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REVIEW

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The plant endosomal system—its structure and role in signal transduction and plant development

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Abstract Endosomes are highly dynamic membrane systems that receive endocytosed plasma membrane proteins and sort them for either degradation or recycling back to the cell surface. In addition, they receive newly synthesised proteins destined for vacuolar/lysosomal compartments. Sorting in the endosomes is necessary for the establishment and maintenance of cell polarity and it is needed to control levels and function of receptors and transporters at the cellular surface. Both processes are crucial for correct cell behaviour during tissue and organ development and for intercellular communication in general. It has therefore become an imperative to investigate structure and function of the endosomal system if we want to obtain a deeper mechanistic understanding of signal transduction and development. This review will compare our current understanding of endosomal trafficking in animals and yeast with what is known in plants, and will highlight some important breakthroughs in our understanding of the role of endosomes in signal transduction and multicellular development in *Drosophila*, as well as in *Arabidopsis*.

Keywords Auxin · Brefeldin A · GNOM · Plant endosomes · Sterol mutants · Vesicle trafficking

Abbreviations ARF: ADP ribosylation factor · BFA: Brefeldin A · EGF: Epidermal growth factor · GEF: GDP/GTP exchange factor · MVB: Multi-vesicular body · PCR: Partially-coated reticulum · PI-3P: Phosphatidylinositol-3-phosphate · TGN: Trans-Golgi network

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Introduction

Cells perceive their environment through an elaborate set of receptor proteins displayed at the plasma membrane, their external surface. This border between the inner and outer world also harbours an extensive set of proteins that set up and maintain the specific homeostasis of ions and solutes, which is fundamental to the correct functioning of the cell. The presence and abundance of these plasma membrane proteins has to be subject to a tight and dynamic control by the cell. In eukaryotes, this is achieved by a complex system of internal membranes that serve to transport proteins to their site of action. These internal membranes represent a large part of the cell volume, sub-dividing the cell into a largely continuous cytosolic space and into a number of membrane-bounded, discontinuous compartments. These compartments are defined by their specific structure, membrane lipid composition and protein content. Exchange of material between them is thought to be achieved by small transport intermediates, the vesicles, which bud off from one membrane and fuse with another in a highly controlled fashion. In this review I want to discuss what is known in plants about a subset of these compartments, the endosomes. Endosomes are the receiving and sorting station for proteins internalised from the cell surface. In theory, cells could control their levels of plasma membrane proteins solely by transcriptional/translational regulation and then constitutively transporting the produced proteins to their site of action and internalising them at a given rate in order to destroy them in the vacuole/lysosome. However, this is far from being the case. In animal and yeast cells, regulated vesicle trafficking to and from the surface is

employed to control the levels and activities of receptors, transporters and other plasma membrane proteins, and this process is shown to be of central importance for the control of plasma membrane protein abundance and, consequently, for cellular interactions and the responses of the cell to the outside world.

In plants, the research on intracellular membrane trafficking in general and on endosomal trafficking in particular is far behind that which is known in animals or yeast. Although deplorable, this leads to a situation where our concepts and models are often derived from drawing analogies with what is known in animals or yeast. Doing so is inevitable and necessary, but carries the danger of designing the wrong experiments or misinterpreting our results when we are dealing with some processes that might be unique to plants or simply regulated differently. Not doing so, on the other hand, might unnecessarily slow down research by attempting to "re-invent the wheel" and to demonstrate that processes in plants are simply identical to what is known from other systems. There is no safe way in-between. However, with this in mind, I will attempt to carefully compare the animal/yeast concepts and models with what is experimentally known in plants, trying not to blur the border between our experimental knowledge and our projections.

I will start with an overview of the latest concepts concerning the mechanism and function of endosomes in animals and then review the old and recent literature about endocytosis and endosomes in plants. Finally, I will discuss the importance of endosomal trafficking for signalling and development—a topic that has experienced an incredibly dynamic development in animals in recent years, but where exciting discoveries are also being made in plants.

The endosomal system of animals

In animals, most plasma membrane proteins become internalised by adaptor-mediated selective recruitment into clathrin-coated pits, which eventually form vesicles by budding from the membrane (Motley et al. 2003). These vesicles then fuse with a highly mobile and dynamic organelle, the early endosome (also referred to as a sorting endosome). Early endosomes have an inconspicuous, pleiomorphic appearance, often described as "vesiculo-tubular" (Geuze et al. 1983). This contrasts with the organisation of the Golgi, with its easily recognisable, ordered array of cisternae. The Golgi apparatus, specifically the associated trans-Golgi network (TGN) is regarded as an important sorting station of proteins in the cell (Keller and Simons 1997; Keller et al. 2001). However, early endosomes are an at least as important center for sorting of cellular proteins. They receive from the TGN not only newly synthesised proteins that are destined for late endosomes/lysosomes (Ludwig et al. 1991) but also, in some cases, proteins that are on their way to the plasma membrane (Futter et al. 1995). Here, they meet with material internalised from the plasma membrane that will either be recycled to the cell surface or be sorted further down the endocytic pathway to eventually end up in the lysosome for degradation (Fig. 1; Geuze et al. 1983). How does this sorting occur? It has become apparent that the endosomal system should not be viewed as an assembly of separate compartments that exchange material among themselves by budding and fusion of vesicles. Instead, early endosomes are divided into membrane sub-domains and are constantly in the process of fusing and separating (Gruenberg 2001; Zerial and McBride 2001). Especially, tubules with a distinct protein composition constantly emanate from the vesicular domains carrying the cargo destined for recycling to the plasma membrane. They apparently move away from the early endosome and fuse with other tubules, thereby forming the recycling endosome. Thus, rather than pre-existing, the recycling endosome can be seen as being continuously formed from early endosomes and being consumed in the process of sorting and recycling. A nice illustration of this is the observation that cytoblasts, cell parts manipulated to exclusively contain early endosomes, appear to spontaneously re-form recycling endosomes, as indicated by the re-appearance of compartments with markers specific for recycling endosomes (Sheff et al. 2002).

Proteins destined for the degradative pathway, on the other hand, will stay in the vesicular region of the early endosome where they are sorted into another sub-domain. This sub-domain will eventually give rise to a multi-vesicular body (MVB), named after its round shape and the presence of numerous internal vesicles (Murk et al. 2003). MVBs end up as being physically separated from early endosomes and their different morphology is again reflected in a protein–lipid composition that is different from that of early endosomes (Fig. 1). The cargo of MVBs is then transported to late endosomes, which, at least in animals, are again a distinct compartment, containing proteins and lipids not found on MVBs (Gruenberg 2003). Proteins exit late endosomes and recycle back to the TGN (Pfeffer 2003). Those that are not transported away will eventually end-up in lysosomes, which are again distinguished from late endosomes by differences in lipid and protein composition (Fig. 1; Gruenberg 2001).

Some molecular mechanisms for endosomal sorting

How can compartments with a specific morphology and protein/lipid composition form from one another and how do they keep their complement of proteins separate when they are part of a contiguous membrane system? An important but controversial concept is the idea of lipid rafts, membrane sub-domains that are rich in sterols and sphingolipids and into which certain membrane proteins preferentially accumulate (Munro 2003). It was put forward that such lipid rafts are instructive to

sort proteins at the TGN and at the plasma membrane, based on their affinity for sterol-rich versus phospholipid-rich sub-domains (Simons and Toomre 2000). Although it is unclear to what extent lipid rafts are important for sorting in endosomes, sterols are definitely enriched in the plasma membrane and in membranes of the late secretory and the endosomal compartments and play an important, although badly understood, role in protein sorting in these membrane systems (Fig. 1). Rab GTPases are thought to be central players in the constant formation and segregation of endosomal membrane domains (Zerial and McBride 2001). Once attached to a membrane in their GTP state, Rabs are able to interact with a large number of "effector" proteins, thereby recruiting them to the membrane. Some of these effectors have the ability to multi-valently interact with each other, which can lead to more or less extended scaffolding of Rab-interacting factors and restrict their lateral diffusion in the membrane. Some others cause the local production of specific lipids in the membrane, which in turn serve as additional binding sites for protein recruitment to the same place. Activators of Rabs, for example, can bind these lipids and set-off a positive feedback loop that eventually can lead to the dynamic assembly of a membrane sub-domain with a specific protein–lipid composition within a larger compartment (Miaczynska and Zerial 2002).

Early endosomes, for example, are defined by the presence of Rab5, which binds and recruits a PI-3 kinase, leading to local production of the lipid PI-3P (phosphatidylinositol-3-phosphate; Zerial and McBride 2001). This enhances recruitment of proteins that bind weakly to both Rab5 and PI-3P (Fig. 1). The Rab5 domain is also competent to receive incoming vesicles

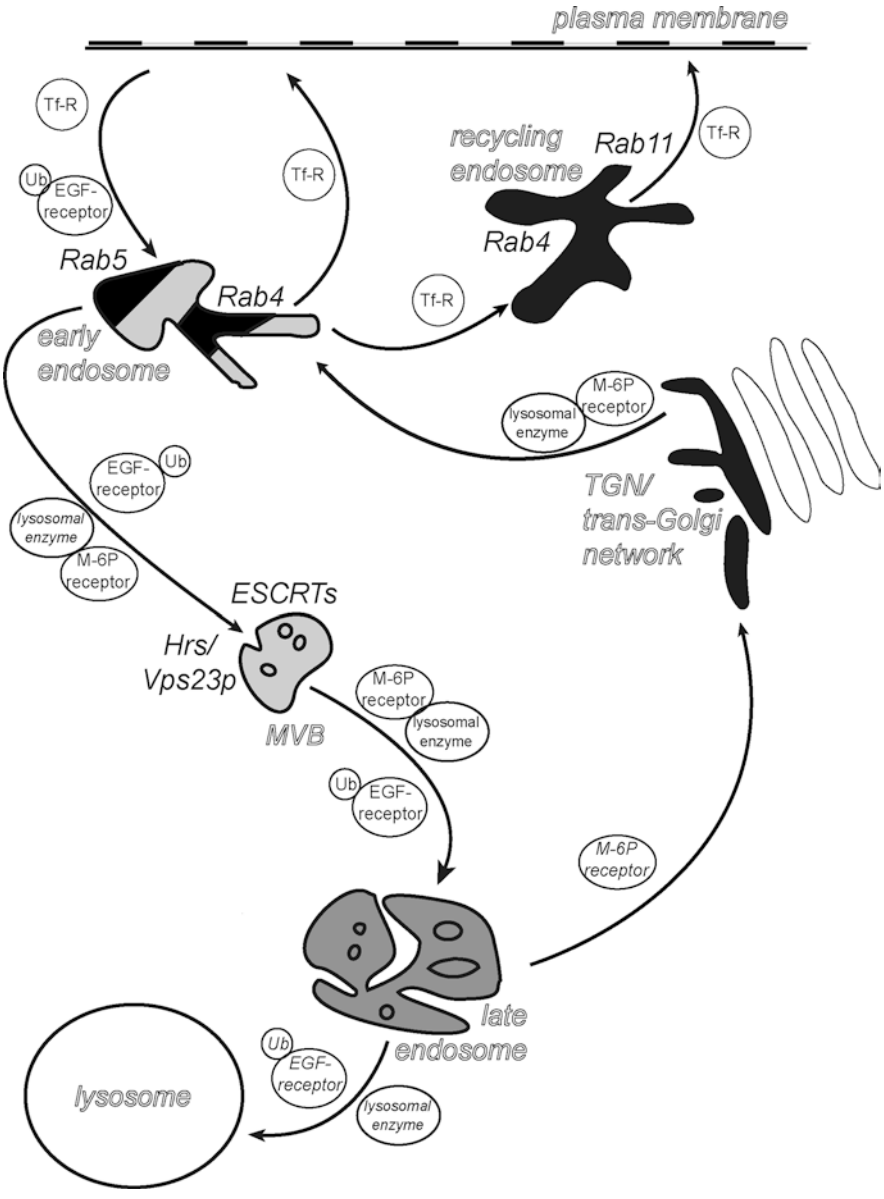

Fig. 1 The endosomal system and trafficking pathways of an animal cell. Compartments and sub-domains marked by specific lipids are *shaded*: PI-3P-rich compartment are shaded *light grey*, sterol-rich compartments are *black*, compartments enriched in lysobisphosphatidic acid (specific for late endosomes) are shaded *dark grey*. Transported marker proteins are *circled in black*; compartmental markers or regulatory factors are *italicised*. *Tf-R* Transferrin receptor, marker for endosomal recycling; *Ub* ubiquitin modification of the EGF-receptor, *M-6P* mannose-6-phosphate

and allows fusion of early endosomes with each other. Recycling tubules, in contrast, contain Rab4 and are negative for PI-3P. Rab4-tubules are thought to be constantly formed from Rab5 domains due to proteins that bind Rab5 and Rab4 simultaneously and thus provide nucleation points for the formation of tubular sub-domains from Rab5 compartments (de Renzis et al. 2002). Rab4-positive tubules are thought to mature into recycling endosomes by separating from Rab5 endosomes and by recruiting Rab11, leading to a compartment from which cargo will eventually recycle back to the plasma membrane (Fig. 1).

The signals and the molecular machinery leading to MVB formation from early endosomes have also recently become unravelled (Katzmann et al. 2002). Mono-ubiquitination of receptors, such as the epidermal growth factor (EGF) receptor, has been recognised as a signal for internalisation and degradation (Fig. 1; Hicke and Riezman 1996). It has now been demonstrated that a ubiquitinated receptor in a PI-3P-containing membrane will cause binding of Hrs/Vps23p. This protein will guide the receptor to the so-called ESCRT-I complex (endosomal complexes required for transport), which, together with other complexes, mediates internal vesicle formation and segregation of the receptor into these vesicles, which is the committed step for degradation for most proteins (Fig. 1). Thus, in a very simplified view, receptor sorting in the early endosome occurs when non-ubiquitinated forms of a receptor are entering the PI-3P-rich environment of early endosomes and then pass on to the rab4-containing recycling tubule, perhaps in a sort of ''default process''. Ubiquitinated proteins, however, will interact with Hrs/Vps23p and thus be retained and segregated away into an ESCRT-complex-containing sub-domain of the endosome, eventually ending up in the internal vesicles of an MVB, forming from the early endosome (Fig. 1).

The endosomal system of plants I—an early charting of the territory

What is known about the endosomal system in plants? In animals, the definition of the endosomal trafficking pathways and compartments is largely derived from studies of receptor-mediated endocytosis, such as the transferrin or the asialoglycoprotein receptor. In plants, the presence of a cell wall obstructs the use of markers employed in animals. In addition, for a long time no cognate, externally applicable, peptide ligand together with a plasma membrane receptor was identified, which could have served as a model for receptor-mediated endocytosis. The first problem was circumvented by making use of protoplasts, where the digestion of the wall renders the plasma membrane proteins readily accessible to externally applied molecules. In the absence of endogenous, detectable ligands, non-specific, electron-opaque markers were used, such as cationised ferritin (reviewed in Robinson and Hillmer 1990). These studies demonstrated uptake of material through clathrin-coated pits, followed by appearance in smooth vesicles and a so-called ''partially-coated reticulum'' (PCR). Later on, the material appears in multi-vesicular bodies and eventually in the vacuole (Fig. 2). In addition, labelling was also observed on Golgi stacks. The significance of this finding is not clear, but might reflect an extensive exchange of material between endocytic and Golgi-based trafficking pathways, which is highlighted by this unspecific probe (Joachim and Robinson 1984; Tanchak et al. 1984, 1988; Hillmer et al. 1986). These early works established a trafficking route of endocytosed material in plant cells, albeit only in wall-less protoplasts where there is always a concern as to how much these reflect the situation of walled cells *in planta*. To address this, uptake of heavy-metal salts was studied, which can also be applied to walled cells. Although heavy-metal salts are potentially problematic because of their toxicity, these studies were able to confirm the results obtained in protoplasts (Hübner et al. 1985). Essentially, the established plant endocytosis route very much resembles the situation in animal cells. In recent years the term ''early endosome'' has gained wide acceptance in the animal literature, as a nice, operational description of this ''tubulo-vesicular'' structure.

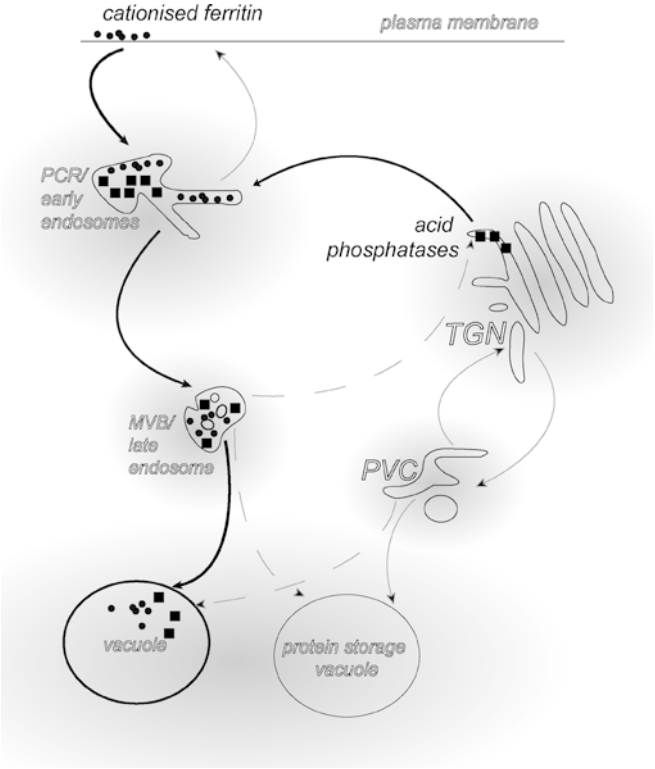

Fig. 2 The endocytic pathway based on early electron-microscopical studies. *Black circles* represent cationised ferritin as an endocytic marker, and *black squares* stand for acid phosphatase activities used to visualise compartments of the biosynthetic vacuolar pathway. Note that these studies did not address the question of vacuolar sub-types. *PVC* Pre-vacuolar compartment

In plants, the morphology-based term PCR was applied. It was initially described by Pesacreta and Lucas as a reticulate structure displaying clathrin budding profiles and shown to be present in a large number of higher-plant species and cell types (Pesacreta and Lucas 1985). PCRs were often observed in proximity to Golgi stacks, but also on their own (Tanchak et al. 1988). In animal cells at least two, functionally distinct, endomembrane compartments fit this description. Clathrin budding profiles can be seen at the TGN as well as at endosomal compartments. Both have a tubular, or reticulate, appearance. However, whereas the TGN is evidently observed to be associated with the trans-side of the Golgi, endosomes are not necessarily found in close proximity. Although the two compartments are constantly exchanging material, they are clearly distinguished by their different function and protein composition. The finding that endocytosed material in plants very soon appears in PCRs very much resembles the situation in animals. Therefore, I would propose that "partially coated reticula" which show an early accumulation of endocytic markers should simply be called "early endosomes". The fact that morphologically very similar compartments exist that are probably the equivalent of the TGN in animals does not mean that these compartments have to be identical to each other, or to be parts of a continuous membrane structure. This has been proposed (Hillmer et al. 1988) and cannot be totally excluded at present. However, I do not think that the available data necessitates such assumptions. From what is known today, the idea of two separate compartments, one for sorting of endocytosed material and another one for sorting of newly synthesised proteins, represents the simpler hypothesis. However, to resolve this question it will be necessary to concomitantly visualise cognate TGN and endosomal markers in plants, preferably with both confocal microscopy and immuno-gold electron microscopy.

The plant endosomal system II—new wine in old wineskins

New visualisations of endosomes—Rab compartments and FM dyes

Recently, there have been a number of publications from groups working on *Arabidopsis* that pertain to the question of endosomal trafficking. Firstly, new endocytic tracers and marker proteins for endosomes and endosomal trafficking were employed. Rab GTPases, for example, are widely used as compartmental markers because of their distinct subcellular localisation. This large protein family can be classed into sub-families, with roles in Golgi, endosomal or vacuolar trafficking. In *Arabidopsis*, the Rab family consists of more than 50 members and, based on structural comparisons with mammalian Rabs, a large sub-family of more than 20 putatively endosomal Rabs can be defined (Pereira-Leal and Seabra 2001; Rutherford and Moore 2002). By transient transfection of *Arabidopsis* protoplasts, two members of this sub-group, ARA6 and ARA7, were localised to small, punctate and fast-moving patches. The identity of these patches was assessed by demonstrating a partial co-localisation of ARA6 or ARA7 with the endocytic tracer FM 4-64 (Fig. 4a; Ueda et al. 2001). This lipophilic molecule only fluoresces upon insertion into the membrane and is widely used in animal and yeast cells as an endocytic tracer. Externally applied FM dyes bind to the plasma membrane and then gradually start to appear in endosomal compartments, eventually highlighting the vacuole/lysosome (Fig. 4a,b). It has been shown that this internalisation is dependent on the vesicle transport machinery (Vida and Emr 1995; Betz et al. 1996) and it is therefore rather certain that in yeast and animal cells, the dye accurately reflects membrane-transport routes down to the vacuole. In plants, a final proof of exclusively membrane-transport-dependent uptake of this dye is lacking. However, sound circumstantial evidence suggests that FM 4-64 (or its analogue FM 1-43) behaves similarly in plants. Specifically, uptake of FM 4-64 in lily pollen tubes was shown to be time- and energy-dependent (Parton et al. 2001), and it was shown for FM 1-43 uptake in tobacco BY-2 cells that two vesicle trafficking inhibitors, Brefeldin A (BFA) and Wortmannin, increase or decrease uptake, respectively (Emans et al. 2002). Thus, the work of Ueda and colleagues defines two Rab proteins as markers for plant endosomal compartments (Fig. 4b). In the future, it will be important to determine if the fast-moving, FM 4-64-positive compartments that they described are identical to the PCR/early endosomal compartments that were previously defined by electron microscopy.

A new use for an old drug—BFA as an inhibito of endosomal recycling

The ability to trace and investigate transport routes of proteins is often dependent on our ability to selectively interfere with protein transport. This is because the steady-state distribution of proteins often does not instruct us about the trafficking pathways the proteins employ to get to their site of action. An obvious illustration of this is the fact that every plasma-membrane-localised trans-membrane protein must have passed at least once through the ER and Golgi. However, in an undisturbed situation the concentration of plasma membrane proteins in the Golgi is most often too low to be detected and one needs to block passage of these proteins through the Golgi in order to prove by their consequent accumulation that they were indeed on passage. The fungal toxin BFA has been widely used for this purpose because it selectively and reversibly blocks membrane trafficking. I want to summarise what is known about this molecule before I discuss what its use has told us about endosomal trafficking of plasma membrane proteins.

Initially investigated for its cytostatic properties (Härri et al. 1963; Ishii et al. 1989), BFA was soon discovered to be an inhibitor of secretion (Misumi et al. 1986), and has since become an important tool to manipulate membrane trafficking. BFA was found to interfere with Golgi-dependent secretion, and also with endosomal/post-Golgi trafficking in mammals (Hunziker et al. 1991; Lippincott-Schwartz et al. 1991; Wood et al. 1991) and in yeast (Gaynor et al. 1998). BFA blocks recruitment of vesicle coat components to membranes by inhibiting activation of ADP ribosylation factor (ARF)-type GTPases (Donaldson et al. 1992; Klausner et al. 1992), thereby compromising cargo selection and vesicle budding from compartments. The molecular targets of BFA were shown to be the ARF GDP/GTP exchange factors (ARF-GEFs; Chardin et al. 1996; Morinaga et al. 1996; Peyroche et al. 1996). It was demonstrated by elegant biochemical, genetic and structural studies that BFA represents a rare case of a non-competitive inhibitor that binds only to a complex between the ARF-GTPase and its exchange factor (Peyroche et al. 1999; Sata et al. 1999; Renault et al. 2003). Certain ARF-GEFs are naturally BFA-resistant and the responsible amino acids map to the BFA binding pocket where they obstruct its binding. These findings now allow us to purposely alter not only BFA-sensitivity, but also to predict how many ARF-GEFs in an organism can be expected to be BFA-resistant (Geldner et al. 2003).

The actual effects of BFA on the endomembrane system are of an awe-inspiring complexity. First, there are various morphological alterations of the compartments, such as tubulation or vesiculation of membranes and aggregation of individual compartments with each other. Although difficult to understand mechanistically at present, they are probably explained by the importance of ARF-GTPases not only for vesicle budding, but also for the maintenance of membrane composition and organelle structure per se (Donaldson and Jackson 2000). A second effect of BFA is the induction of co-localisation of proteins otherwise present on separate compartments. This has been observed for cis-Golgi and ER markers in mammals as well as in plants (Lippincott-Schwartz et al. 1989; Boevink et al. 1998; Batoko et al. 2000) and is thought to reveal a BFA-sensitive pathway between the two compartments that is needed to keep ER- and Golgi-resident proteins separate, despite a constant exchange of material between them. Sometimes, however, instead of being "absorbed" by the ER, Golgi stacks were observed to vesiculate and to agglomerate into so-called "BFA compartments", distinct from the ER (Satiat-Jeunemaitre and Hawes 1992). Possible explanations for these discrepancies, such as differences in the BFA concentrations used or different "sites of action" of BFA were discussed in a number of reviews (Satiat-Jeunemaitre and Hawes 1994; Staehelin and Driouich 1997; Nebenfuhr et al. 2002). It is still worth pointing out, however, that the action of BFA on the endomembrane system is determined by the presence, localisation and relative concentrations of resistant and sensitive ARF/ARF-GEF complexes in the cell—the "sites of action" of BFA. The high number of different ARFs and ARF-GEFs, together with the fact that exchange factors were shown to heterodimerise (Yamaji et al. 2000), adds up to a disconcerting possible complexity of differently sensitive BFA targets. Importantly, there is no apparent selective pressure for or against BFA-resistant residues in exchange factors, since both support functional ARF-GEFs (Peyroche et al. 1999; Geldner et al. 2003). A sensitive exchange factor responsible for a given transport step in one species could well be resistant in another. This is indeed observed when comparing protein sequences between rice and *Arabidopsis*. ARF-GEFs from distinct subgroups can be predicted to be resistant in one species, but sensitive in another (Fig. 3). In addition, not only GEFs but also the ARFs might carry residues that interfere with BFA binding, although those residues are not easily predicted (Jacqueline Cherfils, CNRS, Gif-sur-Yvette, France; personal communication). Therefore, differences in BFA effects between cell types and especially between species are to be expected and should simply be indicated rather than being a matter of dogmatic debate.

The study of BFA effects on plants has long been focussed on its action on the Golgi (Satiat-Jeunemaitre and Hawes 1992; Ritzenthaler et al. 2002). This started to change with the characterisation of the first plant ARF-GEF. It was initially defined by a mutant line, called *gnom* or *emb30*, displaying very strong developmental phenotypes that were shown to be caused by loss-of-function mutations in this ARF-GEF (Shevell et al. 1994; Busch et al. 1996; Steinmann et al. 1999). GNOM is a BFA-sensitive ARF-GEF and is necessary for the ordered localisation of PIN1, a plasma membrane protein and putative auxin-efflux carrier. Although exclusively localised to the plasma membrane in the steady-state, PIN1 rapidly and reversibly accumulates in intracellular compartments upon treatment with BFA (Geldner et al. 2001). These compartments strongly resemble previously described BFA compartments, which were always thought to derive rather exclusively from Golgi membranes (Satiat-Jeunemaitre and Hawes 1992; Wee et al. 1998). However, under the conditions used, individual Golgi stacks could still be observed and it was hypothesised that, in this case, these BFA-induced compartments are rather derived from endosomal compartments. Indeed, GNOM only co-localises with compartments of early FM 4-64 accumulation, but not with ER or Golgi markers (Geldner et al. 2003). Complete co-localisation of GNOM with FM 4-64 and also PIN1 is observed after BFA treatment. When GNOM was rendered BFA-resistant, PIN1 largely remained at the plasma membrane upon drug treatment (Geldner et al. 2003). Thus, the GNOM ARF-GEF appears to be responsible for the recycling of PIN1 from endosomes back to the plasma membrane (Fig. 4b). In addition, ARA7-positive compartments are structurally altered in

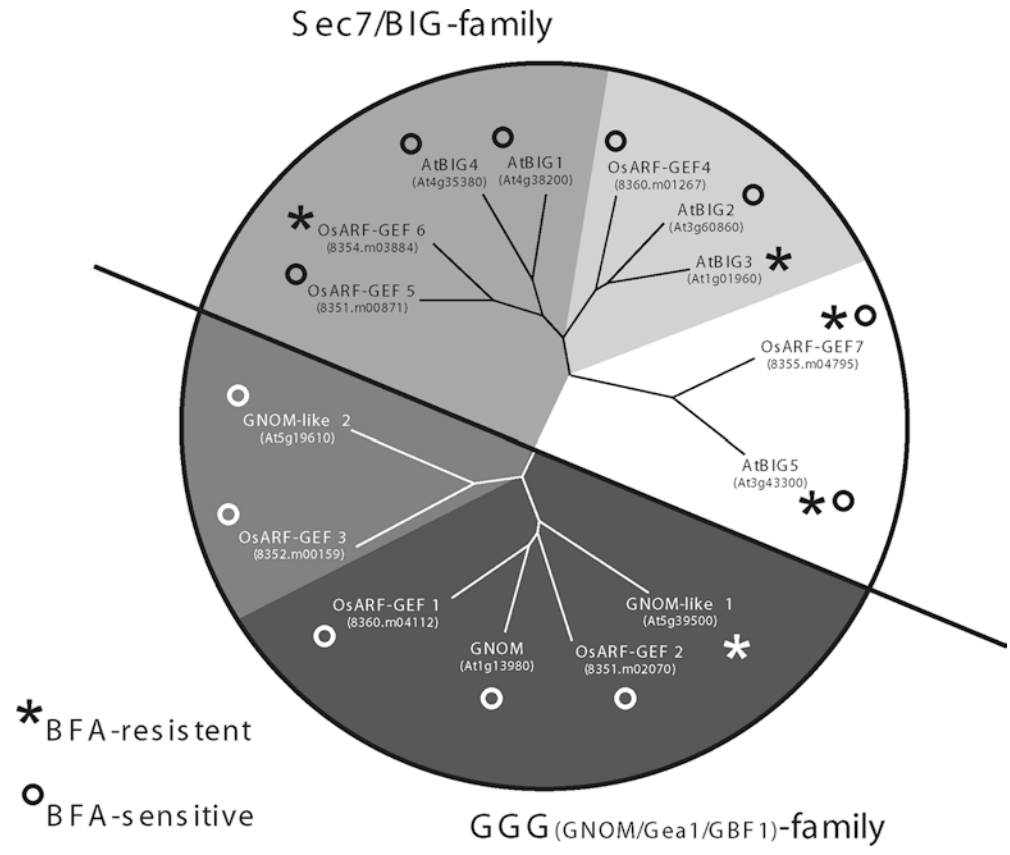

Fig. 3 Phylogenetic tree of ARF-GEF protein sequences from *Arabidopsis thaliana* and rice (*Oryza sativa*). Sequences from both species can be classed into a number of sub-groups. These sub-groups can be seen to represent ARF-GEFs of distinct cellular functions, each group possibly regulating a different trafficking pathway. Proteins marked with *stars* carry residues predicted to lead to BFA-resistance, whereas those marked with *circles* are predicted to be sensitive to BFA (see Geldner et al. 2003 and references therein). Proteins marked with both symbols have ambiguous residues. Note that ARF-GEFs from the same sub-group can be predicted to be sensitive in one species, but being resistant in the other. Nomenclature of *Arabidopsis* ARF-GEFs is as in Cox et al. 2004

gnom mutant cells and appear as larger, ring-shaped structures. This demonstrates that GNOM not only recycles proteins through endosomes, but that it is also important for their structural integrity (Geldner et al. 2003). Concomitant with these studies, other groups independently observed similar effects of BFA on plasma membrane trafficking. It was shown that BFA not only induced intracellular accumulation of plasma membrane markers like PIN1 and PM-ATPase, but also of pectin-epitopes of the cell wall (Baluska et al. 2002). Although in this case a contribution from the biosynthetic pathway cannot easily be excluded, the fact that wall-less protoplasts did not accumulate these epitopes in BFA-compartments suggests that BFA induces uptake of material from the cell wall. The ability of plant cells to internalise sugar-based wall components would provide a surprisingly flexible control of plant cells over their extracellular matrix. Another study investigated trafficking and sub-cellular localisation of sterols in *Arabidopsis* by making use of filipin, a fluorescent marker for sterols that has not been used in plants before (Grebe et al. 2003). Filipin initially labels the plasma membrane, but is then gradually taken up by the cells, eventually highlighting small, punctate intracellular compartments that partially co-localise with the endocytic Rab ARA6 and a trans-Golgi marker. Upon BFA-treatment, filipin label also accumulates in BFA compartments, co-localising with plasma membrane markers and ARA6 (Grebe et al. 2003). This demonstrates that, also in plants, sterols are enriched in the plasma membrane, endosomes and TGN (Fig. 4b). Together, these publications show that, under their specific conditions, BFA induces plasma membrane proteins to co-localise with endosomal markers in BFA-compartments by blocking recycling back to the plasma membrane. Thus, although these compartments have initially been described as being derived from Golgi membranes, the recent studies independently and convincingly show that the ''core'' of the BFA-compartment strongly accumulates plasma membrane and endosomal markers that only partially overlap with trans-Golgi markers. Markers for the cis-part of the Golgi do not accumulate in the ''core'' of the BFA compartment, but rather appear to aggregate around the central ''endosomal'' patch (Baluska et al. 2002; Geldner et al. 2003; Grebe et al. 2003).

Low concentrations of BFA have developmental defects on roots that are completely abolished by the presence of a BFA-resistant variant of GNOM, suggesting that endosomal trafficking is much more sensitive to BFA than the secretory system, at least in *Arabidopsis* roots (Geldner et al. 2003). Elegant FRAP (fluorescence recovery after photobleaching) experiments of Grebe and colleagues provide evidence for this. They show that in the presence of BFA, newly synthesised label re-appears first at the plasma membrane before reaching the BFA compartments (Grebe et al. 2003). This indicates that the secretory pathway in

Arabidopsis is still active at BFA concentrations that inhibit endosomal trafficking. In addition it was found that BFA-treatments of 24 h or more are needed for a secreted green fluorescent protein to accumulate intracellularly, whereas under the same conditions plasma membrane proteins accumulate after 30 min, again illustrating that in *Arabidopsis* roots endosomes are more quickly and readily affected by BFA than the secretory system (Zheng et al. 2004). Thus, BFA has turned out to be a valuable tool for the dissection of endosomal trafficking in *Arabidopsis*.

Biosynthetic vacuolar trafficking and the endosomal pathway—where is the point of convergence?

In yeast as well as in animals, newly synthesised material destined for the vacuole is sorted away from plasma membrane-bound material at the TGN. This sorting is achieved by vacuolar sorting receptors, such as the mannose-6-phosphate receptor in animals or Vps10p in yeast (Keller and Simons 1997; Conibear and Stevens 1998). These TGN-derived vesicles, however, are not directly transported to the vacuole, but instead fuse with compartments of the endosomal pathway (Fig. 1). This convergence of two pathways to the vacuole (the biosynthetic and the endosomal pathway) is nicely illustrated by the analysis of the so-called class-E vps mutants of yeast where both plasma membrane markers and TGN-derived material fail to be delivered to the yeast vacuole and instead end up in a common, prevacuolar hybrid compartment termed the ''class E compartment'' (Conibear and Stevens 1998; Raiborg et al. 2003). The two pathways converge early along the endocytic route, before the formation of MVBs, since some class-E mutants represent genes necessary for MVB sorting, such as Vps27 or factors of the ESCRT complex (see above). Co-localisation of vacuole-bound markers with endocytic material at the level of early endosomes has also been demonstrated biochemically and by electron microscopy (Ludwig et al. 1991) and it was shown that a sorting receptor for vacuolar proteins, the mannose-6-phosphate receptor, enters the endosomal pathway at early endosomes and then recycles back to the TGN from late endosomes (Fig. 1; Hirst et al. 1998; Press et al. 1998). Thus, the MVB sorting pathway of early endosomes is a central point of convergence for transport to the vacuole in both yeast and animals.

In plants, trafficking of newly synthesised material to the vacuole has earned a lot of attention and the extensive literature about this has been well covered by a number of excellent reviews (Neuhaus and Rogers 1998; Bassham and Raikhel 2000; Surpin and Raikhel 2004). In summary, it has been found that plant cells can possess at least two functionally distinct vacuoles, a protein storage vacuole and a lytic vacuole, and a number of different targeting signals in the protein sequences of vacuolar proteins have been identified (Neuhaus and Rogers 1998). In addition, sorting of vacuolar proteins not only occurs at trans-Golgi cisternae, as in animals and yeast, but can even start in cis-Golgi stacks, leading to the formation of non-clathrin-coated, dense vesicles (Hillmer et al. 2001). Alternatively, some storage proteins were shown to entirely bypass the Golgi by a direct ER-to-vacuole pathway (Hara-Nishimura et al. 1998). It is not clear how many different vacuolar pathways actually co-exist in a given species and cell type. In the following I want to focus on TGN-to-vacuole transport pathways and discuss their relationship to the endosomal pathway.

Arabidopsis has a family of seven homologous vacuolar protein-sorting receptors (AtVSR1–AtVSR7) The founding member of this protein family, BP-80, was initially identified in *Pisum sativum* as a transmembrane protein displaying pH-dependent binding activity to the sorting signal of the vacuolar protein barley aleurain (reviewed in Paris and Neuhaus 2002). For *Arabidopsis* VSR1 (ELP), it was demonstrated that it binds to different target signals in vitro, and mutant analysis has shown that it is necessary for delivery of the storage proteins 12S globulin and 2S albumin, but that delivery of a marker for lytic vacuoles, AtALEU, is unaffected (Fig. 4c; Shimada et al. 2003). VSR1/ELP localises to the TGN and to a post-Golgi compartment, defined by the presence of AtPEP12 (AtSYP21; Sanderfoot et al. 1998). At the TGN, VSR1/ELP co-localises with sporamin, but not with barley lectin, which was shown to localise to a different region of the TGN (Ahmed et al. 2000). This demonstrates that different vacuolar sorting pathways issue from the TGN (Fig. 4c). Consistently, vacuolar delivery of barley lectin was shown to be sensitive to inhibition by Wortmannin, whereas sporamin transport is unaffected by this drug (Matsuoka et al.

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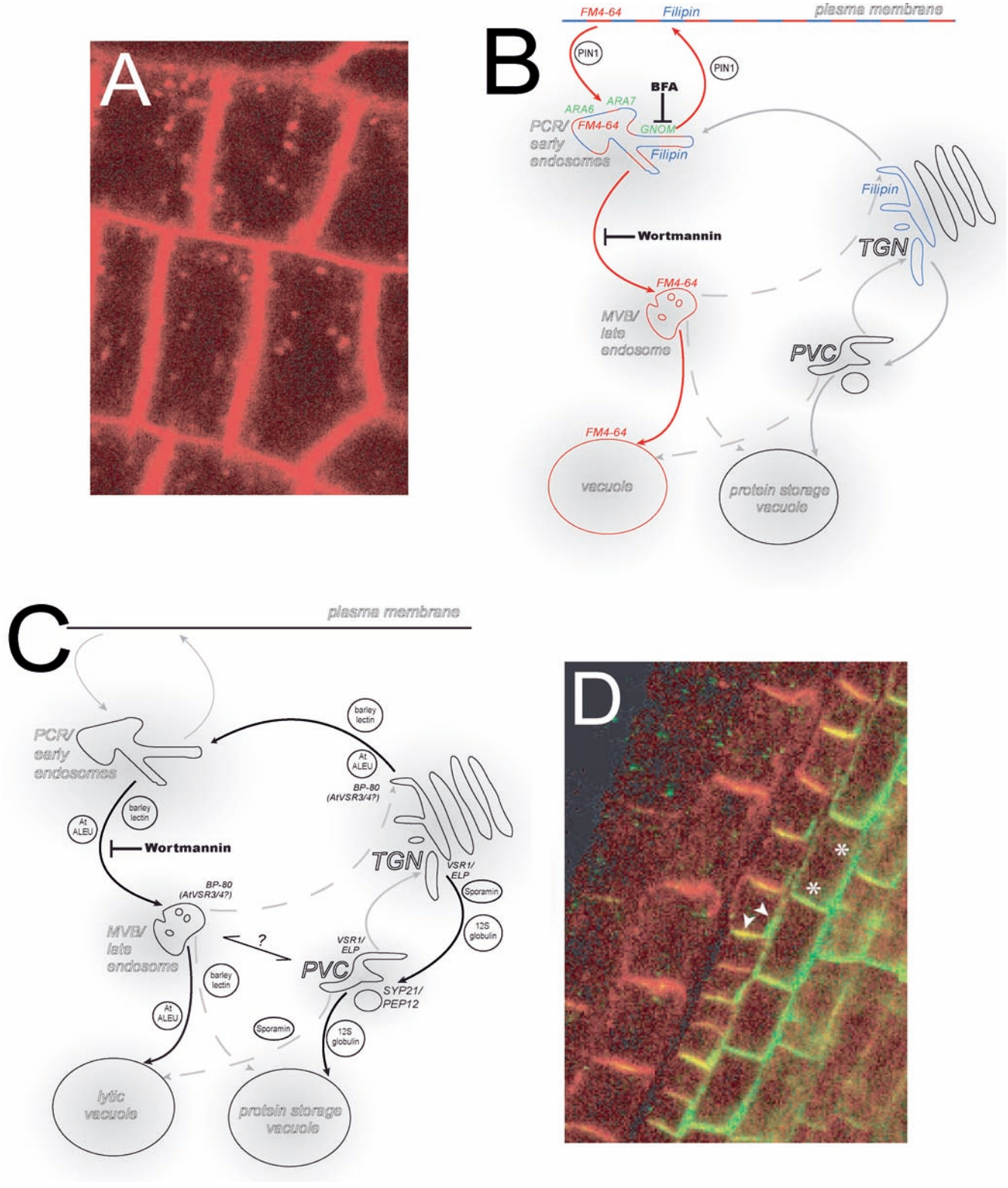
Fig. 4 **a** FM4-64-positive labelled compartments (early endosomes) in *Arabidopsis* root meristem epidermal cells after 10 min of uptake. **b** The endocytic pathway based on recent studies. Compartments highlighted by FM4-64 are marked in *red*. Compartments marked by filipin are in *blue*. Proteins localising to early endosomes are marked in *green*. Transported marker protein (*PIN1*) is *circled in black*. Inhibitors are marked in *bold*, with an indication of their site of action in endosomal trafficking. Wortmannin interferes with PI-3 kinase activity and should therefore block MVB formation from early endosomes. Note that no distinction between early endosomes and recycling endosomes can currently be made in plants (compare with Fig. 1). **c** TGN-to-vacuole transport and its possible relationship to endosomes. Transported marker proteins are *circled in black*, compartmental markers or important factors are *italicised*. Inhibitor is marked in *bold* (see Fig. 3). Note that the exact relationships between vacuolar subtypes and different MVB-dependent and independent pathways remains to be clarified. Also, passage of vacuolar proteins through early endosomes has not yet been demonstrated for plants. *ALEU* Aleurain, *VSR* vacuolar protein-sorting receptor. **d** Double-immunolocalisation of PIN1 (*green*) and plasma membrane (PM)-ATPase (*red*) in *Arabidopsis* root meristem cells. Note the plasma membrane domains labelled with PIN1 only (*green*) or both PIN1 and PM-ATPase (*yellow*; *arrowheads*). Note also the variable and gradual localisation of PIN1 to ''sub-domains'' of the plasma membrane (*asterisks*), arguing against the existence of strict diffusion barriers within the plasma membrane as seen in animal epithelia

1995). Wortmannin inhibits PI-3P production, necessary for MVB formation (see above), suggesting that one of the vacuolar pathways passes through MVBs (Fig. 4c). Indeed, pea storage proteins were shown to localise to MVBs (Robinson et al. 1998). In addition, it was demonstrated that endocytosed cationised ferritin joins compartments with acid phosphatase activity, used to mark the biosynthetic vacuolar pathway (Record and Griffing 1988). Both markers could be co-localised to internal structures of MVBs (Fig. 2). These earlier



findings suggested that endosomal and biosynthetic pathways also converge in plants (Fig. 4c). This view has been beautifully confirmed by a recent publication of Tse et al. (2004), which clearly demonstrates the presence of VSRs on MVBs and shows that their localisation is sensitive to Wortmannin inhibition. Moreover, the authors demonstrate that VSR-positive compartments co-localise with the endocytic tracer FM4-64. Thus, MVBs, as a common passage of endocytic and biosynthetic pathways, are apparently a fundamental, conserved theme in eukaryotes. However, it cannot be excluded that plants contain functionally distinct MVBs for different vacuolar pathways. Moreover, it is not clear whether the AtPEP12/SYP21-positive pre-vacuolar compartment represents a compartment in a linear pathway together with MVBs or whether it is part of an independent, perhaps plant-specific, pathway that delivers material to the vacuole without passing through MVBs (Fig. 4c). The presently available markers and tools in *Arabidopsis* should allow clear dissection and definition of these different pathways. It will be interesting to see, for example, if the AtPEP12/SYP21-positive compartments also receive endocytosed material. Moreover, it will be important to determine if MVBs are the last station before delivery to the vacuole, as suggested by the findings of Tanchak and Fowke, or if an equivalent to late endosomes exists in plants (Tanchak and Fowke 1987). Finally, it remains to be seen if the MVB-dependent pathway can be assigned to either the protein-storage vacuole or the lytic vacuole or if the situation will turn out to be more complicated (Fig. 4c).

The importance of endosomal trafficking for signalling and development—examples from *Drosophila*

Apart from being a fundamental cell biological process that is worth investigating per se, regulated endosomal trafficking is involved in numerous developmental processes that are needed in the context of multicellular development. These include the establishment of morphogen gradients, growth factor signalling or asymmetric cell division. Establishment of morphogen gradients, for example, has long been considered to be merely a problem of creating a localised source, with a stable, graded distribution of the morphogen being simply achieved by passive diffusion together with some constant rate of degradation (Lawrence and Struhl 1996). Wingless, for example, is a secreted protein that is produced by a line of cells and is distributed in a concentration gradient to both sides, instructing the surrounding cells about their position and which fate to adopt. However, it was noted early on that Wingless distribution displays a steeper gradient in one direction from the source than in the other, a behaviour that was difficult to explain at that time (Lawrence 2001). Recently, it has become evident that Wingless is not only perceived by the cells, but is also taken up by receptor-mediated endocytosis and sorted to the multi-vesicular body pathway for degradation (Dubois et al. 2001). It appears that sorting into MVBs occurs at a higher rate in the posterior than in the anterior compartment, thus shortening the morphogen gradient at one side. In addition, Wingless does not freely diffuse in the extracellular matrix, but instead is tightly attached to the membrane. This is explained by the recent finding that Wnt3a, a mammalian Wingless homolog, actually becomes palmitoylated, which would readily explain its tight membrane attachment (Willert et al. 2003). This urges an explanation as to how Wingless then might travel over several cell distances. Again, the explanation might reside in a specialised function of the endosomal pathway. Vesicles in the lumen of MVBs topologically belong to the extracellular space, which means that fusion of an MVB with the plasma membrane would lead to the release of internalised vesicles from the cell. Indeed, lymphocytes employ such a fusion of MVBs with the plasma membrane for the release of so-called exosomes. In addition, retroviruses like HIV also divert the MVB sorting pathway by budding viral particles into MVBs (Gonzalez-Gaitan and Stenmark 2003) and then inducing MVB fusion with the plasma membrane. Such a mechanism of vesicle release into the extracellular space could explain the apparent paradox of a release and spread of Wingless through the tissue while still being tightly attached to the membrane. Co-localisation of wingless with such extracellular carrier vesicles (poetically termed argosomes) has indeed been observed (Greco et al. 2001).

Far from being an exception, a role for vesicle trafficking in morphogen distribution rather might be the rule. Gradient formation of Dpp, another morphogen, was also shown to depend on the presence of its receptor and was compromised when receptor-mediated endocytosis was interfered with (Entchev et al. 2000). This indicates that Dpp distribution through the tissue might come about by successive rounds of endo- and exocytosis from cells. In support of this, interfering with endocytic trafficking by expression of dominant-active forms of Rab5 or Rab7 altered morphogen gradient distribution (Entchev et al. 2000).

Another compelling example of the importance of regulated trafficking during development is the cell fate segregation by asymmetric division during sensory organ development in *Drosophila*. Determination of cell fate between the two daughters of an asymmetric division is dependent on the Notch/Delta system, utilised as a common module in a number of different cell fate decisions during development. Both the membrane-localised ligand Delta and its receptor Notch are present on each of the daughter cells. Negative-feedback loops in this signalling module are then able to amplify small initial differences in Notch signalling, eventually leading to a shut-off of Notch signalling in one cell, causing the adoption of a different cell fate. It is known that asymmetric distribution of the Numb protein into one of the daughter cells during division leads to repression of Notch signalling in this cell, thereby determining its fate.

It has been found that Numb suppresses Notch signalling by recruiting alpha-adaptin to the membrane, a subunit of the endocytic clathrin adaptor AP-2. This induces endocytosis of the Notch receptor (Berdnik et al. 2002) and decreases the level of activatable Notch at the plasma membrane, which ultimately leads to complete suppression of Notch signalling in this cell and to adoption of the according cell fate.

Numb-induced endocytosis of Notch can be seen as a very special case of "desensitisation" of a signalling pathway through receptor-mediated endocytosis. However, instead of only serving as a mechanism for receptor down-regulation, endocytosis can also be necessary to activate a signalling pathway. In the case of the EGF receptor, both mechanisms appear to be the case. Interfering with receptor endocytosis by expressing a dominant mutant form of dynamin (necessary for the pinching-off of endocytic vesicles) leads to increased signalling through the EGF receptor as assessed by its proliferation-inducing activity (Fig. 1; Vieira et al. 1996). However, inhibition of endocytosis suppresses EGF receptor signalling, i.e. the activation of the mitogen-activated protein (MAP) kinase pathway (Vieira et al. 1996). This finding is now understood in molecular terms. It was found that MP1, a protein that binds and organises the different kinases of the MAP-kinase pathway into a complex ("scaffolding"), is recruited to the membrane by virtue of an adaptor protein, p14 (Wunderlich et al. 2001). This adaptor, however, exclusively localises to endosomal membranes. Thus, for sustained and efficient signalling through the MAP-kinase pathway, the EGF receptor has to pass through endosomal membranes. This imposes a temporal delay as well as spatial restriction to EGF signalling and can serve as a mechanism to increase signalling specificity.

The importance of endosomal trafficking for signalling and development—examples from *Arabidopsis*

In plants, molecular genetics in *Arabidopsis* have finally led to an at least partial elucidation of a number of growth factor signalling pathways (McCarty and Chory 2000), making it possible now to investigate how vesicle trafficking and signal transduction intersect in plants. Up to now, evidence for the mere existence of receptor-mediated endocytosis has come from the demonstration of saturable, temperature-dependent uptake of a labelled elicitor fraction into cells (Horn et al. 1989). However, at that time, neither the molecular identity of the receptor, nor the exact nature of the elicitor molecule was known. More recently, it has been demonstrated that the localisation of an *Arabidopsis* homolog of SERK1, a leucine-rich repeat (LRR) receptor-like kinase, is influenced by its ability to be phosphorylated and to interact with KAPP (kinase-associated phosphatase; Shah et al. 2002). It was found that, whereas the normal receptor localises to the plasma membrane, variants mutated at receptor autophosphorylation sites localised to FM4-64-positive compartments. This is a first demonstration that, as in yeast and animals, phosphorylation influences receptor trafficking. However, since these results were obtained by transient transfection of parsley protoplasts and a ligand for this receptor-like kinase has not yet been identified, it remains to be seen how these findings relate to a more physiological situation *in planta*.

In contrast to *Drosophila*, many of the classical developmental mutants characterised in the initial large-scale forward genetic screens have turned out to be important for vesicular trafficking. The first of these genes to be identified, *GNOM*, has already been discussed. The pattern formation defects of *gnom* coincide with severe defects in cell-to-cell alignment (Mayer et al. 1993). Several works have shown that the *gnom* phenotypes are surprisingly well subsumed by defects in auxin transport (Hadfi et al. 1998; Benkova et al. 2003; Geldner et al. 2004). Since GNOM has been demonstrated to recycle auxin-efflux carriers from endosomes to the plasma membrane, it is thought that GNOM is necessary for cells to coordinate their polarity with respect to each other (Geldner et al. 2003, 2004). Indeed polar flow of auxin through the plant tissue can only occur if each cell localises its efflux carrier to the same end, thereby establishing an overall tissue polarity. The constant recycling of efflux carriers and the variability and "sloppiness" of PIN1 polar localisation suggest that plant cells continuously perceive the polarity of their neighbours and re-target their own carriers accordingly (Fig. 4d). Such a dynamic system allows for quick re-direction of carrier polarity which is necessary in processes like lateral root formation where newly forming primordia acquire an axis of polarity orthogonal to the old one (Benkova et al. 2003; Geldner et al. 2004). An even better example of the necessity for vesicle-mediated re-direction of efflux carriers is the gravitropic response of roots. It is thought that the gravitropic stimulus is perceived in the central root cap and translated into an asymmetric distribution of auxin, leading to unequal cell elongation and consequently to a bending of the root (Estelle 1996; Ottenschlager et al. 2003). This response can be recorded after minutes of stimulation. Indeed, it was demonstrated that another auxin-efflux carrier, PIN3, relocates in a matter of minutes from transversal to lateral cell boundaries upon gravistimulation (Friml et al. 2002). This relocation is thought to be responsible for a preferential auxin transport to one side. Although the mechanisms leading to this relocation are not yet known, it is evident that such a quick change upon stimulation can only be achieved by re-directing already present endocytosed carriers.

Another class of genes that illustrate the importance of membrane trafficking for development is represented by mutants like *fackel*, *hydra1*, or *cephalopod*/*orc* (Schrick et al. 2000; Souter et al. 2002; Willemsen et al. 2003). These mutants display a number of severe, but variable phenotypes, such as multiple cotyledons and

shoot meristems, defects in polarity and axis establishment, and disorganised root meristems. All of them have turned out to be affected in sterol biosynthetic genes and were shown to have a strongly altered sterol composition. In yeast, different structural changes in ergosterol, the main yeast sterol, lead to surprisingly specific effects on endosomal trafficking (Heese-Peck et al. 2002). For the *Arabidopsis* mutants it appears that at least some of the defects in the root meristem might be due to defects in auxin transport, and an altered localisation of PIN1 in the root meristem of *orc* mutants has indeed been observed, although it is unclear how directly this defect is linked to altered endosomal trafficking (Willemsen et al. 2003). For *hydra1* it was shown that its root phenotype is strongly improved in a double mutant between *hydra1* and an ethylene-receptor mutant, consistent with the idea that a hyperactive ethylene signal transduction pathway causes part of the phenotype (Souter et al. 2002). A defect in endocytic trafficking caused by altered sterol composition could easily lead to hyperactivity of the receptor. It has to be noted, however, that at present many alternative scenarios could account for the developmental phenotypes of sterol mutants and altered trafficking of signalling and transport components is just one of many plausible explanations. Nonetheless, the above examples from *Arabidopsis* illustrate that understanding the organisation and regulation of plant endosomes is necessary to comprehend how signals are transduced in plants and how individual cells respond to each other in order to integrate into a complex, multicellular organism.

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