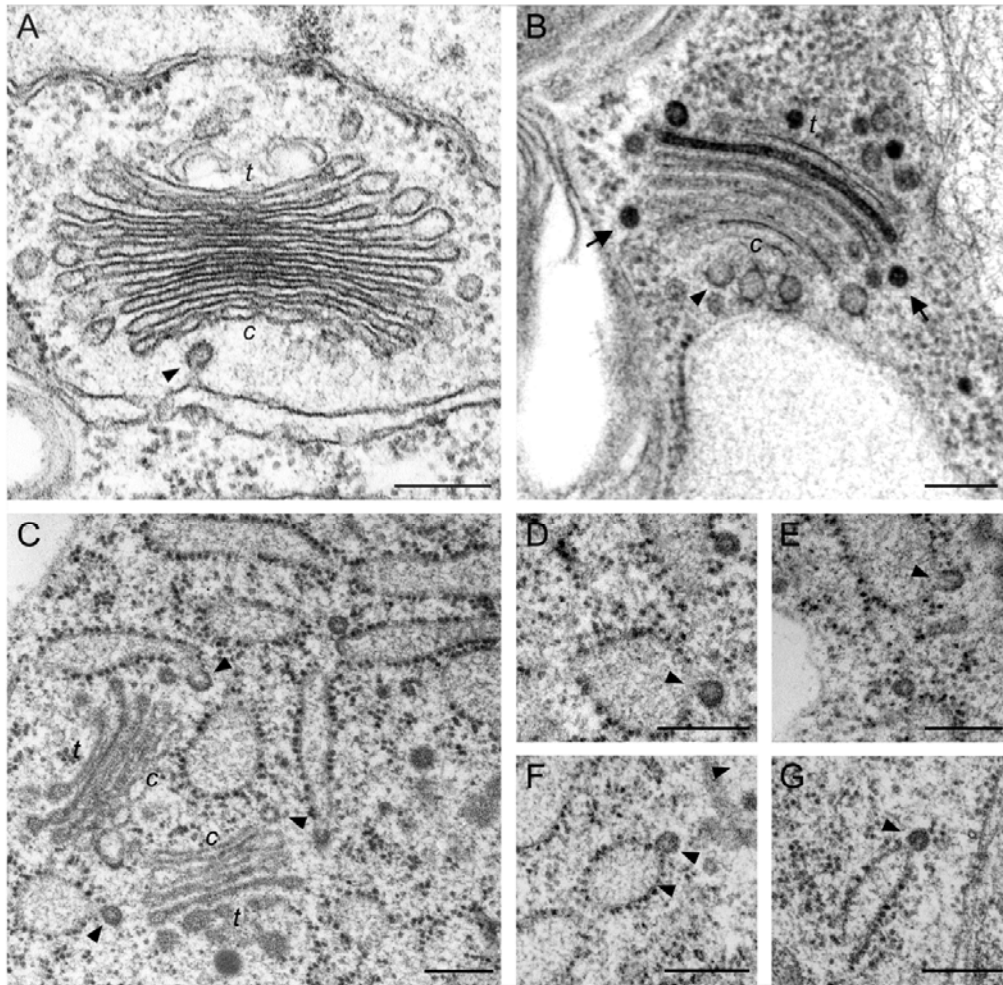


Figure 1

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).

64 profiles and lacking ribosomes. The best examples for this are probably the yeast
 65 *Pichia pastoris* (Mogelsvang et al., 2003), and the algae *Chlamydomonas* (Fig. 1 A,
 66 B; see also Hummel et al., 2007), *Tribonema* and *Melosira* (see Getty Images #
 67 169272449, 128618249). ER vesiculation profiles have often been recorded for

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

75

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

83

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

89

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

102

103

104 **Federica Brandizzi: The “Secretory Units“ model for ER protein transport in**
105 **highly vacuolated cells**

106

107 In live cell imaging analyses, the Arabidopsis COPII coat components (Sec13,
108 Sec23, Sec24 and Sec31) when expressed in highly vacuolated leaf epidermal cells
109 in tobacco and Arabidopsis have been found in punctate structures that are
110 associated with the ER and move with the Golgi stacks (Stefano et al., 2006; Hanton
111 et al., 2007; Sieben et al., 2008; Wei and Wang, 2008; Faso et al., 2009; Hanton et
112 al., 2009; Takagi et al., 2013; Tanaka et al., 2013), which in fully-expanded plant cells
113 are highly dispersed and motile (Boevink et al., 1998; Stefano et al., 2014). The
114 punctae labelled by the COPII coat proteins are commonly indicated as ER exit sites
115 (ERES). With the exception of Sec13 which has also been found at the nuclear
116 envelope (Yang et al., 2005), Sec23, Sec24 and Sec31 are predominantly localized
117 at such areas. Three-dimensional projection reconstruction of confocal images
118 followed by rendering analyses have shown that Sec16 is localized in cup-like
119 structures where the ER assumes a high-degree curvature (Takagi et al., 2013; see
120 also Fig. 2), supporting the intriguing possibility for specific requirements of ER
121 membrane curvature for the ERES establishment and maintenance. In a Sec24A
122 partial loss-of-function mutant, a functional fluorescent protein fusion to Sec24A has
123 been identified also in bright structures of unknown identity (Faso et al., 2009). It has
124 been hypothesized that such structures may represent ERES in formation or protein
125 aggregates (Faso et al., 2009). The COPII recruiting GTPase, Sar1, has been found
126 at the ERES but also over the ER network to a variable degree that may depend on
127 the specific Sar1 isoform (Hanton et al., 2008). The distribution of Sec16, Sar1 and
128 the COPII coat proteins drastically differs from that of the Sar1-GEF, Sec12, which
129 has been found distributed largely at the ER (Bar-Peled and Raikhel, 1997; daSilva
130 et al., 2004; Yang et al., 2005). [Similar to other eukaryotic cells transport of proteins](#)
131 [in plant cells may occur by bulk flow, as demonstrated for soluble proteins \(Crofts et](#)
132 [al., 1999; Phillipson et al., 2001\), as well as in dependence of signals that can be](#)
133 [present in the transmembrane domain \(Brandizzi et al., 2002; Schoberer et al.,](#)
134 [2014\), or through the presence of specific motifs that are recognized by COPII](#)
135 [proteins.](#)

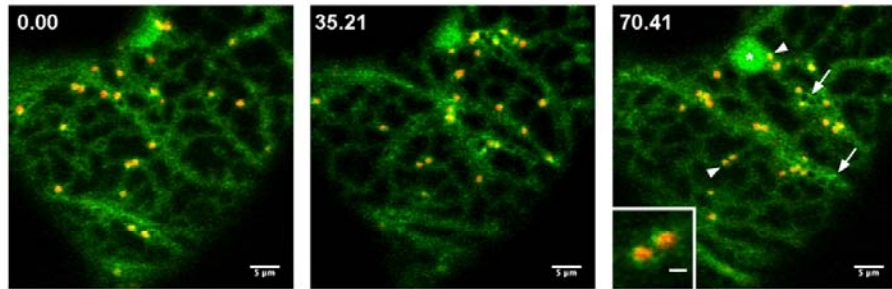


Figure 2

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.

136

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

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

155

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

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

195

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

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

214

215 **Chris Hawes: Let it be tubes**

216

217 *What can EM tell us?*

218

219 Back in the 1970's and 1980's prior to the discovery of COPI and COII vesicles,
220 various EM studies of the plant endomembrane system suggested that there may be
221 direct membrane connections between the ER and Golgi bodies which were often
222 termed dicytosomes in those days (e.g. Harris 1979, Mollenhauer et al. 1975). To
223 enable selective enhancement of the membranes of these organelles and to permit
224 studies in three-dimensions using thick sections and high voltage electron
225 microscopy, osmium impregnation techniques combined with either zinc iodide or
226 potassium ferricyanide, to enhance the deposition of osmium on membranes and
227 within the lumen of EM tubules and ER/Golgi cisternae, were often applied (Harris
228 1979, Hepler 1981). This resulted in a number of reports suggesting that the ER was
229 directly connected to Golgi bodies via membrane bounded tubules and that Golgi
230 bodies themselves presented many tubules at the margins of their cisternae.

231

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

242 was also reported in in developing bean and mung bean seeds (Harris 1979, Harris &
243 Oparka 1983). In wheat these fine tubules were often much thinner than an ER
244 tubule and unless heavily osmium impregnated, under normal contrasting conditions
245 in the EM would unlikely scatter sufficient electrons to be readily visible. More
246 recently we reported ER-Golgi connections in tobacco leaves using osmium
247 impregnation (Brandizzi et al. 2002). Of course the presence of tubules in electron
248 micrographs does not prove they are involved in transport, retrograde or
249 anterograde, but does show perhaps a more intimate relationship between the two
250 organelles than is often assumed. Likewise, it has been shown that the ER surface
251 itself is highly motile (Runions et al. 2006), therefore conceptually there should be no
252 problem in envisaging membrane flow from the ER through to the Golgi
253

254 In contrast to these results there are relatively few reports of vesicles budding from
255 the ER or existing at the interface between the ER and Golgi, although as far back as
256 1980, Schnepf and Christ suggested that a vesicle flow exists in the epithelial cells of
257 the nectaries of *Asclepias curassavica*. Only in a few publications using ultra rapid
258 high pressure freeze-fixation followed by freeze-substitution and electron tomography
259 have shown COPII vesicles budding from the ER (Kang & Staehelin 2008, Donohoe
260 et al. 2013, Hawes 2012), mainly in root meristem cells. An unpublished example in
261 developing arabidopsis endosperm tissue can be seen in Figure 1C-G of this paper.
262 Unfortunately, three-dimensional analysis and reconstruction by electron tomography
263 of data such as these still relies on manual tracing of images as auto-segmentation
264 algorithms cannot as yet differentiate the subtle differences in contrast in such
265 electron micrographs to permit totally unbiased autosegmented reconstructions.
266 However, it is possible to observe tubular connections between ER and *cis*-Golgi in
267 tomograms of osmium impregnated root material (Hawes unpublished, Fig. 3A).). At
268 this stage it should be noted that lack of COPII vesicles is not restricted to plants, but
269 has been reported in several micro-organisms. Likewise depletion of COPII
270 components does not always inhibit cargo transport from the ER in yeasts and
271 animals (see Mironov 2014 for references).

272

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

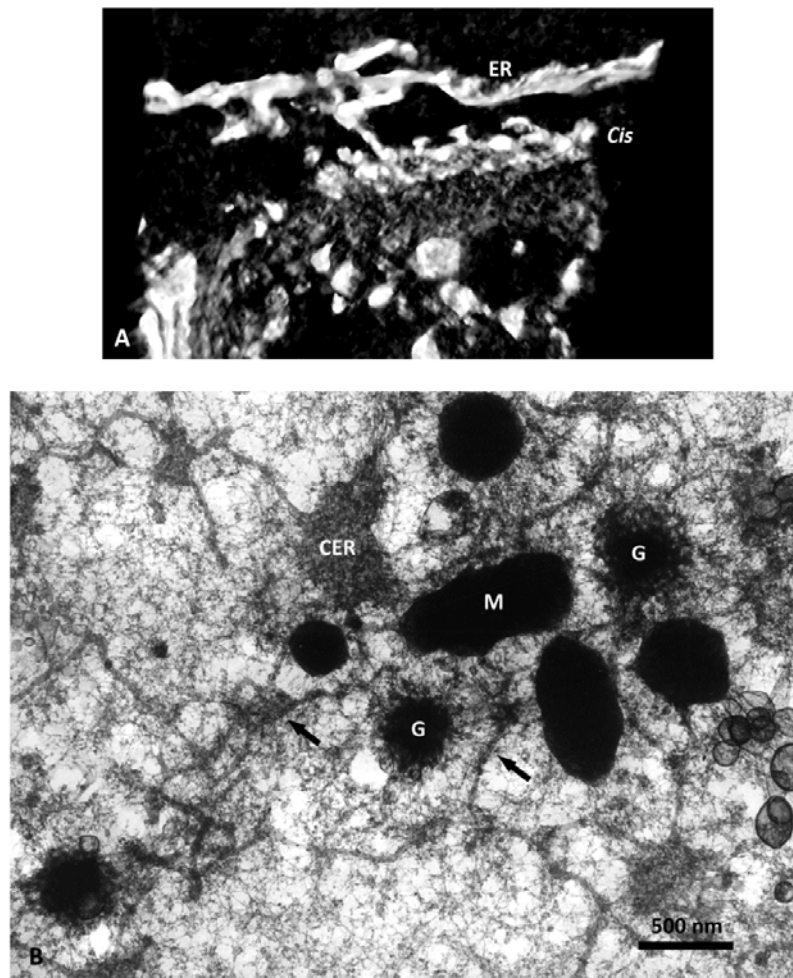


Figure 3

Figure 3: A: Maximum intensity 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). Note the huge difference between the diameter of a Golgi body and ER tubules.

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

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

288

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

310

311 *Has live cell imaging helped?*

312

313 Our initial observations on Golgi and ER in living leaf epidermal cells let us observe,
314 for the first time, the dynamic nature of the organelles and the fact that Golgi bodies
315 in leaves appeared to move over the surface of the ER (Boevink et al 1998). This led
316 us to propose the “hoover model” of Golgi bodies travelling over the ER surface
317 sucking up vesicles produced by the ER, thus making the serious, but all too
318 common, mistake of assuming that the plant ER-Golgi interface would function
319 exactly the same as the mammalian ER in the production of COPII vesicles.
320 However, over the past decade or so we have refined our ideas and developed the
321 “secretory unit” concept of ER exit sites and Golgi bodies travelling as single units
322 around the cell with the motile surface of the ER (daSilva et al 2004, Langhans et al.
323 2012). Such advances were made possible by the revolution in live cell imaging
324 offered by fluorescent protein technology and direct organelle labelling combined with
325 techniques such as photobleaching and photoactivation of fluorescent probes. This
326 enabled a range of experiments to be undertaken on the ER Golgi interface, and
327 contrary to what is often stated it was shown there is no real evidence that transport
328 between the ER and individual Golgi bodies only takes place when stacks are
329 stationary (the stop and go model, Brandizzi et al. 2002). Indeed we demonstrated
330 that in a FRAP experiment, recovery of fluorescence of a Golgi membrane protein
331 could be demonstrated in moving Golgi indicating a continual transfer of protein from
332 ER to Golgi (da Silva et al. 2004). Subsequently laser manipulation of Golgi has
333 demonstrated that when captured and translated through the cytoplasm by an
334 infrared laser beam, Golgi bodies almost always drag a tubule of ER behind them
335 (Sparkes et al. 2009a). One of the conclusions from this work was that the ER and
336 Golgi are closely associated and tethered together not via the cytoskeleton but most
337 likely by a number of structural proteins such as the Golgins/Golgi matrix proteins
338 (Hawes et al. 2008). In studies of Golgi destruction and reformation in tobacco leaf
339 cells we showed that with either a genetic block of secretion or inhibition by Brefeldin
340 A, reabsorption of the Golgi back into the ER was an ordered process, starting with
341 the release of *trans*-located Golgins into the cytoplasm followed by sequential
342 reabsorption of *trans*, medial and *cis*-membranes into the ER. However, a *cis*-
343 located matrix component, CASP remained associated with ER exit sites and we
344 suggest that this protein may be part of the tethering complex holding the Golgi to the
345 ER (Osterrieder et al. 2010, Schoberer et al. 2010). How, this ordered membrane

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

348

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

359

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

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

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

386

387

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

389

390

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

393

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

413

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

434

435 *Why are COPII vesicles difficult to visualize in higher plants?*

436

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

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

456

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

467

468 *Have “gap-spanning“ tubules been observed in the interface between the ER and cis-*
469 *Golgi?*

470

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

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

489

490 *What are the advantages of vesicles?*

491

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

512

513 In contrast, tubular contacts (irrespective of their longevity), or direct contacts
514 between organelles culminating in fusion, then fission (as for example in “hug and
515 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](#)

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

555

556

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

559

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

564

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

573

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

582

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

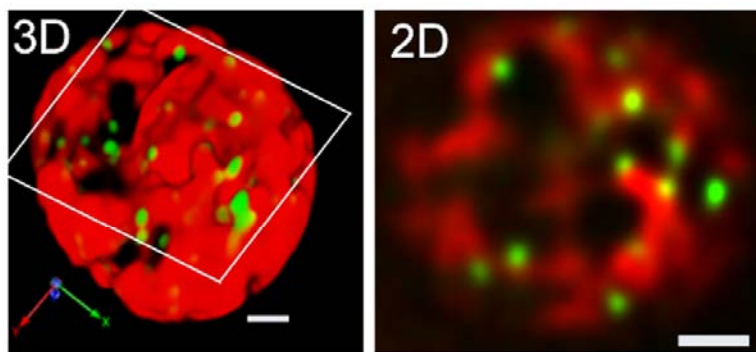


Figure 4

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-dimensional (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).

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

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

625

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

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

633

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

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

641

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

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

653

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

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

666

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

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

671

672 Plant cells have beautiful stacks of the Golgi apparatus, which are largely associated
673 with the ER. To try to address how Golgi stacks are assembled and maintained in
674 tobacco BY-2 cells, we set up a system for live imaging, in which Golgi stacks are
675 disassembled by brefeldin A (BFA) treatment and reformed after BFA removal (Ito et
676 al., 2012). While most of Golgi markers were absorbed into the ER upon BFA
677 treatment as previously reported (Takeuchi et al., 2000, 2002; Ritzenthaler et al.,
678 2002), we realized that some of the *cis*-Golgi markers (SYP31 and RER1B) remained
679 in the cytosol as small punctate structures. Another *cis*-Golgi marker, ERD2, diffuses
680 into the ER together with medial and *trans* markers, suggesting that there are two
681 different classes of *cis*-Golgi proteins. Upon BFA wash, the small punctate structures
682 of SYP31 nucleate to build new cisternae, to which other Golgi proteins then join in a
683 *cis*-to-*trans* sequence. The small punctate structures of SYP31 are in the close
684 vicinity of the ERES but do not completely overlap with them (Ito et al., 2012).

685

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

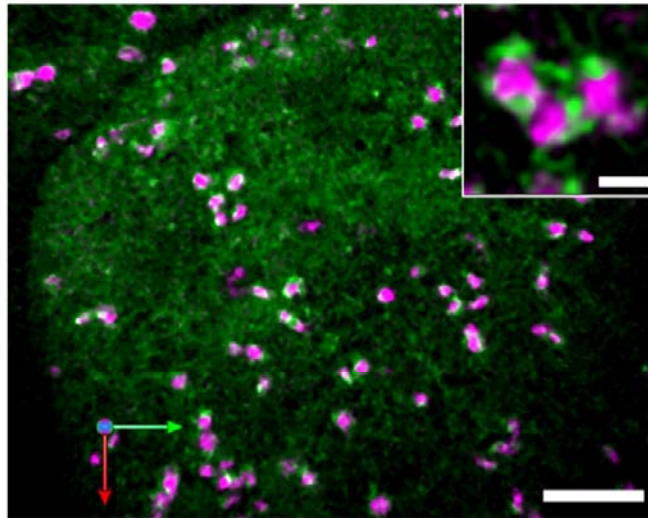


Figure 5

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

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

734

735

736 **Conclusions**

737

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

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

762

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

779

780

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782

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787

788

789 **Legends to Figures**

790

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

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

805
 806 **Figure 2: Golgi cisternae (ST-YFP, red) and the ER exit site marker (SEC16-
 807 GFP, green) visualized in tobacco leaf epidermal cells.** Images from time-lapse
 808 sequence acquired at the cortical region of tobacco leaf epidermal cell with a Zeiss
 809 LSM510 confocal microscope. The Sec16 marker distributes at the peri-Golgi area
 810 (arrowheads) as well as to structures of unknown identity that are not associated with
 811 the Golgi marker (Arrows) (see also Takagi et al., 2013). The structures labelled by
 812 Sec16 can assume a ring-like shape (Takagi et al., 2013). Bars, 5 μm (1 μm , in
 813 inset). Time of frames in the sequence is indicated at the left-hand corner of images
 814 (secs). Asterisk indicates a chloroplast, which is visible through chlorophyll
 815 autofluorescence. Images by Dr. Luciana Renna, DOE-MSU Plant Research Lab,
 816 Michigan State University, East Lansing, USA.

817
 818 **Figure 3: A: Maximum intensity projection in negative contrast of a stack of thin
 819 sections from a tomogram of a pea root tip Golgi body and associated ER
 820 impregnated by the osmium zinc iodide (ZIO) technique. The reconstruction is
 821 presented at an angle to show a clear tubular connection between the ER and *cis*-
 822 Golgi. (Reconstruction courtesy of Louise Hughes). B: Inside face view of a dry-
 823 cleaved carrot suspension culture cell. The cell had been fixed on a coated EM grid,
 824 dehydrated and critical-point dried prior to dry-cleaving on double sided tape. The
 825 view onto the plasma membrane shows dark mitochondria (M), complete Golgi
 826 stacks in face view (G), plus cisternal (CER) and tubular ER (arrows). Not the huge
 827 difference between the diameter of a Golgi body and ER tubules.**

828
 829

830 **Figure 4: Organization of the ER exit sites in the budding yeast *Saccharomyces***
831 ***cerevisiae*.** (left) Dual-color three-dimensional (3D) image of Sec13–GFP (ERES
832 marker, green) and mRFP–Sec12 (bulk ER marker, red) obtained by SCLIM. (right)
833 Two-dimensional (2D) slice image taken from the 3D data. ERES localize at the high-
834 curvature domains of the ER such as along tubules and at the edge of the sheet. Bar:
835 1 μm . Photograph by Kazuo Kurokawa, Live Cell Super-Resolution Imaging
836 Research Team, RIKEN Center for Advanced Photonics (see also Okamoto et al.,
837 2012).

838
839 **Figure 5: *cis*-Golgi cisternae (mRFP-SYP31, magenta) and the ER exit sites**
840 **(SEC13-YFP, green) visualized in tobacco BY-2 cells.** Confocal images were
841 captured by SCLIM and reconstructed into 3D with deconvolution. A typical trajectory
842 image from a 3D time-lapse movie is shown. Almost all of the Golgi stacks were
843 associated with bright spots of ER exit sites. From the magnified image (inset), we
844 could observe the ER exit sites surrounding the *cis*-Golgi making ring-shaped
845 fluorescent patterns. Bar, 5 μm (1 μm in inset). Photograph by Yoko Ito, Live Cell
846 Super-Resolution Imaging Research Team, RIKEN Center for Advanced Photonics.
847 (see also Ito et al., 2012).

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849

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