Scientific Report on the ESF (LESC) Workshop:

ENDOCYTOSIS IN PLANTS AND FUNGI: TRAFFICKING IN FROM THE BORDERS OF WALLED EUKARYOTIC CELLS

1. Background and aims of the workshop

Endocytosis is a vital process common to all eukaryotes that retrieves membrane material and associated cargo from the plasma membrane for internal utilization or destruction, or for processing and recycling back to the plasma membrane. Thus, endocytosis serves multiple purposes, including cell-cell communication and nutrient uptake. Whereas compartments and mechanisms of endocytosis have been analyzed in detail in animal cells, as judged by PubMed entries, only about 1% of all studies of endocytosis have been carried out on plants, and about 5% on yeasts. Moreover, almost nothing is known about endocytosis in filamentous and dimorphic fungi, despite their economical and ecological importance.

Plant cell biologists generally lag behind their counterparts in animal and yeast cell biology because their arsenal of molecular biology techniques is smaller and plant cells are more difficult to manipulate. The same applies to dimorphic pathogenic and filamentous fungi. In the case of endocytosis, however, this imbalance has been exacerbated because of the commonly held belief among physiologists that turgor pressure in plants and fungi would make endocytosis difficult, if not impossible. This state of affairs has been aggravated even further through the lack of obvious candidates for endocytic cargo molecules. However, the situation is now rapidly changing. The *Arabidopsis* and several fungal genome sequencing projects have revealed a considerable conservation of components of the endocytic machinery. Nevertheless, considering their divergent evolution from single-celled ancestors, the fungal, plant and animal lineages seem to have acquired specific adaptations with regard to endocytosis as a result of their unique cellular organisation. Thus, although the *Arabidopsis* genome encodes homologs of animal or yeast components of the endocytic machinery, the plant family members are often modified such that clear one-by-one orthologs are difficult to identify. In contrast, the genomes of *Neurospora crassa* and *Ustilago maydis* contain endocytosis-related components as implied from a comparison with mammalian cells, including Rab-GTPases, which are not found in the yeast *Saccharomyces cerevisiae* .

Endocytosis is an emerging area in plant and fungal cell biology that holds great potential for a mechanistic understanding of many basic biological processes in these organisms. Therefore, in order to direct future research in this field the most relevant problems have to be identified and appropriate strategies evaluated. To this end we organized a workshop with the financial support of the ESF which not only brought together European plant and filamentous fungal specialists, but also included several prominent yeast experts so that the problems encountered in endocytosis research in plants and other classes of fungi might be better understood and put into the right perspective.

2. Scientific content of the Workshop

Plant-based presentations:

Fernando Aniento (Valencia) addressed the problem of receptor-mediated endocytosis, using two different systems. A human transferrin receptor hTfnR expressed in Arabidopsis protoplasts was shown to be internalised into endomembrane compartments, "possibly endosomes" in a temperature-dependent manner. Its rhodamine-labeled ligand transferring, co-localised with hTfnR in these endosomes. hTfnR internalisation was blocked in the presence of tyrphostin A23, which inhibits the interaction of the tyrosine motif of hTfnR with the □-adaptin of the AP-2 complex. These findings point to the requirement of a functional AP-2 complex for endocytosis in plant cells. In another set of experiments, the uptake of biotinylated BSA and fluorescent biotin into "endosomes" was shown to be temperature- and time-dependent and was also inhibited by tyrphostin A23. To identify a biotin receptor, extracts from Arabidopsis cells were separated by affinity chromatography. A 75 kDa integral membrane protein was specifically eluted with free biotin, making it a candidate for the biotin receptor. These data strongly support the existence of receptor-mediated endocytosis in plants.

Jens Stougaard (Aarhus) described the symbiotic interaction between legume plants and rhizobial bacteria. Following their uptake into root hairs, these bacteria accumulate in a specialized symbiotic organ, the nodule, where they are released into specialised membrane compartments within the target cells. This process requires mutual signaling between bacterium and host plant, and involves plasma membrane-localized receptors. Whereas the signaling pathways have been well-investigated, studies on the actual internalisation process of the bacteria into symbiosomes are still at a rudimentary stage.

Stomatal guard cells undergo large and reversible changes in surface area, which require the addition and removal of plasma membrane. **Ulrike Homann** (Darmstadt) presented evidence from electrophysiology and fluorescent imaging studies which demonstrate that the retrieval and insertion of K^+ channels at the plasma membrane accompanies endocytosis and exocytosis in guard cells. Double labeling with the endocytic tracer FM4-64 and KAT1-GFP revealed large (300 nm diameter) membrane vesicles which carried either of the two markers or both. Evidence was presented suggesting that both constitutive and turgor-driven membrane turnover occurs in guard cells.

Some plant cells preferentially grow at their tip, a phenomenon involving endocytosis and local recycling of membrane material. Prime examples are root hairs and pollen tubes. **Irene Lichtscheidl** (Vienna) presented live-imaging studies of endocytosis and movement of vesicles near the plasma membrane at the tip of the root hair. Endocytosis was visualised with fluorescent tracers FM4-64 and filipin, which labels sterols. However, high concentrations of filipin blocked endocytosis and tip growth as did the actin-depolymerising drug latrunculin B. These results suggest that both sterols and actin filaments are involved in root hair endocytosis. **Ben Kost** (Heidelberg) analysed membrane recycling during pollen tube growth. Endosomes were visualised with fluorescent markers including Rab5 and the PI3P-binding FYVE

domain, which colocalized along the entire pollen tube. Expression of dominant-negative Rab5 or overexpression of the FYVE domain slowed pollen tube growth. Filipin accumulated at specific patches on the flanks of the pollen tube tip, possibly marking sites of endocytosis. High concentrations of filipin or manipulation of sterol biosynthesis inhibited pollen tube growth, indicating a role for sterols in endocytosis. **Rui Malho** (Lisbon) discussed the role of phosphoinositides (PIs) and Ca^{2+} in membrane traffic of the pollen tube. A tip-focussed Ca^{2+} gradient is essential for maintenance of pollen tube growth. Increases in cytoplasmic Ca^{2+} stimulate growth, whereas phosphatidic acid, an end-product of PIP_2 hydrolysis stops growth by causing the tips of pollen tubes to dilate. On the other hand, reduction of phosphatidic acid levels leads to a collapse of the calcium gradient. These results point to a fine tuning between membrane recycling and pollen tube tip growth.

Internalisation at the plant plasma membrane was the focus of two presentations. **Susanne Holstein** (Heidelberg) discussed clathrin binding partners involved in endocytosis. The epsin family protein AP180 interacts with both clathrin heavy chain and □-adaptin of the AP-2 complex. Several interaction motifs of AP180 were experimentally tested and AP180 was shown to reassemble clathrin triskelia in vitro. □-adaptin binds to clathrin heavy chain and overexpression of its truncated form inhibits growth of pollen tubes. Putative cargo proteins of clathrin-coated vesicles include two specific plasma membrane receptors, which were shown to interact with a □-adaptin in pulldown experiments. **Sacco De Vries** (Wageningen) presented evidence that two brassinosteroid receptors are indeed endocytosed from the plasma membrane. Interestingly, these receptors heterodimerise preferentially in endosomes. So far endocytosis has not been demonstrated to be triggered by the ligand brassinosteroid. A related receptor was internalised if a protein phosphatase was coexpressed in the same cell, suggesting that dephosphorylation regulates receptor internalisation.

Endosomes and endocytic transport were discussed in three presentations. **Marcus Grebe** (Umea) analysed sterol endocytic transport in the context of cell polarity in the Arabidopsis root epidermis. With FRAP technology and a large set of fluorescent markers, filipin-labeled sterol was shown to be internalised to early endosomes. This trafficking was actin-dependent and coincided with endocytosis of plasma membrane proteins. **Gerd Jürgens** (Tübingen) presented evidence for a role of ARF-GEFs in the recycling of plasma membrane proteins from endosomes. This process is blocked by BFA. An engineered BFA-resistant ARF-GEF was capable of recycling an auxin-efflux regulator to the plasma membrane in the presence of BFA. **Pankaj Dhonukshe** (Tübingen) described inhibitory effects of the plant hormone auxin on endocytic trafficking of constitutively cycling plasma membrane proteins. This inhibition was direct and not depend on auxin-mediated gene expression. Together, these studies provided strong evidence for membrane cycling between the plasma membrane and endosomes.

Endocytosed proteins that are destined for degradation are targeted to the vacuole. **David Robinson** (Heidelberg) demonstrated that the prevacuolar compartment is the place where the endocytic pathways meets the Golgi-vacuole pathway. By all available criteria, this multivesiculate compartment corresponds to an late endosomal compartment. The vacuolar sorting receptor (VSR) delivers soluble cargo from the trans-Golgi to the prevacuolar compartment and needs to be recycled to the trans-

Golgi. By analogy with yeast, a retromer complex was postulated to recycle the VSR. Several retromer proteins were detected in a complex. In addition, these proteins colocalised with each other and with VSR, and were localised to the prevacuolar compartment. These findings suggest retromer-mediated recycling of VSR in plants.

Fungal-based presentations:

Most of our knowledge on the molecular basis and cellular role of fungal endocytosis is restricted to bakers yeast (*Saccharomyces cerevisiae)*. Therefore, it is not surprising that 9 out of 11 talks dealt with molecular aspects of endocytosis in this fungus. Of particular interest was the relationship between endocytosis and the actin cytoskeleton. **Kathryn Ayscough** (Sheffield) reported on factors that couple fluid-phase endocytosis to actin dynamics. She presented a detailed analysis of deletion mutants for several proteins that interact with the key regulator Sla1, indicating the existence of multiple endocytic pathways. In an elegant *in vitro* approach, **Maribel Geli** (Barcelona) found that type I myosins participate in the formation of actin foci. Using Myo5p that was bound to the surface of Sepharose beads her laboratory investigated the role of this myosin motor in the recruitment of proteins that are known to locate to actin patches *in vivo*. Moreover, her work provided highly interesting insights into the regulatory network that underlies actin patch-dependent endocytosis. **Barbara Winsor** (Strasbourg) presented her ongoing research into the function of SH3domain-containing proteins in endocytic vesicle formation at the plasma membrane. Her group found that about one third of all SH3 domains are able to induce actin polymerization *in vitro*. This strongly implicates these proteins as potential regulators in early steps of endocytosis. A preliminary identification of these interacting proteins was also presented. **Kathleen D´Hondt** (Ghent) provided evidence for a role of End3p, a factor essential for endocytosis, in the formation of endocytic vesicles. She showed that End3p localizes to the plasma membrane and participates in membrane deformation, which might underlie the budding of transport membranes. Interestingly, in other cell types this process is based on clathrin, which suggests that alternative mechanisms may have evolved in *S. cerevisiae.*

Another important area of research concerns the role of membrane lipids in endocytotsis. **Joos Holthius** (Utrecht) presented data suggesting that aminolipids participate in the formation of the protein coat around endocytic transport vesicles. This conclusion is based mainly on an analysis of the putative P-type aminophospholipid translocases Dnf1p and Dnf2p in yeast. Deletion of these transporters changed the lipid distribution, but also led to defects in the uptake of endocytic marker dyes. The importance of lipids in endocytosis was further emphasized by **Howard Riezman** (Geneva). His group has shown that sphingoid base synthesis is a prerequisite for the endocytosis of alpha-factor. Interestingly, an arrest in protein biosythesis increased the formation of sphingolipids, which affects endocytosis, indicating that the accumulation of serine is rate limiting for the synthesis of sphingolipids. Surprisingly, a temperature-sensitive mutant of *pkh2*, a kinase that activates sphingoids, was completely blocked in endocytosis but grew almost normally, indicating that endocytosis is not essential for morphogenesis of yeast cells.

Rosine Haguenauer-Tsapis (Paris) reported recent results on the role of ubiquitylation in endocytosis of integral plasma membrane proteins. Her talk focussed

on the degradation pathway of Fur4p, an uracil permease in *S. cerevisiae*. The uptake of this transporter is triggered by uracil, which binds to Fur4p. This results in early ubiquitylation by the ubiquitin ligase Rsp5p. Subsequently. this Nedd4-member of ligases adds ubiquitin at later steps of Fur4p endocytosis and thereby participates in vacuolar sorting. This emphasizes the importance of ubiquitylation in endocytosis of integral membrane proteins. The degradation of such proteins in the vacuole was also the subject of **Hugh Pelham** (Cambridge) presentation. He reported that polar amino acid residues within the transmembrane domain of receptors have a key role in aggregation and subsequent degradation in the vacuole. Again, ubiquitylation of misfolded proteins induces endocytosis and subsequent degradation. Numerous factors are involved in quality control and among these Bsd2, Rsp5 and Doa10 are found in all eukaryotes. This emphasizes once again that the basic mechanisms of endocytosis are conserved among all taxa. The protein coat of another type of transport vesicle was the subject of the presentation of **Matthew Seaman** (Cambridge). His work has focussed on the role of the retromer complex in late endocytic transport between endosome and the Golgi apparatus. This complex consists of 5 highly conserved proteins that participate in recycling of hydrolase receptors which are essential for the maintenance of lysosome/vacuole formation. The retromer complex is also found in mammalian and plant cells (see above), where it has similar functions.

Bearing in mind that the basic mechanism of endocytosis is conserved between mammalians and yeast cells, it is most surprising that the existence of endocytosis in filamentous fungi is still a matter of debate. **Nick Read** (Edinburgh) gave an excellent overview of the current status of our knowledge on endocytosis in *Neurospora crassa* and *Magnaporthe grisea*. Most evidence for endocytosis in filamentous fungi is based on the use of the dyes FM1-43 and FM4-64. Applying these markers, his group was able to visualize the endocytc pathway in *N. crassa*. In addition, an *in silico* analysis of recently published fungal genome sequences suggested the existence of conserved key proteins involved in endocytosis in numerous species. These data strongly indicate that endocytosis does occur in these fungi, but the exact role of this process in hyphal growth and fungal biology remains elusive. **Gero Steinberg** (Marburg) provided evidence that receptor-mediate endocytosis participates in cell-cell recognition during the early stages of the development of the corn smut fungus *Ustilago maydis*. In mating hyphae the pheromone receptor Pra1 is taken up into early endosomes before it is sent back to the plasma membrane. This endocytic recycling of Pra1 is essential for cell-cell communication, and thereby provides the first example for an essential role of endocytosis in development of a pathogenic fungus. It is important to note that these two reports covered most of the existing and also unpublished results on endocytosis in the group of filamentous fungi.

3. Conclusions, Outlook

The participants were unanimous in their opinion that the workshop was highly successful as a platform for the exchange of new data and ideas between scientific communities which do not normally interact with one another. The wish that this kind of interkingdom update should not remain a singular event was very much evident at the end of the meeting.

Although the presence of a cell wall is a common denominator between plant cells, filamentous fungi and yeasts it is clear that it does not constitute a barrier to endocytosis in these organisms. Nevertheless, the status of research into this process in these three organismal groups is significantly different. Whereas in plants more and more evidence has accrued for the operation of receptor-mediated endocytosis, unequivocal proof for receptor internalization and recycling i.e. the uptake of a receptor-ligand complex via clathrin-coated pits at the the plasma membrane and the transport of the same receptor back to the plasma membrane from an endosomal compartment, still has to be delivered. Nevertheless, detailed information on individual pieces of the jigsaw puzzle, e.g. internalizable receptors, the clathrin machinery, and endosomes is now available. GFP-technology has been successfully introduced into this area in last couple of years, but it is abundantly clear that its value is severely limited through the lack of high quality immunogold electron microscopy, which is needed to identify the fluorescent images being obtained.

In contrast to plants, where a lot of structural data on the endocytic pathway has accumulated over the last years both at the electron and light microscopical levels, the situation in yeast much less clear: there are no good images of the actual internalization event at the plasma membrane and the morphological characterization of the endosomal compartments has not reached the level of quality achieved with higher organisms. Indeed, when challenged with the question as to whether clathrin-coated pits/vesicles actually do mediate receptor-mediated endocytosis in yeast a clear-cut answer could not be provided by the contingent of yeast researchers present at the workshop. Endocytosis research in yeast instead takes place at a level at which the plant community cannot for the moment compete: the identification of key molecular players and the elucidation of their interactions. For these reasons and with only few exceptions, e.g. in the case of retromer, it became apparent that, at this moment of time, effective collaborations between plant and yeast researchers will be difficult to achieve.

On the other hand, fungi are a heterogenous group of organisms and include extremely important human and plant pathogens, and yet almost nothing is known about the role of endocytosis in hyphal growth. This dramatically illustrates how much still needs to be done in this area. Interestingly, but because perhaps tip growth is superficially similar between plant systems and filamentous fungi, the workshop established that a greater synergy (and sympathy in aims) exists between the plant and filamentous fungi researchers in terms of endocytosis, than with the yeast group. Nevertheless, almost all of the workshop participants, including yeast researchers, expressed the desire to remain together as a group for the purpose of a joint research grant application within the framework of the EURO-CORE programme.

4. Final Programme of the Workshop

ENDOCYTOSIS IN PLANTS AND FUNGI: TRAFFICKING IN FROM THE BORDERS OF WALLED EUKARYOTIC CELLS

Held at the Internationale Wissenschaftsforum (IWH) Heidelberg, Germany

From October 7th –10th , 2004

Organizers: **Gerd Jürgens, David G. Robinson, Gero Steinberg**

Thursday, 7. 10. 2004

14.00 - 16.00		Arrival, Registration
16.00	David G. Robinson:	Welcome

ENDOCYTOSIS IN PLANTS: PHENOMENOLOGY (Chair: Gerd Jürgens)

16.15	David G. Robinson:	Introduction: Endocytosis in Plants: an Uphill Struggle
16.30	Fernando Aniento:	Uptake of biotin and biotinylated markers in plant cells: receptor-mediated endocytosis?
17.05 – 17.35		Coffee Break
17.35	Jens Stougaard:	Receptors, signalling and bacterial invasion in legume-rhizobial symbiosis
18.10	Ulrike Homann:	Constitutive endocytosis and pressure driven membrane turnover are associated with retrieval of K^+ channels
18.45- 19.30		Discussion

Friday, 8. 10. 2004

8.00		Breakfast

ENDOCYTOSIS IN TIP-GROWING ORGANISMS (Chair: Ulrike Homann)

8.45	Nick Read:	Endocytosis in filamentous fungi
9.20	Irene Lichtscheidl:	Endocytosis during tip growth of root hairs
9.55 – 10.25		Coffee Break
10.25	Ben Kost:	Membrane recycling during polar pollen tube growth
11.00	Rui Malho:	Pollen tube growth and guidance: The role of Phosphoinositides in apical secretion and modulation of $[Ca^{2+}]$ levels.
11.35 – 12.20		Discussion
12.20 – 13.30		LUNCH Break

INTERNALIZATION AT THE PLANT PLASMA MEMBRANE (Chair: David.G. Robinson)

13.30	Susanne Holstein:	Molecular Dissection of Plant Clathrin Binding Partners involved in Endocytosis
14.05	Sacco de Vries:	Heterodimerization and endocytosis of *Arabidopsis* brassinosteroid receptors BRI1 and AtSERK3 (BAK1)
14.40 – 15.25		Coffee and Discussion

ENDOSOMES AND ENDOCYTIC TRANSPORT – FUNGI (Chair: Nick Read)

15.25	Gero Steinberg:	Microtubule-dependent endosome traffic is essential for receptor-mediated pheromone perception in mating of the plant pathogen *Ustilago maydis*

16.00	Kathryn Ayscough:	Coupling actin dynamics to the endocytic process in *Saccharomyces cerevisiae*
16.35 – 17.05		Coffee Break
17.05	Maribel Geli:	Molecular Dissection of in vitro Generated Actin Patches: Insights into the Mechanism of the Endocytic Uptake in Yeast
17.40	Barbara Winsor:	The SH3 domains of the *S. cerevisiae* proteome : old and new interactions with the actin machinery and their dependence on activators
18.15 – 19.00		Discussion

Saturday, 9. 10. 2004

8.00		Breakfast

ENDOSOMES AND ENDOCYTIC TRANSPORT – PLANTS (Chair: Sacco deVries)

8.45	Markus Grebe:	Endocytic transport and cell polarity in the *Arabidopsis* root epidermis
9.20	Gerd Juergens:	ARF-GEFs and recycling from endosomes in *Arabidopsis*
9.55 – 10.25		Coffee Break
10.25	Pankaj Dhonukshe:	Non-genomic effect of auxin on endocytic protein trafficking
11.00 – 11.45		Discussion
11.45 – 13.00		LUNCH Break

MOLECULAR CHARACTERIZATION OF YEAST ENDOCYTOSIS (Chair: Kathryn Ayscough)

13.00	Joost Holthuis:	P-type ATPase-dependent lipid transport in endocytosis

13.35	Rosine Hagenauer-Tsapis:	Ubiquitylation events in regulated trafficking of plasma membrane transporters in *S. cerevisiae*: the fate of uracil permease
14.10	Howard Riezman:	Roles of sphingoid bases in endocytosis and heat stress
14.45	Kathleen D'Hondt:	End3p interaction with membranes
15.20 – 16.05		Coffee and Discussion

WHERE ENDOCYTIC AND VACUOLAR TRAFFICKING PATHWAYS MEET
(Chair: Howard Riezman)

16.05	Hugh Pelham:	Sorting membrane proteins into the yeast vacuole
16.40	Matthew Seaman:	Retromer and its role in endosome-to-Golgi retrieval
17.15	David G. Robinson:	VSRs, retromer and the plant prevacuolar compartment
17.50		General Discussion and Closing Remarks
18.35		Conference Dinner: Buffet at the IWH

Sunday, 10. 10. 2004

8.00	Breakfast
9.00	Departure or Sightseeing (optional)

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6. Statistical Information on the Participants

Name	Gender	Status	Country	Age
Invited participants				
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D`Hondt	F	YS	Belgium	40
De Vries	M	ES	Netherlands	50
Dhonukshe	M	PD	Germany	29
Geli	F	ES	Spain	38
Grebe	M	YS	Sweden	37
Haguenauer-Tsapis	F	ES	France	50 - 60
Holstein	F	YS	Germany	42
Holthuis	M	ES	Netherlands	46
Homann	F	YS	Germany	40
Jürgens	M	ES	Germany	55
Kost	M	YS	Germany	40
Lichtscheidl	F	ES	Austria	49
Malho	M	ES	Portugal	37
Pelham	M	ES	UK	50
Read	M	ES	UK	50
Riezman	M	ES	Switzerland	51
Robinson	M	ES	Germany	57
Seaman	M	YS	UK	38
Steinberg	M	ES	Germany	43
Stougaard	M	ES	Denmark	51
Winsor	F	YS	France	45 - 55
Observers				
Baluska	F	ES	Germany	47
Belgareh	M	PD	France	25 - 30
Friant	F	PD	France	34
Rato	F	GS	Portugal	25
Russinova	F	PD	Netherlands	36
Schumacher	F	YS	Germany	38
Thiel	M	ES	Germany	47

ES = Established Scientist
YS = Young Scientist
PD = PostDoc
GS = Graduate Student