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1 **An Arabidopsis reticulon and the atlastin homologue *RHD3-like2* act together in**

2 **shaping the tubular endoplasmic reticulum**

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13 **Summary**

14 • The endoplasmic reticulum (ER) is a network of membrane sheets and tubules
15 connected via three-way junctions. A family of  proteins, the reticulons, are
16 responsible for shaping the tubular ER. Reticulons interact with other tubule-
17 forming proteins (Dp1 and Yop1p) and the GTPase atlastin. The Arabidopsis
18 homologue of Dp1/Yop1p is HVA22.

19 • We show here that a seed-specific isoform of HVA22 labels the ER in tobacco
20 cells but its over-expression does not  alter ER morphology. The closest plant
21 homologue of atlastin is RHD3. We show that RHD3-like 2 (RL2), the seed-
22 specific isoform of RHD3, locates to the ER without affecting its shape or
23 Golgi  mobility. Expression of RL2 bearing mutations within its GTPase
24 domain induces the formation of large ER  strands, suggesting that a
25 functional GTPase domain is important for the  formation of three-way
26 junctions.

27 • Co-expression of the reticulon RTNLB13 with RL2 resulted in a dramatic
28 alteration of the ER network. This alteration did not depend on an active

GTPase domain but required a functional reticulon, as no effect on ER morphology was seen when RL2 was co-expressed with a non-functional RTNLB13. RL2 and its GTPase mutants co-immunoprecipitate with RTNLB13.

- These results indicate that RL2 and RTNLB13 act together in modulating ER morphology.

**Key words:**

Plant endoplasmic reticulum, reticulon, atlastin, RHD3, plant secretory pathway, organelle architecture, derlin, YOP1.

**Introduction**

The plant endoplasmic reticulum (ER) is the gateway to the secretory pathway and oversees a large number of biological processes, among which are the synthesis, quality control and export of secretory proteins. The ER, a very dynamic organelle, is continuous with the nuclear envelope, comprises a network of tubules and cisternae much of which is cortical, and in plants are associated mainly with the actin cytoskeleton (Sparkes *et al.*, 2009). A fundamental yet unsolved question is how the shape of the ER informs its biosynthetic capacity. Very recent research in both animals and plants has begun to elucidate which factors are involved in conferring and maintaining ER shape (reviewed in (Sparkes *et al.*, 2009; Sparkes *et al.*, 2011). We have recently shown that a large family of integral membrane proteins, the reticulons (RTN), are sufficient to induce curvature of the plant ER membrane and generate tubules (Tolley *et al.*, 2008; Sparkes *et al.*, 2010; Tolley *et al.*, 2010), in a manner similar to their mammalian and yeast counterparts (Voeltz *et al.*, 2006; Hu *et al.*, 2008). In plants, RTN are represented by large gene families, with the Arabidopsis family having 21 members (Oertle *et al.*, 2003; Nziengui *et al.*, 2007). In

yeast and mammals DP1/Yop1, proteins with similar transmembrane topology to RTN and comparable function, work in concert with reticulons both *in vivo* (Voeltz *et al.*, 2006) and *in vitro* (Hu *et al.*, 2008). The closest DP1/Yop1 homologue in plants is HVA22, one isoform of which has been recently shown to localise to the ER (Chen *et al.*, 2011), but it is not yet clear whether it can shape the ER membrane in the same way as its animal and yeast relatives.

Membrane fusion events within the secretory pathway are generally mediated by SNARE proteins. While plant ER SNAREs have been characterised, they appear to regulate vesicle fusion within the bi-directional trafficking between ER and Golgi, and have yet to be shown to be involved in homotypic ER membrane fusion (Bubeck *et al.*, 2008; Lerich *et al.*, 2012). Recently a family of large, dynamin-like GTPases, the atlastins, was proposed to mediate ER membrane fusion in a GTP-dependent manner (Hu *et al.*, 2009; Orso *et al.*, 2009). Atlastins are integral membrane proteins with a large N-terminal cytosolic region containing the GTPase domain, two transmembrane domains and a cytosolic C-terminus of variable length. Very recent structural analysis of the atlastin cytosolic domain (Bian *et al.*, 2011) and mutagenesis studies (Moss *et al.*, 2011; Pendin *et al.*, 2011) point towards a mechanistic model where dimerisation of the atlastin GTPase domains and a subsequent conformational change, possibly driven by GTP hydrolysis, bring two membranes into close contact and facilitate their fusion (Moss *et al.*, 2011; Pendin *et al.*, 2011). As a consequence, atlastin mutants with impaired nucleotide-binding activity are incapable of promoting liposome fusion *in vitro* (Bian *et al.*, 2011) and several mutations *in vivo* present an unbranched ER network, where tubules form large cable-like bundles instead of anastomosing into the normally observed three-way junctions (Hu *et al.*, 2008; Pendin *et al.*, 2011).

1   Atlastins have been shown to interact with RTN and DP1/YOP1 in animal and yeast

2   cells (Hu *et al.*, 2009; Orso *et al.*, 2009). It is therefore possible that the interplay

3   between reticulons and atlastins, i.e. between membrane-shaping and membrane-

4   fusing proteins, may underpin the shared, distinctive cortical ER network architecture

5   among eukaryotic cells. The closest plant homologue of atlastin is RHD3 (Wang *et al.*,

6   1997; Zheng *et al.*, 2004). Three isoforms of this protein are present in the

7   *Arabidopsis* genome (Hu *et al.*, 2003) and recently the most abundant isoform of

8   RHD3 (At3g13870), which is mostly expressed in vegetative tissues, was shown to be

9   localised to the tubular ER and to complement the *rhd3* mutation. A GTPase domain

10  mutant was shown to phenocopy the *rhd3* mutation (Chen *et al.*, 2011) and an intact

11  C-terminal domain was also shown to be necessary for RHD3 function (Stefano *et al.*,

12  2012). While RHD3 has a role in the formation of ER junctions, an *rhd3* null mutant

13  is still capable of forming tubular fusions in the peripheral ER, indicating that this

14  protein is important but not essential for this process (Stefano *et al.*, 2012). The

15  question remains as to whether RHD3 also interacts with reticulons.

16  Our previous work has focussed on a reticulon isoform, RTNLB13, which is predicted

17  to be expressed solely in embryos of maturing seeds (Sparkes *et al.*, 2011). In this

18  report we characterise the DP1/Yop1 homologue HVA22b and one isoform of the

19  atlastin homologue RHD3 (At5g45160, recently renamed RHD-like2 (RL2) (Chen *et*

20  *al.*, 2011), which are both predicted to be present at the same developmental stages as

21  RTNLB13 (Supporting Information Fig. S1). RL2 was recently shown to be

22  functionally interchangeable with the more widely expressed RHD3 (Chen *et al.*,

23  2011).

24  We asked whether HVA22b and RL2 have ER-shaping properties and whether they

25  have the capacity to interact with RTNLB13. Here we show, using transient

1     expression in tobacco epidermal cells, that while HVA22b and wild-type RL2 have no

2     apparent effect on the ER membrane and network, GTPase domain mutations in RL2

3     can affect cortical ER structure. RTNLB13 and RL2 proteins physically interact and,

4     when co-expressed, can induce major alterations to the architecture of the cortical ER

5     network. We also show that this additive effect requires a functional reticulon protein.

6     **Materials and Methods**

7     *Expression constructs*

8     The coding sequences of RHD3-like2 (At5g45160) and HVA22b (At5g62490) were

9     amplified from genomic DNA using the following primers: RL2 forward 5'-

10    GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCCGCCAATGGGTGAAAAT

11    GATGATGGATGCTCAACTCAAC-3'       and       RL2       reverse       5'-

12    GGGGACCACTTTGTACAAGAAAGCTGGGTCCATCTGACTAATCTCACTCT

13    CTTGCACGTTG-3'       and       HVA22b       forward       5'-

14    GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCCGCCAATGAGTTCCGGAA

15    TCGGAAG-3'       and       HVA22b       reverse       5'-

16    GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGTAGATATAGGCGTCAT

17    C-3'.

18    All cloned products were cloned into the Gateway entry vector pDONR207 and then

19    into their respective destination vectors: pB7RWG2 for C-terminal mRFP required

20    and pB7WGF2 for N-terminal eGFP (Karimi *et al.*, 2005). Binary constructs were

21    transformed into the *Agrobacterium tumefaciens* strain GV1301.

22    GFP or RFP fused to the ER retrieval signal HDEL (GFP-HDEL or RFP HDEL

23    respectively) were used as an ER marker for localization experiments. The signal

anchor sequence from a rat sialyl transferase fused to green fluorescent protein (ST-GFP (Boevink *et al.*, 1998) was used as a Golgi marker.

For the construction of the Derlin 1 reporter, the cDNA encoding AtDer1 (At4g29330) was obtained from the Nottingham Arabidopsis Stock Centre (NASC). The cDNA was amplified by PCR to add ClaI and KpnI sites at its 5' and 3', respectively, and cloned downstream of the 35S promoter in pDE109 (Denecke *et al.*, 1990). The cDNA for YFP was cloned in frame with the 3' of the derlin coding sequence using KpnI and XbaI, upstream of the nos terminator cassette of pDE. The whole expression cassette was then extracted from pDE with EcoRI and HindIII and cloned into the same sites of pGREEN0029 (Hellens *et al.*, 2000). The binary vector was introduced into *Agrobacterium tumefaciens* C58::pSOUP (Hellens *et al.*, 2000).

The construction of RTNLB13-YFP and RTNLB13-ΔTM4 has already been described (Tolley *et al.*, 2008; Tolley *et al.*, 2010). RLD2-K53A, RL2-R171Q and RL2-S54N were generated by QuickChange mutagenesis (Kunkel, 1985) using the following primers: forward RL2-K53A 5'-CCTCAATCTTCTGGAGCGTCTACTCTTTTGAAC-3' and reverse 5'-GTTCAAAAGAGTAGACGCTCCAGAAGATTGAGG-3'. RL2-R171Q forward 5'-CTTTTGTTTGTGATCCAAGATAAGACCAAAACT-3'. and reverse 5'-AGTTTTGGTCTTATCTTGGATCACAAACAAAAG-3'. RL2-S54N forward 5' CAATCTTCTGGAAAGAATACTCTTTTGAACCATTTG-3' and 5' CAAATGGTTCAAAAGAGTATTCTTTCCAGAAGATTG-3'.

*Transient expression and confocal microscopy*

*Nicotiana tabacum* cv Petit Havana SR1 was grown as described previously (Sparkes *et al.*, 2006). *Agrobacterium tumefaciens* cultures were infiltrated at the following

OD$_{600}$ ST-GFP 0.04, GFP-HDEL/RFP HDEL 0.04, RL2-RFP, the GTPase mutants (RL2-K53A-RFP, RL2-S54N-RFP , and RL2-R171Q-RFP) and GFP- or -HVA22b 0.05. Segments of infiltrated leaves were observed after 3-4 days with a Leica TCS SP5 confocal microscope equipped with a x 63 (1.3NA) water immersion objective. GFP was excited at 488 nm and detected in the 495 to 520 nm range. YFP was excited at 514 nm and detected in the 525 to 550 nm range. RFP was excited at 561 nm and detected in the 571 to 638 nm range. Simultaneous detection of YFP and RFP was performed by combining the settings indicated above in the sequential scanning facility of the microscope, as instructed by the manufacturer. The confocal microscope settings were kept constant throughout experiments.

Data acquisition and subsequent analysis using Volocity software version 3 (Perkin Elmer) for Golgi body tracking was carried according to (Sparkes *et al.*, 2008) and (Avisar *et al.*, 2009).

*Co-immunoprecipitation*

*Nicotiana tabacum* leaves were agroinfiltrated with both myc-RTNLB13 and RL2-RFP or its relative GTPase mutant constructs. Leaf sectors were homogenised in homogenisation buffer (150mM Tris-HCl pH7.5, 150mM NaCl, 1.5% (v/v) Triton X-100, supplemented immediately before use with 'Complete' protease inhibitor cocktail (Boehringer Mannheim, UK) and subjected to immunoprecipitation with anti-myc monoclonal antibody 9E10 and Protein A Sepharose beads. The beads were subsequently washed three times using NET-Gel buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA, 0.1% (v/v) Nonidet P-40, 0.25% (w/v) gelatine, 0.02% (w/v) NaN3) and resuspended in SDS PAGE sample buffer. Immunoselected

1 polypeptides were resolved by SDS-PAGE, transferred to nitrocellulose filters and

2 subjected to immunoblotting with either anti-myc or anti-DsRed antibody (Invitrogen).

3

## Results and Discussion

*HVA22b labels the cortical ER but does not affect its morphology*

6 As a first step towards the characterisation of HVA22b and RL2 proteins, we studied

7 their intracellular location and effect on the ER network in the transient expression

8 system of agroinfiltration in tobacco epidermal cells. AtHVA22 is the closest

9 homologue of DP1/Yop1, a protein structurally similar to RTN in yeast and

10 mammalian cells (Voeltz *et al.*, 2006). There are five isoforms of HVA22 in

11 Arabidopsis (Chen *et al.*, 2002), with HVA22b (At5g62490) having a seed-limited

12 expression pattern comparable to RTNLB13 and RL2 (Supporting Information Fig.

13 S1). We generated fusions between HVA22b and GFP. When expressed in tobacco

14 epidermal cells by agroinfiltration (Sparkes *et al.*, 2006), GFP-HVA22b labels the ER

15 uniformly (Fig. 1, A-C) and, unlike RTN, it is also found in ER cisternae and the

16 nuclear envelope (Fig. 1, D-F). No apparent constriction of ER tubules, as normally

17 observed with overexpressed RTNLB13 (Tolley *et al.*, 2008), can be seen. This

18 localisation matches that reported for a different isoform, AtHVA22d (Chen *et al.*,

19 2011). These results may indicate that HVA22 is not serving an ER-shaping structural

20 role in plants. Indeed, sequence homology with Yop1 is relatively low (33.6%

21 similarity, 18.9% identity with EMBOSS Needle tool at the European Bioinformatics

22 Institute, www.ebi.ac.uk). More importantly, HVA22b has a predicted transmembrane

23 topology that is different from the reticulon-like 'w' topology of Yop1 (Supporting

Information Fig. S2), which may explain its lack of ability to induce membrane curvature and/or constriction of ER tubules.

*RL2 labels the ER network and mutations in the GTPase domains affect ER morphology*

We generated a fusion between RL2 and RFP and expressed it in tobacco epidermal leaf cells. Fig. 1(G-I) shows that, like HVA22b, RL2-RFP labels the whole of the ER network and appears to have no apparent structural effect on tubular ER. Therefore it seems that constitutive overexpression of RL2 does not induce obvious morphological changes in tobacco epidermal cells.

Given that the GTPase activity of atlastin (Stefano *et al.*, 2012) is key to its capacity to oligomerise and facilitate ER membrane fusion (Hu *et al.*, 2009; Bian *et al.*, 2011; Moss *et al.*, 2011; Pendin *et al.*, 2011), we tested whether RL2 localisation and effect on ER shape was affected by mutations within its predicted GTPase domain. We introduced the following point mutations into RL2-RFP: K53A (corresponding to K80A in ATL1; (Hu *et al.*, 2009)), S54N in the P-loop region (G1, same as Chen *et al.*, 2011), and R171Q in the G4 guanosine binding motif. In tobacco leaf epidermal cells, all mutants still labelled the cortical ER network (Fig. 2) but its morphology appeared to be altered. A large number of cable-like structures were present in cells expressing K53A and R171Q (Fig. 2, panels C-D and G-H), at the expense of the normal network of three-way junctions and the occasional, thicker bundles observed upon expression of wild-type RL2-RFP (Fig. 1H and Fig. 2, A-B). The appearance of these large ER strands is reminiscent of the ER phenotype observed in the *rhd3-1* mutant (Zheng *et al.*, 2004). This likely indicates that the GTPase mutations have a dominant negative effect outcompeting the native protein. Surprisingly, a second

mutation within the G1 motif, S54N, located to punctate structures on the ER (Fig. 2, E-F). When S54N was co-expressed with the ER luminal marker GFP-HDEL, the cortical ER appeared to more cisternal in structure (Supporting Information Fig S3, D-F) – an effect reminiscent of the phenotype observed upon overexpression of the ER membrane marker GFP-calnexin (Irons *et al.*, 2003) and Supporting Information Fig. S6, D-F).

When wild-type atlastin is overexpressed in mammalian cells, the ER assumes an abnormal, punctate appearance (Moss *et al.*, 2011). Normal ER morphology is restored when expressing mutants impaired in membrane fusion (Moss *et al.*, 2011). This is compatible with the predicted fusogenic function of atlastin. Ours and others' observations (Chen *et al.*, 2011) seem to indicate a different behaviour for the plant atlastin homologues. Overexpression of wild-type RL2 does not affect ER morphology, whereas both downregulation and expression of GTPase mutants results in ER morphology alterations. Intriguingly, the vegetative isoform of RHD3 cannot complement a mutant lacking Sey1p, the yeast atlastin homologue (Chen *et al.* 2011) perhaps indicating functional divergence of the protein or of essential interacting partners.

We also tested whether overexpression of wild-type or mutant RL2 had an effect on Golgi body motility, as indicated for the vegetative form of RHD3 (Chen *et al.*, 2011). We co-expressed RL2 and its relative mutants with the Golgi marker ST-GFP (Boevink *et al.*, 1998). We tested the effects in at least 20 cells taken from 3 independent experiments from cells co-expressing the Golgi and RL2 marker. Golgi bodies display a range of movements such as saltatory, stop-go, fast, and uni/birectional. In order to quantify the effects of RL2 on Golgi dynamics, velocity, displacement rate and meandering index were monitored. Displacement is the shortest

straight-line distance between the beginning and end of a track, while the meandering

index is calculated by dividing the displacement rate with the velocity, and therefore

provides a measure of Golgi trajectory. For example, a meandering index of 1

corresponds to a Golgi body moving with a straight trajectory, whereas a lower

number indicates a more random motion. All of these parameters were calculated for

more than 280 Golgi bodies per construct and plotted as a cumulative distribution

frequency (Fig. 3, Sparkes et al. 2008). Comparisons between CDF curves were

generated using the Kolmogorov-Smirnov statistical test (KS test). The velocities and

displacement rates of Golgi bodies in cells expressing wild type RL2 or the S54N

mutant do not appear to be significantly different to control cells only expressing the

Golgi marker. However, velocity and displacement rate CDF plots for RL2 mutants

K53A and R171Q mutants were significantly different to the control ($p < 0.06$). As can

be seen from the meandering index plot (D/V), Golgi bodies in cells expressing the

RL2 mutant K53A display a more random motion than under control and other

conditions. As it has been proposed that Golgi bodies move over or with the ER

network in an actin dependent manner (DaSilva *et al.*, 2004; Sparkes *et al.*, 2008),

such differences in movement between the expression of mutant RHD3 and the wild

type could be ascribed to the reorganisation of the network into a more cable-like

form witnessed under mutant RLD2 expression.

*Co-expression of RL2 and RTNLB13 affects the morphology of the cortical ER network*

Our results indicate that RL2 labels the cortical ER network but mutations that affect

its nucleotide-binding capacity lead to an apparent increase in the number of large ER

1   strands and a reduction of ER regions presenting normal three-way junctions (Fig. 2).

2   This hints at a role of RL2 in organising the classic geometrical network of the

3   cortical ER possibly by mediating homotypic fusion of ER tubules. In mammalian

4   and yeast cells, atlastins and reticulons have been shown to interact (Hu *et al.*, 2009).

5   We therefore tested whether the overexpression of both RL2-RFP and RTNLB13-

6   YFP affected ER morphology. When both constructs were co-infiltrated into tobacco

7   leaves, at low magnification cells co-expressing both proteins presented very bright

8   fluorescent spots, not present when RL2-RFP is co-expressed with GFP-HDEL

9   (Supporting Information Fig. S4; compare a-c with b-d). At higher magnification,

10  these bright areas appeared to be part of a severely altered cortical ER network (Fig.

11  4A-C, compare with adjacent cell expressing RTNLB13 only, asterisk). The number

12  of three way junctions was drastically reduced and large membrane cables and

13  aggregates became apparent (Fig. 4, D-F). It is possible that this effect is a

14  consequence of the overexpression of two ER membrane proteins and bears no

15  relation to the function of either RL2 or RTNLB13. Therefore we tested whether the

16  same phenotype could be caused by co-expressing RL2 and a 'neutral' ER membrane

17  marker. Given the current paucity of polytopic ER membrane proteins available as

18  markers in plants, we produced a new marker by generating a YFP fusion to the

19  Arabidopsis homologue of derlin 1. Human derlin proteins 1-3 (Lilley & Ploegh, 2004;

20  Oda *et al.*, 2006) are homologues of *Saccharomyces cerevisiae* Der1p (Knop *et al.*,

21  1996). All derlins function in the ER membrane as components of a larger complex

22  where they may facilitate the recognition and retro-translocation of certain misfolded

23  proteins from the ER to the cytosol in a protein quality control pathway. In

24  Arabidopsis, there are 3 derlin homologues: one (At4g29330) shows homology to

25  mammalian Derlin-1 and is annotated as Derlin 1; two other genes (At4g21810 and

At4g04860) have closer homology to Der2 and are named Derlin 2.1 and Derlin 2.2, respectively (Supporting Information Fig. S5A). Arabidopsis Derlin1 is closer to its human counterpart than to Derlin 2.1 and 2.2 (Supporting Information Fig. S5a). When expressed in tobacco protoplasts alongside a plant ERAD substrate, ricin A chain (RTA; (Di Cola *et al.*, 2001), Derlin 1 does not seem to affect the degradation kinetics of RTA (Supporting Information Fig. S5b). More importantly for the present work, when expressed in tobacco leaves together with the luminal marker RFP-HDEL, derlin 1-YFP labelled the ER network without apparently affecting its shape, unlike more popular markers such as GFP-calnexin, which induces ER cisternae (Irons *et al.*, 2003; Runions *et al.*, 2006), and various reticulon isoforms, which induce constrictions in ER tubules (Sparkes *et al.*, 2010) (Supporting Information Fig. S6). Therefore, while derlin 1 may not function in the ERAD pathway its apparent lack of effect on ER morphology makes it an attractive potential polytopic (Supporting Information Fig S5C) ER membrane marker.

When Derlin1-YFP was co-expressed with RL2, the ER network appeared normal (Fig. 4, G-I). It is therefore unlikely that the drastic remodelling observed upon RL2 and RTNLB13 co-expression is a non-specific effect arising from the simultaneous overexpression of two ER membrane proteins.

The disruption of the ER was also observed when RTNLB13 was co-expressed with the RL2 GTPase mutants (Fig. 5, A-I). In the case of the most severe mutant, S54N, loss of network architecture was further aggravated by the membrane fragmentation phenotype observed with S54N alone (Fig 5, G-I). This indicates that the effect of co-expressing RTNLB13 and RL2 may be additive.

Given that the severe morphological alteration of the ER persists when GTPase mutants of RL2 are expressed, we asked whether this additive effect depended on the reticulon protein being functional. We therefore co-expressed RHD3 with a mutant of RTNLB13 (ΔTM4; (Tolley *et al.*, 2010), in which each transmembrane segment was shortened to match the 'standard' length (17 residues) of an ER membrane protein (Brandizzi *et al.*, 2002). We have previously shown that ΔTM4, while still residing in the ER membrane, is no longer capable of inducing tubule constrictions, or converting ER sheets into tubules (Tolley *et al.*, 2010). Indeed, when ΔTM4 was co-expressed with RL2, the ER network appeared unperturbed (Fig. 5, J-L). Given that the ΔTM4 mutant protein appears to have comparable stability to wild-type RTNLB13, this indicates that the major disruption of tubular ER specifically arises from the combined action of RL2 and RTNLB13 and that such additive effect requires a functional reticulon.

In mammalian cells, interaction between ATL1 atlastin and the reticulon isoforms Rtn3c or Rtn4a appears to be mediated by their respective transmembrane domains: accordingly, interaction persists in ATL1 mutants with altered nucleotide binding activity (Hu *et al.*, 2009). Our results seem to corroborate this observation because the additive effect of RL2 and RTNLB13 is maintained in the GTPase mutants, while shortening of the TMD of RTNLB13 prevents the additive effect seen when coexpressed with RL2.

*RL2 interacts with RTNLB13*

Given the spectacular ER disruption phenotype observed by the simultaneous expression of RL2 and RTNLB13, we hypothesised that these proteins must interact physically. To test this, we performed co-immunoprecipitation experiments on

infiltrated leaf sectors expressing a myc-tagged version of RTNLB13 (Tolley *et al.*, 2008) and the RFP-tagged RL2. Figure 6 shows that RL2-RFP can be co-immunoprecipitated with RTNLB13-myc. Co-immunoprecipitation with RTNLB13-myc is also observed for each of the GTPase mutants (Fig. 6). This further confirms the hypothesis that interaction between reticulon and atlastin homologues does not require a functional GTPase domain.

In conclusion, the data presented here confirm that the plant homologues of atlastin the RHD3 family, are involved in the geometrical organisation of the ER network, perhaps through regulation and formation of three-way junctions between tubules. This role may be regulated by an interaction with the membrane curving reticulon proteins, although such an interaction is not dependent on the GTPase activity of RL2. The exact nature of this interaction and the potential role of RL2 in ER tubule fusion remain to be ascertained.

**Acknowledgements**

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**Figure legends**

**Figure 1. HVA22b-YFP  and RL2-RFP localise to the endoplasmic reticulum but do not induce membrane constrictions.**
Tobacco leaves were infiltrated with agrobacteria containing the indicated expression constructs. Epidermal cells were analysed by CLSM after 3 days. Scale bars, 10 μm.

**Figure 2. Mutations affecting the GTPase domain of RL2 lead to altered cortical ER network morphology**
Tobacco leaves were infiltrated with agrobacteria containing the indicated expression constructs. Epidermal cells were analysed by CLSM after 3 days. Scale bars, 10 μm.

**Figure 3. RL2 and its GTPase mutants do not drastically affect Golgi movement**
Golgi body dynamics in tobacco epidermal cells transiently expressing STGFP alone (control) or in combination  with mRFP fusions to RHD3 variants, were monitored and quantified. Cumulative distribution frequency plots (CDF) of velocity, displacement rates and meandering index (D/V) of Golgi bodies were calculated with numbers below the meandering index plot denoting the number of Golgi bodies tracked per condition.

**Figure 4. Co-expression of RL2 and RTNLB13 affects the morphology of the cortical ER network**

Tobacco leaves were infiltrated with agrobacteria containing the indicated expression constructs. The asterisks indicate a control cell expressing only RTNLB13-YFP. Epidermal cells were analysed by CLSM after 3 days. Scale bars, 10 μm.

**Figure 5. The effect of RL2 and RTNLB13 on ER morphology is independent of GTPase activity and requires a functional reticulon**
Tobacco leaves were infiltrated with agrobacteria containing the indicated expression constructs. Epidermal cells were analysed by CLSM after 3 days. Scale bars, 10 μm.

**Figure 6. RL2 and RTNLB13 co-immunoprecipitate.**
Tobacco leaves were infiltrated with agrobacteria containing the indicated expression constructs. Infiltrated sectors were homogenised and samples resolved by SDS-PAGE (total) or subjected to immunoprecipitation (IP) with anti-myc antibody. Total extracts and immunoselected polypeptides were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted (IB) with the indicated antibodies.

**Supporting Information**

**Fig. S1**. eFP browser expression predictions for RL2 and HVA22b .
Anatomical expression maps were generated using the Arabidopsis eFP browser (Winter *et al.*, 2007) ( http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

**Fig. S2. A.** Comparison of the transmembrane topologies of HVA22b and yeast Yop1p, as predicted by TOPCONS (http://topcons.cbr.su.se/). Red segments indicate predicted cytosolic regions, blue segments luminal (extracellular) regions. **B**. Sequence alignment between Yop1p and HVA22b, generated by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/ ).

**Fig. S3**. Effect of GTPase mutants of RL2 on ER morphology. Tobacco leaves were infiltrated with agrobacteria containing the indicated expression constructs. Epidermal cells were analysed by CLSM after 3 days. Scale bars, 10 μm.

**Fig. S4.** Co-expression of RL2 and RTNLB13 affects the morphology of the cortical ER network.
Tobacco leaves were infiltrated with agrobacteria containing the indicated expression constructs. Epidermal cells were analysed by CLSM after 3 days. Scale bars, 20 μm.


**Fig. S5**. **A**. Evolutionary relationships of Arabidopsis (At) and human (Hs) derlin proteins. The amino acid sequences from the indicated derlins were aligned with ClustalW. The tree was produced with MEGA4.1 using the minimum evolution method with 1000 bootstrap repetitions. Yeast Der1p was used as the outgroup. Bootstrap test results are shown where higher than 50.
**B**. Tobacco mesophyll protoplasts were transfected with plasmids encoding the indicated constructs. Protoplasts were pulse labelled for 1 h with $^{35}$S-methionine and cysteine, then chased for the indicated times. Cell homogenates were subjected to immunoprecipitation with anti-RTA antiserum. Immunoselected polypeptides were resolved by 15% SDS-PAGE and revealed by autoradiography. Numbers to the left of the autoradiogram indicate molecular weight markers (kDa). A representative result is shown.
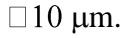
**C**. transmembrane topology of  Arabidopsis derlin1, as predicted by TOPCONS (http://topcons.cbr.su.se/). Red segments indicate predicted cytosolic regions, blue segments luminal (extracellular) regions.

**Fig. S6.** Derlin 1-YFP labels, but does not perturb, the ER network. Tobacco epidermal cells were infiltrated with agrobacteria containing the indicated constructs. A□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□ □□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□bar□□ □10 μm.