Comparative analysis of a membrane trafficking regulator in flowering plants

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1 Summary

The transport of secreted and membrane-bound cargo molecules within a plant cell takes place in membrane-coated vesicles. Vesicle formation at the donor compartment is, amongst other factors, regulated by ADP-ribosylation factor-guanine nucleotide exchange factors (ARF-GEFs). There are 8 large ARF-GEFs in *Arabidopsis thaliana* that mediate vesicle transport between the different endomembrane compartments and with the plasma membrane. The two closely-related ARF-GEFs GNOM and GNOM-like 1 (GNL1) are both involved in retrograde vesicle transport between the Golgi apparatus and the endoplasmic reticulum. In contrast to GNL1, GNOM also fulfils a function in the endosomal recycling of the auxin efflux carrier PIN1 to the plasma membrane. GNOM and GNL1 have the same domain architecture and their sequences are largely conserved, which begs the question of how the functional diversification between GNOM and GNL1 could be explained.

By investigating chimeric proteins with GNOM-GNL1 domain swaps, this study was able to show that because of the heterotypical interaction of the N-terminal DCB domain of GNOM with the three adjacent domains HUS, SEC7 and HDS1, critical motifs are likely exposed on the protein surface of GNOM. The recognition of these motifs then leads to its recruitment to endosomes, whereas GNL1 is recruited to the Golgi membrane. These differences in the protein localization are a requirement for the functional diversification of GNOM and GNL1.

The study was further able to show that the functional divergence between GNOM and GNL1 has evolved in the green plant kingdom. The expression of GNOM and GNL1 paralogues from other species in Arabidopsis *gnom* mutants made it plain that only one of the two proteins of other species is able to replace the GNOM function completely. The expression of the two poplar proteins showed an interesting result: While one of the proteins was able to rescue the *gnom* phenotype completely, the other one was at least able to rescue it partially. This suggests that the functional divergence in poplar has started, but is not complete.

Previous experiments in our lab showed that the interaction between the N-terminal DCB domains of two GNOM proteins probably prevents the formation of GNOM-GNL1 heterodimers (Manoj Singh, Sabine Brumm, Hauke Beckmann, personal communication). It is likely that this is a means to keep GNL1 away from the putative recycling endosome, as it cannot fulfil the required function there. Analogous experiments were performed with the DCB domains of GNOM and GNL1 homologs from several plant species, using the lack of heterodimerization as an indicator for functional diversification. First results indicate that the specialisation in function was initiated with the emergence of the eurosids.

The N-terminal DCB domain can mediate GNOM dimer formation in two ways: DCB-DCB interaction and DCB-ΔDCB interaction. Yeast experiments with mutated DCB domains showed that a charge reversal from the conserved amino acid lysine120 to aspartate ($K_{120}D$) prevents the interaction between two mutated DCB domains; however, the interaction of the mutated DCB domain with GNOM ΔDCB was still possible. This behaviour of the mutant GNOM DCB domain is analogous to the GNL1-DCB domain, which only interacts with ΔDCB. Future experiments in *Arabidopsis thaliana* are going to show the effects of the $K_{120}D$ mutation on the dimerization of GNOM full-length proteins.

2 Zusammenfassung

Der Transport von sezernierten oder membrangebundenen Frachtmolekülen innerhalb einer pflanzlichen Zelle findet in membranumhüllten Vesikeln statt. Die Vesikelbildung am Donorkompartiment wird unter anderem von Adenosyl-Ribosylierungs-Faktor-Guanin-Nukleotid-Austausch-Faktoren (ARF-GEFs) reguliert. In *Arabidopsis thaliana* gibt acht große ARF-GEFs, die den Vesikeltransport zwischen unterschiedlichen Endomembrankompartimenten sowie mit der Plasmamembran vermitteln. Die beiden nahe verwandten ARF-GEFs GNOM und GNOM-like 1 (GNL1) sind beide am retrograden Vesikeltransport zwischen dem Golgi-Apparat und dem endoplasmatischen Retikulum beteiligt. Zusätzlich erfüllt GNOM eine Rolle im endosomalen Recycling des Auxin-Transporters PIN1 zur basalen Plasmamembran, welche nicht von GNL1 ersetzt werden kann. GNOM und GNL1 haben dieselbe Domänenarchitektur, und ihre Sequenzen sind weitgehend konserviert, weshalb noch offen ist, wie die funktionale Diversifizierung zwischen GNOM und GNL1 erklärt werden kann.

Durch die Untersuchung von chimären Proteinen mit GNOM-GNL1-Domänenaustauschen konnte in dieser Arbeit gezeigt werden, dass wahrscheinlich aufgrund der heterotypischen Interaktion der N-terminalen DCB-Domäne von GNOM mit den drei benachbarten Domänen HUS, SEC7 und HDS1 kritische Motive auf der Proteinoberfläche von GNOM präsentiert werden. Die Erkennung dieser Motive führt dann zu dessen Rekrutierung zu Endosomen, wohingegen GNL1 zur Golgi-Membran rekrutiert wird. Diese Unterschiede in der Proteinlokalisierung sind Bedingung für die funktionelle Diversifizierung von GNOM und GNL1.

Des Weiteren konnte gezeigt werden, dass die funktionale Divergenz zwischen GNOM und GNL1 im Reich der grünen Pflanzen evolviert ist. Die Expression von GNOM- und GNL1-Paralogen aus anderen Spezies in Arabidopsis *gnom* Mutanten machte deutlich, dass jeweils nur eines der beiden artfremden Proteine die GNOM-Funktion vollständig ersetzen kann. Die Expression der beiden Pappelproteine zeigte ein interessantes Ergebnis: Während ein Protein den

gnom Phänotyp vollständig rettete, konnte das zweiten Protein zumindest partiell retten, was darauf hindeutet, dass die funktionale Divergenz in Pappel zwar schon begonnen hat, aber noch nicht vollständig abgeschlossen ist.

Vorherige Experimente in unserem Labor zeigten, dass wahrscheinlich die Interaktion der N-terminalen DCB-Domänen zweier GNOM-Proteine die Bildung von GN-GNL1-Heterodimeren verhindert (Manoj Singh, Sabine Brumm, Hauke Beckmann, persönliche Kommunikation). Möglicherweise ist dies ein Mechanismus, um GNL1 vom putativen Recycling-Endosom fernzuhalten, da es dort keine Funktion erfüllen kann. Analoge Experimente mit den DCB-Domänen weiterer Pflanzenhomologe von GNOM und GNL1 wurden durchgeführt, wobei das Fehlen der Heterodimerisierung als Indikator für funktionale Diversifizierung verwendet wurde. Erste Ergebnisse deuten darauf hin, dass die Funktionsspezialisierung mit dem Auftreten der Eurosiden initiiert wurde.

Die N-terminale DCB-Domäne vermittelt die Bildung von GNOM-Dimeren auf zwei Weisen: DCB-DCB-Interaktion und DCB-ΔDCB-Interaktion. Hefeexperimente mit mutierten DCB-Domänen von GNOM zeigten, dass ein Ladungsaustausch der konservierten Aminosäure Lysin 120 zu Aspartat ($K_{120}D$) die Interaktion mit einer zweiten mutierten DCB-Domäne verhindert, wohingegen die Interaktion mit GNOM-ΔDCB immer noch möglich ist. Dieses Verhalten der mutierten DCB-Domäne von GNOM ist analog zur GNL1-DCB-Domäne, die nur mit ΔDCB interagieren kann. Die Auswirkungen der $K_{120}D$ Mutation auf die Dimerisierung von GNOM-Volllängeproteinen werden zukünftige Experimente in *Arabidopsis thaliana* zeigen.

3 Introduction

3.1 The endomembrane system

A single eukaryotic cell is organized in different organelles with specific functions, comparable to the organs constituting an organism. Each of these organelles is surrounded by a membrane to keep them spatially and functionally separate and the entirety of these membrane-bounded organelles is called the endomembrane system. The main compartments of the endomembrane system were investigated via imaging using fluorescently tagged proteins and immunolabeling (Brandizzi et al., 2002; Stierhof & El Kasmi, 2010). It comprises the endoplasmic reticulum (ER), the Golgi apparatus (Golgi), the *trans*-Golgi network (TGN), multivesicular bodies (MVBs) and vacuoles.

The ER surrounds the nucleus and stretches across the entire cell. The Golgi stack can be divided into a *cis*-end, facing the ER, and a *trans*-end. It is believed that the *trans*-most Golgi-cisterna sloughs off, resulting in a cluster of large vesicles forming the TGN which can move independently and is only transiently associated with *trans*-side of the Golgi stack (Viotti et al., 2010). MVBs are big vesicles containing numerous smaller, intraluminal vesicles packed with cargo destined for degradation (Cui et al., 2016). In plant cells, two different types of vacuoles exist: the storage vacuole and the lytic vacuole. The first type is mainly confined to embryonic cells and, after seed germination, the storage vacuoles are converted into lytic vacuoles. The lytic vacuoles in meristematic cells also undergo big changes during differentiation from multiple smaller vacuoles, forming a tubular network to a large central vacuole that occupies more than 90% of the cell volume (Krüger & Schumacher, 2017).

3.2 Trafficking routes

The different compartments of the endomembrane system are connected via membrane trafficking, which is crucial for many aspects of plant life, such as cell

growth, pathogen response or nutrient uptake. There a two major trafficking routes: the secretory and the endocytic pathway (Rojo & Denecke, 2008).

In the secretory pathway, mainly proteins are transported in vesicles from the location of their biosynthesis, the ER, via anterograde vesicle traffic to the Golgi (Fig.1, I) and further on to the TGN. (Fig.1, II) (Viotti et al., 2010). Proteins without any sorting signal are then delivered to the plasma membrane and, if soluble, secreted to the apoplast via the default pathway (Fig.1, III) (Crofts et al., 1999; Denecke et al., 1990). Other proteins are targeted for degradation by vacuolar sorting receptor (VSR) proteins in the ER and the Golgi and are released in the TGN (Früholz et al., 2018; Künzl et al., 2016). From there on, the VSR ligands progress further on to the vacuole, a process that is mediated by maturation of MVBs from a subcompartment of the TGN (Fig.1, IV) (Scheuring et al., 2011; Singh et al., 2014). After the fusion of the MVBs with the vacuole, the proteins are finally released and degraded in the vacuole (Fig.1, V).

In contrast, the other major pathway, the endocytic pathway, does not deal with newly synthesized macromolecules, but with proteins that are internalized at the plasma membrane (PM). These vesicles first reach the TGN, which is therefore also called an early endosome (EE) (Fig. 1, VI). Some of these proteins are then sorted to the vacuole, passing the MVB/PVC (Fig.1, IV and V), which can also be described as a late endosome (LE). Other proteins (mainly carriers, receptors or other transmembrane proteins) cycle back to the PM (Fig. 1, VII). It is assumed that this transport is mediated by a subcompartment of the TGN, called recycling endosome (RE). However, this is poorly documented so far (Geldner & Jürgens, 2006).

The TGN/EE serves as the compartment that merges the secretory and the endocytic pathway (Dettmer et al., 2006). In addition to these two main trafficking routes, there is also retrograde vesicle traffic from the Golgi stacks to the ER. As mentioned above, the ER and the Golgi stacks are transiently physically linked. It was postulated that the *cis*-most Golgi cisternae originates from the ER (Kang & Staehelin, 2008; Y. D. Yang et al., 2005). The retrieval of proteins from the *cis*-most Golgi-cisterna into the ER (Fig.1, VIII) through the retrograde trafficking pathway is important for the homeostasis of membrane

export from the ER (Brandizzi, 2017) as well as for the protein quality control that takes place there.

Finally, during cell division, a reprogramming of normal trafficking pathways takes place. All newly synthesized and endocytosed proteins are sorted by the TGN to the plane of cell division where they fuse to form a cell plate, which then matures to a new plasma membrane and attached cell wall separating the two daughter cells. This is specific for the plant kingdom (Müller & Jürgens, 2016; Richter et al., 2014).

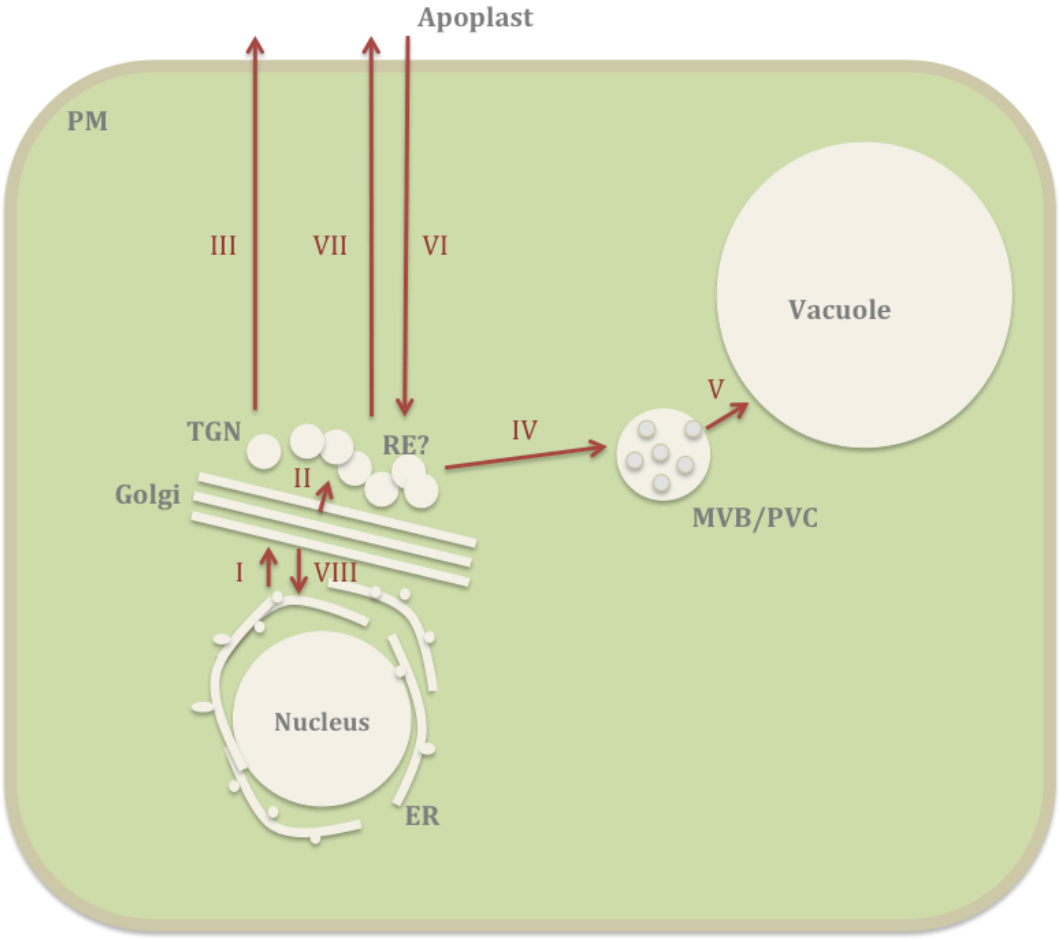

Fig.1 Various trafficking pathways link the different organelles with each other. In the secretory pathway, newly synthesized proteins are transported in vesicles from the ER to the Golgi (I), followed by the TGN (II). From there on, proteins are either delivered to the PM and secreted to the apoplast (III) or sent via the MVB/PVC (IV) to the vacuole (V) where they are degraded. In the endocytic pathway, internalized proteins from the PM reach first the TGN (VI) and can be recycled back to the PM (VII) or delivered via the MVB (IV) to the vacuole (V). In addition to the anterograde ER-Golgi transport (I) a retrograde Golgi-ER pathway exists as well (VIII).

3.3 Regulation of Vesicle Budding

Vesicular traffic is a complex mechanism, which consists of four different steps: 1) Formation and budding of vesicles from the membrane of the donor organelle, 2) vesicle transport along the cytoskeleton of the cell, 3) membrane tethering with the acceptor compartment and 4) the fusion with the acceptor membrane (Stalder & Antonny, 2013). As this thesis deals with key players in vesicle budding, the first step in vesicular traffic, its regulation is explained in more detail in this chapter (see also Fig. 2).

Major regulators in vesicle budding are ADP-ribosylation factors (ARFs). These proteins can act as molecular switches. In their inactive, cytosolic form, they are bound to GDP. After recruitment to the donor membrane, GDP is converted to GTP, leading to an active ARF molecule, which then recruits further effectors for vesicle budding (Jadhav et al., 2015). ARFs are also called ARF-GTPases and belong to the family of G proteins. In contrast to other G proteins that have a lipid modification in the C-Terminus, ARF-GTPases harbor an N-terminal amphipathic helix consisting of a myristoyl group that is inserted into the membrane of the donor compartment while activated (Gillingham & Munro, 2007). For the ARF family member Sar1p, it was shown that the amphipathic helix can contribute to lipid bilayer bending during vesicle budding (M. C. Lee et al., 2005).

ARF activation is mediated by ARF Guanine Exchange Factors (ARF-GEFs), which also alternate between a cytosolic and a membrane-bound form (Donaldson & Jackson, 2000; Jackson & Casanova, 2000). A common feature amongst all ARF-GEFs is their catalytic domain, also called SEC7 domain, which is capable of activating ARF-GTPases. The molecular structure of the SEC7 domain while binding an ARF substrate was solved by crystallization (Goldberg, 1998; Mossessova et al., 2003; Renault et al., 2003). It was shown that two conserved regions in the SEC7 domain act together, forming the active site that borders a hydrophobic groove where the GDP to GTP exchange takes place.

After cargo recruitment and coat assembly (Bremser et al., 1999), the activated ARF-GTP is then hydrolyzed to ARF-GDP again (Nickel et al., 1998; Nie & Randazzo, 2006; Weiss & Nilsson, 2003). Recruitment of coat proteins to the donor membrane is an essential step in vesicle budding, as they force the

membrane in a curved structure leading to a local protuberance of the membrane (Beck et al., 2008; Krauss et al., 2008). The forming coated vesicles are then pinched off from the membrane (Beck et al., 2011; M. C. Lee et al., 2005; Sweitzer & Hinshaw, 1998). For some ARF-GEFs, their direct interaction with coat proteins was proven as well. For example, the human ARF-GEF GBF1 binds to GGA (Lefrancois & McCormick, 2007), whereas the yeast protein Sec7p interacts with COPI (coat of vesicles in retrograde Golgi-ER traffic) and COPII (coat of vesicles in anterograde ER-Golgi traffic) (Deitz et al., 2000).

As mentioned above, it is important for vesicle budding to hydrolyze the GTP bound to ARF to obtain non-activated ARF-GTPase in the cytosol, which can be recruited to the membrane again. ARFs have a very low intrinsic rate of hydrolysis, making it necessary to have GTPase-activating proteins (GAPs) (Kahn & Gilman, 1986). The key players of vesicle budding, ARF-GTPases, ARF-GEFs, ARF-GAPs and coat proteins, will be introduced in more detail in the following chapters.

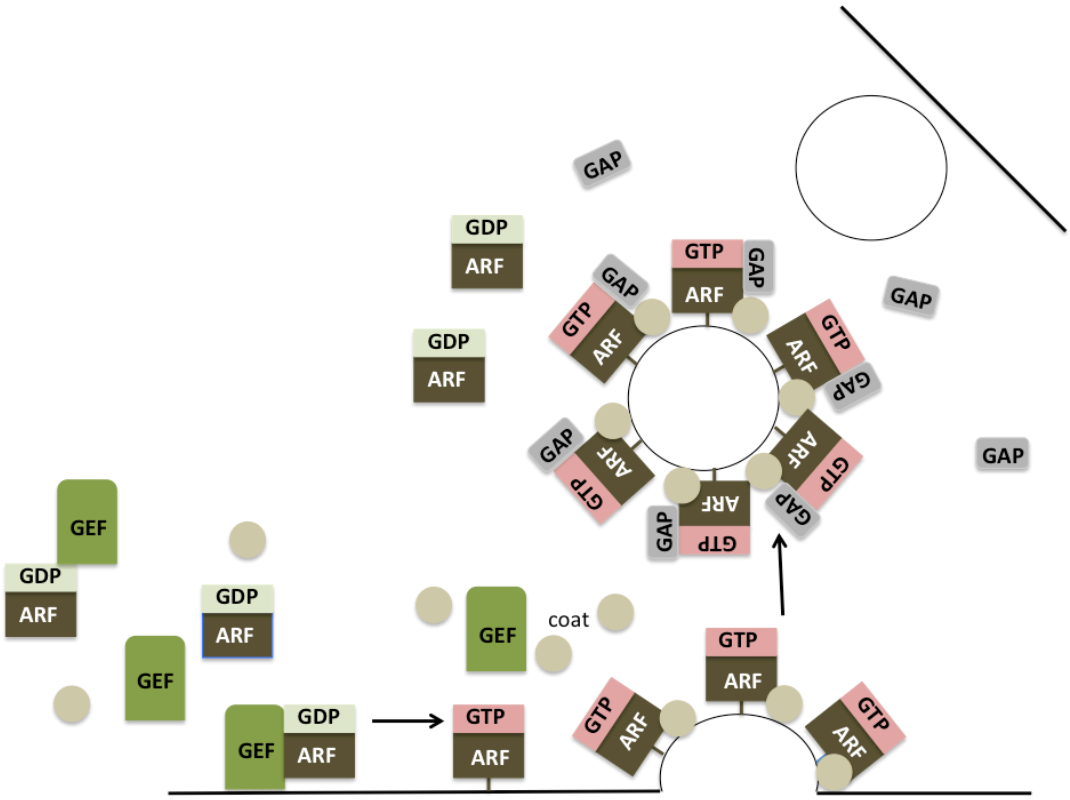

Fig.2 One critical step during vesicle budding is the recruitment of GDP-bound, inactive ARF-GTPase and its exchange factor, ARF-GEF, to the membrane of the donor compartment. After ARF activation via GDP to GTP exchange, the ARF-GEF is released back to the cytosol. The active ARF-GTP assembles coat proteins on the surface of the donor organelle, which leads to membrane curvature and vesicle formation. The vesicle is then pinched off the donor compartment and is transported to its destination. GTP is hydrolyzed by ARF GTPase with the assistance of ARF-GAP and the resulting ARF-GDP falls off the membrane. The inactive ARF-GDP can then serve in another round of vesicle budding.

3.4 ARF-GTPases

Together with the Rab, Ran, Ras and Rho family, ARF-GTPases constitute the Ras superfamily (Wennerberg et al., 2005). Arfs were first identified as cofactors for cholera toxin that affects the ADP-ribosylation of G proteins. Consequently, these cofactors were termed ADP-ribosylation factor GTPases as they are GTP-binding proteins themselves (Kahn & Gilman, 1986; O'Neal et al., 2005). It was only later on that their major task as key players in vesicle budding was discovered. In addition to the already mentioned myristoyl group, which is important for membrane anchoring of the ARF protein, another important structural feature are the so-called switch regions. These regions are the effector binding sites. After GTP binding, a loop of β-sheet, the so called interswitch, moves away from the GTP binding site and causes the myristoylated anchor to insert into an adjacent lipid bilayer (Goldberg, 1998; Pasqualato et al., 2002).

The presence of ARF proteins is conserved amongst eukaryotes, but there are differences between various kingdoms. In mammalians, three ARF classes are present (Class I, II and III), comprising six Arf proteins that localize to different compartments, therefore clearly catalyzing vesicle budding in different trafficking pathways. In contrast, *Saccharomyces cerevisae* has three Arf proteins, which are orthologs of human Arfs from the Class I and III. Surprisingly, in *Arabidopsis thaliana*, two new classes have evolved over time, ARF A and ARF B, and the only ARF orthologs of yeast and mammals belong to the ARF I class. Arabidopsis has nine *ARF* genes in total, six of them belonging to the ARF I class, two making up class A and one class B (Jürgens & Geldner, 2002). The role of the

additional plant specific classes is unknown so far, but it is speculated that they act in trafficking pathways that are special to the plant kingdom.

It was shown that Arabidopsis ARF 1 is involved in different trafficking pathways as it localizes to the Golgi and post-Golgi structures, implying a role in ER-Golgi traffic (Lee et al., 2002; Takeuchi et al., 2002) as well as endocytosis and/or recycling traffic (Naramoto et al., 2010; Tanaka et al., 2014; Xu & Scheres, 2005). Matheson et al. (2008) showed that the destination of ARF B is the plasma membrane (PM). In the same paper, motifs for membrane recruitment were studied in more detail. For ARF 1, this motif was narrowed down to the first 18 amino acids, whereas it was shown that the localization of ARF B to the PM is determined by an interplay of various domains which could not be identified (Matheson et al., 2008). Overall, membrane recruitment to specific organelles seems to be very complex as ARF1 localizes to different compartments.

ARFs seem to act redundantly as single ARF protein knockdowns did not affect Golgi organization, whereas altering the expression of two ARFs simultaneously led to an impairment of specific steps along the secretory and endocytic pathway. This might add some specificity to vesicle budding (Volpicelli-Daley et al., 2005).

In addition to the mentioned ARFs, the mammalian protein Sar1 and Arf-like proteins (Arls) are clustered in a larger Arf family. Apart from their sequence similarity, they do not share much common ground as they are lacking ARF activity (Kahn et al., 2006; Pasqualato et al., 2002).

The substrate specificity between Arf GTPases and their exchange factors was investigated in the mammalian field, bringing up the hypothesis that one Arf can only be activated by its corresponding Arf-GEF (Gillingham & Munro, 2007). A similar model for Arabidopsis is missing so far.

3.5 ARF-GEFs

The involvement of ARF-GEFs in the activation of ARF GTPases was first mentioned in 1996 (Chardin et al., 1996; Morinaga et al., 1996; Peyroche et al.,

1996). The critical domain for GDP to GTP exchange, the SEC7 domain, is conserved amongst yeast, mammal and plant ARF-GEFs (Chardin et al., 1996). It was labeled SEC7 domain, as it is also present in Sec7p, a yeast ARF-GEF involved in secretion (Franzusoff et al., 1991).

In the active site of the catalytic SEC7 domain, a conserved glutamate residue promotes GDP dissociation of the ARF substrate, followed by GTP-binding and release of the ARF1-GTP (Renault et al., 2003; Robert et al., 2004). Furthermore, the SEC7 domain harbors a binding site for the fungal toxin Brefeldin A (BFA), which stabilizes the intermediate state of ARF-GEF – ARF-GDP interaction (Cherfils & Melancon, 2005; Renault et al., 2003; Robineau et al., 2000). This affects secretion by inhibiting ER-Golgi transport and re-distributing Golgi-resident membrane proteins to the ER (Doms et al., 1989; Donaldson et al., 1990; Lippincott-Schwartz et al., 1989). Another BFA-effect is the aggregation and fusion of TGN and endosomal compartments, whereas vesicle cycling between plasma membrane and endosome is unaffected (Lippincott-Schwartz et al., 1991; Wood et al., 1991).

Critical amino acids acting as a BFA-target were first identified in yeast (Peyroche et al., 1999; Sata et al., 1999). In Arabidopsis, some ARF-GEFs are naturally sensitive to BFA, whereas others are resistant to the BFA effect (see Fig. 3). Geldner et al. (2003) rendered GNOM, the best-investigated ARF-GEF in Arabidopsis, BFA-resistant by exchanging one of the BFA targeting sites, methionine, at position 696 for leucine (Geldner et al., 2003). Blocking single ARF-GEFs by engineering their BFA-binding sites was used as a tool to investigate their role in specific trafficking routes (Geldner et al., 2003; Richter et al., 2007; Richter et al., 2014).

ARF-GEFs are classified due to their protein size in small (~40-80 kDa), middle (~100-150 kDa) and large (~170-200 kDa) ARF-GEFs (Cox et al., 2004; Mouratou et al., 2005). Only in mammals, all three classes exist, comprising the six ARF-GEF families GBF1, BIG, IQSEC, cytohesins, and FBX08. Sec12 is a special Arf-GEF which exclusively activates Sar1. The large Arf-GEFs in *Saccharomyces cerevisae* can be clustered in three large Arf-GEFs, Sec7p, Gea1p and Gea2p, and two medium-sized Arf-GEFs, Syt1p and Yel1p (Cox et al., 2004; Gillingham & Munro, 2007). In Arabidopsis, there are fewer classes of ARF-GEFs as there are

fewer classes of ARF-GTPases: compared to mammals, plants lack two classes. Here, only the large ARF-GEFs are present. They can be separated in two families: The GBF1-related family with the members GNOM, GNL1 and GNL2 is related to human GBF1 and Gea1/2p from yeast, and the BIG-family comprising the proteins BIG1-5, which are putative orthologs of human BIG1 and BIG2 as well as yeast Sec7p (Fig. 3).

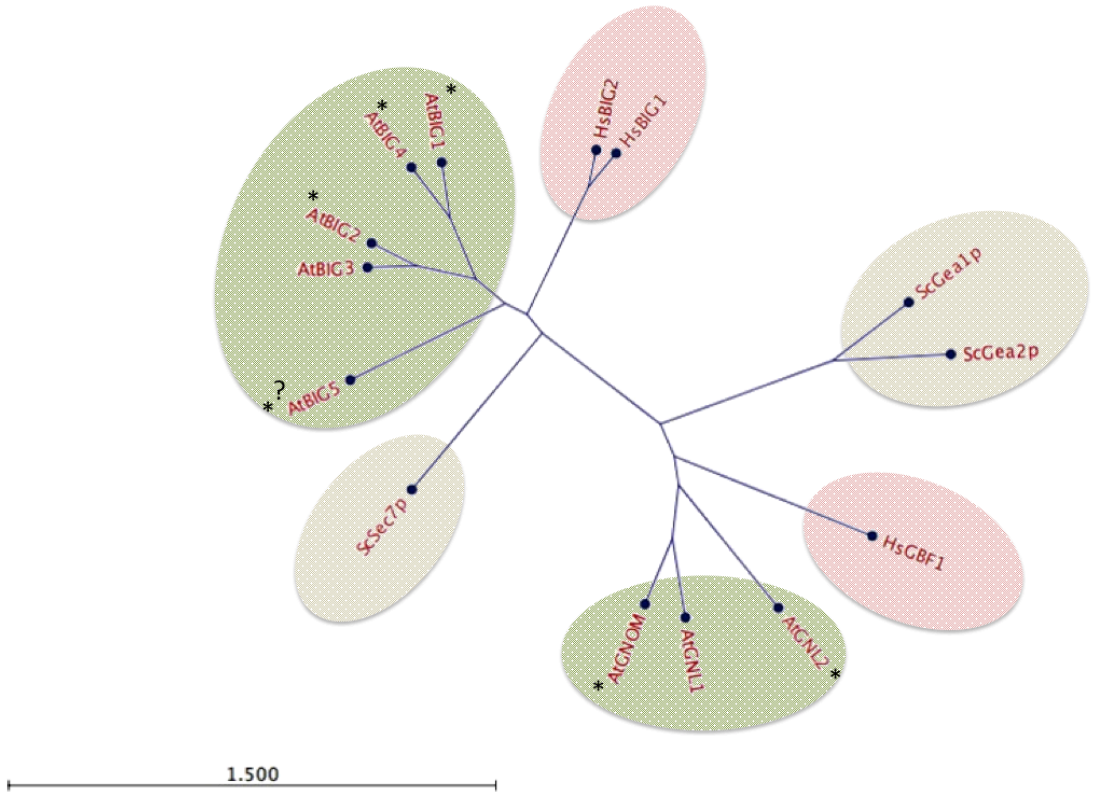

Fig.3 Phylogenetic tree depicting all large ARF-GEFs from *Homo sapiens* (Hs, pink), *Saccharomyces cerevisiae* (Sc, brown) and *Arabidopsis thaliana* (At, green). The upper clade represents the BIG family, the lower part shows the GBF1-related family. In accordance to already described critical amino acids for BFA binding, BFA-sensitive proteins in Arabidopsis are marked by an asterisk. The situation in BIG5 is contradictory.

Most of the Arabidopsis ARF-GEFs have been studied in more detail and the following section will give a brief overview of the most important findings. GNOM was identified in an EMS mutagenesis screen aiming to find key players of embryonic development (Mayer et al., 1991; Mayer et al., 1993). Later on, it was classified as a member of the ARF-GEF family with a role in the recycling of the

auxin efflux carrier PIN1 and endosomal localization (Friml et al., 2003; Geldner et al., 2003; Geldner et al., 2004; Kleine-Vehn et al., 2008; Steinmann et al., 1999). The involvement of GNOM in secretion together with GNL1 was demonstrated by investigating their role in retrograde Golgi-ER transport (Richter et al., 2007; Teh & Moore, 2007). Teh et al. (2007) propose that GNL1 acts in the endocytosis of specific proteins as well.

While GNOM and GNL1 are expressed ubiquitously, the third member of the GGG family, GNL2, is only expressed in pollen. It was shown that GNL2 mediates pollen tube and root hair growth by mediating polar recycling. Additionally, GNL2 can replace GNOM´s function in recycling and secretion in somatic cells when expressed under the GNOM promoter (Richter et al., 2011).

Richter et al. (2014) showed that BIG 1-4 act together in post-Golgi trafficking. All four proteins localize to the TGN where they mediate the transport of newly synthesized proteins to the PM. Both secreted and endocytosed proteins are delivered to the plane of cell division by the help of BIG 1-4 during cytokinesis (Richter et al., 2014).

The most divergent member of the BIG family, BIG5, localizes to an early endocytic compartment where it is involved in the endocytosis of PM proteins. A further involvement in plant immunity was shown, as BIG5 is degraded after pathogen infection (Nomura et al., 2006; Nomura et al., 2011; Tanaka et al., 2009; Xin et al., 2016).

Based on multiple sequence alignments, six domains were identified for large ARF-GEFs (Cox et al., 2004; Mouratou et al., 2005). SEC7 is probably the best investigated of these domains, whereas the adjacent domains still have to be analyzed in more detail. Because of a lack of knowledge about their functions, these domains were called ‘homology domain upstream of SEC7’ (HUS) and ‘homology domain downstream of SEC7 1, 2 and 3’ (HDS 1-3).

The most N-terminal domain is called ‘Dimerisation and Cyclophilin-Binding Domain’ (DCB), as it mediates dimerization between two ARF-GEF proteins (Grebe et al., 2000). Unfortunately, it is questionable whether the DCB domain really binds to Cyclophilin *in planta* (Anders & Jürgens, 2008), whereas the homodimerization of two DCB domains was studied in much detail: Critical amino acids in this process were analyzed in the human ARF-GEF GBF1 (Bhatt et

al., 2016; Ramaen et al., 2007). In Arabidopsis, a so called heterotypic interaction of the DCB domain with the HUS and SEC7 domain within the same molecule was demonstrated and amino acids that are involved in this interaction were identified (Anders et al., 2008). In addition, the DCB domain is required for membrane association, but it is not sufficient for membrane binding (Monetta et al., 2007). However, a mutant allele of GNOM, *gnom*^{SIT4}, lacking only the domains HDS 1 to 3 is then able to fulfill GNOM function nearly completely (Geldner et al., 2004).

Fig. 4 Domain architecture of large ARF-GEFs

3.6 ARF-GAPs

The fusion of vesicles with their target membrane requires coat dissociation that is triggered by the hydrolysis of ARF-GTP to ARF-GDP (Spang et al., 2010). As ARFs have a very low intrinsic rate of hydrolysis, GTPase-activating proteins (GAPs) are required to catalyze this reaction (Kahn & Gilman, 1986). Furthermore, hydrolysis of active ARF-GTPases triggers coat dissociation. The timing of the ARF-GAP activity is critical because the coat subunits have to assemble into a vesicle coat first.

Two regulatory mechanisms were proposed to control the mammalian Arf-GAP1 (Spang et al., 2010): In the first model, the activity of Arf-GAPs depends on the coat protein subunits and is inhibited by cargo proteins (Goldberg, 2000; S. Y. Lee et al., 2005; Weiss & Nilsson, 2003; J. S. Yang et al., 2002). In the second model, Arf-GAP1 is able to sense membrane curvature through specific motifs called Arf-GAP lipid-packing sensor (ALPS) (Drin et al., 2007). Compared to the relatively flat surface around the forming vesicle, the curvature leads to a local bud and is a result of coat protein polymerization. (Bigay et al., 2005; Bigay et al., 2003; Drin et al., 2007; Mesmin et al., 2007; Y. D. Yang et al., 2005). Ongoing

investigations which are dealing with these two models strengthen our understanding of ARF-GAP regulation.

A common feature of all ARF-GAPs is a Cys_4Zn-finger motif in the N-terminal GAP homology domain. The C-terminus, on the other hand, is not conserved and might target ARF-GAPs to different endomembranes (Cukierman et al., 1995).

The number of ARF-GAP genes varies amongst the different kingdoms. Mammals harbor 31 genes encoding proteins with an Arf-GAP catalytic domain (Kahn et al., 2008), whereas yeast only has six proteins which potentially hydrolyze Arf1 and Arf2 proteins (Kawada et al., 2015). 15 ARF-GAPs are present in Arabidopsis, called Arabidopsis GAP domain proteins (AGDs) (Singh & Jürgens, 2017). Plenty of data is still missing to understand the function of ARF-GAPs in more detail, but it is already clear that they are important regulators in membrane trafficking.

3.7 Coat proteins

Coat proteins are essential for the formation of transport vesicles. A large number of individual coat proteins can bind to each other as well as lipids in the membrane surface of donor compartments. In addition, they can interact with cargo proteins, thereby aiding in cargo sorting.

The role of coat proteins during vesicle transport can be structured in four functions: Cargo collection, induction of membrane bending which leads to a coated bud, membrane scission and disassembly of the coat to allow fusion with the target membrane (Faini et al., 2013).

The main coats COPI, COPII and clathrin are recruited to the membranes by active ARF-GTPases and are involved in different trafficking routes. Clathrin acts in the late secretory and in the endocytic pathway. COPII-coated vesicles are transported between the ER and the Golgi, whereas COPI is the coat for vesicles in the retrograde Golgi-ER traffic as well as in the intra-Golgi transport (Faini et al., 2013).

Clathrin, COPI and COPII coats share a common organization as they can be divided into an adaptor and a cage complex. In the case of clathrin and COPII, the

adaptor complexes are first recruited to the membrane, followed by the cage complexes. These cage complexes are able to interact with each other via subdomains and form a "cage" that eventually leads to membrane curvature. It was shown that different clathrin adaptor complexes are associated with different membranes, comprising the adaptor proteins (APs) AP1-5, the γ-ear containing Arf-binding proteins (GGAs) and AP180 (Morris et al., 1993; Nakayama & Wakatsuki, 2003). Clathrin heavy chains and light chains serve as the cage complex (Heuser & Kirchhausen, 1985). The COPII adaptor complex consists of Sec23 and Sec 24, whereas Sec13 and Sec31 form the cage complex (Barlowe et al., 1994).

In case of COPI, the adaptor and cage complexes are associated with each other, building up a heptameric complex which is recruited to the membrane *en bloc.* The seven core subunits of the COPI coat, also called coatomer, are α-COP, β-COP, β'-COP, γ-COP, δ-COP, ε-COP and ζ-COP (Hara-Kuge et al., 1994).

4 Aim of this thesis

Intracellular vesicle transport is essential for many aspects in plant life, like cell growth, communication between cells and pathogen response. Many proteins are involved in the regulation of vesicle formation, amongst them, ADP-ribosylation factor-guanine nucleotide exchange factors (ARF-GEFs).
The family of large ARF-GEFs in *Arabidopsis thaliana* comprises eight members, of which GNOM is the best-characterized. GNOM was shown to mediate endosomal PIN1 recycling (Geldner et al., 2003; Steinmann et al., 1999) as well as retrograde vesicle trafficking from the Golgi apparatus to the endoplasmic reticulum (Richter et al., 2007). The latter function is also regulated by the closely related Arabidopsis protein GNL1 (Richter et al., 2007) and is conserved in the eukaryotes mammals and yeast (Peyroche et al., 2001; Spang et al., 2001; Zhao et al., 2006).
This thesis focused on the characterization of the functional diversification of GNOM of GNL1 using different experimental approaches.

The analysis of chimeras with GNOM and GNL1 domain swaps should help in the identification of critical domains for recruitment of the GNOM protein to the membrane of the putative recycling endosome.
The next aim was the analysis of the functional diversification in the course of evolution. Rescue experiments expressing GNOM and GNL1 homologs from different plant species in the *gnom* and *gnl1* mutant Arabidopsis background should help in understanding whether GNL1 has simply lost the ability of endosomal recycling during evolution.
The same question was addressed in a different experimental setup: Due to their specialization in endosomal recycling and retrograde Golgi-ER trafficking respectively, GNOM and GNL1 do not form heterodimers. The heterodimer formation of different species of the Viridiplantae kingdom should be tested in yeast two-hybrid assays. The prevention of heterodimer formation could be used as an indicator for a likely functional diversification of the two tested GNOM-GNL1 related proteins.

The importance of the interaction between two N-terminal DCB domains for the formation of GNOM dimers should be analyzed in this thesis as well. Yeast two-hybrid interaction studies should help to test point mutations in the GNOM DCB domain in order to find critical amino acids for this interaction. Future experiments expressing full-length GNOM proteins with these DCB mutations *in planta* could further deepen our understanding of the GNOM homodimer.

5 Draft Manuscripts

5.1 Draft Manuscript 1:

The membrane specificity of the large ARF-GEF GNOM is a result of heterotypic interaction between four domains

Contributions:

The GNOM-GNL1 chimeras were cloned by several people:

DCB-swap = GNOM$^{GNL1\ DCB}$:	Hanno Wolters
HUS-swap = GNOM$^{GNL1\ HUS}$:	Hanno Wolters
SEC7-swap = GNOM$^{GNL1\ SEC7}$:	Shinobu Tanaka
HDS-swap = GNOM$^{GNL1\ HDS1-3}$:	Shinobu Tanaka
DCB-HUS-SEC7-swap = GNOM$^{GNL1\ DCB-HUS-SEC7}$:	Marika Kientz
HUS-SEC7 swap = GNOM$^{GNL1\ HUS-SEC7}$:	Nadine Anders
SEC7-HDS swap = GNOM$^{GNL1\ SEC7-HDS1-3}$:	Kerstin Huhn

Plant transformation and crosses in the *gnom* (*sgt*) and *gnl1* background respectively were performed by Sandra Richter, Alice Wagner and Kerstin Huhn.

Except of the characterization of the DCB swap, all rescue experiments were performed by Alice Wagner under the supervision of Kerstin Huhn.

The Co-IP experiments were performed by Manoj Singh, Alice Wagner and Kerstin Huhn.

The interpretation of the results as well as the writing of the manuscript was done by Kerstin Huhn.

Draft Manuscript 1:

The membrane specificity of the large ARF-GEF GNOM is a result of heterotypic interaction between four domains

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ABSTRACT

Membrane traffic is essential within a eukaryotic cell. It depends on the function of guanine nucleotide exchange factors regulating vesicle formation at a donor compartment. The two closely related large ARF-GEF proteins in *Arabidopsis thaliana*, GNOM and GNL1, localize at the Golgi and jointly regulate retrograde Golgi-ER trafficking. In addition, GNOM mediates vesicle formation at the putative recycling endosome and thereby regulates the polar recycling of the auxin efflux carrier PIN1. Since GNOM and GNL1 share 63% sequence identity, this raises the question of how membrane specificity is mediated. Our analysis of different GNOM-GNL1 chimeras suggests that the GNOM-specific heterotypic interaction between four domains is critical for its endosomal recycling function. The exchange of these critical domains might lead to the exposure of different amino acids on the surface of the protein. This, in turn, could prevent the recruitment of these chimeras to the putative recycling endosome.

INTRODUCTION

Membrane trafficking is controlled by ADP-ribosylation factor (ARF) GTPases. These small G proteins cycle between an inactive, cytoplasmic GDP-bound form and a membrane-bound GTP-associated active form. This conversion is catalyzed by ARF guanine nucleotide exchange factors (ARF-GEFs) (D'Souza-Schorey & Chavrier, 2006; Gillingham & Munro, 2007). The eight ARF-GEFs in *Arabidopsis thaliana* are either related to human GBF1, namely GNOM, GNL1 and GNL2, or to human BIG1 and 2 (BIG1-5 in Arabidopsis). Depending on their subcellular localization, ARF-GEFs mediate vesicle formation from different compartments. It was shown that the functionally redundant ARF-GEFs BIG1-4 are recruited to the *trans*-Golgi network (TGN), where they function in post-Golgi secretory traffic (Richter et al., 2014). Conversely, the closely related ARF-GEFs GNOM and GNL1 only partially overlap in localization and function. They both share the ancestral role in retrograde Golgi-ER transport, which is conserved amongst eukaryotes (Richter et al., 2007). In addition, GNOM performs a plant-specific role in the recycling of the auxin efflux carrier PIN1 to the basal plasma membrane from the putative recycling endosome (Geldner et al., 2003; Steinmann et al., 1999). Thus, whereas GNOM shows a dual localization at Golgi and endosomes, GNL1 localizes specifically to the Golgi.

In summary, it appears that ARF-GEFs are recruited to distinct compartments and thereby mediate specifically different trafficking pathways. However, the mechanism of membrane recruitment is still poorly understood. In yeast, the C-terminus of the ARF-GEF Sec7p, the homolog of Arabidopsis BIG1-5, might mediate TGN localization. Activated ARF substrate then leads to a positive feedback loop by recruiting more Sec7 to the membrane. This mechanism seems to be specific for ARF-GEFs involved in post-Golgi traffic. Yeast Gea1p, for example, which is associated with the Golgi, is not recruited to the membrane by GTP-bound Arf1 (Richardson et al., 2012). Another mechanism of membrane recruitment was proposed for Sec71, an ortholog of human BIG1/2 in *Drosophila melanogaster*. Affinity chromatography showed that Sec71 directly interacts with the activated form of the ARF-like protein Arl1 (Christis & Munro, 2012). However, in Arabidopsis it is unlikely that interaction with the ARF-GTPase

alone mediates the recruitment to the correct membrane as we demonstrated recently that GNOM and GNL1 interact with the same ARF1 substrate (Singh and Richter et al., manuscript in revision). These results suggest that critical motifs in the ARF-GEF proteins are recognized by membrane receptors.
Here, we analyze how membrane specificity of the closely related ARF-GEFs GNOM and GNL1 is achieved. Domain swaps of GNOM and GNL1 suggest that the GNOM-specific heterotypic interaction between four domains is critical for endosomal recycling function of GNOM at the putative recycling endosome.

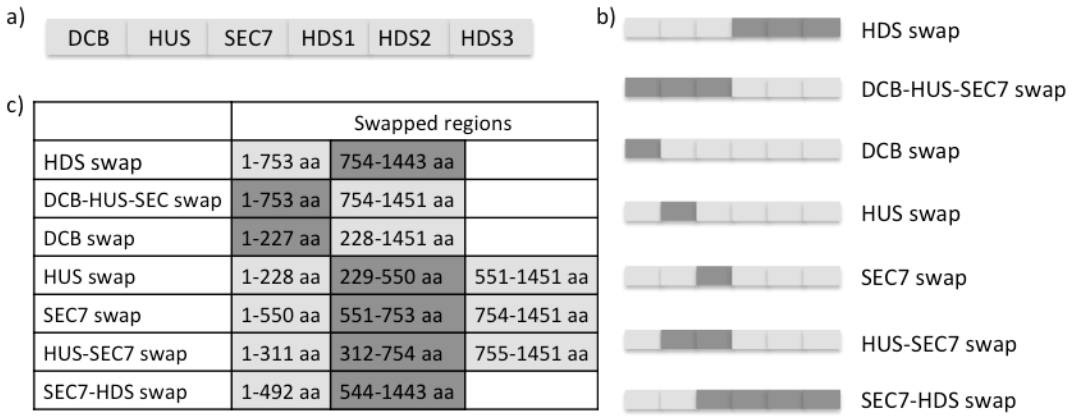
RESULTS

GNOM-GNL1 chimeras were used as a tool to investigate membrane specificity

Large ARF-GEFs share a common domain architecture, which is highly conserved amongst eukaryotes (Bui et al., 2009; Cox et al., 2004; Mouratou et al., 2005) (Fig. 1a). The N-terminal domain, the DCB domain, was shown to be involved in dimerization of GNOM protein (Grebe et al., 2000). In addition to the interaction between two DCB domains, the DCB domain of GNOM can also bind to a GNOM protein lacking the DCB domain (GNOM ΔDCB). Mutations in the adjacent domain of DCB, the HUS domain, as well as the catalytic SEC7 domain were shown to abolish this interaction (Anders et al., 2008). Past studies in our lab characterized critical amino acids in the HDS1 domain as having the same effect (Beckmann, 2015). Together with the HDS2 and HDS3 domain, this domain makes up the C-terminal half of an ARF-GEF protein.

Domain swaps between GNOM and GNL1 were cloned in order to identify domains which are involved in the recruitment of ARF-GEF to specific membranes. Since GNOM can take over the GNL1 function (Richter et al., 2007), the different chimeras were cloned under the GNOM regulatory elements, introduced into a *gnom* deletion mutant background (*sgt* lacks *GNOM* and four additional genes on either side) and analyzed for their ability to replace GNOM function.

The DCB, HUS or SEC7 domain of GNL1 replaced their homologous domain in the GNOM backbone. Exchanging the domain(s) mediating specific membrane recruitment of GNOM should prevent endosomal localization and endosomal recycling function of the chimera, but should not affect its ability to mediate retrograde Golgi-ER trafficking. In addition to swapping single domains in the N-terminal half (DCB-swap = $GNOM^{GNL1\ DCB}$, HUS-swap = $GNOM^{GNL1\ HUS}$, SEC7-swap = $GNOM^{GNL1\ SEC7}$), half-half chimeras were cloned as well (HDS-swap = $GNOM^{GNL1\ HDS1\text{-}3}$ and DCB-HUS-SEC7-swap = $GNOM^{GNL1\ DCB\text{-}HUS\text{-}SEC7}$). Lastly, two adjacent domains in the middle region (HUS-SEC7 swap = $GNOM^{GNL1\ HUS\text{-}SEC7}$) and domains 3-6 (SEC7-HDS swap = $GNOM^{GNL1\ SEC7\text{-}HDS1\text{-}3}$) were also swapped (Fig. 1b). Fig. 1c shows the exact boundaries of the swapped segments.



c)

	Swapped regions		
HDS swap	1-753 aa	754-1443 aa	
DCB-HUS-SEC swap	1-753 aa	754-1451 aa	
DCB swap	1-227 aa	228-1451 aa	
HUS swap	1-228 aa	229-550 aa	551-1451 aa
SEC7 swap	1-550 aa	551-753 aa	754-1451 aa
HUS-SEC7 swap	1-311 aa	312-754 aa	755-1451 aa
SEC7-HDS swap	1-492 aa	544-1443 aa	

Fig. 1 Overview of the GNOM-GNL1 chimeras analyzed

a) Schematic of the conserved domain architecture of large ARF-GEFs; DCB, dimerization domain; HUS, Homology Upstream of SEC7; SEC7, catalytic domain; HDS, Homology Downstream of SEC7. b) Schematic of chimeras (light grey: GNOM, dark grey: GNL1); c) Table showing amino acid positions of swapped regions (light grey: GNOM, dark grey: GNL1), Note: The SEC7-HDS swap lacks the linker region between HUS(GNOM) and SEC7(GNL1).

Swapping the C-Terminus or the first two single domains of GNOM does not impair its function

Exchanging the HDS1-3, DCB or HUS domain(s) did not affect the endosomal recycling function of GNOM since these domain swaps were able to rescue the *gnom* mutant embryo phenotype completely (Fig. 2a, Table S1). The presence of

the transgene and the *gnom* mutant background were verified by genotyping (Table S1). Postembryonic functions of GNOM are, for example, gravitropism and lateral root initiation for which two independent DCB swaps ($GNOM^{GNL1\ DCB}$) in a *gnom* mutant background were analyzed. As expected, these two physiological responses were rescued as well (Fig. 2b and c). BFA traps the abortive complex of ARF-GEF and ARF-GDP at the membrane, thereby preventing ARF activation, which results in a cessation of vesicle formation (Cherfils & Melancon, 2005; Renault et al., 2003; Robineau et al., 2000). GNOM is naturally sensitive to BFA, whereas GNL1 is BFA-resistant due to a critical amino acid in the catalytic SEC7 domain. Compared to the BFA-resistant GNOM-ML control or the seedlings investigated without BFA treatment, the DCB swap did not lead to a rescue of gravitropism and lateral root initiation in the presence of BFA (Fig. 2b and c). This is surprising to some extent, as the $GNOM^{GNL1\ DCB}$ chimera could in principle form heterodimers with endogenous GNL1 (Suppl. table S2). Chimeras were also tested in a primary root growth assay. Primary root growth heavily relies on secretory trafficking. A block of the retrograde Golgi-ER trafficking leads indirectly to a breakdown of the secretory pathway. Thus, measurement of the primary root growth served as a test of whether the chimera could still act in the retrograde Golgi-ER transport in concert with GNL1. After BFA treatment, BFA reduced the primary root growth of Col0 almost by half (compared to no BFA-treatment) since BFA-sensitive GNOM is blocked. Compared to the BFA-resistant GNOM-ML control, the DCB swaps rescue secretion not fully, but they have some positive effect (Fig.2c).

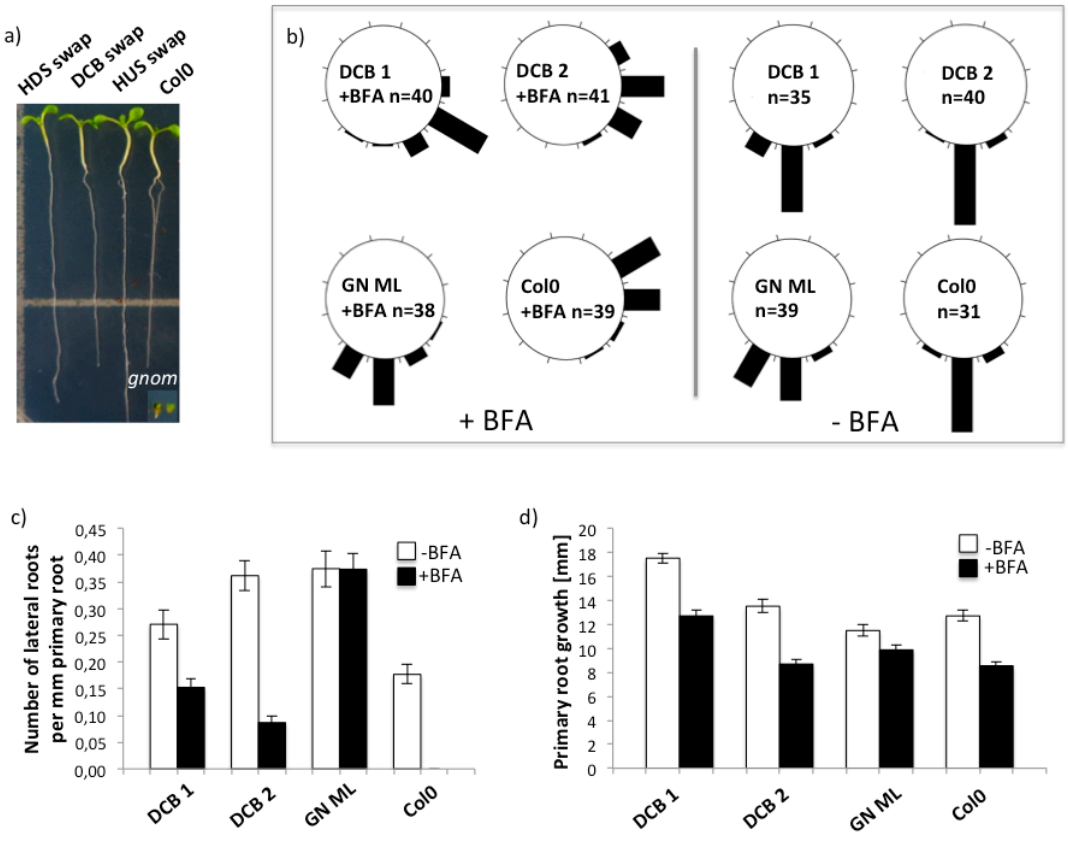


Fig. 2 Chimeras with HDS, DCB or HUS domain swapped each rescue *gnom* embryos and can replace GNOM in postembryonic development

a) *gnom* (*sgt*) mutant seedlings expressing the chimeras as indicated; Col0, wild-type control. Gravitropic response (b), lateral root initiation (c) and primary root growth (d) were (largely) rescued by DCB chimeras in the absence of BFA only; GN ML, BFA-resistant GNOM. (b-d: chimeras in a *sgt/+* background)

Swapping the SEC7 or HUS+SEC7 domain interferes with GNOM function in embryo development to different extents

As swapping single domains that act in dimerization and partly in intramolecular interaction did not impair GNOM function, the catalytic SEC7 domain was swapped next (GNOM$^{GNL1\ SEC7}$). A slightly abnormal seedling phenotype was observed, with cotyledons more or less fused and the root shortened compared to the wildtype (Fig. 3a). As only one line in the *sgt* background had been analyzed to that point, three more lines were crossed in this mutant background. Compared to the SEC7 swap, a similar phenotype was observed for the HUS-

SEC7 swap (GNOM$^{GNL1\ HUS\text{-}SEC7}$) at the seedling stage (Fig. 3a). As an adult plant, the HUS-SEC7 swap (GNOM$^{GNL1\ HUS\text{-}SEC7}$) showed a more severe phenotype compared to the SEC7 swap (GNOM$^{GNL1\ SEC7}$), with only one short stem, which nevertheless produced numerous siliques (Fig. 3b). After seven weeks, the SEC7 swap (GNOM$^{GNL1\ SEC7}$), however, was delayed in development compared to the wildtype, with fewer branches and curled rosette leaves (Fig. 3b). This phenotype resembled a complemented *gnom* phenotype called *b4049/emb30*. A heterodimer consisting of the two mutant proteins GNOM-B4049 and GNOM-emb30 produces fertile plants, developing slowly with downwards-bent leaves (Fig. 3b) (Anders et al., 2008; Busch et al., 1996). As the SEC7 domain of GNL1 is naturally resistant to BFA, rescue of gravitropism was tested in the heterozygous *gnom* background, blocking the endogenous GNOM function with 10 µM BFA. The two chimeras responded to the shift in gravity in a similar manner to GNOM (Fig. 3c+d). In addition, the initiation of lateral roots was again comparable between BFA-treated and untreated seedlings (suppl. Fig. 2a), thereby confirming that the SEC7 swap (GNOM$^{GNL1\ SEC7}$) aids in PIN1 recycling. The role of chimeras in secretion was investigated by measuring the primary root growth. In comparison to the control without BFA or the GN-ML control, no difference was observed (suppl. Fig. 1b).

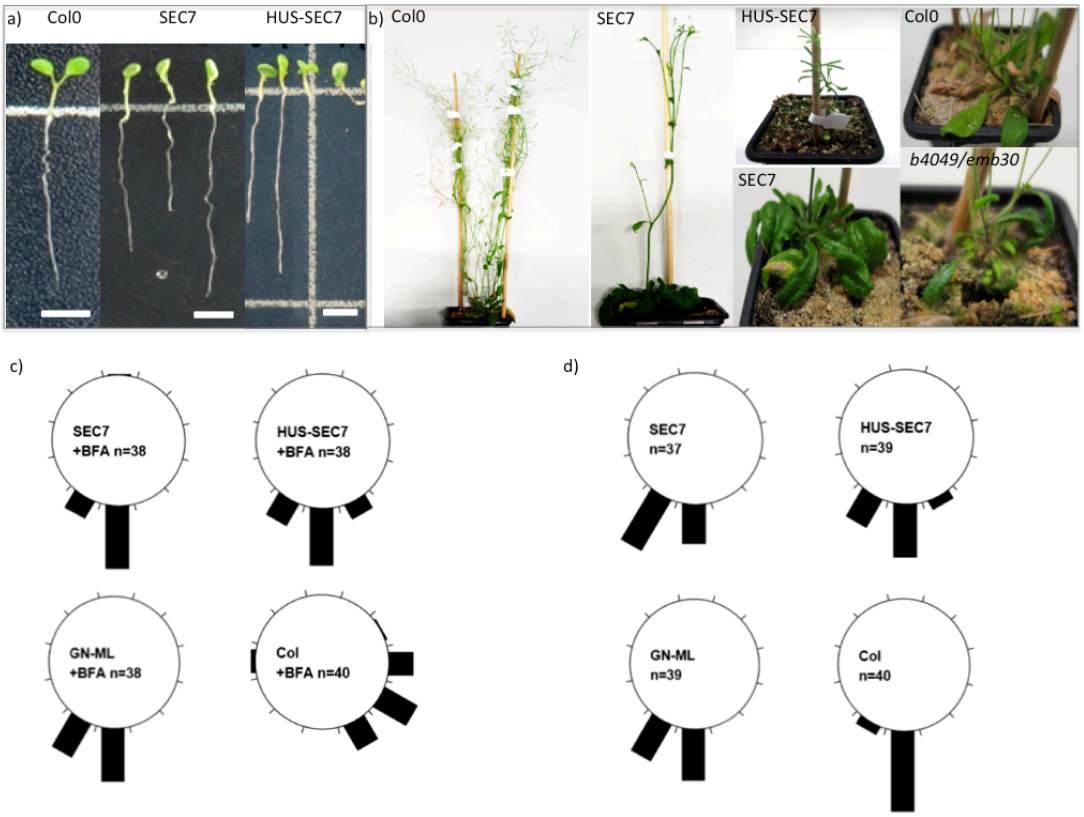


Fig. 3 Chimeras with SEC7 or HUS and SEC7 domains swapped largely rescue *gnom* plant development and mediate BFA-resistant gravitropic response

a) *gnom* (*sgt*) mutant seedlings expressing GNOM$^{GNL1\ SEC7}$ (SEC7 domain swapped) or GNOM$^{GNL1\ HUS\text{-}SEC7}$ (HUS-SEC7 domains swapped) chimeras show fused cotelydons and slightly shorter roots at the seedling stage compared to wildtype. Scale bar, 5 mm; b) Postembryonic development is delayed, adult plants are shorter and have fewer siliques compared to wildtype (Col0). The rosette leaves of the SEC7 swap resemble the *b4049/emb30* (weak *gnom* mutant) phenotype; c-d) Root gravitropism is not impaired in the chimeras in the presence of 10 µM BFA (c) nor in the absence of BFA (d). (c-d: SEC7 swap in a *sgt/-* background, HUS-SEC7 swap in a *sgt/+* background)

Domains that are involved in the heterotypic interaction of GNOM seem to be critical for its membrane specificity

Because swapping one or two domains in the N-terminal half did not strongly affect the *gnom* rescue, we next analyzed a chimera with the N-terminal half of GNL1 (DCB-HUS-SEC7 swap = GNOM$^{GNL1\ DCB\text{-}HUS\text{-}SEC7}$) and a chimera which consists mainly of GNL1 (SEC7-HDS swap = GNOM$^{GNL1\ SEC7\text{-}HDS1\text{-}3}$).

Interestingly, there was hardly any function of a chimera in endosomal recycling with the first three domains of GNL1 and the C-terminal half of GNOM ($GNOM^{GNL1\ DCB\text{-}HUS\text{-}SEC7}$). These seedlings looked like *gnom,* except for a short root protruding from the basal end (Fig. 4a), resembling a mutant GNOM allele, called GNOM-R5, a truncated GNOM protein, missing the last 86 amino acids. The chimera consisting mostly of GNL1 with only DCB and HUS domains from GNOM ($GNOM^{GNL1\ SEC7\text{-}HDS1\text{-}3}$) was unable to fulfill the GNOM function during embryogenesis, resulting in seedlings without forming shoots and roots (Fig. 4a). Whether this rescuing inability of the chimera is due to the lack of a linker between the HUS domain of GNOM and the SEC7 domain of GNL1 (aa493-543 are missing; see Fig. 1c) or is caused by the SEC7 and HDS1 domains of GNL1 needs to be elucidated. Its partial gravitropic response in the presence of BFA suggests that this chimeric protein is at least partially functional (Fig. 4b). As both chimeras harbor the BFA-resistant SEC7 domain of GNL1, the functional experiments were performed with BFA. In the presence of the drug, both swaps showed an intermediate rescue of gravitropism compared to Col0 and GN-ML. Both chimeras showed an intermediate rescue of gravitropism compared to the GN-ML and wildtype control (Fig. 4b). Seedlings without BFA treatment showed a response to the shift in gravity, which is expected, due to the heterozygous *gnom* background (Fig. 4c). The lateral root initiation was rescued only partially as well. While the SEC7-HDS swap ($GNOM^{GNL1\ SEC7\text{-}HDS}$) produced half of the lateral roots on BFA compared to the control plates, the DCB-HUS-SEC7 swap ($GNOM^{GNL1\ DCB\text{-}HUS\text{-}SEC7}$) had hardly any lateral roots (Suppl. Fig. 1a). Primary root growth was hardly disturbed when expressing either chimeras, showing that both proteins are functional (Suppl. Fig. 1b). These results indicate that exchanging the domains that are involved in homo- and heterotypic interaction of the GNOM protein (DCB, HUS, SEC7 and HDS1) with the respective GNL1 domains affects the endosomal recycling function of the chimera, suggesting that recruitment of the chimera to the putative recycling endosome is affected.

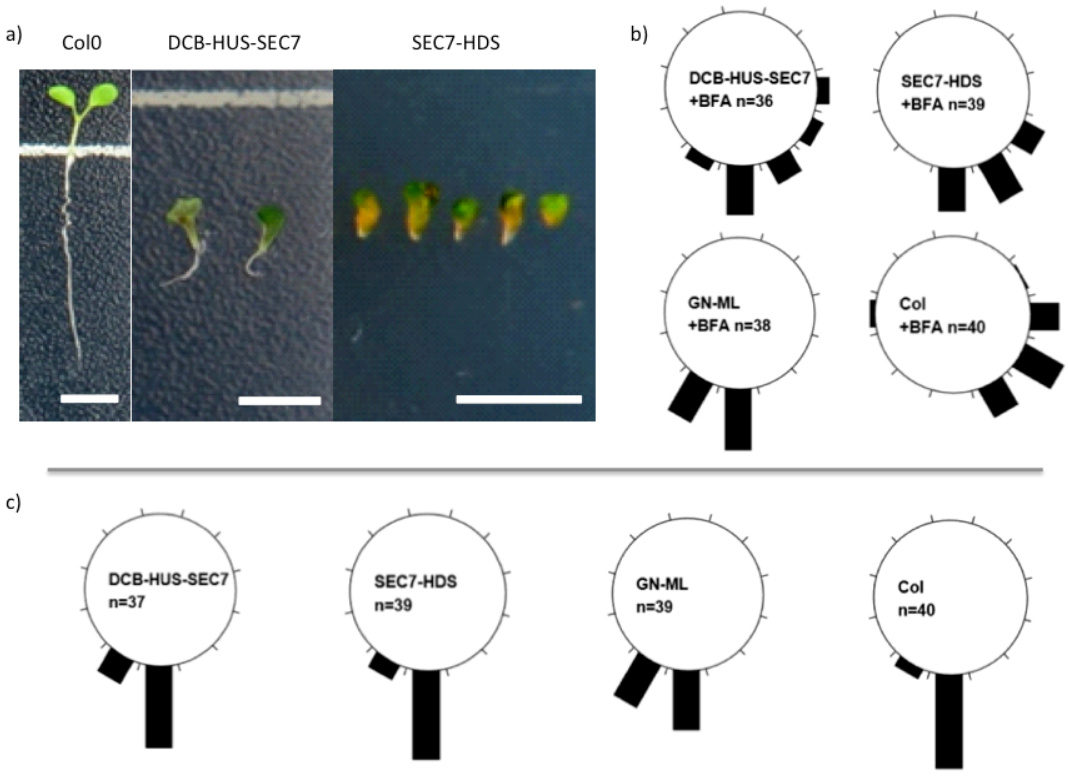


Fig. 4 The DCB, HUS, SEC7 and HDS1 domains are important for GNOM function

a) *gnom* (*sgt*) seedlings are not rescued by the GNOM$^{GNL1\ SEC7\text{-}HDS}$ chimera (SEC7-HDS3 swapped) whereas the GNOM$^{GNL1\ DCB\text{-}HUS\text{-}SEC7}$ chimera with DCB-HUS-SEC7 swapped produced a short primary root (similar to the weak *gnom-R5* mutant). b) Both chimeras only partially rescued root gravitropism on BFA plates (10 µM BFA) in a sgt/+ background. c) Control: gravitropic response in the absence of BFA.

DISCUSSION

The two closely related ARF-GEFs GNOM and GNL1 fulfill a redundant function in promoting membrane trafficking from Golgi to ER. GNOM has an additional, plant-specific role in PIN1 recycling from endosomes. Surprisingly, GNL1 is not able to act in this specific trafficking pathway (Richter et al., 2007), probably because it is not recruited to the endosomes. In contrast to small- and medium-sized ARF-GEFs which possess a membrane-binding PH domain, large ARF-GEFs – the only class in Arabidopsis – lack a characterized membrane binding domain (Casanova, 2007). Apart from the catalytic SEC7 domain and the N-terminal dimerization domain, the other domains have been poorly investigated so far. It

is speculated that they play a role in subcellular localization and membrane association (Anders & Jürgens, 2008). In order to find critical regions for the membrane specificity, chimeras with swapped GNOM and GNL1 domains were tested for their ability to rescue the *gnom* phenotype in early and late development. To understand the behavior of certain chimeras *in planta*, it might be useful to introduce the current knowledge of GNOM and GNL1 dimers first. In our lab, we demonstrated that two GNOM proteins dimerize via their N-terminal DCB domain (Grebe et al., 2000). This so-called homotypic interaction between two DCB domains is special for GNOM, as the GNL1 DCB domain does not dimerize via DCB-DCB interaction (Hauke Beckmann, personal communication). Nevertheless, both proteins interact with domains downstream of DCB (ΔDCB) (Hauke Beckmann, personal communication). It was also demonstrated that the GNOM DCB-ΔDCB interaction is dependent on critical amino acids in the HUS domain (D468), the SEC7 domain (D579) and the HDS1 domain (D857, E858, K868) (Anders et al., 2008; Beckmann, 2015). Mutating these critical amino acids abolished the heterotypic DCB-ΔDCB interaction in GNOM. Fig. 5 shows models of the interaction between two GNOM and two GNL1 proteins respectively, based on genetic experiments in our lab. We propose that the heterotypic interaction of GNOM with the HUS, SEC7 and HDS1 domain of a second GNOM protein is critical for membrane recruitment to the putative recycling endosome and that swapping these domains interferes with the GNOM function.

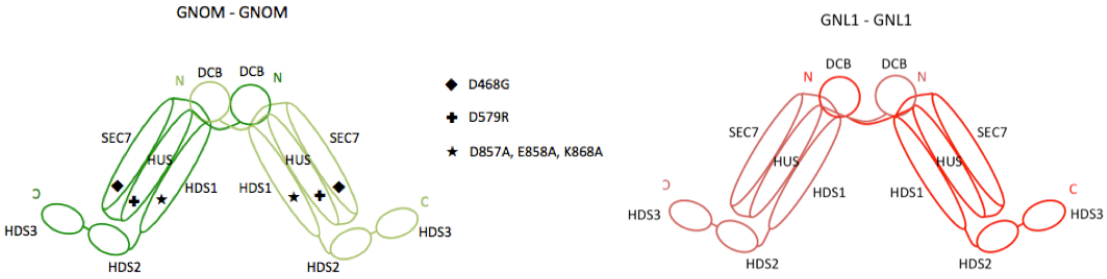


Fig. 5 Domain organization and interaction of ARF-GEFs GNOM and GNL1 (model based on genetic data)

The DCB domain of GNOM interacts with itself and with a bundle of 3 physically linked domains (HUS, SEC7, HDS1). The heterotypic interaction is disrupted by the mutations indicated in the middle. The SEC7 domain also contains the catalytic site E_{658} for GDP-GTP exchange, residue M_{696} conferring sensitivity to the fungal inhibitor brefeldin A (BFA), and the ARF-binding site loop>J (E_{744}IRT). Domains HDS2, HDS3 appear non-essential. The DCB of GNL1 does not interact with a second DCB domain, but with the domains downstream of the DCB domain of a second GNL1 protein (GNL1 ΔDCB). The mechanism of DCB-ΔDCB interaction of GNL1 has not been investigated so far, but due to the high sequence identity with GNOM (63 %) we propose a similar behavior. (Left: Interaction of one GNOM protein (dark green) with a second GNOM protein (light green). Right: Interaction of one GNL1 protein (pink) with a second GNL1 protein (red))

Expressing a chimera with the C-terminal half of GNL1, including HDS1-3 ($GNOM^{GNL1\ HDS1\text{-}3}$) in the *gnom* background did not obviously affect the GNOM function (Fig. 2a). This is in agreement with the assumption that the heterotypic GNOM DCB-ΔDCB is critical for the correct membrane recruitment, as the swap consists of the GNOM HUS and SEC7 domain and only one critical domain (HDS1) has GNL1 identity. In addition, the rescue was consistent with the report by Geldner at al. (2004) demonstrating that the deletion of the HDS domains 2 and 3 in the *gnom-sit4* mutant allele does not disturb GNOM function in embryogenesis (the mutation caused truncation after aa 983 – 10 aa downstream of the start of the HDS2 domain). Phenotypically, *gnom-sit4* resembled *gnom-R5* in which only about 80 aa at the C-terminus are deleted (Geldner et al., 2004).

Swapping the DCB domain also rescued the *gnom* phenotype completely. Co-IP experiments in our lab demonstrated that the DCB swap dimerizes with

endogenous GNL1 as well as endogenous GNOM (Suppl. table S2), which is modeled in Fig. 6. However, due to the rescue of embryogenesis in the mutant *gnom* background, we suggest that the chimera interacts preferentially with the ΔDCB part of GNOM (of a second chimera), but we can only speculate about the reason. The expression of the chimera is under control of the GNOM promoter and therefore the chimeras might be translated at a different subpopulation of ribosomes compared to endogenous GNL1. Other possibilities are a higher affinity of GNL1 DCB towards GNOM ΔDCB in comparison to GNL1 ΔDCB or a stronger protein expression of the chimera. In addition, trimer or tetramer formation between DCB swaps and endogenous GNL1 cannot be excluded as well. However, importantly, the DCB domain of GNL1 did not disturb the recruitment of the chimera to the putative recycling endosome. Also swapping the HUS domain in GNOM completely rescue the *gnom* phenotype.

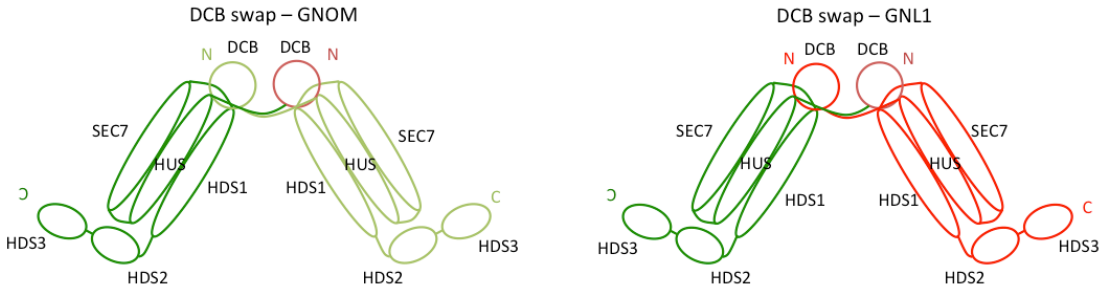


Fig. 6 Possible interactions between the DCB swap and GNOM and GNL1 respectively
In accordance with the models of GNOM and GNL1 dimers (Fig. 5) and our Co-IP data, the DCB domain of the DCB swap might interact with the physically linked HUS, SEC7 and HDS1 domains of GNOM and GNL1 respectively. (Left: The DCB swap (dark green, pink) interacts with GNOM (light green). Right: The DCB swap (green, pink) interacts with GNL1 (red))

A mild phenotype concerning the rescue of the *gnom* phenotype was observed after swapping the SEC7 domain ($GNOM^{GNL1\ SEC7}$) (Fig. 3a+b). Therefore, it is likely that the SEC7 domain is involved in the recruitment of GNOM to the correct donor membrane. This defect was slightly more obvious after additionally swapping the HUS domain ($GNOM^{GNL1\ HUS\text{-}SEC7}$) (Fig. 3a+b). Anders et al. (2008) suggested that a heterotypic interaction of the DCB domain with the

HUS and SEC7 domain of GNOM is critical for a closed conformation of the protein in the cytosol, which is essential for membrane association.

Swapping the whole N-terminus of GNOM (GNOM$^{GNL1\ DCB\text{-}HUS\text{-}SEC7}$) resulted in a very weak residual activity of the chimera in endosomal recycling (Fig.4, Suppl. Fig. S1). In this case, nearly all domains involved in the heterotypical interaction have GNL1 identity (except for the HDS1 domain). It is possible that the resulting tertiary structure of the chimeric protein does not present critical motifs for the correct membrane association. In addition, Co-IP experiments demonstrated, a possible interaction with endogenous GNL1 (Manoj Singh, personal communication) (model in Fig. 7) and could in principle interfere with a role of the chimera in endosomal recycling by recruiting a subpopulation of the chimera to the Golgi.

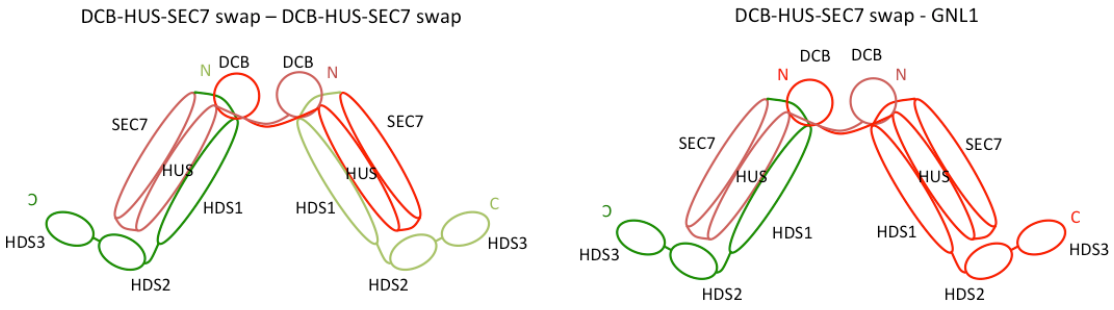


Fig. 7 Possible interactions of the DCB-HUS-SEC7 swap with a second DCB-HUS-SEC7 swap and GNL1 respectively

Similar to Fig. 5, the DCB domain of the DCB-HUS-SEC7 swap might interact with a second DCB-HUS-SEC7 swap or with endogenous GNL1 respectively. (Left: The DCB-HUS-SEC7 swap (dark green, pink) interacts with a second chimera of the same type (light green, red). Right: The DCB-HUS-SEC7 swap (green, pink) interacts with GNL1 (red))

The last analyzed chimera, the SEC7-HDS swap (GNOM$^{GNL1\ SEC7\text{-}HDS1\text{-}3}$), harbored only the first two domains of GNOM (DCB, HUS). It might thus well be that the interaction mechanism of the chimeric protein is different compared to GNOM, as two critical domains (SEC7, HDS1) and the C-terminal part (HDS2, HDS3) are from GNL1. This chimera was not able to rescue the *gnom* seedling phenotype (Fig. 4a). Co-IP experiments with the SEC7-HDS swap (GNOM$^{GNL1\ SEC7\text{-}HDS1\text{-}3}$)

demonstrated heterodimers with endogenous GNOM (Suppl. table. S2). In the absence of GNOM in the mutant *gnom* background, the dimer formation between two chimeras is likely, but has not been demonstrated so far (see model in Fig. 8). In these putative chimera dimers, the interaction of the GNOM DCB domain with GNOM HUS and GNL1 SEC7 and HDS1-3 might result in a different structure compared to GNOM, thereby presenting different amino acids on the protein surface. This difference compared to the GNOM protein might prevent its recruitment to the putative recycling endosome.

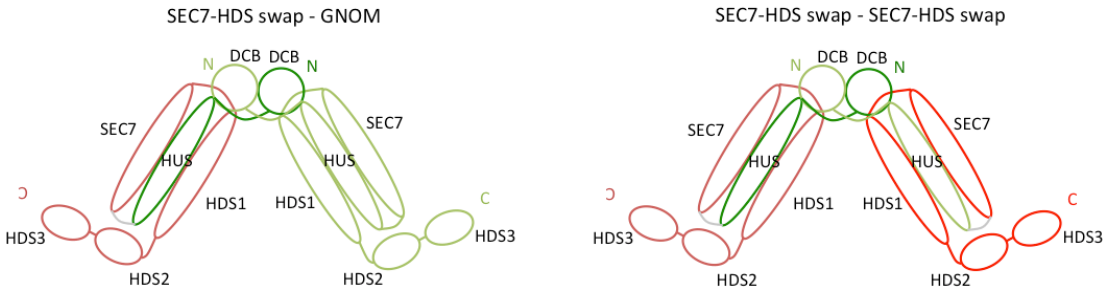


Fig. 8 Possible interactions between the SEC7-HDS swap and GNL1 respectively and between two molecules of the SEC7-HDS swap

The SEC7-HDS swap could interact with GNOM and probably with a second SEC7-HDS chimera (Left: The SEC7-HDS swap (dark green, pink) interacts with GNOM (light green). Right: The SEC7-HDS swap (dark green, pink) is thought to interact with a second molecule of the same type (light green, red))

In summary, it was shown that the heterotypic interaction of the GNOM DCB domain with the HUS, SEC7 and HDS1 domain probably leads to the presentation of motifs that are critical for the recruitment of the protein to the putative recycling endosome. Future studies will hopefully shed more light on the nature of these motifs.

To test this theory, more chimeras need to be investigated. Introducing the SEC7 and HDS1 domains of GNOM into the GNL1 backbone or even GNOM DCB, SEC7 and HDS1 would be helpful in getting a better understanding of critical motifs for membrane specificity. Localization studies will further underscore the genetic data. In addition, missing Co-IP experiments to test for interaction with full-length GNOM and GNL1 are desirable.

SUPPLEMENTAL DATA

Table S1: Results of genotyped seedlings of the different chimeras

swap	n	Phenotype	*gnom* PCR	Transgene PCR	*GNOM* PCR
HDS	9	Wildtype	homo	Positive	Negative
	11	Wildtype	WT, hetero		
DCB	9	Wildtype	homo	Positive	Negative
HUS	3	Wildtype	homo	Positive	Negative
	10	Wildtype	WT, hetero		
SEC7	4	Fused cotyledons	homo	Positive	Negative
	15	Wildtype	WT, hetero		
HUS-SEC7	14	Fused cotyledons, short root	homo	Positive	Negative
	20	Wildtype	WT, hetero		
DCB-HUS-SEC7	Pool	*gnom* with root tip	homo	Positive	Negative
	15	Wildtype	WT, hetero		
SEC7-HDS	Pool	*gnom*	homo	Positive	Negative
	15	Wildtype	WT, hetero		

GNOM PCR = endogenous *GNOM* gene

gnom PCR = *sgt* deletion

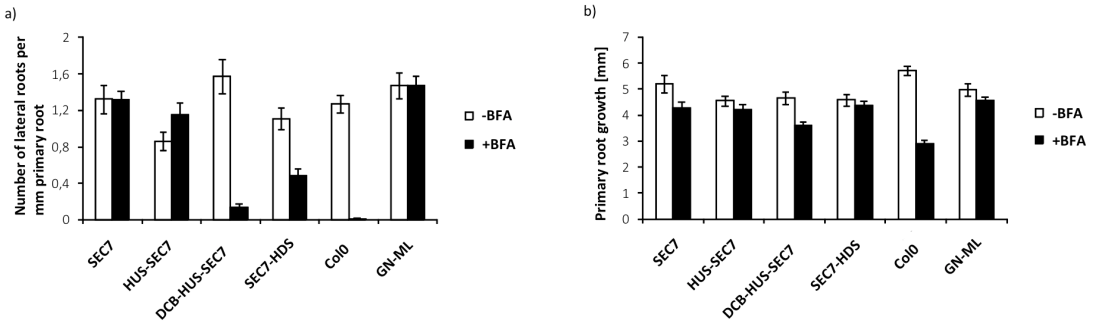


Fig. S1: Diagrams showing the number of lateral roots and the primary root growth of selected chimeras

Neither the SEC7 (GNOM$^{GNL1\ SEC7}$) nor the HUS-SEC7 swap (GNOM$^{GNL1\ HUS\text{-}SEC7}$) are impaired in the formation of lateral roots. The DCB-HUS-SEC7 swap (GNOM$^{GNL1\ DCB\text{-}HUS\text{-}SEC7}$) has nearly no lateral roots, whereas the number of lateral roots is nearly halved in the SEC7-HDS swap (GNOM$^{GNL1\text{-}SEC7\text{-}HDS1\text{-}3}$) when blocking the endogenous GNOM by BFA (10 µM BFA); b) All chimeras with a GNL1 SEC7 domain can replace the endogenous GNOM in retrograde Golgi-ER trafficking, indicated by primary root growth on BFA plates (10 µM BFA).

(a+b: SEC7 swap in a sgt/- background, all other chimeras in a *sgt/+* background)

Table S2: Results of interaction studies of selected chimeras with full-length GNOM and GNL1 respectively

swap	GNOM	GNL1
HDS	?	?
DCB	Yes	Yes
HUS	?	?
SEC7	?	?
HUS-SEC7	Yes	?
DCB-HUS-SEC7	No	Yes
SEC7-HDS	Yes	?

DCB swap: GNOM$^{GNL1\ DCB}$-myc was crossed with tagged full-length GNOM and GNL1 respectively

HUS-SEC7 swap: GNOM$^{GNL1\ HUS\text{-}SEC7}$-myc was enriched using myc-beads and tested for the presence of endogenous GNOM using a GNOM-specific SEC7 antibody

DCB-HUS-SEC7 swap: GNOM$^{GNL1\ DCB\text{-}HUS\text{-}SEC7}$-myc was crossed with tagged full-length GNOM and GNL1 respectively

SEC7-HDS swap: GNOM$^{GNL1\ SEC7\text{-}HDS1\text{-}3}$-myc was enriched using myc-beads and tested for the presence of endogenous GNOM using a GNOM-specific SEC7 antibody

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5.2 Draft Manuscript 2:

Analysis of the functional diversification of two membrane trafficking regulators during evolution

Contributions:

All constructs were cloned by Kerstin Huhn.

All lines were generated by Kerstin Huhn; Marika Kientz aided in the genotyping of individual seedlings.

All experiments were performed by Kerstin Huhn.

The interpretation of the results as well as the writing of the manuscript was done by Kerstin Huhn.

Draft Manuscript 2:

Analysis of the functional diversification of two membrane trafficking regulators during evolution

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ABSTRACT

The intracellular transport of cargo molecules is an important process that is mediated by membrane trafficking. Amongst other key players, ADP-ribosylation factor guanine exchange factors (ARF-GEFs) are important regulators of vesicle formation. The two closely related Arabidopsis ARF-GEFs GNOM and GNL1 promote different subcellular trafficking pathways: While both proteins regulate retrograde Golgi-ER trafficking, only GNOM has an additional role in endosomal recycling of the auxin efflux carrier PIN1. Ancient plant species have only one protein related to GNOM and GNL1 which we suppose to be involved in retrograde Golgi-ER traffic (to mediate protein secretion) as well as PIN1 recycling (to direct the auxin flow). During evolution, genome duplication led to multiple GNOM and GNL1 paralogs in species of the Viridiplantae kingdom. In this work, we asked how the functional diversification of GNOM and GNL1 evolved and whether Arabidopsis GNL1 simply lost its role in endosomal PIN1 recycling. Rescue experiments in Arabidopsis *gnom* and *gnl1* mutant plants expressing the homologous proteins of the dicots *Brassica napus* and *Populus trichocarpa* indicate that the Brassicaceae family has undergone functional diversification as well and that this process is ongoing in poplar. Rescue experiments with the two proteins from *Oryza sativa* suggest that a functional diversification of GNOM-related proteins has also occurred in monocots.

INTRODUCTION

Membrane trafficking within a eukaryotic cell has to be strongly controlled. The first step in membrane trafficking, the vesicle formation, is regulated by a machinery of different proteins, amongst them ADP-ribosylation factor guanine exchange factors (ARF-GEFs).

In *Arabidopsis thaliana*, the eight ARF-GEFs form two subfamilies. The members of the BIG subfamily are mainly involved in post-Golgi secretion (Richter et al., 2014), whereas the early secretory route is controlled by the joint action of GNOM and GNL1 of the ARF-GEF subfamily related to human GBF1 (Richter et al., 2007; Teh & Moore, 2007). This task is evolutionary conserved as the homologous proteins GBF1 from *Homo sapiens* and Gea1/2p from *Saccharomyces cerevisiae* mediate the retrograde vesicle transport between the ER and Golgi apparatus as well (Garcia-Mata et al., 2003; Peyroche et al., 1996; Spang et al., 2001; Zhao et al., 2006). In addition, GNL2, the third plant member of this subfamily, is expressed only in the pollen and aids in pollen tube growth (Richter et al., 2011).

Interestingly, GNOM exerts another role in polar recycling of the auxin efflux carrier PIN1 from the putative recycling endosome to the basal plasma membrane (Geldner et al., 2003; Steinmann et al., 1999) and this role cannot be taken over by GNL1. We therefore asked the question of whether Arabidopsis GNOM has gained an additional role in endosomal recycling or whether GNL1 has lost this function during evolution.

GNOM and GNL1 originate from one ancestral protein and are the result of several genome duplications. It is therefore very likely that the ancestral protein exerted both tasks in subcellular trafficking, the retrograde ER-Golgi transport to mediate protein secretion as well as recycling of PIN1, which is important for a directed auxin flow in multicellular organisms. Consequently, we speculated that the role in endosomal recycling was lost during the evolution of Arabidopsis GNL1.

In this study, we tested this hypothesis by expressing GNOM and GNL1 homologs from different species in a mutant *gnom* background lacking the GNOM protein completely (*GNOM* allele *sgt*). Due to the interference with the auxin flow, the

embryogenesis is disturbed in the *gnom* mutants, resulting in abnormally shaped seedlings, lacking a proper root and fully-grown cotyledons (Geldner et al., 2003; Mayer et al., 1993; Steinmann et al., 1999). In contrast to this, in the weak *gnom-R5* allele, a GNOM protein lacking the last 86 amino acids is expressed, which leads to seedlings displaying more or less fused cotyledons and a shorter root compared to the wild type (Geldner et al., 2004). In our experiments, we analyzed the rescue of *sgt*. When the *sgt* phenotype was only partially rescued, we compared it to the $gnom^{R5}$ phenotype to get a better impression of the rescue ability of the proteins from other species. In addition to a putative role in endosomal PIN1 recycling during embryogenesis, we also tested for the rescue of post-embryonic development using gravitropism and lateral root initiation experiments. The minimal functional requirement of the tested proteins, however, was the rescue of retrograde Golgi-ER trafficking, which we tested by the rescue of the stunted Arabidopsis *gnl1* phenotype and rescue of primary root growth.

Our results suggest that the functional diversification of Arabidopsis GNOM and GNL1 occurred indeed during evolution as the two proteins in *Brassica napus*, *Populus trichocarpa* and *Oryza sativa* differ in their ability to rescue the *gnom* phenotype.

RESULTS

Phylogenetic analysis of GNOM and GNL1 homologs was used to identify candidate genes for the Arabidopsis *gnom* and *gnl1* rescue experiments

GNOM, GNL1 and GNL2 make up the GBF1-related subfamily of ARF-GEFs in Arabidopsis. GNOM and GNL1 have a redundant function in retrograde Golgi-ER transport (Richter, 2007). In addition, GNOM has a unique role in endosomal PIN1 recycling which cannot be exerted by the closely related ARF-GEF GNL1 (Geldner et al., 2003; Richter et al., 2007; Steinmann et al., 1999). GNL2 is expressed solely in pollen and is involved in its germination and pollen tube growth. However, GNL2 can functionally replace GNOM if expressed like GNOM (Richter et al., 2011). These findings raised the question of why GNL1 is not able

to fulfill a role in endosomal recycling and whether this is a result of loss of function during the evolution of Arabidopsis GNL1.

A phylogenetic tree consisting of the protein sequences of numerous homologous protein sequences of Arabidopsis GNOM, GNL1 and GNL2 from the green plants further supported this hypothesis (Fig. 1). Basal plants only have one GNOM-GNL1-GNL2 related protein, while all flowering plants have one GNOM-GNL1 homolog and additionally a GNL2 homolog. All these GNL2 homologous proteins are present in the same clade of the phylogenetic tree depicting GNOM, GNL1 and GNL2 paralogs from the Viridiplantae kingdom (Fig. 1). While the basal flowering plant *Amborella trichopoda* only has one GNOM-GNL1 related protein, all other species that evolved later have at least two GNOM and GNL1 homologs. (Suppl. Fig. S1 shows a phylogenetic tree depicting the evolution of the model species used for the phylogenetic tree in Fig. 1).

Strikingly, only the GNOM-GNL1 paralogs of the Brassicaceae family and related species are separated into two clades, while all other GNOM-GNL1 related proteins are represented on the same clade as Arabidopsis GNOM. A separation in two clades might indicate that the proteins on the GNL1 clade have lost its role in endosomal recycling as it was demonstrated for *Arabidopsis thaliana* before (Richter et al., 2007).

To address this question, homologous proteins from selected species were expressed in a mutant Arabidopsis background. Rescue of the *gnom* phenotype showed a function in endosomal recycling. As the retrograde Golgi-ER transport in eukaroytes is conserved, it was assumed that the homologous proteins are at least able to rescue the Arabidopsis *gnl1* phenotype.

Brassica napus (rapeseed), *Populus trichocarpa* (poplar) and *Oryza sativa* (rice) were chosen as candidate organisms because the two rapeseed proteins are separated on the two clades, while poplar and rice serve as examples for dicotyledonous and monocotyledonous species, respectively, with both proteins displayed on the same clade, suggesting functional similarity.

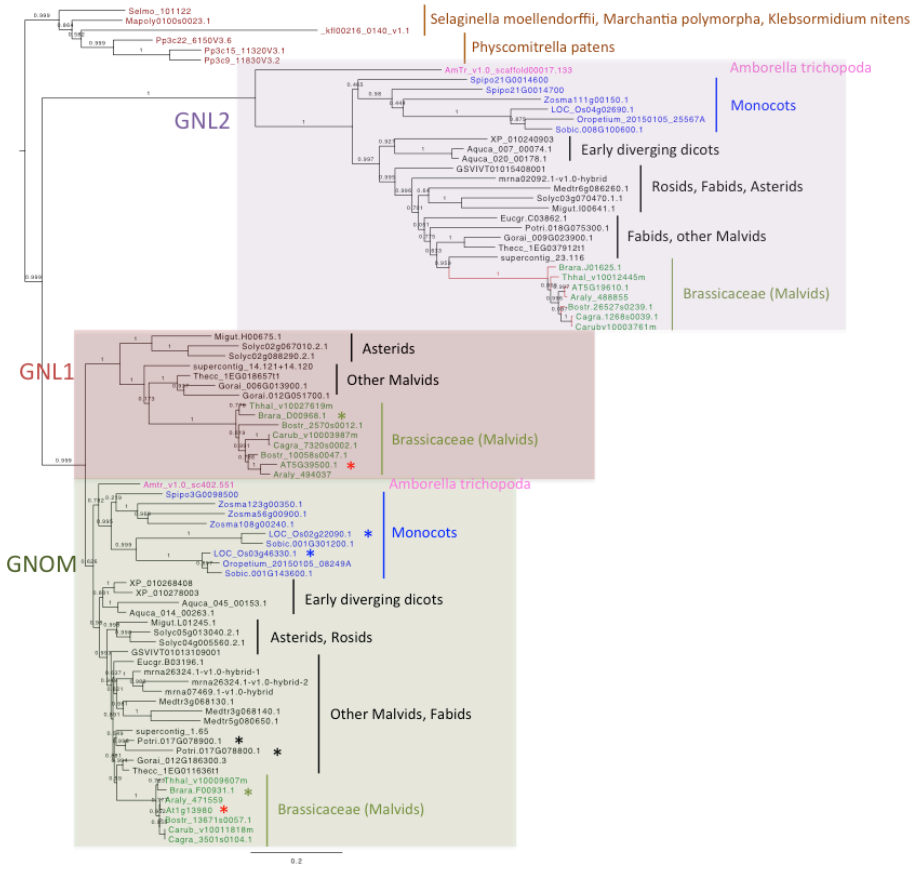

Fig. 1: Phylogenetic tree of the plant GBF1-related ARF-GEF family

The purple box highlights the GNL2 clade. Arabidopsis GNOM and GNL1 are separated into two clades (the pink box marks the clade of Arabidopsis GNL1, the green box marks the clade of Arabidopsis GNOM). Related species or plant families are displayed in different colors (monocots: Alismatales: *Spirodela polyrhiza*, *Zostera marina*; grasses: *Oryza sativa*, *Sorghum bicolor*, *Oropetium thomaeum*. Early diverging dicots: *Nelumbo nucifera*, *Aquilegia coerula*. Asterids, Rosids: *Mimulus guttatus*, *Solanum lycopersicum, Vitis vinifera*. Other Malvids, Fabids: *Eucalyptus grandis*, *Fragaria vesca*, *Medicago truncatula*, *Carica papaya*, *Populus trichocarpa*, *Gossypium raimondii*, *Theobroma cacao*. Brassicaceae (Malvids): *Eutrema salsugineum, Brassica rapa, Arabidopsis lyrata*, *Arabidopsis thaliana*, *Boechera stricta*, *Capsella rubella*, *Capsella grandiflora*.) The asterisks mark the proteins expressed in Arabidopsis in order to investigate the rescue of *gnom* and *gnl1*: *Brassica napus* (green), *Populus trichocarpa* (black), *Oryza sativa* (blue). GNOM and GNL1 from *Arabidopsis thaliana* are marked as a reference (red asterisks).

The two *Brassica napus* proteins show functional diversification

Brassica napus is a close relative of Arabidopsis and belongs to the Brassicaceae family. This species is the result of crossing *Brassica rapa* and *Brassica oleracea*.

The two *Brassica rapa* proteins were separated into two different clades (Fig. 1), suggesting that also the two *Brassica napus* proteins have diversified in their functions. Indeed, only the GNOM homolog, which we named BnGNOM (Brassica napus GNOM), rescued the *gnom* seedling phenotype when expressed from Arabidopsis *GNOM* 5'- and 3'-UTR sequences. Fig. 2a shows seedlings expressing BnGNOM in a *sgt* homozygous background, suggesting that the *gnom* phenotype is rescued completely, maybe showing a slightly shorter root compared to *gnom* heterozygous and wildtype seedlings expressing the transgene. The other Brassica protein, which we named BnGNL1, was represented on the GNL1 clade. If expressed in the *sgt* background, the seedlings showed the typical mutant phenotype of round seedlings missing cotyledons and a root (Fig. 2a). However, this protein was able to rescue the *gnl1* bushy phenotype (Suppl. Fig. 2a) and was strongly expressed (Suppl. Fig. S4).

In accordance with the rescue of the seedling phenotypes, the two rapeseed proteins differed in their localization. BnGNL1 labeled the Golgi stacks (Fig. 2d) as shown for AtGNL1 before (Richter et al., 2007; Teh & Moore, 2007), while BnGNOM behaved exactly as its Arabidopsis homolog (Geldner et al., 2003). The localization studies were performed in the presence of the fungal toxin Brefeldin A (BFA), which stabilizes the intermediate interaction of BFA-sensitive ARF-GEFs with their inactive ARF-GTPase substrates at the donor membrane (Cherfils & Melancon, 2005; Renault et al., 2003; Robineau et al., 2000). In *Arabidopsis thaliana*, GNOM is BFA-sensitive and GNL1 is resistant to BFA-treatment. The same situation applies for *Brassica napus*. After BFA-treatment, BnGNOM co-localized with the endocytic tracer FM4-64 (Fig. 2b) which labels endosomal compartments.

After BnGNL1 was negatively tested for its ability to substitute for AtGNOM during embryogenesis, we sought to investigate a possible role in post-embryonic functions, such as gravitropism and lateral root initiation. For this purpose, seedlings expressing BnGNL1 in a *gnom* heterozygous background, were germinated on MS plates and, after six days, transferred to BFA-containing plates. For the gravitropism experiment, the plates were rotated by 135°. For the test of lateral root initiation, the ratio between the primary root length and lateral root number was determined after several days on BFA.

Both experiments revealed that BnGNL1 played hardly any role in post-embryonic PIN1 recycling. Fig. 2e displays that blocking endogenous GNOM by BFA in a *gnom* heterozygous background expressing BnGNL1 led to agravitropic roots, similar to a wild type control without a transgene (Col0). In contrast, the expression of a BFA-resistant version of GNOM (GN-ML) (Geldner et al., 2003) rescued the gravitropism in the presence of BFA. Furthermore, the lateral root initiation was not rescued in *gnom/+* seedling expressing BnGNL1 after blocking endogenous GNOM by BFA (Fig. 2f).

However, the participation of BnGNL1 in retrograde Golgi-ER trafficking was demonstrated by the rescue of the bushy *gnl1* phenotype (Suppl. Fig. S2). In addition, BnGNL1 promoted primary root growth on BFA plates to the same extent as GN BFAres (Suppl. Fig. S3). The measurement of the primary root serves as an index for secretion that is dependent on Golgi-ER trafficking.

In summary, only the BnGNOM protein was able to rescue the AtGNOM function and BnGNL1 lost the ability of endosomal recycling like shown before for the putative homologous proteins in Arabidopsis (Richter et al., 2007).

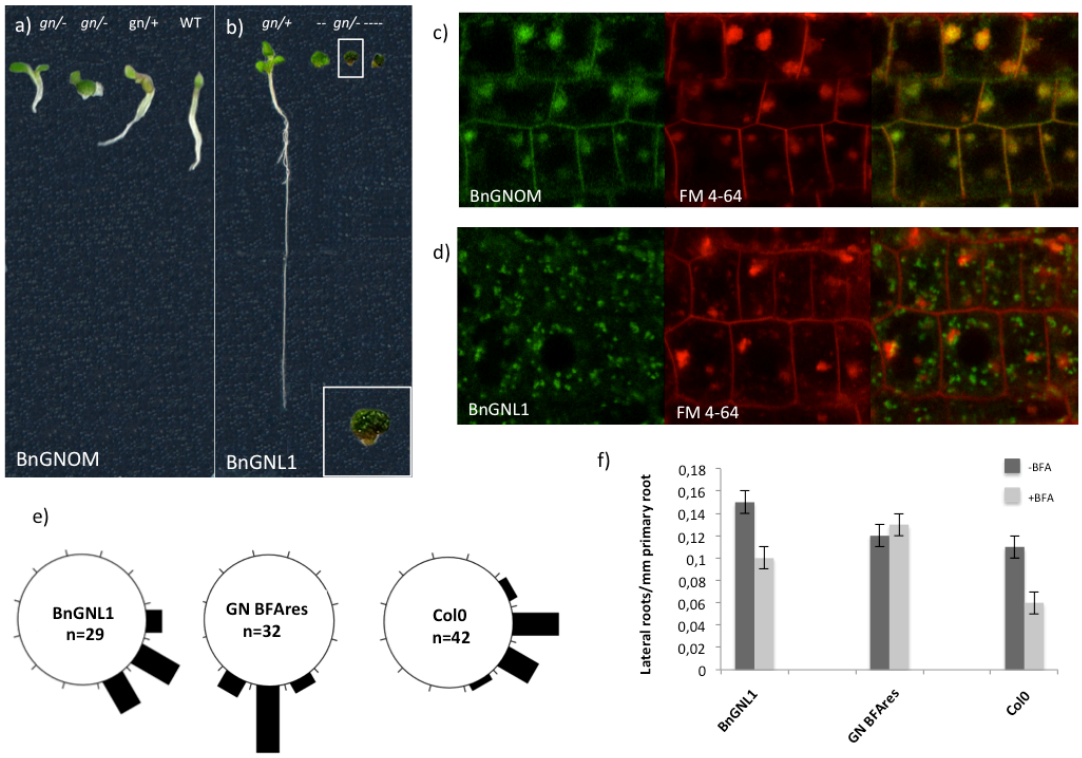

Fig. 2: Physiological and localization studies of the two putative AtGNOM and AtGNL1 homologs of *Brassica napus*

a) Seedling phenotypes of AtGN::BnGN-YFP:AtGN expressed in homozygous (*sgt/-*) and heterozygous (*sgt/+*) *gnom* and wildtype (WT) seedlings respectively (individual seedlings were PCR-checked). b) Seedling phenotype of AtGN::BnGNOM-YFP:AtGN in heterozygous (*sgt/+*) and homozygous (*sgt/-*) seedlings respectively (see inset for non-rescued *gnom* seedling at higher magnification). c) Subcellular localization of BnGNOM-YFP and FM4-64 after BFA treatment (1h, 50 µM BFA). d) Subcellular localization of BnGNL1-YFP and FM4-64 after BFA treatment (1h, 50 µM BFA). e) Gravitropic response on BFA plates (~16h, 10 µM BFA) (BnGNL1 is BFA-resistant); f) Lateral root density with and without BFA (5 days +/- BFA; 10 µM BFA) (c-f: Mother plant was in a *sgt/+* background)

The two poplar proteins differ in their ability to rescue endosomal recycling in Arabidopsis

In contrast to *Brassica napus*, both GNOM-related proteins of *Populus trichocarpa* were located in the same clade as AtGNOM (Fig. 1). Judging from their accession number, the two genes are tandem replicates, suggesting that they might share regulatory elements and act redundantly. In addition, both proteins share more sequence identity with AtGNOM than with AtGNL1 (Potri.017G078900.1: 83% identity with AtGNOM, 63% identity with AtGNL1; Potri.017G078800.1: 76 % identity with AtGNOM, 59% identity with AtGNL1). As the two poplar proteins were represented on the same clade, they were named as *Populus trichocarpa* GN-GNL1-related proteins (PtGR). In the following, Potri.017G078900.1 is abbreviated as PtGR1 and Potri.017G078800.1 as PtGR2 respectively.

Although both poplar proteins grouped in the GNOM-clade, PtGR1 and PtGR2 differed in their ability to rescue the *gnom* phenotype. While PtGR1 could rescue the *gnom* phenotype completely (Fig. 3a), PtGR2 was only able to rescue partially, resulting in seedlings with more or less fused cotelydons and a shorter root compared to the wildtype (Fig. 3b). These defects led to the death of the seedling after a few weeks. The expression strength of both proteins was comparable (Suppl. Fig. S4).

Similarly to the different behavior in rescuing of the *gnom* phenotype, the localization of PtGR1 and PtGR2 also varied between the two proteins. While PtGR1 co-localized with F4-64 in agglomerates consisting of endosomal

compartments after BFA-treatment (Fig. 3c), PtGR2 localized at the Golgi since it displays a donut-like structure (marked by asterisks) that is typical for Golgi-localized proteins. (Fig. 3d).

Although PtGR2 and AtGNOM differ in their subcellular localization, a minor role of PtGR2 in endosomal recycling cannot be excluded because the *gnom* seedling phenotype was rescued partially by expression of the poplar transgene (Fig. 3b). In order to quantify the role of PtGR2 in PIN1 recycling, its rescue of gravitropism and lateral root initiation was analyzed using wildtype-looking heterozygous *gnom* seedlings expressing the PtGR2, which is BFA-resistant. Surprisingly, in the presence of BFA, PtGR2 did not play a major role in both post-embryonic GNOM functions when compared with the positive control expressing the BFA-resistant GNOM protein and the Col0 negative control (Fig. 3e+f), meaning that PtGR2 cannot promote PIN1 recycling during gravitropism and lateral root initiation. However, a function in retrograde ER-Golgi transport was demonstrated by the complete rescue of the Arabidopsis *gnl1* phenotype (Suppl. Fig. S2) as well as the rescue of primary root growth (Suppl. Fig. S3), indicating that PtGR2 can replace Arabidopsis GNL1 in retrograde Golgi-ER trafficking.

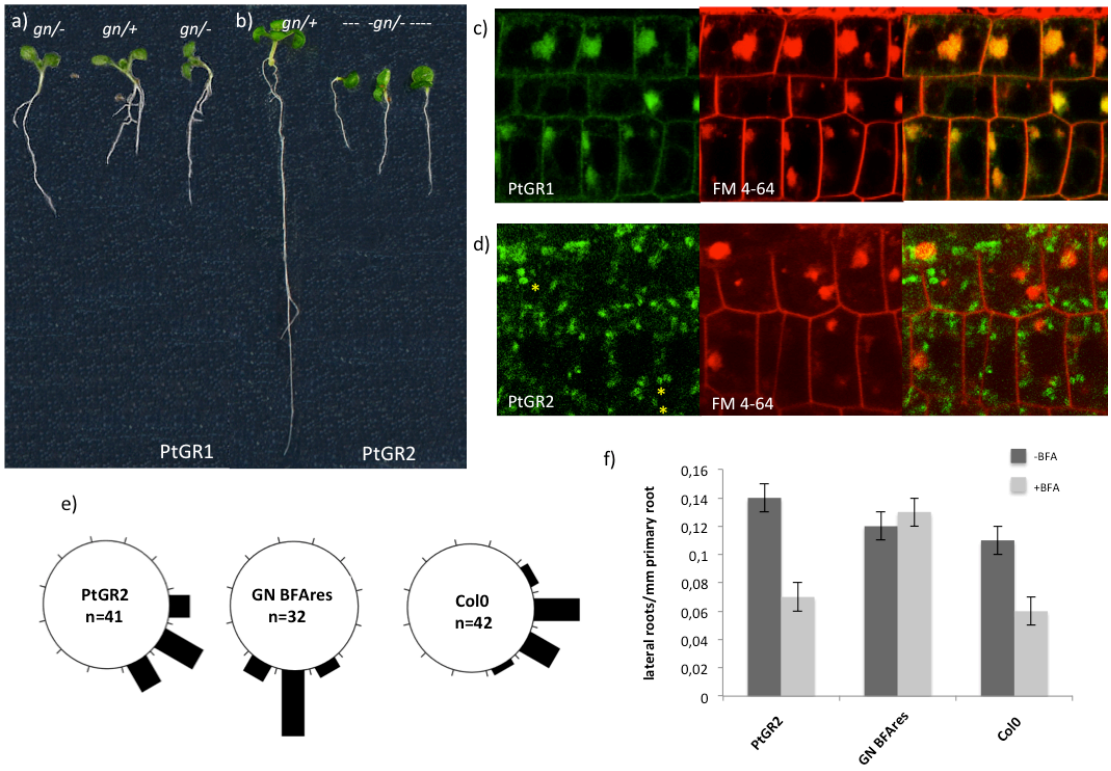

Fig. 3: Rescue experiments and localization studies of the AtGNOM and AtGNL1 relatives from *Populus trichocarpa*

a) Seedling phenotypes of AtGN::PtGR1-YFP:AtGN expressed in homozygous (*sgt/-*) and heterozygous (*sgt/+*) *gnom* seedlings respectively (individual seedlings were PCR-checked). b) Seedling phenotype of AtGN::PtGR2-YFP:AtGN expressed in heterozygous (*sgt/+*) and homozygous (*sgt/-*) *gnom* seedlings respectively (see inset for the partially rescued *gnom* seedling at higher magnification; rescue is similar to the $gnom^{R5}$ phenotype). c) Subcellular localization of PtGR1-YFP and FM4-64 after BFA treatment. (1h, 50 µM BFA). d) Subcellular localization of PtGR2-YFP and FM4-64 after BFA treatment. Yellow asterisks mark donut-like structures that represent Golgi stacks (1h, 50 µM BFA); e) Gravitropic response on BFA plates (~16h, 10 µM BFA); f) Lateral roots per mm with and without BFA (5 days +/- BFA; 10 µM BFA) (c-f: Mother plant was in a *sgt/+* background)

The two GNOM-related ARF-GEFs of *Oryza sativa* have also undergone functional diversification

All GNOM and GNL1-related ARF-GEFs from monocotyledonous plants are represented in the same clade. Both proteins from rice cluster together with

ARF-GEFs from other monocots in the GNOM clade (Fig. 1), suggesting that the two GNOM and GNL1 homologs of the monocots are able to promote Golgi-ER transport as well as endosomal PIN1 recycling. This hypothesis was again tested by expressing the two GNOM-GNL1 homologous proteins from *Oryza sativa* in *gnom* and *gnl1* mutants of Arabidopsis.

Considering their appearance on the same clade in the phylogenetic tree, the rice proteins were named OsGR1 (LOC_Os02g22090.1) and OsGR2 (LOC_Os03g46330.1). Both amino acid sequences share a higher identity with AtGNOM (OsGR1-AtGNOM 62%, OsGR2-AtGNOM 75%) than with AtGNL1 (OsGR1-AtGNL1 75%, OsGR2-AtGNL1 60%).

OsGR1 hardly rescued the *gnom* phenotype, producing seedlings with one fused cotyledon and a very short root (Fig. 4a). The expression of OsGR1 was below detection limit in the Western Blot. However, a weak fluorescent signal was detectable by confocal microscopy. The resulting images show that the localization of OsGR1 was comparable to Arabidopsis GNOM as it co-localizes with FM4-64 to endosomes after BFA-treatment (Fig. 4c). Rescue experiments of *gnl1* plants indicate that the very low amount of OsGR1 protein is still enough to rescue the mutant bushy phenotype (Suppl. Fig. S2).

Gene expression of *OsGR2* in the *gnom* mutant background revealed that the rice protein was able to compensate for AtGNOM completely (Fig. 4b). This finding indicated that OsGR2 mediated endosomal recycling, which was consistent with the localization data. Although the expression could not be detected in the Western blot, a fluorescent signal of OsGR2 was detected in root cells by confocal microscopy. After BFA-treatment, OsGR2 was stabilized on endosomal membranes and co-colocalized with FM4-64 in so-called BFA compartments (Fig. 4d), which are mainly made up of the aggregated *trans*-Golgi network/early endosomes and surrounded by Golgi stacks. Fig. 4d implies that a subpopulation of OsGR2 was recruited to the Golgi stacks at the periphery of BFA compartments. In addition, a role of OsGR2 in retrograde Golgi-ER traffic was demonstrated by the phenotypic rescue of the *gnl1* stunted phenotype (Suppl. Fig. 2).

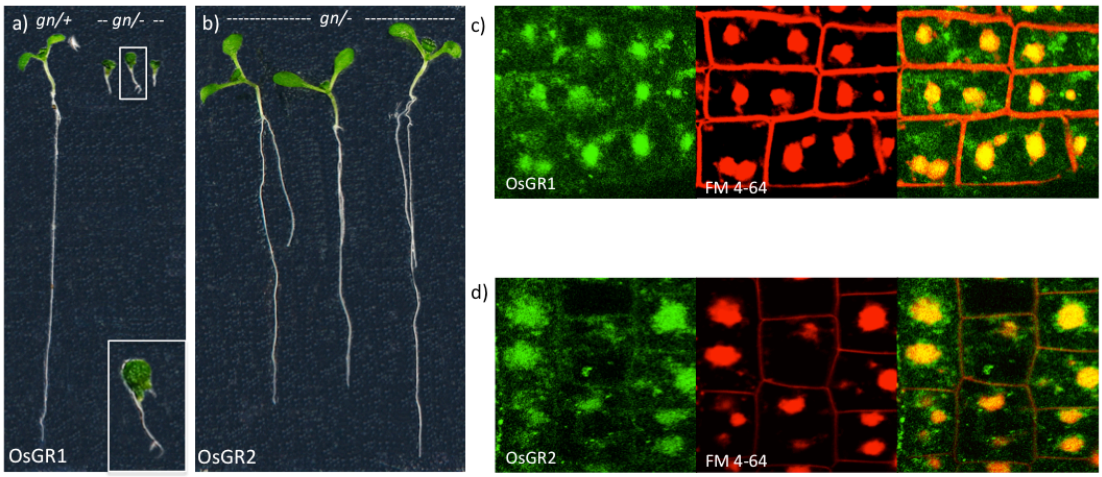

Fig. 4: Rescue tests of *gnom* seedlings by expressing *Oryza sativa* GNOM-related ARF-GEFs in *Arabidopsis thaliana* and localization studies

a) Seedling phenotypes of AtGN::OsGR1-YFP:AtGN expressed in homozygous (*sgt/-*) and heterozygous (*sgt/+*) *gnom* seedlings respectively (genotype of individual seedlings is indicated above). OsGR1 rescued *gnom* homozygous seedlings only partially (see inset for a higher-magnification). b) Seedling phenotype of AtGN::OsGR2-YFP:AtGN in a homozygous *gnom* background (*sgt/-*). (c, d) Subcellular localization of (c) OsGR1-YFP or (d) OsGR2-YFP with FM4-64 after BFA treatment (50 µM BFA for 1 h). The parental generation of the localization studies in c and d was heterozygous for *gnom.*

DISCUSSION

The functional diversification of Arabidopsis GNOM and GNL1 led to the hypothesis of subfunctionalization of duplicated gene products. This model suggests that after genome duplications, GNL1 has lost the ancient GNOM role in endosomal recycling and has specialized in the additional task of GNOM, the promotion of retrograde Golgi-ER trafficking. The investigation of phylogenetic trees representing the homologous proteins of AtGNOM, AtGNL1 and AtGNL2 showed separate clades only for the GNOM and GNL1 related proteins of the Brassicaceae family and closely related species from other families, whereas the homologous proteins of most other species were represented in the GNOM clade, indicating that they are more similar to AtGNOM than to AtGNL1 (Fig. 1).

In order to investigate whether the separation in two clades reflects specialized tasks in subcellular trafficking, we expressed the two GNOM/GNL1 homologs of *Brassica napus*, *Populus trichocarpa* and *Oryza sativa* in the *gnom* and *gnl1* mutant background of Arabidopsis. In addition, we analyzed the subcellular localization of the transgenes because AtGNOM is recruited to the endosomes, while AtGNL1 localizes to the Golgi stacks.

Of the two Brassica ARF-GEFs, only the putative GNOM ortholog BnGNOM rescued the *gnom* mutant seedling phenotype (Fig. 2a) and co-localized with the endocytic tracer FM4-64 (Fig. 2c) which labels endosomes. In contrast, BnGNL1 did not replace the function of AtGNOM, neither during embryo development (Fig. 2b) nor in post-embryonic processes (Fig. 2e+f). However, the involvement of BnGNL1 in secretion was demonstrated by the rescue of Arabidopsis *gnl1* plants (Suppl Fig. S2), its localization to the Golgi apparatus (Fig. 2c) and the rescue of primary root growth on BFA plates (Suppl. Fig. S3). These results indicate that the functional diversification of the two closely related proteins GNOM and GNL1 is not specific for *Arabidopsis thaliana,* but also present in *Brassica napus*, another species of the Brassicaceae family.

The two poplar proteins have only a partly redundant function in endosomal recycling as their expression in the Arabidopsis *gnom* background leads to a complete or partial rescue of the mutant phenotype, respectively. PtGR2 localizes to the Golgi (Fig. 3d) and cannot rescue the analyzed post-embryonic GNOM defects, gravitropism and lateral root initiation (Fig. 3e+f). Nevertheless, the partial rescue of the *gnom* phenotype indicates that this protein has not completely lost the ability of endosomal recycling (Fig. 3b). Poplar seems to be a species in which PtGR1 is the major player in endosomal recycling and PtGR2 is on its way to specialize in retrograde Golgi-ER traffic, indicating that this species maps close to the evolutionary time point at which the functional diversification of GNOM-related ARF-GEFs was initiated. Still, the functional diversification is not complete as the two poplar proteins are located on the same clade in the phylogenetic tree depicted in Fig. 1.

Oryza sativa is a species that is more distantly related to *Arabidopsis thaliana* and the monocots are separated from the dicots on the “GNOM-clade”. Although localizing to endosomes (Fig. 4c), OsGR1 can hardly substitute for Arabidopsis

GNOM when expressed in the latter's mutant background, pointing out that OsGR1 is incapable of promoting endosomal recycling in Arabidopsis. It still remains unclear whether this is a result of expressing the rice protein in a heterologous system or whether the GNOM-related ARF-GEFs have undergone the same evolution towards subfunctionalization in monocotyledonous plants as it was suggested for the Brassicaceaes. Since OsGR1 is able to rescue the Arabidopsis *gnl1* phenotype completely, it is unlikely that the rice and Arabidopsis proteins are too diverse to replace each other. In addition, OsGR2 can fully rescue the *gnom* phenotype (Fig. 4b) and shows the same subcellular localization as AtGNOM (Fig. 4d). However, the alignment of the two protein sequences revealed that OsGR2 lacks the first 271 amino acids comprising the DCB domain, which was demonstrated to be involved in dimerization of two GNOM proteins (Grebe et al., 2000). Nevertheless, an AtGNOM protein lacking the DCB domain was shown to dimerize with AtGNL1 *in planta*. Furthermore, the resulting heterodimer behaved like GNOM because it rescued the *gnom* mutant phenotype (Sabine Brumm, personal communication). Future Co-IP experiments could reveal whether the truncated OsGR2 protein forms heterodimers with endogenous AtGNL1 as well. Nevertheless, a putative heterodimer of OsGR2 and AtGNL1 was shown to be involved in endosomal recycling, as the expression of OsGR2 in the homozygous *gnom* background fully rescued the mutant phenotype. This indicates that OsGR2 can replace GNOM in endosomal recycling.

A reason for the specialization of GNL1 in retrograde Golgi-ER transport might be that it enables the cell to secrete large amounts of macromolecules into the apoplast, which is necessary during cell growth. In addition, GNOM can further aid in this process. However, we suppose that GNOM is more specialized in endosomal PIN1 recycling. It was shown before that full-length AtGNOM and AtGNL1 do not form heterodimers (Manoj Singh, personal communication), and we hypothesize that this is a means to keep the non-recycling ARF-GEF GNL1 protein off the putative recycling endosome. In case of redundancy between the two GNOM-GNL1 related proteins of other plant species, heterodimers should be formed because both proteins are recruited to the endosomes. Consequently, future Co-IP experiments should test for the heterodimer formation between

two GNOM and GNL1 orthologs as well as their ability to form dimers with Arabidopsis GNOM and GNL1.

Homologous proteins that are able to interact with Arabidopsis GNOM could also be analyzed in additional Arabidopsis *gnom* alleles. The experiments that were introduced in this work were performed in the GNOM *sgt* allele, which lacks the GNOM locus and neighboring genes due to a deletion of 37 kb caused by transposon mutagenesis. In contrast, two different full-length *GNOM* alleles with point-mutations are available. GNOM-emb30 is catalytically inactive, but it could be complemented by dimerization with another GNOM protein. Rescue of *emb30* seedlings would reveal that the homologous protein can heterodimerize with the GNOM-emb30 protein and that it is catalytically active. In addition, rescue experiments in the *b4049* allele could demonstrate whether the protein from the heterologous system can associate with the donor membrane as GNOM-b4049 proteins cannot bind to the membrane on their own.

In conclusion, *Arabidopsis thaliana* and *Brassica napus* each have two ARF-GEFs that have specialized in their function during evolution, leading to a loss of promoting the ancestral function of endosomal recycling in one of the two paralogs. This is a process happening in other species as well. This functional diversification is probably also ongoing in *Populus trichocarpa*. In the future, it would be interesting to investigate more species that are closely related to poplar to get a better idea of the subfunctionalization of the GNOM and GNL1 orthologs.

SUPPLEMENTAL DATA

Fig. S1: Phylogenetic tree with plant model species highlighted

The phylogenetic tree gives an overview of the evolution of green plants, showing orders and some model species (Source: Stevens, P. F. (2001 onwards). (Angiosperm Phylogeny Website http://www.mobot.org/MOBOT/research/APweb/. Version 14, July 2017 [and more or less continuously updated since]).

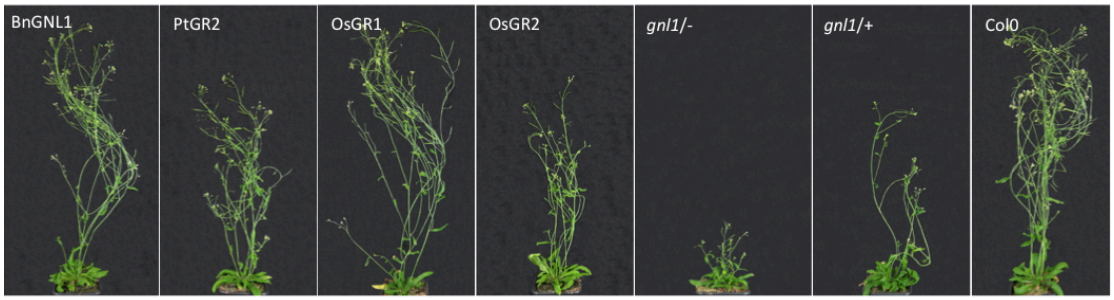

Fig. S2: GNOM-GNL1 homologous proteins from different species can rescue the *gnl1* mutant phenotype of *Arabidopsis thaliana*

GNOM and GNL1 paralogs from *Brassica napus, Populus trichocarpa* and *Oryza sativa* rescued the stunted phenotype of *gnl1* homozygous plants.

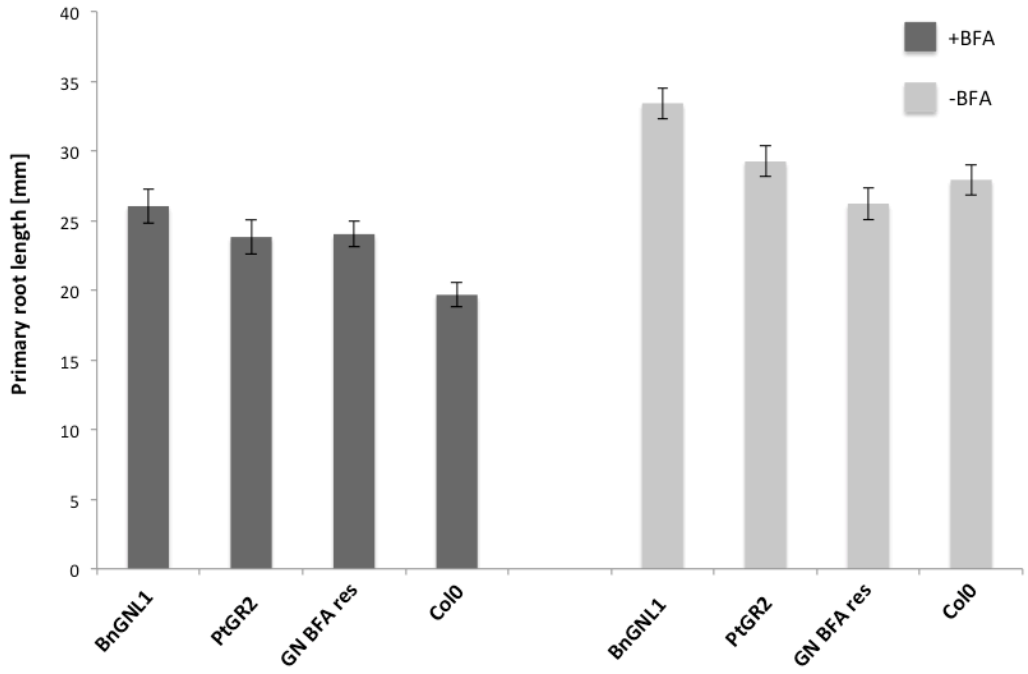

Fig. S3: BFA effect on primary root length of seedlings expressing BFA-resistant GNOM-related ARF-GEFs from Brassica and poplar in *Arabidopsis thaliana* compared to BFA-resistant GNOM

The seedlings in the *sgt/+* background were grown for 5 days on MS plates and then transferred to +/- 10 µM BFA plates for three days

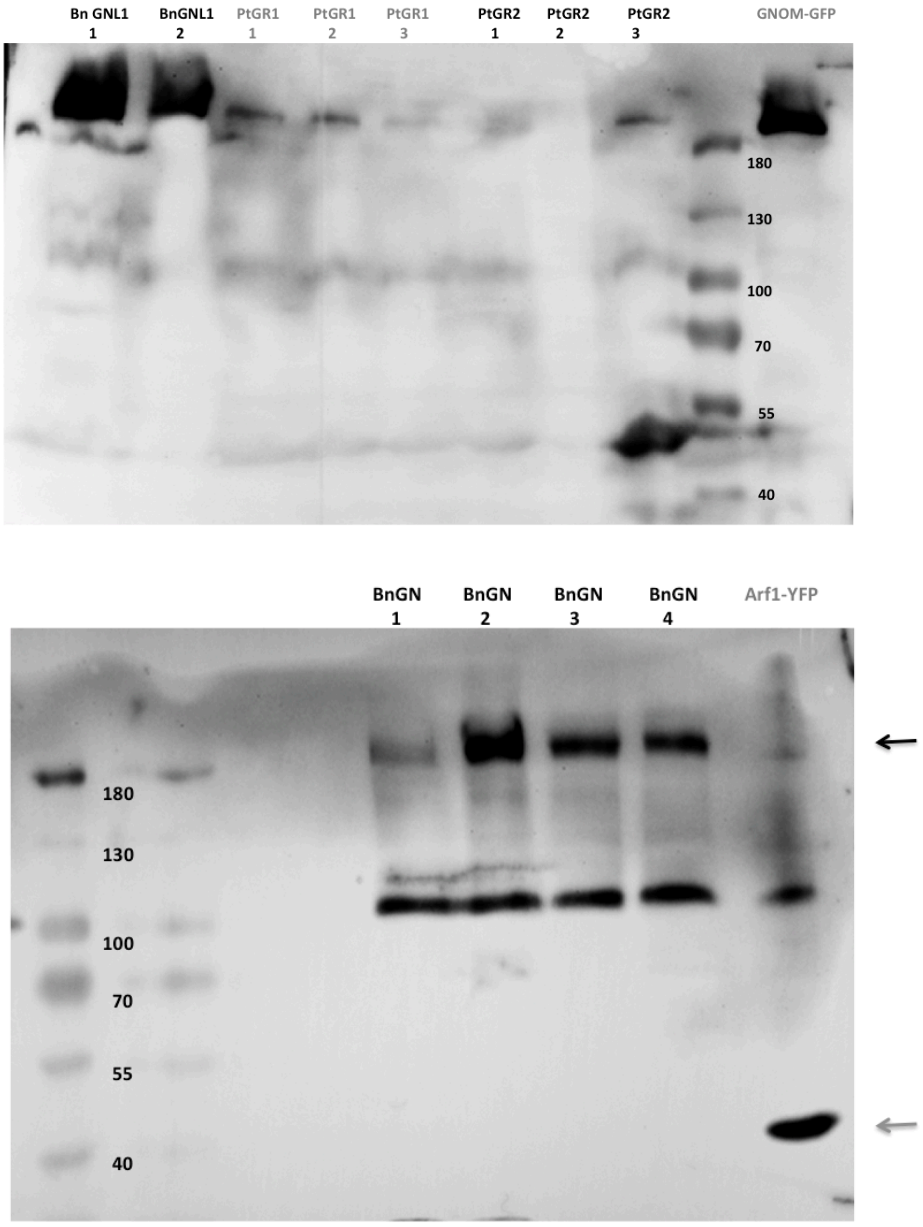

Fig. S4: Protein expression

Western Blots with anti-GFP antiserum, showing the expression of the tested proteins in at least two independent lines. Upper Blot: The expected protein size of the YFP-tagged proteins is about 190 kDa, which is similar to the GN-GFP positive control. Lower Blot: The black arrow indicates YFP-tagged BnGNOM proteins, the grey arrow indicates the Arf1-YFP positive control.

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5.3 Draft Manuscript 3:

Interaction studies of the conserved dimerization domain of two closely related membrane trafficking regulators revealed their functional diversification during evolution

Contributions:

The cloning of the constructs with the DCB constructs of GNOM and GNL1 homologs was a joint effort of Marika Kientz, Moritz Peters and Kerstin Huhn.

The yeast-two hybrid interaction studies depicted in this work were performed by Marika Kientz and Moritz Peters, supervised by Kerstin Huhn.

The quantitative ONPG interaction assay was performed by Kerstin Huhn.

The interpretation of the results as well as the writing of the manuscript was done by Kerstin Huhn.

Draft Manuscript 3:

Interaction studies of the conserved dimerization domain of two closely related membrane trafficking regulators revealed their functional diversification during evolution

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ABSTRACT

The two closely related membrane trafficking regulators of *Arabidopsis thaliana*, GNOM and GNL1, are homologs of human ARF guanine-nucleotide exchange factor (ARF-GEF) GBF1 and as such act redundantly in retrograde vesicle transport between the Golgi apparatus and the endoplasmic reticulum. However, GNOM has an additional function in endosomal PIN1 recycling. Here, we address whether GNL1 represents the evolutionarily ancient function and GNOM gained an additional function during plant evolution or whether GNOM was the ancient plant-specific GBF1-like ARF-GEF and GNL1 lost the recycling function during plant evolution. In yeast two-hybrid studies, the dimerization domain (DCB domain) of both GNOM and GNL1 can interact with the rest of the protein (ΔDCB), but only the GNOM DCB can interact with itself, which might prevent heterodimer formation of GNOM with GNL1 in Arabidopsis plants. Using the yeast two-

hybrid interaction assay, we investigated the DCB domains from several species of the Viridiplantae kingdom in regard to their ability to interact with themselves, the other DCB domain (if two paralogs were present in the same species), DCB(GNOM), DCB(GNL1) and ΔDCB(GNOM). DCB domains of GNOM-related proteins (GNRs) from algae and lower plants behaved like DCB(GNOM). The inability of DCB domains of paralogous GNRs in angiosperm species to form heterodimers was taken as the initial step towards functional diversification of the two proteins. Our preliminary results suggest that this diversification was initiated within the eurosids of the dicotyledonous flowering plants.

INTRODUCTION

The transport of cargo molecules within a eukaryotic cell has to be strongly regulated. One major step in membrane trafficking is the vesicle budding which is initiated after the activation of ADP-ribosylation factor GTPases (ARF-GTPases) by their Guanine Exchange Factors (ARF-GEFs) (Beraud-Dufour et al., 1999).

Arabidopsis thaliana comprises eight different ARF-GEFs which regulate different routes interconnecting the organelles. Earlier publications revealed that GNOM and GNL1 act in the early secretory trafficking pathway (Richter et al., 2007; Teh & Moore, 2007), and BIG 1-4 in the late secretory pathway as well as in cytokinesis (Richter et al., 2014). Furthermore, GNOM and GNL2 are involved in polar recycling (Geldner et al., 2003; Richter et al., 2011; Steinmann et al., 1999), but GNL2 was shown to be expressed only in the pollen where it aids in pollen tube growth (Richter et al., 2011). GNOM, GNL1 and GNL2 are members of the GBF1-subfamiliy of large ARF-GEFs.

Algae, lower plants or the basal flowering plant *Amborella trichocarpa* encode only one homolog of GNOM and GNL1. It is speculated that GNOM and GNL1 in *Arabidopsis thaliana* are a result of genome duplication events during evolution and that the progression of more specialized species led to the functional diversification of these two ARF-GEFs. Arabidopsis GNOM and GNL1 share a common role in retrograde trafficking between the Golgi apparatus and the endoplasmic reticulum, but only GNOM is capable of promoting endosomal recycling (Richter et al., 2007).

Recent experiments in our lab suggest that the N-terminal dimerization domain of GNOM, called DCB domain, acts in the prevention of GNOM-GNL1 heterodimer formation, because only after deleting the DCB domain of GNOM, a GNOM-GNL1 heterodimer forms *in planta* (Manoj Singh and Sabine Brumm, personal communication), probably through the interaction of the DCB(GNL1) domain with the ΔDCB(GNOM) protein. In addition, yeast two-hybrid experiments indicate that DCB(GNOM) domain forms homodimers by interaction with another DCB(GNOM) and that DCB(GNL1) is not able to interact with another

DCB domain, neither with its own nor with the DCB(GNOM) (Hauke Beckmann, personal communication).

We therefore presumed that the dimerization of two DCB(GNOM) domains is a means to prevent heterodimerization of GNOM with GNL1 because the two proteins have undergone subfunctionalization during evolution. Yeast two-hybrid studies were used to investigate the mode of DCB dimerization in GNOM-related proteins from other plant species. We reasoned that functional diversification of GNOM-related (GNR) proteins was likely in the tested organisms if the DCB domains were only to homodimerize, but not to heterodimerize. In addition, we also tested the interaction of DCB(GNR) with DCB domain of Arabidopsis GNOM and GNL1 to test for the conservation of the DCB domain. Although some data is still missing, our results suggest that the DCB-DCB dimerization is a common feature amongst different species and was lost during evolution, probably when the GNOM and GNL1 paralogs of certain species started to diversify in function. Our studies revealed such change within the eurosids of the dicotyledonous flowering plants.

RESULTS

The DCB domains of GNOM-related proteins from the tested algae and liverwort species behave similarly to DCB(AtGNOM)

In our studies, we first tested whether the DCB domain of putatively ancient homologs of GNOM and GNL1 dimerized with the DCB domains of the GNOM and GNL1 and whether two different DCB domains of the same species were able to interact. We used *Klebsormidium nitens* as a representative of the charophyte algae and the liverwort *Marchantia polymorpha* as a representative of lower land plants. Both species have only one homolog of Arabidopsis GNOM and GNL1, and regarding our hypothesis, their DCB domain should be able to interact with DCB(GNOM), but not with DCB(GNL1) and should also dimerize with itself. Indeed, the Klebsormidium DCB interacted with the DCB(GNOM) and with itself (Fig. 1, lane 1+4). DCB(GNL1) in the bait vector also interacted with the algal DCB domain (Fig. 1, lane 2). However, no interaction was detectable when

DCB(GNL1) was in the prey vector (Fig. 1, lane 3). Auto activation of DCB(GNL1) in the bait vector had already been observed several times, therefore we assumed that the interaction in lane 2 was a false positive result. The liverwort Marchantia showed very weak interaction with DCB(GNOM) (Fig. 1, lane 7) and no interaction with DCB(GNL1) (Fig. 1, lane 8). An interaction between two Marchantia DCBs was not detectable as well (Fig. 1, lane 9). To test for the integrity of the Marchantia DCB domain, we included ΔDCB(GNOM) protein in the assay as well. As both DCB(GNOM) and DCB(GNL1) interact with the complementary ΔDCB fragment of both proteins (Hauke Beckmann, personal communication), we assume that this interaction is conserved and can serve as an indicator of the integrity of DCB domain constructs. In our experiment, only 8 out of 12 colonies showed blue color in the interaction test between Klebsormdium DCB and ΔDCB(GNOM) (Suppl. Fig. S1). Expression tests of these colonies are going to be performed to verify whether the white colonies simply did not express the Marchantia DCB domain.

Interaction	Bait vector pEG202	Prey vector pJG4-5	
	GN DCB	Klebsormidium DCB	1
	GNL1 DCB	Klebsormidium DCB	2
	Klebsormidium DCB	GNL1 DCB	3
	Klebsormidium DCB	Klebsormidium DCB	4
	Klebsormidium DCB	-	5
	-	Klebsormidium DCB	6
	GN DCB	Marchantia DCB	7
	GNL1 DCB	Marchantia DCB	8
	Marchantia DCB	Marchantia DCB	9
	ΔDCB	Marchantia DCB	10
	-	Marchantia DCB	11
	Marchantia DCB	-	12

Fig. 1: DCB interaction studies of representatives of algae and lower plants

The DCB(GNR) domain of *Klebsormidium nitens* behaved like DCB(AtGNOM) in regard to DCB-DCB interaction. The DCB(GNR) domain of liverwort *Marchantia polymorpha* interacted only weakly with DCB(AtGNOM) and did not show a clear-cut result for the interaction with GNΔDCB. The table shows the coloring of two representative colonies each of 10 colonies tested per assay. The whole plates are depicted in Suppl. Fig. S1.

The DCB(GNR) from the basal flowering plant *Amborella trichopoda* dimerizes with itself and with the DCB domain of Arabidopsis GNOM, but not GNL1

Next, we tested the DCB domain of the single GNOM-related protein (GNR) from the basal flowering plant *Amborella trichopoda*. This DCB(GNR) was able to dimerize with DCB(GNOM) (Fig. 2, lane 1+2) and with itself (Fig 2, lane 5). An interaction with ΔDCB(GNOM) was demonstrated as well (Fig. 2, lane 6+7), but no interaction with DCB(GNL1) was detectable (Fig. 2, lane 3+4). The analysis of DCB(GNR) from the monocot *Oryza sativa* is still ongoing, but the preliminary results indicate that at least one protein behaves like Arabidopsis GNOM, meaning that DCB(Oryza 1) interacted with itself (Fig.2, lane 14), with DCB(GNOM) (Fig. 2, lane 10+12) and ΔDCB(GNOM) (Fig. 2, lane 15+16), but not with DCB(GNL1) (Fig. 2, 12+13). The interaction was weak (probably due to the assay plates used for this experiment), therefore the Y2H studies of Oryza 1 as well as all the experiments with Oryza 2 are going to be repeated. The original experiments of Oryza 2 suggested that DCB(GNR2) cannot dimerize with the DCB domain of Arabidopsis GNOM, but is able to interact with the ΔDCB(GNOM) protein. However, in several experiments, no expression of the DCB domain of Oryza 2 was detected (data not shown), therefore a thorough investigation is still missing.

Interaction	Bait vector pEG202	Prey vector pJG4-5	
	Amborella DCB	GN DCB	1
	GN DCB	Amborella DCB	2
	Amborella DCB	GNL1 DCB	3
	GNL1 DCB	Amborella DCB	4
	Amborella DCB	Amborella DCB	5
	Amborella DCB	GN ΔDCB	6
	GN ΔDCB	Amborella DCB	7
	Amborella DCB	-	8
	-	Amborella DCB	9
	Oryza 1 DCB	GN DCB	10
	GN DCB	Oryza 1 DCB	11
	GNL1 DCB	Oryza 1 DCB	12
	Oryza 1 DCB	GNL1 DCB	13
	Oryza 1 DCB	Oryza 1 DCB	14
	Oryza 1 DCB	GN ΔDCB	15
	GN ΔDCB	Oryza 1 DCB	16
	Oryza 1 DCB	-	17
	-	Oryza 1 DCB	18
	Oryza 2 DCB	GN DCB	19
	Oryza 2 DCB	GN ΔDCB	20

Fig. 2: DCB interaction studies of the basal flowering plant *Amborella trichopoda* and the monocotyledonous plant *Oryza sativa* (rice)

DCB(GNR) of *Amborella trichopoda* behaved like DCB of Arabidopsis GNOM. One of the two rice proteins tested behaved like GNOM whereas the other needs to be investigated in more detail. The table shows the coloring of two representative colonies each of 10-15 per assay. The whole plates are depicted in Suppl. Fig. S2.

The DCB domains of both GNR proteins from the legume *Medicago truncatula* can dimerize with DCB(GNOM), with each other and with themselves

The basal dicotyledonous plants *(Aquilegia coerulea)* appeared very early during Angiosperm evolution. Preliminary results of our Y2H studies suggest that at least one of the two GNOM-related (GNR) proteins behaves like Arabidopsis GNOM, as the tested Aquilegia DCB interacted with DCB(GNOM) but not with DCB(GNL1) (Fig. 3, lane 1+2). More detailed studies are still ongoing. However, more data is available is for *Medicago truncatula*, a representative for the

Fabidae order. The DCB domain of both GNOM-related proteins was able to interact with Arabidopsis DCB(GNOM) but not with DCB(GNL1) (Fig. 3, lane 4-8). In addition, DCB-DCB interaction was strong for Medicago 1 and weaker for Medicago 2 (Fig. 3, lane 9+10), indicating that both *Medicago truncatula* proteins behave like Arabidopsis GNOM. However, the test for heterodimer formation between the two Medicago DCBs is still missing. In addition to the dimerization studies with the DCB domains, the interaction with ΔDCB(GNOM) protein proved the integrity of the two Medicago DCB domain constructs (Fig. 3, lane 11+12).

Interaction	Bait vector pEG202	Prey vector pJG4-5	
	GN DCB	Aquilegia 1 DCB	1
	GNL1 DCB	Aquilegia 1 DCB	2
	-	Aquilegia 1 DCB	3
	GN DCB	Medicago 1 DCB	4
	GNL1 DCB	Medicago 1 DCB	5
	GN DCB	Medicago 2 DCB	6
	GNL1 DCB	Medicago 2 DCB	7
	Medicago 2 DCB	GNL1 DCB	8
	Medicago 1 DCB	Medicago 1 DCB	9
	Medicago 2 DCB	Medicago 2 DCB	10
	GN ΔDCB	Medicago 1 DCB	11
	GN ΔDCB	Medicago2 DCB	12
	-	Medicago 1 DCB	13
	Medicago 1 DCB	-	14
	-	Medicago 2 DCB	15
	Medicago 2 DCB	-	16

Fig. 3: DCB interaction studies of the basal dicotyledonous plant *Aquilegia coerulea* and the legume *Medicago truncatula*

Preliminary results suggest that at least one GNR protein of *Aquilegia coerulea* and both GNR proteins of *Medicago truncatula* behave like Arabidopsis GNOM concerning DCB-DCB dimerization. The table shows the coloring of two representative colonies each of 10-15 per assay. Whole plates are depicted in Suppl. Fig. S3.

The two DCBs from *Populus trichocarpa* behave differently in regard to DCB-DCB dimerization

We detected a difference in the behavior of DCB-DCB interaction for the first time in our interaction studies when we tested the two poplar proteins. *Populus trichocarpa* represent the order of Malpighiales, which like the Brassicales order including *Arabidopsis thaliana* is part of the eurosids. Fig. 4 shows that the DCB domain of one poplar protein only interacted with ΔDCB(GNOM) (Fig. 4, lane 13+14), but neither with DCB(GNOM) (Fig. 4, lane 1+2) or DCB(GNL1) (Fig. 4, lane 5+6) nor with itself (Fig. 4, lane 9) or the DCB domain of the other poplar protein (Fig. 4, lane 11+12). Conversely, the other poplar protein interacted with DCB(GNOM) (Fig. 4, lane 3+4) and with itself (Fig. 4, lane 10), but not with DCB(GNL1) (Fig. 4, lane 7+8) or the DCB domain of first poplar protein (Fig. 4, lane 11+12). The negative controls with the empty vector controls were tested several times before in independent experiments and are not shown here. The interaction with ΔDCB(GNOM) served as a control for the integrity of the DCB constructs (Fig. 4, lane 13-16).

Interaction	Bait vector pEG202	Prey vector pJG4-5	
	Populus 1 DCB	GN DCB	1
	GN DCB	Populus 1 DCB	2
	Populus 2 DCB	GN DCB	3
	GN DCB	Populus 2 DCB	4
	Populus 1 DCB	GNL1 DCB	5
	GNL1 DCB	Populus 1 DCB	6
	Populus 2 DCB	GNL1 DCB	7
	GNL1 DCB	Populus 2 DCB	8
	Populus 1 DCB	Populus 1 DCB	9
	Populus 2 DCB	Populus 2 DCB	10
	Populus 1 DCB	Populus 2 DCB	11
	Populus 2 DCB	Populus 1 DCB	12
	Populus 1 DCB	GN ΔDCB	13
	GN ΔDCB	Populus 1 DCB	14
	Populus 2 DCB	GN ΔDCB	15
	GN ΔDCB	Populus 2 DCB	16

Fig. 4: Interaction studies with DCB(GNR) domains from of *Populus trichocarpa*

One poplar protein (GNR2) shows the same DCB interaction behavior as AtGNOM, whereas the other protein (GNR1) behaves like AtGNL1. The table shows the coloring of two representative colonies each of 10-15 per assay. The whole plates are depicted in Suppl. Fig. S4.

Only one of the two GNOM-related proteins from *Brassica napus* homodimerizes via its DCB domain, but the two Brassica DCBs heterodimerize

Brassica napus is the closest Arabidopsis homolog we used in this study. The two species both belong to the Brassicaceae family and the GNOM-related proteins are expected to behave similarly concerning their DCB-DCB dimerization. However, only the putative GNOM ortholog, in his study called Brassica 1, behaved like its relative in Arabidopsis concerning the interaction of its DCB domain with DCB(GNOM) (Fig. 5, lane 1+2), and with itself (Fig. 5, lane 9). We observed an interaction of DCB(Brassica 1) and DCB(GNL1) when the latter protein was in the bait vector (Fig. 5, lane 6), but this was probably again a false positive result. Brassica 2, the GNL1 ortholog, only behaved like the related Arabidopsis protein concerning DCB dimerization with itself (Fig. 5, lane 10) and DCB(GNL1) (Fig. 5, lane 7+8). Surprisingly, we observed a heterotypic interaction with DCB(Brassica 1) (Fig. 5, lane 11+12). In addition, we tested both Brassica DCBs for interaction with ΔDCB(GNOM). Both proteins were able to interact with ΔDCB(GNOM) (Fig. 5, lane 13-16), indicating that the proteins did not act abnormally in this regard. Next, we tested our results independently with a quantitative yeast interaction assay (ONPG assay), which gave similar results (Suppl. Fig. S6). As the heterodimerization of the two Brassica proteins was unexpected, we are going to test their interaction with full-length Arabidopsis GNOM and GNL1 proteins in the near future *in planta*.

Interaction	Bait vector pEG202	Prey vector pJG4-5	
	Brassica 1 DCB	GN DCB	1
	GN DCB	Brassica 1 DCB	2
	Brassica 2 DCB	GN DCB	3
	GN DCB	Brassica 2 DCB	4
	Brassica 1 DCB	GNL1 DCB	5
	GNL1 DCB	Brassica 1 DCB	6
	Brassica 2 DCB	GNL1 DCB	7
	GNL1 DCB	Brassica 2 DCB	8
	Brassica 1 DCB	Brassica 1 DCB	9
	Brassica 2 DCB	Brassica 2 DCB	10
	Brassica 1 DCB	Brassica 2 DCB	11
	Brassica 2 DCB	Brassica 1 DCB	12
	Brassica 1 DCB	GN ΔDCB	13
	GN ΔDCB	Brassica 1 DCB	14
	Brassica 2 DCB	GN ΔDCB	15
	GN ΔDCB	Brassica 2 DCB	16
	Brassica 1 DCB	-	17
	-	Brassica 1 DCB	18
	Brassica 2 DCB	-	19
	-	Brassica 2 DCB	20

Fig. 5: Interaction studies with DCB(GNR) domains of *Brassica napus*

Only one of the two rapeseed DCB(GNR) domains homodimerized, but both DCB domains interacted with each other and DCB(GNOM). The table shows the coloring of two representative colonies each of 10-15 per assay. The whole plates are depicted in Suppl. Fig. S5.

DISCUSSION

The two closely related ARF-GEFs GNOM and GNL1 both promote retrograde Golgi-ER traffic in *Arabidopsis thaliana*. In addition, GNOM has a unique function in the recycling of the auxin efflux carrier PIN1. We think that this is an ancient function which was lost during flowering plant evolution and tried to narrow down this time point. In previous yeast two-hybrid experiments, we showed that only the N-terminal DCB domain of Arabidopsis GNOM and not of GNL1 could interact with itself (Hauke Beckmann, personal communication), and we propose

that this DCB-DCB interaction might prevent heterodimerization between GNOM and GNL1. We repeated the same experiments using the DCB domains of GNOM-related proteins from various species of the Viridiplantae kingdom. We analyzed their ability to perform homotypic DCB-DCB interaction with the help of Y2H studies, using two DCB domains of the same kind (DCB GNR1-GNR1), the DCB domains of two orthologous proteins (DCB GNR1-GNR2) or tested against the DCB domain of Arabidopsis GNOM (DCB GNR-GNOM) and GNL1 (DCB GNR-GNL1). Although a detailed analysis is not completed yet, we saw a trend for GNR proteins from lower species to dimerize via their DCB domain and to interact with Arabidopsis GNOM. We showed this for the charophyte algae *Klebsormidium flaccidum* (Fig. 1) and the basal flowering plant *Amborella trichocarpa* (Fig. 2), which have both only a single GNOM-related ARF-GEF, disregarding a GNL2 ortholog in Amborella. During angiosperm evolution, a split between monocots and eudicots took place. In our experiments, we used *Oryza sativa* as a monocot representative and *Aquilegia coerulea* as a basal dicot. Both species have two GNOM-related proteins, which is a result of genome duplications, but their analysis has not yet been completed. Currently, a DCB-DCB interaction could only be proposed for one Aquilegia (Fig. 3) and one Oryza ortholog (Fig. 2) respectively. Interestingly, the test of three dicot species revealed a difference in their DCB dimerization behavior: While the DCB domains of both orthologs of the legume *Medicago truncatula* could interact with another DCB domain (Fig. 3), the two poplar proteins behaved like Arabidopsis GNOM and GNL1 (Fig. 4): The DCB domain of one poplar protein (GNR1) could neither interact with itself, nor with the DCB of GNR2, GNOM or GNL1. However, we discovered a point mutation in the poplar GNR1 clone leading to an amino acid exchange (H87N). Therefore, we are going to verify our results with the correct clone in future experiments. Surprisingly, the two rapeseed DCB domains could interact with each other, although no interaction between two Brassica GNR2 DCBs and between the DCBs of GNR2 and GNL1 was observed (Fig. 5). Further experiments *in planta* will investigate whether the interaction can also be observed between full-length Brassica GNR2 protein with Arabidopsis GNOM and GNL1.

Taken together, our results suggest that, indeed, ancient plant species with only one GBF1-related protein behaved similarly to Arabidopsis GNOM regarding DCB dimerization. This feature is conserved amongst eukaryotes as the human GNOM homolog GBF1 was shown to perform DCB-DCB interaction as well (Bhatt et al., 2016; Ramaen et al., 2007). As mentioned above, we assume that plant proteins which dimerize via DCB-DCB interaction could perform both GNOM functions in retrograde Golgi-ER traffic and endosomal recycling. As the role in endosomal PIN1 recycling is special for the plant kingdom, an interaction test between the DCB of Arabidopsis GNOM and its human homolog GBF1 would be very interesting. Our findings indicate as well that a function in endosomal recycling started already with the evolution of charophytes, represented by *Klebsormidium nitens*. In future experiments, the DCB domain of a chlorophyte algae should be tested as well. After genome duplications during the angiosperm evolution, both proteins probably acted redundantly in endosomal recycling and retrograde traffic. However, the observation that the two poplar proteins do not heterodimerize via their DCB domains suggest that a functional diversification was initiated within the eurosids, a group that comprises, amongst other species, *Populus trichocarpa*, *Brassica napus* and *Arabidopsis thaliana*. Future yeast studies with more species of the eurosids could provide a deeper insight in the evolution of GNOM and GNL1 and the development of their functional diversification. Interesting candidate genes should then be expressed in the mutant *gnom* and *gnl1* background of *Arabidopsis thaliana* to verify the yeast data *in planta*.

SUPPLEMENTAL DATA

Bait vector pEG202	Prey vector pJG4-5	
GN DCB	Klebsormidium DCB	1
GNL1 DCB	Klebsormidium DCB	2
Klebsormidium DCB	GNL1 DCB	3
Klebsormidium DCB	Klebsormidium DCB	4
Klebsormidium DCB	-	5
-	Klebsormidium DCB	6
GN DCB	Marchantia DCB	7
GNL1 DCB	Marchantia DCB	8
Marchantia DCB	Marchantia DCB	9
ΔDCB	Marchantia DCB	10
-	Marchantia DCB	11
Marchantia DCB	-	12

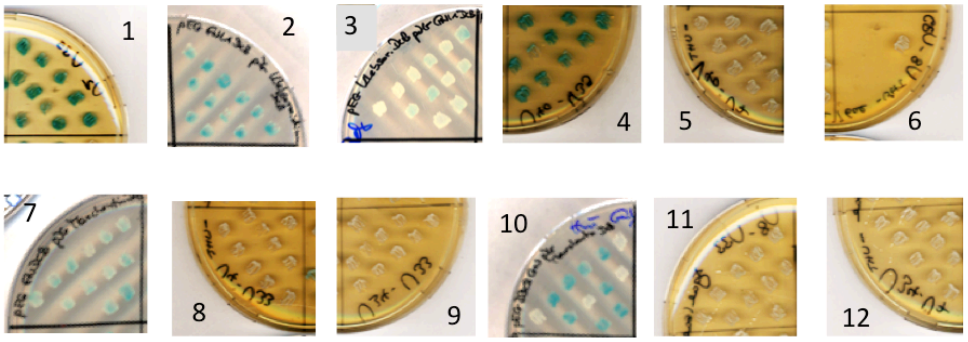

Fig. S1: DCB interaction studies of representatives of algae and lower plants

For the interaction studies, in total 10 colonies of each transformation event were tested on X-Gal plates.

Bait vector pEG202	Prey vector pJG4-5	
Amborella DCB	GN DCB	1
GN DCB	Amborella DCB	2
Amborella DCB	GNL1 DCB	3
GNL1 DCB	Amborella DCB	4
Amborella DCB	Amborella DCB	5
Amborella DCB	GN ΔDCB	6
GN ΔDCB	Amborella DCB	7
Amborella DCB	-	8
-	Amborella DCB	9
Oryza 1 DCB	GN DCB	10
GN DCB	Oryza 1 DCB	11
GNL1 DCB	Oryza 1 DCB	12
Oryza 1 DCB	GNL1 DCB	13
Oryza 1 DCB	Oryza 1 DCB	14
Oryza 1 DCB	GN ΔDCB	15
GN ΔDCB	Oryza 1 DCB	16
Oryza 1 DCB	-	17
-	Oryza 1 DCB	18
Oryza 2 DCB	GN DCB	19
Oryza 2 DCB	GN ΔDCB	20

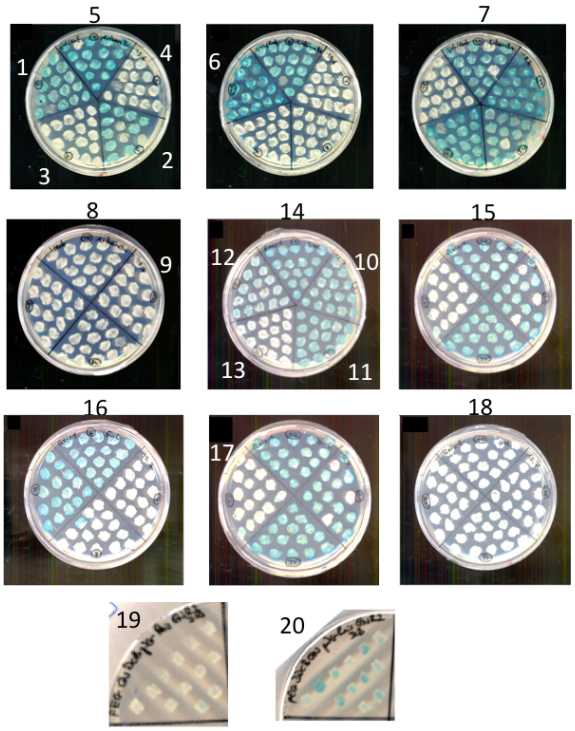

Fig. S2: DCB interaction studies of the basal flowering plant *Amborella trichopoda* and the monocotyledonous plant *Oryza sativa* (rice)

For the interaction studies, in total 10-15 colonies of each transformation event were tested on X-Gal plates.

Bait vector pEG202	Prey vector pJG4-5	
GN DCB	Aquilegia 1 DCB	1
GNL1 DCB	Aquilegia 1 DCB	2
-	Aquilegia 1 DCB	3
GN DCB	Medicago 1 DCB	4
GNL1 DCB	Medicago 1 DCB	5
GN DCB	Medicago 2 DCB	6
GNL1 DCB	Medicago 2 DCB	7
Medicago 2 DCB	GNL1 DCB	8
Medicago 1 DCB	Medicago 1 DCB	9
Medicago 2 DCB	Medicago 2 DCB	10
GN ΔDCB	Medicago 1 DCB	11
GN ΔDCB	Medicago2 DCB	12
-	Medicago 1 DCB	13
Medicago 1 DCB	-	14
-	Medicago 2 DCB	15
Medicago 2 DCB	-	16

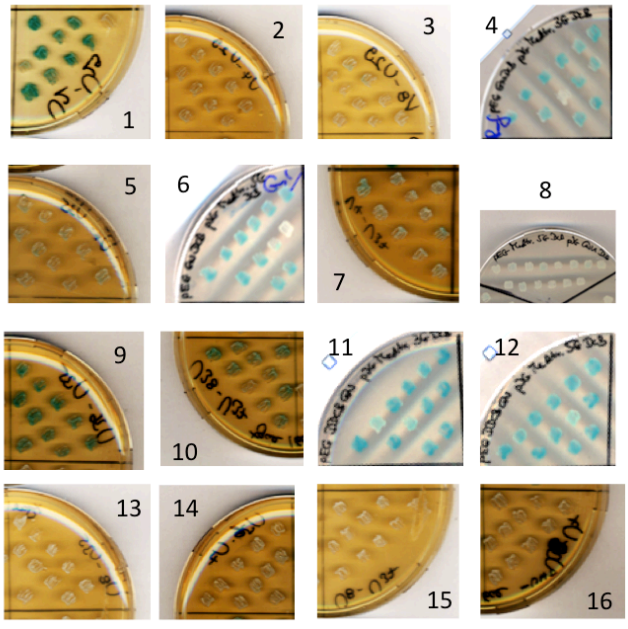

Fig. S3: DCB interaction studies of the basal dicotyledonous plant *Aquilegia coerulea* and the legume *Medicago truncatula*

For the interaction studies, in total 10-15 colonies of each transformation event were tested on X-Gal plates.

Bait vector pEG202	Prey vector pJG4-5	
Populus 1 DCB	GN DCB	1
GN DCB	Populus 1 DCB	2
Populus 2 DCB	GN DCB	3
GN DCB	Populus 2 DCB	4
Populus 1 DCB	GNL1 DCB	5
GNL1 DCB	Populus 1 DCB	6
Populus 2 DCB	GNL1 DCB	7
GNL1 DCB	Populus 2 DCB	8
Populus 1 DCB	Populus 1 DCB	9
Populus 2 DCB	Populus 2 DCB	10
Populus 1 DCB	Populus 2 DCB	11
Populus 2 DCB	Populus 1 DCB	12
Populus 1 DCB	GN ΔDCB	13
GN ΔDCB	Populus 1 DCB	14
Populus 2 DCB	GN ΔDCB	15
GN ΔDCB	Populus 2 DCB	16

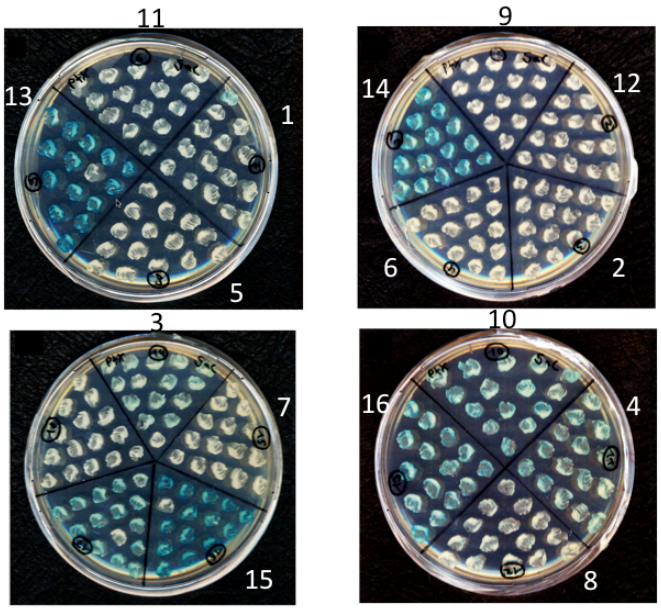

Fig. S4: Interaction studies with DCB(GNR) domains from of *Populus trichocarpa*

For the interaction studies, in total 15 colonies of each transformation event were tested on X-Gal plates.

Bait vector pEG202	Prey vector pJG4-5	
Brassica 1 DCB	GN DCB	1
GN DCB	Brassica 1 DCB	2
Brassica 2 DCB	GN DCB	3
GN DCB	Brassica 2 DCB	4
Brassica 1 DCB	GNL1 DCB	5
GNL1 DCB	Brassica 1 DCB	6
Brassica 2 DCB	GNL1 DCB	7
GNL1 DCB	Brassica 2 DCB	8
Brassica 1 DCB	Brassica 1 DCB	9
Brassica 2 DCB	Brassica 2 DCB	10
Brassica 1 DCB	Brassica 2 DCB	11
Brassica 2 DCB	Brassica 1 DCB	12
Brassica 1 DCB	GN ΔDCB	13
GN ΔDCB	Brassica 1 DCB	14
Brassica 2 DCB	GN ΔDCB	15
GN ΔDCB	Brassica 2 DCB	16
Brassica 1 DCB	-	17
-	Brassica 1 DCB	18
Brassica 2 DCB	-	19
-	Brassica 2 DCB	20

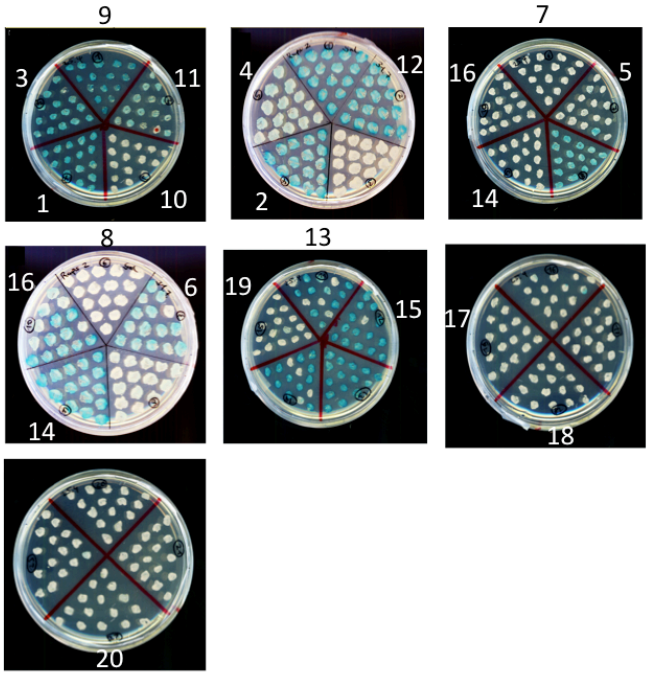

Fig. S5: Interaction studies with DCB(GNR) domains of *Brassica napus*

For the interaction studies, in total 15 colonies of each transformation event were tested on X-Gal plates.

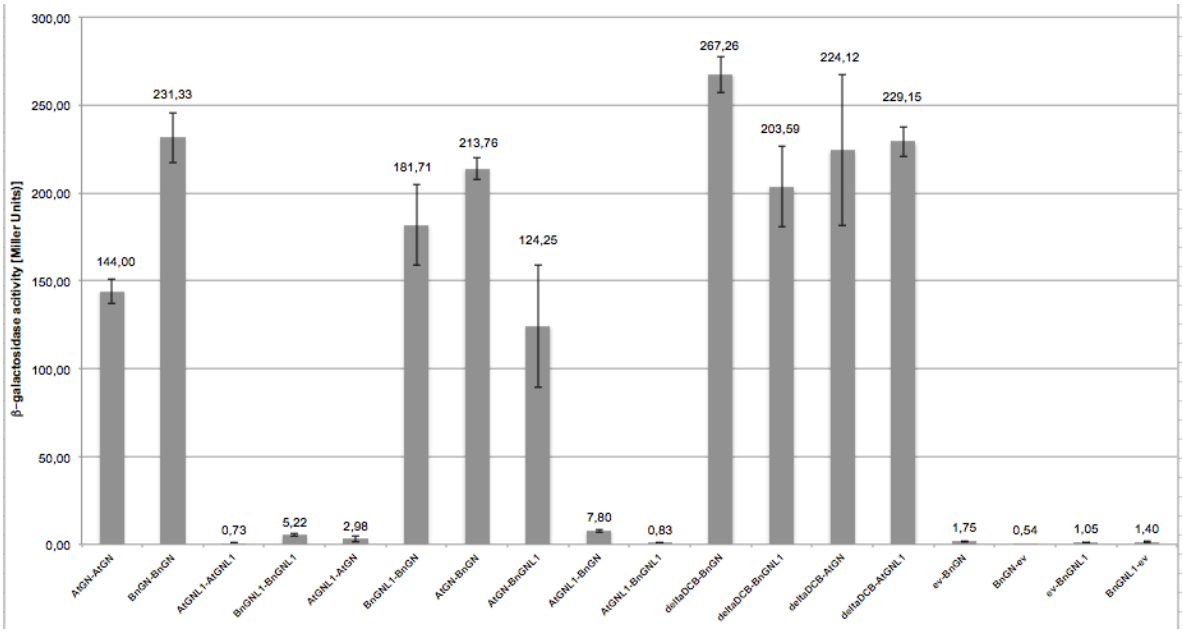

Fig. S6: Quantitative ONPG interaction assay of the *Brassica napus* and *Arabidopsis thaliana* DCB(GNR) domains

Brassica DCB(GNR1 = BnGN) can interact with itself, with Brassica DCB(GNR2 = BnGNL1) and with DCB(AtGNOM) and GN-ΔDCB. Brassica DCB(GNR2 = BnGNL1) can interact with Brassica DCB(GNR1 = BnGN), DCB(AtGNL1) and DCB(AtGNOM).

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5.4 Draft Manuscript 4:

Two conserved amino acids contribute to GNOM dimerization, but act differently compared to other eukaryotes

The mutant DCB constructs for the yeast assay were cloned by Kerstin Huhn.

All yeast two-hybrid studies were performed by Kerstin Huhn.

The plant vectors for future studies were cloned by Kerstin Huhn (wildtype and mutant DCB with different tags).

Marika Kientz generated plant lines expressing the various versions of the GNOM DCB.

The interpretation of the results as well as the writing of the manuscript was done by Kerstin Huhn.

Draft Manuscript 4:

Two conserved amino acids contribute to GNOM dimerization, but act differently compared to other eukaryotes

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ABSTRACT

Dimerization of large ADP-Ribosylation Factor Guanine Exchange Factors (ARF-GEFs) was genetically demonstrated 22 years ago (Busch et al., 1996), and the N-terminal DCB domain identified as its critical domain in the *Arabidopsis thaliana* ARF-GEF GNOM (Grebe et al., 2000). However, the mechanism of dimerization has not been elucidated so far. Here, we address this question, using yeast two-hybrid interaction assays. Our results reveal the essential role of two conserved amino acids K120 and E159 in the interaction between two GNOM DCB domains. A charge reversal of K120 abolished the DCB-DCB interaction of GNOM, but dimerization with a second protein was still possible by interaction with the DCB-adjacent domains. A similar behavior was already demonstrated for GNL1, a close homolog of GNOM. It is suggested that the DCB-DCB interaction of GNOM prevents heterodimerization with GNL1 as the two proteins show functional redundancy. Future experiments *in planta* will clarify whether the K120D mutation could indeed prevent the GNOM DCB-DCB interaction and will reveal consequences for the formation of GNOM dimers and potential heterodimers with GNL1.

INTRODUCTION

Protein dimerization or oligomerization is a widespread mechanism in biological systems, playing an essential role in numerous cellular processes. There are many advantages for proteins to associate with each other, for example enhanced stability, increased enzyme activity by concentrating the active site and transmitting signals across membranes (Marianayagam et al., 2004).

The importance of dimerization was also shown for a key regulator in membrane trafficking, namely GNOM, in *Arabidopsis thaliana*. GNOM belongs to a family of ADP-Ribosylation Factor Guanine Exchange Factors (ARF-GEFs) which are involved in vesicle budding by activating ARF GTPases, followed by the recruitment of coat proteins, membrane curvature and scission of the vesicle (Chardin et al., 1996; Springer et al., 1999).

By using structural and biochemical analyses, the dimerization of ARF-GTPases was demonstrated before (Amor et al., 1994; Greasley et al., 1995; Zhao et al., 1999). Large ARF Guanine Exchange Factors across different kingdoms were shown to dimerize as well (Anders et al., 2008; Grebe et al., 2000; Ramaen et al., 2007). Large ARF-GEFs consist of two families: the GBF1-related family, to which GNOM is assigned, and the BIG family, sharing a conserved domain architecture (Bui et al., 2009; Cox et al., 2004; Mouratou et al., 2005). It was demonstrated that the most N-terminal domain of large ARF-GEFs, the DCB domain, is the one mediating dimerization and was therefore termed the DCB and Cyclophilin Binding domain (Grebe et al., 2000). More recent studies in *Saccharomyces cerevisiae* and *Thielavia terrestris* proposed an ARF-GEF of the BIG family to dimerize via the C-terminal HDS4 domain (Richardson et al., 2016). However, this mechanism can hardly apply in Arabidopsis GNOM, as this protein is lacking an HDS4 domain. In addition, the authors were not able to show dimerization via the C-terminus for Gea2, a yeast ortholog of GNOM (Richardson et al., 2016).

In addition to the dimerization via DCB-DCB interaction, it was shown for GNOM and the human large ARF-GEFs GBF1 and BIG1 that the DCB domain also supports intramolecular interaction with adjacent domains (Anders et al., 2008; Ramaen et al., 2007). Critical amino acids for this interaction in the ΔDCB part were described for GNOM in much detail (Anders et al., 2008; Beckmann, 2015).

However, information about amino acids in the DCB domain involved in the inter- and intramolecular interactions is still missing. Sequence alignments, including all large ARF-GEFs of *Saccharomyces cerevisiae*, *Homo sapiens* and *Arabidopsis thaliana,* revealed several conserved amino acids in the DCB domain. Two of these amino acids, namely lysine120 and glutamate159 in GNOM, were shown to be key regulators of dimerization in orthologous proteins. The mutation of the conserved lysine to alanine led to the loss of dimerization in the human GBF1 DCB with both the wildtype and the mutated DCB domain, whereas a glutamate to alanine exchange abolished the same interaction as well as the interaction with the HUS domain of GBF1 (Ramaen et al., 2007). Separation on a Blue Native Gel showed that a full-length protein harboring these lysine and alanine mutations was affected in dimerization (Bhatt et al., 2016). The authors proposed that dimerization only serves the purpose of protein stability, as the mutated proteins still co-localized with the Golgi marker Golgin-245 in HeLa cells. Nevertheless, the existence of monomers in these experiments is questionable, as endogenous GBF1 is still able to interact with the mutated proteins and could aid in Golgi localization. An interesting new role of the conserved lysine was proposed by Galindo et al. (2016): They crystallized the human ARF-GEF BIG1 together with Arl1, a member of the ARF superfamily which is supposed to target BIG1 to the *trans*-Golgi membrane (Christis & Munro, 2012). After introducing a charge reversal by exchanging the lysine to aspartate, the interaction between BIG1 and Arl1 was abolished, but dimerization was unaffected. Instead, two other amino acids further downstream in the DCB domain were found to mediate dimerization. This is in agreement with the divergent evolution of ARF-GEF dimerization between members of the GGG and the BIG family as already mentioned above (Richardson et al., 2016).

Here, we analyze the role of the conserved amino acid residues lysine and glutamate in GNOM dimerization, using yeast two-hybrid assays. Our results confirm the involvement of both amino acids in DCB-DCB interaction, but at the same time highlight differences, suggesting that the underlying mechanisms of intra- and intermolecular interactions of ARF-GEFs are not conserved between different kingdoms.

RESULTS

The conserved amino acids K120 and E159 of GNOM are within α helices

In 2016, the crystal structure of DCB domain was solved twice. Galindo et al. published a complex, consisting of the mammalian BIG1 DCB domain together with Arl1 (Galindo et al., 2016) and Richardson et al. declared the DCB and HUS domain of Sec7 from *Thielavia terrestris* as one structural unit that forms a single continuous armadillo repeat (Richardson et al., 2016). However, this interpretation was questioned, as the junction between the DCB and HUS domain involve two helices that project out of the solenoid and are connected by a poorly conserved linker (Galindo et al., 2016). In addition, it seems confusing that a DCB-HUS fragment in *Thielavia terrestris* was crystallized as a monomer while the same fragment is dimeric in *Saccharomyces cerevisiae*. Nevertheless, both papers agree on eight antiparallel helices that form the DCB domain and are arranged in a twisted array. Fig. 1 shows a sequence alignment of the N-terminal part of the DCB domain, including all large ARF-GEFs from Arabidopsis, yeast and human. The highly conserved amino acids lysine and glutamate (K120 and E159 in GNOM) are located in helix 4 and the beginning of helix 6, respectively (Fig.1). The impact of these two amino acids on inter- and intramolecular DCB interaction was tested in yeast two-hybrid (Y2H) experiments. First, the charged amino acids lysine and glutamate were replaced by the neutral, small amino acid alanine (analogous to Ramaen et al., 2007). Next, both mutations were introduced in the same DCB molecule as published by Bhatt et al. (2016). Finally, the work of Galindo et al. (2016) served as an example of how the charge reversal could influence the behavior of the DCB domain. Consequently, DCB^{K120D} and $DCBE^{159K}$ were tested as well.

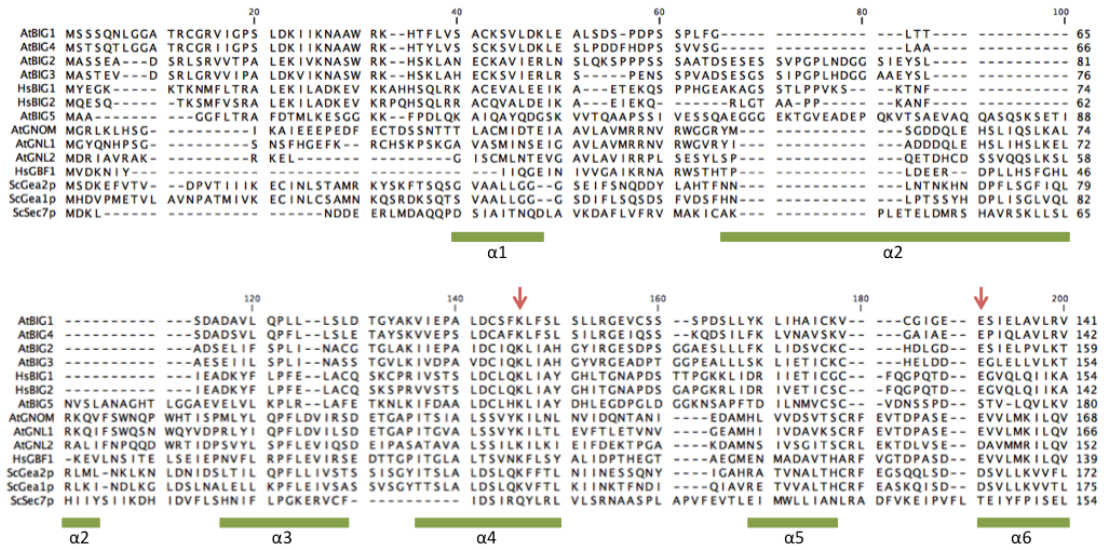


Fig. 1 Alignment of the N-terminal part of plant, yeast and human ARF-GEFs

An alignment with the first 300 amino acids of the large ARF-GEFs from *Arabidopsis thaliana* (At), *Homo sapiens* (Hs) and *Saccharomyces cerevisiae* (Sc) was performed. Only the important first part of the alignment is shown here. The green lines show six of the eight α helices that were predicted by Galindo et al., 2006. The red arrows show the highly conserved amino acids lysine and glutamate that were mutated in the experiments reported here.

Single point mutations of lysine120 and glutamate159 to alanine affect GNOM homodimerization only weakly

Y2H experiments revealed that neither K120 nor E159 alone are responsible for the DCB dimerization (Fig. 2 and Suppl. Fig. S1). Both mutated DCB domains were tested for interaction with the modified and the wildtype DCB domain. The GNOM minimal fragment needed for dimerization was demonstrated to end at amino acid position 246 (Grebe et al., 2000). After introducing the mutations into this construct, dimerization was hardly impaired in the interaction test with the wildtype DCB. Compared to this, the interaction of the respective two mutated DCB domains was slightly weaker (only K120A, not E159A). The intermolecular, also called heterotypic interaction with the rest of the protein (ΔDCB) was also not affected by the two single mutations. This was surprising to some extent, as the same mutations abolished the interaction with the mutated and wildtype DCB in human GBF1. In addition, the glutamate was shown to be the more critical amino acid, as its mutation prevented the DCB-DCB and DCB-HUS

interaction (Ramaen et al., 2007). Obviously, replacing K120 and E159 with a neutral amino acid is not enough to affect dimerization in GNOM.

a)

Interaction	Bait vector pEG202	Prey vector pJG4-5	
	DCB	DCB	1
	DCB	DCB^{K120A}	2
	DCB^{K120A}	DCB	3
	DCB^{K120A}	DCB^{K120A}	4
	ΔDCB	DCB	5
	DCB	ΔDCB	6
	ΔDCB	DCB^{K120A}	7
	DCB^{K120A}	ΔDCB	8
	Empty vector	DCB	9
	DCB	Empty vector	10
	Empty vector	DCB^{K120A}	11
	DCB^{K120A}	Empty vector	12
	Empty vector	ΔDCB	13
	ΔDCB	Empty vector	14
	Empty vector	Empty vector	15

b)

Interaction	Bait vector pEG202	Prey vector pJG4-5	
	DCB	DCB	1
	DCB	DCB^{E159A}	2
	DCB^{E159A}	DCB	3
	DCB^{E159A}	DCB^{E159A}	4
	ΔDCB	DCB	5
	DCB	ΔDCB	6
	ΔDCB	DCB^{E159A}	7
	DCB^{E159A}	ΔDCB	8
	Empty vector	DCB	9
	DCB	Empty vector	10
	Empty vector	DCB^{E159A}	11
	DCB^{E159A}	Empty vector	12
	Empty vector	ΔDCB	13
	ΔDCB	Empty vector	14
	Empty vector	Empty vector	15

Fig. 2: Results of the Y2H tests using GNOM DCB^{K120A} and DCB^{E159A}

a) Overview of the interaction studies between GNOM DCB^{K120A} and wildtype DCB or ΔDCB respectively on X-Gal plates. The left panel shows sections of two out of 15 tested colonies (for whole plates, see Suppl. Fig. S1). The two middle panels show the bait and prey constructs used for the assay. (Positive controls: 1, 5 and 6, negative controls: 9-15); b) Overview of the interaction studies between GNOM DCB^{E159A} and wildtype DCB or ΔDCB respectively on X-Gal plates. For further description, see a) (Whole plates are depicted in Suppl. Fig. S2)

The double-mutated GNOM DCB can still interact with wildtype DCB of GNOM, but is impaired in self-interaction

Next, the same interaction studies were performed with a DCB domain that comprised both mutations in one molecule. Compared to the positive control (WT DCB – WT DCB interaction), the interaction with the WT domain was again not affected (Fig. 3, lanes 1-3 and Suppl. Fig. S3). Interestingly, the interaction between two double-mutated DCB domains was strongly affected, resulting only in a weak coloring on X-Gal plates (Fig. 3, lane 4 and Suppl. Fig. S3). This result confirmed that lysine120 and glutamate 159 are indeed involved in the GNOM dimerization. As shown in Fig. 3, lane 7+8 and Suppl. Fig. S3, only the DCB-DCB

interaction was affected, whereas the interaction with the ΔDCB protein was not perturbed. The simultaneous exchange of the two charged amino acids lysine and glutamate to the small, neutral amino acid alanine indeed strongly weakened the interaction between two mutated DCB domains, but was not sufficient to totally abolish the DCB-DCB dimerization or affect the interaction with the wildtype domain.

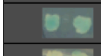
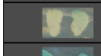
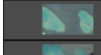
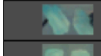
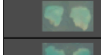
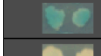
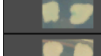
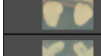
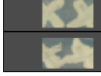
Interaction	Bait vector pEG202	Prey vector pJG4-5	
	DCB	DCB	1
	DCB	$DCB^{K120A+E159A}$	2
	$DCB^{K120A+E159A}$	DCB	3
	$DCB^{K120A+E159A}$	$DCB^{K120A+E159A}$	4
	ΔDCB	DCB	5
	DCB	ΔDCB	6
	ΔDCB	$DCB^{K120A+E159A}$	7
	$DCB^{K120A+E159A}$	ΔDCB	8
	Empty vector	DCB	9
	DCB	Empty vector	10
	Empty vector	$DCB^{K120A+E159A}$	11
	$DCB^{K120A+E159A}$	Empty vector	12
	Empty vector	ΔDCB	13
	ΔDCB	Empty vector	14

Fig. 3: Results of the Y2H interaction studies with the mutated GNOM $DCB^{K120A+E159A}$ protein
15 colonies of each transformation (column 2 and 3) were tested on X-Gal. Two representatives each are depicted in the first column. (For whole plates, see Suppl. Fig. S3) (Positive controls: 1, 5 and 6, negative controls: 9-14)

Charge reversal of lysine to aspartate in position 120 prevents the interaction of two mutated DCB molecules

In order to totally abolish the interaction between two DCB domains, the positively charged amino acid K120 was exchanged with a negatively charged aspartate in accordance with the published data from Christis & Munro (2012). As the mutated amino acid is within an α-helix, partly giving structure to the DCB domain, it was doubtful whether reversing the charge would interfere with protein folding. The interaction with the ΔDCB protein served as a test to prove the integrity of the mutated DCB protein. This test was positive (Fig. 4, lane 7+8

and Suppl. Fig. S4+S3), possibly showing an even stronger interaction than wildtype DCB with ΔDCB (Fig. 4, lane 5+6 and Suppl. Fig. S4+S3). In contrast to the unaffected intramolecular binding between DCB^{K120D} and ΔDCB, the intermolecular interaction was almost not detectable with the wildtype DCB domain and totally abolished in the test with another mutated protein (Fig. 4, lane 2-4 and Suppl. Fig. S4+S3). This result emphasized again that K120 plays a key role in the DCB-DCB dimerization of two GNOM proteins.

Interaction	Bait vector pEG202	Prey vector pJG4-5	
	DCB	DCB	1
	DCB	DCB^{K120D}	2
	DCB^{K120D}	DCB	3
	DCB^{K120D}	DCB^{K120D}	4
	ΔDCB	DCB	5
	DCB	ΔDCB	6
	ΔDCB	DCB^{K120D}	7
	DCB^{K120D}	ΔDCB	8
	Empty vector	DCB	9
	DCB	Empty vector	10
	Empty vector	DCB^{K120D}	11
	DCB^{K120D}	Empty vector	12
	Empty vector	ΔDCB	13
	ΔDCB	Empty vector	14
	Empty vector	Empty vector	15

Fig. 4: The K120D mutation strongly affects the intermolecular DCB-DCB interaction
Columns 2 and 3 lists the transformed bait and prey vectors for each interaction test, column 1 shows the respective result of two representative colonies on X-Gal plates (for whole plates, see Suppl. Fig. S4). (Positive controls: 1, 5 and 6, negative controls: 9-15)

The E159K mutation only inhibits the dimerization with K120D, but does not affect the interaction with wildtype DCB or with DCB^{E159K}

As the charge reversal of K120 strongly inhibited the DCB-DCB interaction, the negative charge of E159 was substituted for the positively charged amino acid lysine as well. The glutamate at position 159 is at the very beginning of an α-helix (as predicted by Galindo et al. in 2016), making it again necessary to prove the proper folding of the mutated protein. As the interaction with the ΔDCB

protein was not abolished (Fig. 5, lane 7+8 and Suppl. Fig. S5), the other results were reliable. However, the E159K mutation did not interfere with the DCB-DCB interaction, neither with the wildtype nor with the mutated DCB (Fig. 5, lane 2-4 and Suppl. Fig. S5). This was surprising to some extent, as the participation of E159 in the dimerization of two GNOM molecules was demonstrated before (see Fig. 3). A role of E159 in DCB-DCB interaction is nevertheless very likely, as there was no obvious interaction between DCB^{K120D} and DCB^{E159K} (Fig. 5, lane 15+16 and Suppl. Fig. S5), whereas DCB^{K120D} still interacted weakly with wildtype DCB (Suppl. Fig. S5).

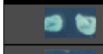
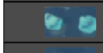
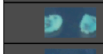
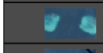
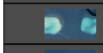
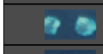
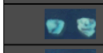
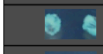
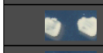
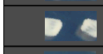
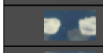
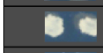
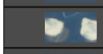
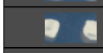
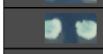
Interaction	Bait vector pEG202	Prey vector pJG4-5	
	DCB	DCB	1
	DCB	DCB^{E159K}	2
	DCB^{E159K}	DCB	3
	DCB^{E159K}	DCB^{E159K}	4
	ΔDCB	DCB	5
	DCB	ΔDCB	6
	ΔDCB	DCB^{E159K}	7
	DCB^{E159K}	ΔDCB	8
	Empty vector	DCB	9
	DCB	Empty vector	10
	Empty vector	DCB^{E159K}	11
	DCB^{E159K}	Empty vector	12
	Empty vector	ΔDCB	13
	ΔDCB	Empty vector	14
	DCB^{E159K}	DCB^{K120D}	15
	DCB^{K120D}	DCB^{E159K}	16

Fig. 5: Y2H interaction studies of DCB^{E159K}

The dimerization behavior of DCB^{E159K} was tested against itself, WT DCB and ΔDCB of GNOM. The left column shows the relative interaction strength on X-Gal plates of two exemplary colonies (see Supp. Fig. S5 for whole plates). The two middle columns show the tested construct combinations. (Positive controls: 1, 5 and 6, negative controls: 9-15).

DISCUSSION

Numerous yeast two-hybrid interaction tests confirmed a role of the two amino acids lysine120 and also glutamate159 in the DCB-DCB interaction of two GNOM proteins. However, the mode of interaction is not conserved amongst homologous ARF-GEFs of different organisms. For the human ortholog GBF1, which belongs to the same class of large ARF-GEFs, a single amino acid substitution to the small, neutral amino acid alanine was enough to inhibit the interaction with wildtype DCB domain. The E159A mutation even prevented the interaction with the HUS domain (Ramaen et al., 2007). The analogous mutations did not affect dimerization of GNOM (Fig. 2 and Suppl. Fig. S1+2), which was surprising, but not unexpected as the intramolecular interaction differs as well between GBF1 and GNOM: The GBF1 DCB domain, on the one hand, interacts only with the HUS domain, in addition to other smaller fragments (Ramaen et al., 2007). On the other hand, the GNOM DCB binds to a minimal fragment consisting of the GNOM amino acids 303-869 (HUS-HDS1) (Beckmann, 2015). Nevertheless, a certain consensus of DCB-DCB dimerization amongst different eukaryotes was still demonstrated, as the $DCB^{K120A+E159A}$ protein could hardly interact with another mutated DCB domain (Fig. 3 and Suppl. Fig. S3) and a charge reversal of lysine120 to aspartate totally abolished the dimerization of two modified DCB domains (Fig. 4 and Suppl. Fig. S4). Lysine120 lies within a predicted α-helix (Fig. 1), therefore it was necessary to verify the integrity of the mutated DCB^{K120D} domain. The interaction with the GNOM ΔDCB protein was used as a demonstration of the integrity of the protein (Fig. 4). Another advantage of the K120D mutation not affecting intramolecular interaction is that a mutated full-length protein can be used for functional studies *in planta*. Interference with the intramolecular interaction of the DCB domain with the rest of the protein leads to non-viable seedlings because GNOM is then affected in membrane binding (Anders et al., 2008), which is not desirable for further experiments. Future studies could reveal how full-length proteins with a DCB^{K120D} mutation behave *in planta* as they are lacking the ability of DCB-DCB interaction. Past studies in our lab suggest that this so called homotypic interaction is a means to prevent heterodimerization with the closely related ARF-GEF GNL1, which dimerizes via

heterotypic interaction (DCB-ΔDCB). Albeit their high sequence similarity, GNOM and GNL1 are functionally diverse with both proteins acting in retrograde Golgi-ER traffic and an additional role of GNOM in endosomal PIN1 recycling. We suspect that the prevention of the GNOM-GNL1 heterodimer is a means to keep the two proteins spatially separated. Expression of the mutated GNOM DCB^{K120D} could help in the investigation of a possible GNOM-GNL1 heterodimer. Furthermore, functional data *in planta* could hopefully demonstrate the meaning of the GNOM DCB-DCB interaction. If, in the future, the crystal structure of GNOM is going to be solved, it will facilitate the interpretation of the data that was described here. It remains still elusive how glutamate159 aids in DCB-DCB interaction. It is likely that K120 interacts with E159, as a double mutation nearly totally abolishes the interaction with another copy of the mutated protein (Fig. 3). In addition, no binding between DCB^{K120D} and DCB^{E159K} was detectable (Fig. 5). This is clearly a recessive effect because it is not enough to mutate only one of the two DCB molecules. The eight predicted α helices possibly produce a tertiary structure in which K120 and E159 come in close proximity, resulting in an interaction between the positively charged lysine and the negatively charged glutamate from the other molecule. The solved crystal structures of GBF1 and Sec7 from *Homo sapiens* and *Thielavia terrestris*, respectively, predicted eight α-helices to form a parallel structure bringing the amino acids in the helices close together (Galindo et al., 2016; Richardson et al., 2016). Modelling of the GNOM DCB domain, using the published structures as a matrix, can be used to investigate this hypothesis.

The importance of GNOM forming dimers was demonstrated by interallelic complementation studies using different *gnom* alleles, resulting in a rescue of the *gnom* phenotype (Busch et al., 1996). It was also shown that the presence of the DCB domain is crucial for protein function as the deletion of the first 294 aa in GBF1 resulted in a loss of membrane association (Mansour et al., 1999) and that yeast expressing Gea1p without its DCB domain in the *Δgea1 Δgea2* mutant background did not grow at 30°C (Ramaen et al., 2007). This result indicates that the DCB domain of Gea1p is essential for yeast viability (Ramaen et al., 2007). Moreover, recent data in Arabidopsis validated these findings since seedlings expressing GNOM ΔDCB in the *gnom gnl1* mutant background are non-viable

(Sabine Brumm, personal commiunication). Obviously, the DCB domain has to be characterized further as this domain is crucial for the function of ARF-GEFs and the mechanism of the inter- and intramolecular interactions is still not completely solved. In addition, the requirement of DCB-DCB interaction for the function of the full-length GNOM protein has not been analyzed so far, whereas it was demonstrated that abolishment of the DCB domain with the SEC7 domain interferes with its membrane association (Anders et al., 2008).

SUPPLEMENTAL DATA

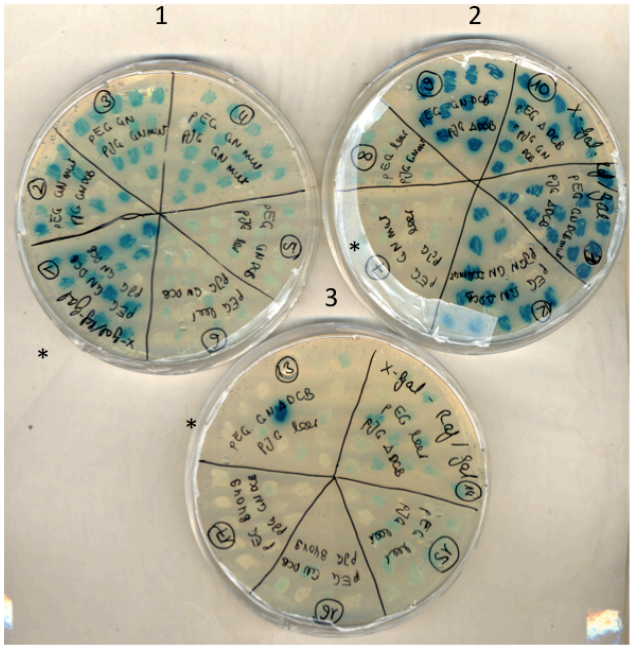


Plate 1:

	Bait vector pEG202	Prey vector pJG4-5
1	DCB	DCB
2	DCB	DCB^{K120A}
3	DCB^{K120A}	DCB
4	DCB^{K120A}	DCB^{K120A}
5	Empty vector	DCB
6	DCB	Empty vector

Plate 2:

	Bait vector pEG202	Prey vector pJG4-5
7	Empty vector	DCB^{K120A}
8	DCB^{K120A}	Empty vector
9	ΔDCB	DCB
10	DCB	ΔDCB
11	ΔDCB	DCB^{K120A}
12	DCB^{K120A}	ΔDCB

Plate 3:

	Bait vector pEG202	Prey vector pJG4-5
13	Empty vector	ΔDCB
14	ΔDCB	Empty vector
15	Empty vector	Empty vector
16	$GNOM^{b4049}$	ΔDCB
17	ΔDCB	$GNOM^{b4049}$

Fig. S1: Whole plates of the Y2H interaction studies using DCB^{K120A}

The picture on the left shows the colonies grown over night on X-Gal plates substituted with galactose; the glucose plates did not show blue colors (data not shown). The tables on the right show the bait and prey vectors of each section on the plate. Asterisks mark the lowest number on each plate, the numbers on the plate increase clockwise and correspond to the numbers in the tables.

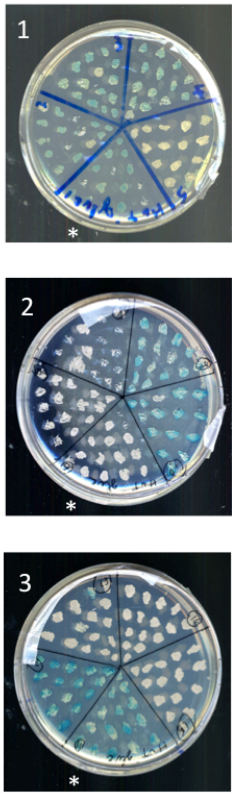


Plate 1:

	Bait vector pEG202	Prey vector pJG4-5
1	DCB	DCB
2	DCB	DCB^{E159A}
3	DCB^{E159A}	DCB
4	DCB^{E159A}	DCB^{E159A}
5	Empty vector	DCB

Plate 2:

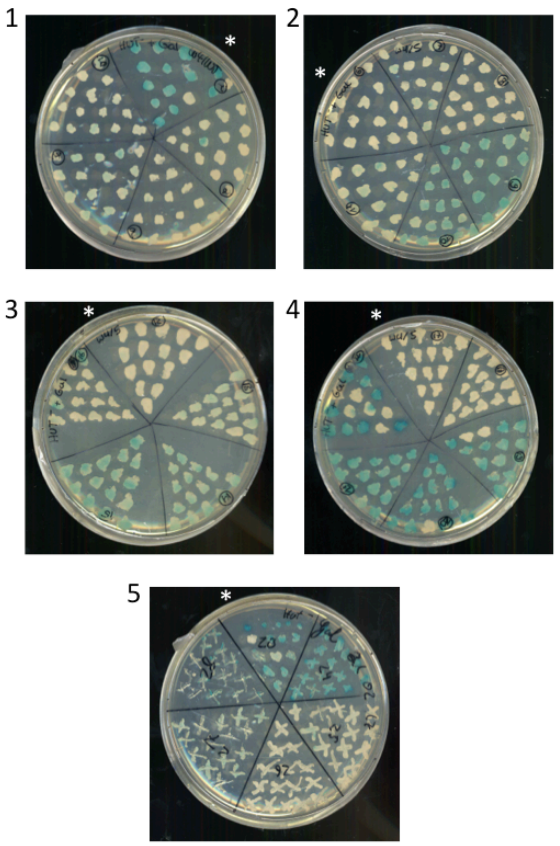
	Bait vector pEG202	Prey vector pJG4-5
6	DCB	Empty vector
7	Empty vector	DCB^{E159A}
8	DCB^{E159A}	Empty vector
9	ΔDCB	DCB
10	DCB	ΔDCB

Plate 3:

	Bait vector pEG202	Prey vector pJG4-5
11	ΔDCB	DCB^{E159A}
12	DCB^{E159A}	ΔDCB
13	Empty vector	ΔDCB
14	ΔDCB	Empty vector
15	Empty vector	Empty vector

Fig. S2: Whole plates of the Y2H interaction studies using DCB^{E159A}

The picture on the left shows the colonies grown over night on X-Gal plates substituted with galactose; the glucose plates did not show blue colors (data not shown). The tables on the right show the bait and prey vectors of each section on the plate. Asterisks mark the lowest number on each plate, the numbers on the plate increase clockwise and correspond to the numbers in the tables.



		Bait vector pEG202	Prey vector pJG4-5
1	1	DCB	DCB
	2	Empty vector	DCB
	3	DCB	Empty vector
	4	GNL1 DCB	GN DCB
	5	GN DCB	GNL1 DCB
2	6	GNL1 DCB	Empty vector
	7	Empty vector	GNL1 DCB
	8	GNL1 DCB	GNL1 DCB
	9	DCB	$DCB^{K120A+E159A}$
	10	$DCB^{K120A+E159A}$	DCB
	11	Empty vector	$DCB^{K120A+E159A}$
3	12	$DCB^{K120A+E159A}$	Empty vector
	13	$DCB^{K120A+E159A}$	$DCB^{K120A+E159A}$
	14	DCB	DCB^{K120D}
	15	DCB^{K120D}	DCB
	16	Empty vector	DCB^{K120D}
4	17	DCB^{K120D}	Empty vector
	18	DCB^{K120D}	DCB^{K120D}
	19	ΔDCB	$DCB^{K120A+E159A}$
	20	$DCB^{K120A+E159A}$	ΔDCB
	21	ΔDCB	DCB^{K120D}
	22	DCB^{K120D}	ΔDCB
5	23	ΔDCB	DCB
	24	DCB	ΔDCB
	25	Empty vector	ΔDCB
	26	ΔDCB	Empty vector

Fig. S3: Whole plates of the Y2H interaction studies using $DCB^{K120A+E159A}$

The picture on the left shows the colonies grown over night on X-Gal plates substituted with galactose; the glucose plates did not show blue colors (data not shown). The tables on the right show the bait and prey vectors of each section on the plate. Asterisks mark the lowest number on each plate, the numbers on the plate increase clockwise and correspond to the numbers in the tables.

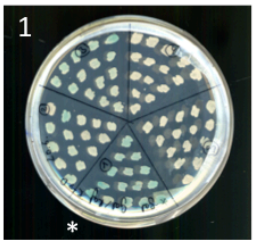


Plate 1:

	Bait vector pEG202	Prey vector pJG4-5
1	DCB	DCB
2	DCB	DCB^{K120D}
3	DCB^{K120D}	DCB
4	DCB^{K120D}	DCB^{K120D}
5	Empty vector	DCB

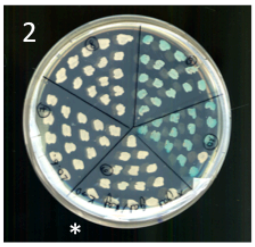


Plate 2:

	Bait vector pEG202	Prey vector pJG4-5
6	DCB	Empty vector
7	Empty vector	DCB^{K120D}
8	DCB^{K120D}	Empty vector
9	ΔDCB	DCB
10	DCB	ΔDCB

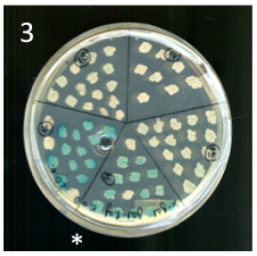
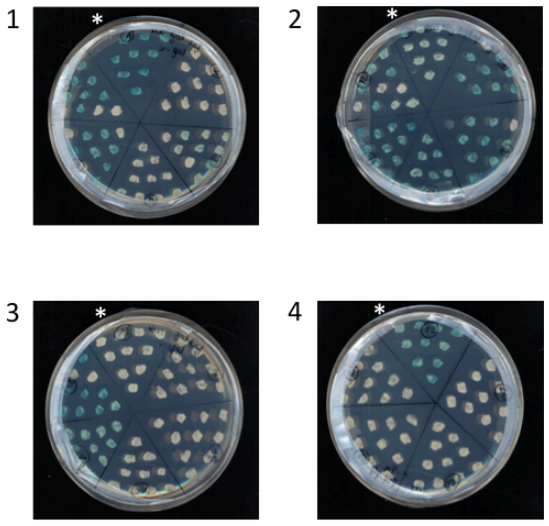


Plate 3:

	Bait vector pEG202	Prey vector pJG4-5
11	ΔDCB	DCB^{K120D}
12	DCB^{K120D}	ΔDCB
13	Empty vector	ΔDCB
14	ΔDCB	Empty vector
15	Empty vector	Empty vector

Fig. S4: Whole plates of the Y2H interaction studies using DCB^{K120D}

The picture on the left shows the colonies grown over night on X-Gal plates substituted with galactose; the glucose plates did not show blue colors (data not shown). The tables on the right show the bait and prey vectors of each section on the plate. Asterisks mark the lowest number on each plate, the numbers on the plate increase clockwise and correspond to the numbers in the tables.



		Bait vector pEG202	Prey vector pJG4-5
1	1	DCB^{E159K}	DCB^{E159K}
	2	DCB^{K120D}	DCB^{K120D}
	3	DCB^{E159K}	DCB^{K120D}
	4	DCB^{K120D}	DCB^{E159K}
	5	DCB^{E159K}	DCB
2	6	DCB	DCB^{E159K}
	7	DCB^{K120D}	DCB
	8	DCB	DCB^{K120D}
	9	DCB^{E159K}	ΔDCB
	10	ΔDCB	DCB^{E159K}
	11	DCB^{K120D}	ΔDCB
3	12	ΔDCB	DCB^{K120D}
	13	DCB^{E159K}	Empty vector
	14	Empty vector	DCB^{E159K}
	15	DCB^{K120D}	Empty vector
	16	Empty vector	DCB^{K120D}
	17	DCB	DCB
4	18	DCB	ΔDCB
	19	ΔDCB	DCB
	20	DCB	Empty vector
	21	Empty vector	DCB
	22	ΔDCB	Empty vector
	23	Empty vector	ΔDCB

Fig. S5: Whole plates of the Y2H interaction studies using DCB^{E159K}

The picture on the left shows the colonies grown over night on X-Gal plates substituted with galactose; the glucose plates did not show blue colors (data not shown). The tables on the right show the bait and prey vectors of each section on the plate. Asterisks mark the lowest number on each plate, the numbers on the plate increase clockwise and correspond to the numbers in the tables.

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6 Discussion

The transport of secreted and membrane-bound cargo molecules within a eukaryotic cell is strongly regulated by the interplay of numerous membrane trafficking regulators. Amongst them, ARF-GEFs are key players in the initiation of vesicle formation. The two major trafficking pathways, the secretory and the recycling pathway, are regulated by two ARF-GEF subfamilies in *Arabidopsis thaliana*: four members of the BIG class are involved in the late secretory pathway from the TGN to the plasma membrane as well as TGN-vacuole trafficking (Richter et al., 2014), while GNOM and GNL1 of the GBF1 class jointly act in the early secretory pathway by promoting retrograde Golgi-ER traffic (Richter et al., 2007; Teh & Moore, 2007). GNOM was shown before to be involved in endosomal recycling (Geldner et al., 2004; Kleine-Vehn et al., 2008; Steinmann et al., 1999), a function that can only be replaced by the pollen-specific ARF-GEF GNL2 if expressed under the GNOM regulatory elements (Richter et al., 2011), but not by the more closely related ARF-GEF GNL1 (Richter et al., 2007). GNOM is recruited to endosomal compartments, which was demonstrated after stabilizing the protein at the donor membrane by BFA treatment (Geldner et al., 2003), and there it fulfills its role in recycling. GNL1, on the other hand, is only involved in Golgi-ER traffic and is therefore recruited to the Golgi apparatus. This specificity in membrane recruitment is a consequence of the functional diversification between GNOM and GNL1 and was investigated in more detail in the first project of this thesis.

We approached the question of membrane specificity by searching for domains that are critical for membrane recruitment of GNOM to the putative recycling endosome. To this end, we created several chimeras between GNOM and GNL1 and tested their ability to rescue the *gnom* mutant. A half-half swap with the N-terminus of GNOM rescued the *gnom* defect in embryogenesis fully, whereas a swap with the C-terminal half of GNOM showed hardly any rescue of the *gnom* phenotype. These findings suggest that critical motifs for the recruitment of GNOM to endosomal compartments lie in the three N-terminal domains DCB, HUS and SEC7.

Swapping only the DCB or HUS domain of GNOM and GNL1 produced normal-looking plants that were *gnom* mutant, whereas swapping the SEC7 domain led to partially rescued *gnom* seedlings and a mild, curled leaf phenotype. These results indicate that critical motifs for the recruitment to endosomal membranes require the interplay of two or more N-terminal domains of GNOM. Furthermore, this hypothesis was underscored by the incomplete rescue of a chimera with the HUS and SEC7 domain of GNL1. A swap mainly consisting of the DCB and HUS domain of GNOM, followed by domain SEC7 to HDS3 from GNL1, could not rescue the *gnom* phenotype at all.

Our data suggested that the HUS and SEC7 domain of GNOM are critical for its recruitment to the putative recycling endosome, but that more than just those two domains are involved. Yeast two-hybrid studies in our lab demonstrated that the DCB domain of GNOM interacts with a GNOM protein that lacks the DCB domain (GN ΔDCB) and that this interaction is critical for membrane association (Anders et al., 2008). Point mutations in GN ΔDCB revealed that this so called heterotypic interaction is dependent on critical amino acids in the HUS, SEC7 and HDS1 domain (Anders et al., 2008; Beckmann, 2015), indicating an additional role of the GNOM HDS1 in the recruitment of GNOM to the membrane of endosomes. The interaction of DCB(GNOM) with ΔDCB(GNL1) was demonstrated as well in yeast, but it is still unclear whether the same motifs as in ΔDCB(GNOM) are critical in ΔDCB(GNL1) for this interaction. We therefore propose that the HUS, SEC7 and HDS1 domain of GNOM are important for "shaping" the ΔDCB(GNOM) part, thereby presenting critical amino acids for the recruitment to the putative recycling endosome.

Further evidence comes from the yeast field. A conserved motif in the HUS domain of the GNOM homolog Gea2p reduced its membrane association (Park et al., 2005) and a truncated Sec7p protein missing the HDS1-4 domain resulted in complete mislocalization (Richardson et al., 2012). This was further confirmed by Meissner et al. (2018), who recently showed that a mutation in the HDS1 domain of human GBF1 reduced the binding affinity to specific membrane lipids in vitro. In addition, experiments with a GBF1 protein harboring point mutations in the HDS2 domain suggest that this domain is also involved in membrane binding (Pocognoni et al., 2018). Furthermore, it was recently speculated that

the Sec7p tertiary structure is a result of the N-terminal part of the protein interacting with the C-terminus, thereby influencing the conformation of the catalytic domain or its position at the membrane surface (Halaby & Fromme, 2018).

The analysis of more chimeras, one with the HUS, SEC7 and HDS1 from GNOM and the other domains from GNL1, and one with only the SEC7 and HDS1 domain from GNOM could help in an improved characterization of the endosomal localization of GNOM in future experiments. In addition, localization studies of the chimeras are necessary to underpin the physiological experiments.

Our study gives new insights into the regulation of membrane association of large ARF-GEFs. Past studies focused on effector proteins that aid in membrane recruitment. It was, for example, broadly discussed that the ARF substrate could be involved. Mammalian Arfs can be divided into three classes and differ in their localization to the Golgi, endosomes and the plasma membrane (Gillingham & Munro, 2007), enabling them to recruit specific Arf-GEFs to certain donor membranes. Indeed, substrate specificity was demonstrated for the human Arf-GEF GBF1 which catalyzes the activation of Arf5, and also Arf1 and Arf3 under low Mg^{2+} concentrations (Claude et al., 1999). However, *in planta,* the major ARF substrates belong to the ARF1 class and target both the Golgi and post-Golgi structures (Stefano et al., 2006). Therefore, it is hard to imagine that substrate specificity can lead to membrane specificity. In addition, Co-IP experiments in our lab revealed that both Arabidopsis GNOM and GNL1 are able to interact with the same ARF1 substrate (Manoj Singh, personal communication). The work by Lowery et. al (2011) also contradicts this theory, as they showed that mutations in a helix within the catalytic SEC7 domain of GBF1, BIG2 and the small ARF-GEF ARNO abolished the binding to ARF-GDP, but not the membrane binding. This indicates that membrane association is independent of the ARF substrate (Lowery et al., 2011). The search for membrane receptors or specific lipids labeling the donor membrane resulted in the Golgi proteins Gmh1p and Drs2p that interact with Gea2p, the GNOM/GNL1 homolog in yeast (Chantalat et al., 2003; Chantalat et al., 2004). It was also postulated that Rab GTPases have a function in marking different compartments in addition to their known role in regulating membrane trafficking (Jedd et al., 1995; Segev, 2001; Zerial &

McBride, 2001). A model by McDonold and Fromme (2014), for example, suggests that Ypt1 (Rab1 in mammals) and Ypt31/32 (Rab11 family in mammals) act on the recruitment of yeast Sec7 to the Golgi stacks and stimulate its exchange activity. In this scenario, Arf1 and Arl1 would also play a major role in the Sec7 location (McDonold & Fromme, 2014). It is still unclear whether membrane recruitment in *Arabidopsis thaliana* is similar. However, we propose that ARF-GEFs themselves harbor critical motifs for the recognition of proteins that lead them to donor membrane. Additionally, these motifs can help in the recognition of receptors or the lipid composition of a compartment-specific membrane.

The second strategy for explaining the functional diversification of Arabidopsis GNOM and GNL1 was the analysis of their homologs in other species of the Viridiplantae kingdom. The investigation of several phylogenetic trees, including different species respectively, led to the assumption that, in ancient plant species, only one GBF1-related plant protein was able to promote retrograde Golgi-ER traffic as well as endosomal PIN1 recycling like shown previously for Arabidopsis GNOM (Geldner et al., 2003; Steinmann et al., 1999). During evolution, several genome duplications led to several copies of this ancestral gene and most of the redundant genes were lost over time leading to two GNOM-GNL1 paralogs in many species of the Viridiplantae. Interestingly, the analysis of our phylogenetic trees showed that separation of the GNOM and GNL1 homologs in two clades can only be determined for the Brassicaceae family as well as for closely related species, whereas the homologous proteins of most analyzed species were grouped in the same clade as Arabidopsis GNOM. This led to the idea that the two clades might represent diversified functions and that species with both proteins in separate clades might have undergone functional diversification like already shown for *Arabidopsis thaliana* (Richter et al., 2007). To test this assumption, we expressed the homologous proteins of *Brassica napus*, *Populus trichocarpa* and *Oryza sativa* in the *gnom* and *gnl1* mutant background of Arabidopsis and tested them for rescue of the mutant phenotype as well as for their subcellular localization.

The two rapeseed proteins showed diverse functions, indicating that the GNOM and GNL1 orthologs in the Brassicaceae family indeed specialized in their roles in membrane trafficking. Both poplar proteins differed in their rescue of the *gnom* phenotype. While one protein showed complete rescue, the second protein rescued the *gnom* phenotype only partially during embryogenesis and could not rescue the post-embryonic development. Both rice proteins show diversified functions as well. While one rice protein could completely replace Arabidopsis GNOM, the other protein hardly rescued the *gnom* mutant phenotype.

Taken together, our results suggest that the functional diversification of GNOM and GNL1 is not special for the Brassicaceae, but has started before the emergence of this family in evolution and might have been initiated in the monocots and dicots independently. The evolution of land plants brought a lot of changes in plant architecture, making a strong regulation of auxin transport necessary. During the evolution of *Arabidopsis thaliana,* a lot of genome duplication events took place and there is still a debate going on about the number and timing of these events. Still, there is a general agreement for a genome doubling event after the eudicot divergence, splitting monocotyledons and dicotyledons, as well as a polyploidization event after divergence of the cotton lineage. The divergence of the GNOM-related proteins in *Populus trichocarpa* happened after this genome duplication, but it is speculated that another round of polyploidization occurred between 20 and 60 million years ago, after the split of *Arabidopsis thaliana* and the Brassica lineage (Adams & Wendel, 2005; Blanc & Wolfe, 2004). Each duplication event might have advanced the subfunctionalization of Arabidopsis GNOM and GNL1.

Further investigation of more species is therefore desirable. Especially the analysis of more closely related species of *Populus trichocarpa* might help in narrowing down the time point of the functional diversification of GNOM and GNL1 in the angiosperm evolution. In addition, further analysis of the rapeseed, poplar and rice GNOM-related proteins in Arabidopsis bearing other *gnom* mutant alleles could aid in the further characterization of these proteins. All rescue experiments so far were performed in the *gnom-sgt* allele which lacks the GNOM protein completely. In case of partially rescued seedlings, a classification of the residual GNOM activity is difficult. Rescue experiments in different *gnom*

mutant backgrounds could solve this issue. The *emb30* allele of GNOM expresses a full-length, but catalytically inactive protein, whereas the *b4049* point mutation leads to an impairment of the full-length mutant protein in membrane association. Both mutant alleles can complement each other, which was demonstrated in previous studies (Anders et al., 2008). Complete rescue of these mutant alleles by expressing the homologous rapeseed, poplar and rice proteins would demonstrate that the respective proteins have GNOM identity, as they could heterodimerize with the mutant GNOM proteins and would further serve as a proof that they are catalytically active and can be recruited to the correct donor membrane, respectively.

We used yeast two-hybrid assays as a fast tool to test more GNOM and GNL1 homologs with regards to their dimerization behavior. In past experiments, we learned that the N-terminal DCB domain of GNOM interacts with the DCB domain of another GNOM protein, but not with the DCB domain of GNL1 (Hauke Beckmann, personal communication). In GBF1, the human homolog of GNOM and GNL1, the interaction of two DCB domains was demonstrated as well (Bhatt et al., 2016; Ramaen et al., 2007), indicating that this is a conserved mechanism and that Arabidopsis GNL1 has lost the ability to perform this homotypic interaction. We propose that this is a mechanism to separate GNOM and GNL1 that might be necessary due to their functional diversification. As mentioned above, we assume that this feature evolved over time and that the paralogous GNOM and GNL1 proteins from more ancient species act redundantly, while the specialization of GNOM and GNL1 in endosomal recycling and retrograde recycling during evolution made the prevention of heterodimers necessary. Indeed, the preliminary results of our yeast two-hybrid studies with the DCB domains of green plant species before the monot-dicot separation suggest that they dimerize via the DCB-DCB interaction, analogous to GNOM. We also observed the same mechanism in ancient angiosperm species and in *Medicago truncatula,* which originated earlier than the Brassicacea. However, we demonstrated that the two *Populus trichocarpa* proteins cannot heterodimerize via their DCB domains, supporting our previous findings in which we showed that only one poplar protein could rescue the Arabidopsis *gnom* phenotype fully.

These preliminary results corroborate our hypothesis that an ancient GBF1-related protein behaved similarly to GNOM in regard to the DCB-DCB interaction and the dual function in Golgi-ER traffic and endosomal recycling. Genome duplications in angiosperm evolution led to the presence of two GNOM-GNL1 homologs and first results led us to speculate that during the eurosid evolution, the heterodimerization of these two proteins was prevented for the first time. A detailed investigation is still ongoing and further experiments testing more species of the Brassicales would be helpful to verify the recent data. Furthermore, yeast two-hybrid experiments with the DCB domain of human GBF1 and Arabidopsis GNOM and GNL1 would improve our understanding of the conservation of the DCB-DCB interaction. Finally, the results obtained in these yeast assays are useful to find more candidate proteins for rescue experiments *in planta* to further confirm our data.

Yeast-two hybrid experiments demonstrated that both GNOM and GNL1 form dimers, but that their dimerization domains behave differently. The DCB domain of GNL1 interacts with the ΔDCB part of another GNL1 protein. The DCB domain of GNOM, on the other hand, has two interaction sites: one for another DCB domain and another for the ΔDCB part of GNOM (Hauke Beckmann, personal communication). We wanted to analyze the reason for the DCB-DCB interaction of GNOM in more detail by first identifying critical amino acids that prevent the DCB-DCB interaction and do not affect the interaction with the ΔDCB protein. We took advantage of studies on mammalian large ARF-GEFs, where two critical amino acids were identified already (Bhatt et al., 2016; Galindo et al., 2016; Ramaen et al., 2007). The respective amino acids in the GNOM DCB, lysine at position 120 and glutamate at position 159 affected neither the DCB-DCB interaction nor the DCB- ΔDCB interaction if substituted with the small, neutral amino acid alanine. This was surprising and contradicts the data published for human GBF1 and BIG1 (Bhatt et al., 2016; Ramaen et al., 2007). However, when reversing the charge of lysine120 to aspartate, the dimerization with another mutated GNOM DCB domain was prevented, but the interaction with ΔDCB was not affected, thereby ensuring that the charge reversal does not disturb the tertiary structure of the mutated DCB domain. In addition, because of the

DCB^{K120D}-ΔDCB interaction, the formation of a GNOM dimer was still possible. We assume that dimer formation is critical for the ARF-GEF function, which is supported by complementation experiments expressing different *gnom* alleles (Anders & Jürgens, 2008; Busch et al., 1996).

A full-length mutant GNOM protein bearing the $K_{120}D$ mutation should form dimers like GNL1, and it would be of interest to determine whether the inability to interact by DCB-DCB interaction would compromise its function. We showed in Co-IP experiments that full-length GNOM and GNL1 proteins do not heterodimerize (Manoj Singh, personal communication). However, deleting the DCB of GNOM enables the formation of heterodimers of ΔDCB(GNOM) with full-length GNL1 that rescue the *gnom* phenotype (Sabine Brumm, personal communication). Y2H experiments demonstrated that an interaction between the DCB domain of GNOM and the ΔDCB part of GNL1 is possible (Hauke Beckmann, personal communication), but we speculate that the DCB-DCB interaction of two GNOM proteins directly after translation prevents this interaction *in planta*, thereby keeping GNOM and GNL1 apart. As already mentioned, this heterodimer formation is possible only in an artificial situation with the GNOM protein lacking the DCB domain and shows no harmful effects, at least in plant chamber conditions. We could use the GNOM DCB^{K120D} mutation to test the effect of a full-length GNOM protein which has lost the ability of DCB-DCB interaction to see whether this homotypic interaction indeed prevents heterodimerization with GNL1. In case of heterodimer formation, we could further test this GNOM-GNL1 heterodimer for its role in endosomal recycling in order to improve our understanding of functional diversification of GNOM and GNL1.

7 Material and Methods

Plant material and growth conditions

Seeds were sterilized with chlorine gas for between 4 to 17 hours and then sown out on agar plates containing 0.2% (w/v) Murashige and Skoog medium, 1% (w/v) sucrose and 0.8% (w/v) agar. After two days of stratification the plants were transferred to the growth chambers running at continuous light conditions and 23°C. The seedlings were transferred to soil after eight to ten days and grown at the same light and temperature conditions like the agar plates.

All transgenes were selected on Phosphinotricin containing plates (15 mg/l), the *gnom* seedlings were selected on Kanamycin (50 mg/l) and the *gnl1* seedlings on Hygromycin (200 mg/l).

Cloning

All plant lines expressed their transgenes using the pGREENII binary vector containing the Arabidopsis 5'- and 3'UTR. Genomic DNA and available vectors were used as a template for the cloning of the chimeras (Draft manuscript 1).

For the cloning of the GNOM/GNL1-related genes, *Brassica napus* genomic DNA was provided bei Prof. Dr. Ulrike Zentgraf, and was used as a template for BnGNL1. BnGNOM was synthesized as a coding sequence flanked by NotI restriction sites by the company baseclear. The poplar plant material was provided by Prof. Dr. Markus Grebe. PtGR2 is based on genomic DNA whereas PtGR1 is missing the intron between the DCB and HUS domain. Both rice genes are based on genomic DNA (Draft manuscript 2).

The DCB constructs used for the yeast two-hybrid interaction assays were inserted in the pEG202 and pJG4-5 vectors using the EcoRI and XhoI restriction sites.

The full-length constructs of rapeseed, poplar and rice in the pGREENII vectors were used to amplify their DCB domains while the DCBs of the other investigated species were synthesized by the company baseclear (Draft manuscript 3).

The mutations were introduced by primer extension PCR using pre- existing GNOM DCB constructs as templates. The full-length constructs of rapeseed,

poplar and rice in the pGREENII vectors were used to amplify their DCB domains while the DCBs of the other investigated species were synthesized by the company baseclear (Draft manuscript 4).

Physiological tests

Physiological root experiments were performed with seedlings grown for five days on MS plates and then transferred to 10 µM BFA containing plates. For the gravitropism experiments the plates were rotated by 135° for ~16h. For the primary root growth assay, the plates were scanned after three additional days on plates with and without BFA; the lateral root initiation was investigated after 5 additional days on +/- BFA plates.

Images of the plates for taken using the Epson Expression 1600 Pro Scanner. The ImageJ Software served as a tool to measure the root lengths and root angles.

Co-Immunoprecipitation

2-3 g of plant material were homogenized using a mortar and a pestle and the proteins were then solubilized for 30 min in the extraction buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and protease inhibitor cocktail (Roche). After 30 min of centrifugation at 4°C at maximum speed the supernatant was incubated for 4h at 4°C using 40 µl of rabbit anti-Myc agarose beads (Sigma-Aldrich).

The beads were then washed three times with wash buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0,1 % Triton X-100 and two times in wash buffer without detergent. After the final washing step, the beads were eluted in 50 µl 2x Lämmli buffer containing 3% β-Mercaptoethanol (also see (Anders et al., 2008).

Western Blot

Proteins were resolved using SDS-PAGE and blotted overnight on a PVDF membrane using a wet blot transfer system. After blocking in TBS-T containing 5% milk powder the membranes were incubated for 1 hour in TBS-T with 1% milk powder and the first (anti-myc 9E10 1:1000, Santa Cruz; anti-GNOM SEC7, 1:2000, custom-made; anti-GFP, 1:2000, Roche) and the second (anti-mouse AP

1:5000, Novagen; anti-rabbit HRP 1:5000, Merck Milipore; anti-mouse AP 1:5000, Novagen) antibody respectively. BM Chemiluminescence Western Blotting Substrate (Roche) or CDP-Star (Tropix) were used respectively as substrates for the detection.

Yeast two-hybrid interaction assays

The yeast strain EGY48 was pre-transformed with the reporter plasmid pSH18-34 harboring the β-Galactosidase coding sequence, followed by a double transformation with the bait vector (pEG202) and the prey vector (pJG 4-5) (Grebe et al., 2000). Since the bait construct is expressed from the inducible GAL1 promoter, the assay was performed on X-Gal containing plates supplemented with Glucose or Galactose as a sugar source respectively.

ONPG assay

500 µl of the yeast culture is harvested in their log-phase. The cell walls are broken by freeze-thaw cycles and the pellet is then resuspended in 800 µl Z-Buffer (40mM $Na_2HPO_4 \bullet 7H_2O$, 60mM $NaH_2PO_4 \bullet H2O$, 10 mM KCl, 1mM $MgSO_4 \bullet 7H_2O$ and 50mM β-Mercaptoethanol). After adding 160µl ONPG substrate, the samples are incubated at 30 °C until a yellow coloring is visible. After stopping the reaction with1M Na_2CO_3 the OD is measured at 420 mm. The interaction strength is calculated in miller units.

Sequence alignment

The sequence alignment using the DCB domains of GNOM homologous proteins was performed using the CLC Main Workbench 8.

Phylogenetic tree

Full-length protein sequences of Arabidopsis GNOM, GNL1 and GNL2 orthologs were downloaded from the phytozome (phytozome.jgi.doe.gov/pz/portal.html) and ncbi (blast.ncbi.nlm.nih.gov/Blast.cgi) homepage respectively. The phylogenetic tree was drawn up by Prof. Dr. Richard Neher.

Imaging

The YFP-tagged GNOM/GNL1-related proteins were localized using the confocal laser scanning microscope TCS-SP8 from Leica with the 63x water-immersion objective and the Leica software.

For BFA treatment, the seedlings were incubated for 1h in liquid medium containing 0.2% (w/v) Murashige and Skoog medium, 1% (w/v) sucrose, 50 μM BFA and FM4-64 (1,7 μg/ml).

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9 Appendices

9.1 Primers and oligonucleotides

	Primer name	Nucleotide sequence
1	GNOM-Promotor-Seq	AGGTTCCATTAATTGGGCCT
2	Br-GNOM-Seq1	GCCAACAGATCTGAAATC
3	Br-GNOM-Seq2	CGCTGGATGGCTTAATTG
4	BrGNOM-Seq3	GCATAAATCCAAGAAAAC
5	Br-GNOM-Seq4	CTGCCTGGAATTGCTAAT
6	PtGR1-Seq1	GCGCTGAACAGACATTGG
7	PtGR1-Seq2	GGATGGCTGAAAGGATAG
8	PtGR1-Seq3	TGATCTAATGCACAAGTC
9	PtGR1-Seq4	CTGCCTTGAATTGCTCAT
10	BrGNOM-Seq1a	GGTGTATCTTCTCCCATCT
11	GNOM-Prom-KpnI-S	TTTTTGGTACCGGTGTGTATGATAATGAATATTG
12	GNOM-Prom-ApaI-AS	TTTTTGGGCCCTTAATCTGCTCAAATCTTCAGCCAG
13	GNOM-YFP-SacI-AvrII-S	TTTTTGAGCTCCCTAGGATGGTGAGCAAGGGCGAGG
14	GN-UTR-SacI-AS	TTTTTGAGCTCAATCGAAATCCGTCTCCCGGAGC
15	PtGR1-CDS-ApaI-S	TTTTGGGCCCATGGGGAGATTAAAGCTAAACACTG
16	PtGR1-CDS-NotI-AS	TTTTGCGGCCGCACCTCCTGTGCCAGCAACTTCAGCA
17	PtGR2-CDS-ApaI-S	TTTTGGGCCCATGGGGAGAATAAAGCTACAGTCTG
18	PtGR2-CDS-NotI-AS	TTTTGCGGCCGCACCTCCAGTGCCAGCAGCACATTCAG
19	BrGNL1-1 Bra040986 ApaI-S	TTTTGGGCCCATGGGGTATCAGAATCACCATCCAT
20	BrGNL1-1 Bra040986 NotI-AS	TTTTGCGGCCGCGTTTTCCGGTACCACCGGAGTATCC
21	PmeI-GN-Prom-intern-F	AAACTTATTCCATCGGATATAG
22	Hyb-R (GN-HUSLInk-GNL1-S7)	CAAAAATTAGGATCTCCGTAATTCTGCATTGCAGACAAAGGACAG T
23	Hyb-F (GN-HUS-Link-GNL1-S7)	ACTGTCCTTTGTCTGCAATGCAGAATTACGGAGATCCTAATTTTT G
24	gnom-Intron-F	GAATAATTTTCCCAACAAATGCTGGATTTTAAG
25	OsGR1-ApaI-F	TTTTGGGCCCATGGGCGGCCTGAGGGCAGCGTCGC
26	OsGR1-NotI-R	TTTTGCGGCCGCAACATTCACGCCTTCAGATTGTGCT
27	OsGR2-ApaI-F	TTTTGGGCCCATGCTACTTGTGCAGATGGGTGGC
28	OsGR2-NotI-R	TTTTGCGGCCGCGTCCCGGGACTCAAC
29	BnGNL1-Seq1	ATCGTATTATGATTTATCTG
30	BnGNL1-Seq2	GTGATATAACATGCAGTAATG
31	BnGNL1-Seq3	GATTCAGATGATCCCAGATC
32	BnGNL1-Seq4	AGATAAAGCTAGTGCTGTC
33	BnGNL1-Seq5	GCTAGACGAACTGCTCGCTG
34	PtGR2-Seq1	GTAACATCTCTGCTCTCAATG
35	PtGR2-Seq2	GATTTGCACCTCATGACATTG
36	PtGR2-Seq3	CGGAGCTGATCACTTCAATC
37	PtGR2-Seq4	ATTGGATGCTGTAGTTGTG
38	PtGR2-Seq5	TTGAGGTCACTGCAACTTG
39	PtGR2-Seq6	GAAGGGACACTTGTCATTGC
40	OsGR1-Seq1	CCTTCCTGTAAAGATTTCG
41	OsGR1-Seq2	TGTGTTATCATAAGACTTGG
42	OsGR1-Seq3	AGTAATCATGCTCAACACAG
43	OsGR1-Seq4	ATTGTATTTAGATGCTGAAG
44	OsGR1-Seq5	GTGAATTGTCTTACACGCTG
45	OsGR2-Seq1	TCTTGCTCTGGAAGGTCTG

	Primer name	Nucleotide sequence
46	OsGR2-Seq2	GTCGAAGTCAACATCTCTG
47	OsGR2-Seq3	TGTCTGGAACTGCTAATCGC
48	OsGR2-Seq4	ACTTACTAGAGATCAGCCAG
49	GN-Prom-Seq2	GATTGAGTTTGGCCTCTATC
50	BnGNL1-Ins-R	ATGCTATAGGGTTAGATCTG
51	PtGR1-Seq1-2	GCATGGTAGAGATATTTCAT
52	site directed DCB K120A -F	GTCATCAGTTTACGCGATCTTAAACCTG
53	site directed DCB K120A -R	CAGGTTTAAGATCGCGTAAACTGATGAC
54	GN-DCB E159A-F	GATCCTGCATCAGAAGCGGTTGTGCTAATG
55	GN-DCB-E159A-R	CATTAGCACAACCGCTTCTGATGCAGGATC
56	GN-DCB-K120D-F-neu	GTCATCAGTTTACGACATCTTAAACCTG
57	GN-DCB-K120D-R-neu	CAGGTTTAAGATGTCGTAAACTGATGAC
58	PtGR1-ohneIntron-Bst1107I-F	TCTGAATCGCTGGAGAAACAGT
59	PtGR1-ohneIntron-BstEII-R	GGACTTGTGCATTAGATCAATCCA
60	YFP-R	TAGCCGAAGGTGGTCAC
61	GN-DCB-ApaI-F	TTTTGGGCCCATGGGTCGCCTAAAGTTG
62	GN-DCB-Not-R	TTTTGCGGCCGCTTGTTTGATGCTACCAGC
63	AtGNOM-DCB-XhoI-R	TTTTCTCGAGTTGTTTGATGCTACCAGC
64	myc-tag-annealed oligo-XhoI-F	TCGAGGAACAAAAACTTATTTCTGAAGAGGATCTTCCC
65	myc-tag-annealed oligo-SmaI-R	GGGAAGATCCTCTTCAGAAATAAGTTTTTGTTCC
66	GN-DCB-E159K-F	GATCCTGCATCAGAAAAGGTTGTGCTAATG
67	GN-DCB-E159K-R	CATTAGCACAACCTTTTCTGATGCAGGATC
68	BnGN-DCB-EcoRI-F	TTTTGAATTCATGGGCCGACTTAAGTTGCATT
69	BnGN-DCB-XhoI-R	TTTTCTCGAGTTACTCTTGATTGATGATTTCAG
70	BnGNL1-DCB-EcoRI-F	TTTTGAATTCATGGGGTATCAGAATCACCATCCA
71	BnGNL1-DCB-XhoI-	TTTTCTCGAGTTATGTTCCCACCTTATCGCCGA
72	PtGR2-DCB-EcoRI-F	TTTTGAATTCATGGGGAGAATAAAGCTA
73	PtGR2-DCB-XhoI-R	TTTTCTCGAGTTACCCCCCACTCTCCTGTTT
74	P1-DCB-EcoRI-F-neu	ATAGAATTCATGGGGAGATTAAAGCTAAACACTGG
75	P1-DCB-XhoI-R-neu	TTCTCGAGttaCCCACCAATCTCATGTTTGTGAGAAG

9.2 Vectors

1	pGII-GNOM-5'UTR (KpnI/ApaI)
2	pGII-GN 5'UTR (KpnI/ApaI)-YFP-GN 3'UTR (SacI)
3	Vector 2 + genomic BnGNL1 (ApaI/NotI)
4	Vector 2 + PtGR1-CDS (ApaI/NotI)
5	Vector 2 + genomic PtGR2 (ApaI/NotI)
6	Vector 2 + genomic OsGR1 (ApaI/NotI)
7	Vector 2 + genomic OsGR2 (ApaI/NotI)
8	pGII-GN 5'UTR (KpnI/ApaI) – GN-3'UTR (SacI)
9	pGII GN genomic 5'UTR-GNOM DCB-HUS – GNL1 SEC7-HDS3 -3xmyc (AvrII) – GN3'UTR
10	pJG 4-5 GNOM DCB K120A 1-246aa (EcoRI/XhoI)
11	pEG 202 GNOM DCB K120A 1-246aa (EcoRI/XhoI)
12	pJG 4-5 GNOM DCB E159A 1-246aa (EcoRI/XhoI)
13	pEG202 GNOM DCB E159A 1-246aa (EcoRI/XhoI)
14	pJG 4-5 GNOM DCB K120A + E159A 1-246aa (EcoRI/XhoI)
15	pEG 202 GNOM DCB K120A + E159A 1-246aa (EcoRI/XhoI)
16	pJG 4-5 GNOM DCB K120D 1-246aa (EcoRI/XhoI)
17	pEG 202 GNOM DCB K120D 1-246aa (EcoRI/XhoI)
18	pJG 4-5 GNOM DCB E159K 1-246 aa (EcoRI/XhoI)
19	pEG202 GNOM DCB E159K 1-246 aa (EcoRI/XhoI)
20	pJG 4-5 Brassica 1 DCB 1-246aa (EcoRI/XhoI)
21	pEG 202 Brassica 1 DCB 1-246aa (EcoRI/XhoI)
22	pJG 4-5 Brassica 2 DCB 1-243aa (EcoRI/XhoI)
23	pEG 202 Brassica 2 DCB 1-243aa (EcoRI/XhoI)
24	pJG 4-5 Populus 1 DCB 1-250aa (EcoRI/XhoI)
25	pEG 202 Populus 1 DCB 1-250aa (EcoRI/XhoI)
26	pJG 4-5 Populus 2 DCB 1-248aa (EcoRI/XhoI)
27	pEG202 Populus 2 DCB 1-248aa (EcoRI/XhoI)
28	pJG 4-5 Oryza 1 DCB 1-250 aa (EcoRI/XhoI)
29	pEG 202 Oryza 1 DCB 1-250 aa (EcoRI/XhoI)
30	pJG 4-5 Oryza 2 DCB (LOC_Os03g46325) 1-251aa (EcoRI/XhoI)
31	pEG 202 Oryza 2 DCB (LOC_Os03g46325) 1-251aa (EcoRI/XhoI)
32	pJG 4-5 Amborella DCB 1-250aa (EcoRI/XhoI)
33	pEG 202 Amborella DCB 1-250aa (EcoRI/XhoI)
34	pJG 4-5 Klebsormidium DCB 1-233aa (EcoRI/XhoI)
35	pEG 202 Klebsormidium DCB 1-233aa (EcoRI/XhoI)
36	pJG 4-5 Marchantia DCB 1-256aa (EcoRI/XhoI)
37	pEG 202 Marchantia DCB 1-256aa (EcoRI/XhoI)
38	pJG 4-5 Aquilegia 1 DCB (Aqcoe6G027) 1-245aa (XhoI)
39	pJG 4-5 Medicago 1 DCB (Medtr3g068140.1) 1-251aa (EcoRI/XhoI)
40	pEG 202 Medicago 1 DCB (Medtr3g068140.1) 1-251aa (EcoRI/XhoI)
41	pJG 4-5 Medicago 2 DCB (Medtr5g080650.1) 1-248aa (EcoRI/XhoI)
42	pEG 202 Medicago 2 DCB (Medtr5g080650.1) 1-248aa (EcoRI/XhoI)

9.3 Plant lines

Konstrukt	**Background**
SEC7-HDS-swap-3xmyc	*sgt/+*
BnGNOM-YFP	*sgt/+* (T2)
BnGNL1-YFP	*sgt/+*
BnGNL1-YFP	*gnl1/gnl1*
PtGR1-YFP	*sgt/+* (T2)
OsGR1-YFP	*sgt/+*
OsGR1-YFP	*gnl1/gnl1*
OsGR2-YFP	*sgt/+*
OsGR2-YFP	*gnl1/gnl1*