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Kriechbaumer, VC, Botchway, SW, Frigerio, L, Oparka, K and Hawes, CR

Reticulomics: Protein-protein interaction studies with two plasmodesmata-localised reticulon family proteins identify binding partners enriched at plasmodesmata, ER and the plasma membrane

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1 Running title: Identifying interaction partners of reticulons

2

3 Author for correspondence:

4 Dr Verena Kriechbaumer

5 Plant Cell Biology

6 Biological and Medical Sciences

7 Oxford Brookes University

8 Gipsy Lane, Headington

9 Oxford OX3 0BP, UK

10 Reticulomics: Protein-protein interaction studies with two plasmodesmata-localised reticulon  
11 family proteins identify binding partners enriched at plasmodesmata, ER and the plasma  
12 membrane

13

14 Verena Kriechbaumer<sup>1</sup>, Stanley W. Botchway<sup>2</sup>, Susan E Slade<sup>3</sup>, Kirsten Knox<sup>4</sup>, Lorenzo  
15 Frigerio<sup>5</sup>, Karl Oparka<sup>4</sup>, Chris Hawes<sup>1</sup>

16

17 <sup>1</sup> Plant Cell Biology, Biological and Medical Sciences, Oxford Brookes University, Oxford OX3  
18 0BP, UK

19 <sup>2</sup> Central Laser Facility, STFC Rutherford Appleton Laboratory, RCaH, Didcot OX11 0QX, UK

20 <sup>3</sup> WPH Proteomics Facility RTP, School of Life Sciences, University of Warwick, Coventry CV4  
21 7AL, UK

22 <sup>4</sup> Institute of Molecular Plant Sciences, University of Edinburgh, Edinburgh EH9 3JR, UK

23 <sup>5</sup> School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK

24

25

26

27 Summary: Protein interactions for two plasmodesmata-localised reticulon proteins suggest  
28 that these proteins, in addition to a role in ER modelling, may play important roles in linking  
29 ER and plasma membrane.

30

31

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35

36

37 Author for correspondence: vkriechbaumer@brookes.ac.uk

38 Abstract

39 The ER is a ubiquitous organelle that plays roles in secretory protein production, folding,  
40 quality control, and lipid biosynthesis. The cortical ER in plants is pleomorphic and structured  
41 as a tubular network capable of morphing into flat cisternae, mainly at three way junctions,  
42 and back to tubules. Plant reticulon (RTNLB) proteins tubulate the ER by dimer- and  
43 oligomerization, creating localised ER membrane tensions that result in membrane curvature.  
44 Some RTNLB ER-shaping proteins are present in the plasmodesmal (PD) proteome  
45 (Fernandez-Calvino et al., 2011) and may contribute to the formation of the desmotubule, the  
46 axial ER-derived structure that traverses primary PD (Knox et al., 2015). Here we investigate  
47 the binding partners of two PD-resident reticulon proteins, RTNLB3 and RTNLB6, that are  
48 located in primary PD at cytokinesis (Knox et al., 2015). Co-immunoprecipitation of GFP-  
49 tagged RTNLB3 and RTNLB6 followed by mass spectrometry detected a high percentage of  
50 known PD-localised proteins as well as plasma-membrane proteins with putative membrane  
51 anchoring roles. FRET-FLIM assays revealed a highly significant interaction of the detected  
52 PD proteins with the bait RTNLB proteins. Our data suggest that RTNLB proteins, in addition  
53 to a role in ER modelling, may play important roles in linking the cortical ER to the plasma  
54 membrane.

## 55 **Introduction**

56  
57 The endoplasmic reticulum (ER) is a multifunctional organelle (Hawes et al., 2015) and is the  
58 site of secretory protein production, folding and quality control (Brandizzi et al., 2003) and lipid  
59 biosynthesis (Wallis and Browse, 2010), but is also involved in many other aspects of day-to-  
60 day plant life including auxin regulation (Friml and Jones, 2010) and oil and protein body  
61 formation (Huang, 1996; Herman, 2008). The cortical ER network displays a remarkable  
62 polygonal arrangement of motile tubules that are capable of morphing into small cisternae,  
63 mainly at the three way junctions of the ER network (Sparkes et al., 2009). The cortical ER  
64 network of plants has been shown to play multiple roles in protein trafficking (Palade, 1975;  
65 Vitale and Denecke, 1999) and pathogen responses (reviewed in Pattison and Amtmann,  
66 2009; Beck et al., 2012).

67  
68 In plants, the protein family of reticulons (RTNLBs) contributes significantly to tubulation of the  
69 ER (Tolley et al., 2008, 2010; Chen et al., 2012). RTNLBs are integral ER membrane proteins  
70 that feature a C-terminal reticulon homology domain (RHD) that contains two major  
71 hydrophobic regions. These regions form two “V”-shaped transmembrane wedges joined  
72 together via a cytosolic loop, with the C- and N-terminus of the protein facing the cytosol.  
73 RTNLBs can dimerize or oligomerize creating localised tensions in the ER membrane,  
74 inducing varying degrees of membrane curvature (Sparkes et al., 2010). Hence, RTNLBs are  
75 considered to be essential in maintaining the tubular ER network.

76  
77 The ability of RTNLBs to constrict membranes is of interest in the context of cell-plate  
78 development and the formation of primary PD (Knox et al., 2015). PD formation involves  
79 extensive remodelling of the cortical ER into tightly furled tubules to form the desmotubules,  
80 axial structures that run through the PD pore (Ehlers and Kollmann, 2001; Overall and  
81 Blackman, 1996). At only 15 nm in diameter, the desmotubule is one of the most constricted  
82 membrane structures found in nature, with no animal counterparts (Tilsner et al., 2011). PD  
83 are membrane-rich structures characterized by a close association of the plasma membrane  
84 with the ER. The forces that model the ER into desmotubules, however, are poorly  
85 understood. RTNLBs are excellent candidates for this process and can constrict fluorescent  
86 protein labelled ER membranes into extremely fine tubules (Sparkes et al., 2010). We have  
87 shown recently that two of the RTNLBs present in the PD proteome, RTNLB3 and RTNLB6  
88 (Fernandez-Calvino, 2011), are present in primary PD at cytokinesis (Knox et al., 2015).  
89 However, nothing is known of the proteins that interact with RTNLBs identified in the PD  
90 proteome, or that may link RTNLBs to the plasma membrane (PM). To date, the only protein

91 shown to bind to plant RTNLBs is RHD3-like2, the plant homologue of the ER tubule fusion  
92 protein, atlastin (Lee et al., 2013).

93  
94 Here we used a dual approach to identify interacting partners of RTNLB3 and RTNLB6  
95 (Fernandez-Calvino et al., 2011; Knox et al., 2015). First, we used GFP-immunoprecipitation  
96 assays coupled to mass spectrometry to identify proteins potentially binding to RTNLB3 and  
97 RTNLB6. Second, from the proteins we identified, we conducted a detailed FRET-FLIM  
98 (Förster Resonance Energy Transfer by Fluorescence Life Time Imaging) analysis to confirm  
99 prey-bait interactions *in vivo*.

100  
101 The application of time resolved fluorescence spectroscopy to imaging biological systems has  
102 allowed design and the implementation of Fluorescence Lifetime Imaging Microscopy (FLIM).  
103 The technique allows measuring and determining the space map of picosecond fluorescence  
104 decay at each pixel of the image through confocal single and multiphoton excitation. The  
105 general fluorescence or Förster resonance energy transfer (FRET) to determine co-  
106 localisation of two colour chromophores can now be improved to determine physical  
107 interactions using FRET-FLIM using protein pairs tagged with appropriate GFP-fluorophores  
108 and monomeric red fluorescent protein. FRET-FLIM measures the reduction in the excited  
109 state life time of GFP (donor) fluorescence (in the presence of an acceptor fluorophore (e.g.  
110 mRFP) which is independent of the problems associated with steady state intensity  
111 measurements. the observation of such a reduction is an indication that the two proteins are  
112 within a distance of 1 to 10 nm thus indicating a direct physical interaction between the two  
113 protein fusions (Osterrieder et al., 2009, Sparkes et al., 2010, Schoberer and Botchway,  
114 2014). It was previously shown that a reduction of as little as ~200 ps in the excited state  
115 lifetime of the GFP labelled protein represents quenching through a protein-protein interaction  
116 (Stubbs et al., 2005).

117  
118 Our interaction data identified a large percentage (40%) of ER proteins, including other  
119 RTNLB family members. However, we also found a relatively large number (25%) of proteins  
120 present in the published PD proteome (Fernandez-Calvino et al., 2011), and a surprisingly high  
121 proportion (35%) of plasma membrane proteins. Of the PD-resident proteins we identified, a  
122 significant number were shown previously to be targets of viral movement proteins (MPs) or  
123 proteins present within lipid rafts, consistent with the view that PD are lipid-rich microdomains  
124 (Bayer et al., 2014). Additional proteins identified suggested roles for RTNLBs in transport and  
125 pathogen defence. We suggest that RTNLBs may play key roles in anchoring and/or signalling  
126 between the cortical ER and PM.

127

128

129 **Results**

130

131 *Identification of proteins that interact with RTNLB3 and RTNLB6*

132 The reticulon proteins RTNLB3 and RTNLB6 are found in the PD proteome (Fernandez-  
133 Calvino et al., 2011). We have shown recently that when both RTNLBs are co-expressed  
134 transiently in tobacco epidermal leaf cells with the viral movement protein (MP) of tobacco  
135 mosaic virus (TMV) there is significant co-localisation (Knox et al., 2015). Both these RTNLBs  
136 are located to the developing cell plate at cytokinesis, and are therefore strong candidates for  
137 proteins that model the cortical ER into desmotubules (Knox et al., 2015).

138

139 As it is likely that RTNLBs form protein complexes with proteins in the plasma membrane and  
140 desmotubule in order to stabilise the desmotubule constriction and to allow gating in PD (see  
141 model in Knox et al., 2015) we searched for potential interacting proteins capable of carrying  
142 out these tasks.

143

144 To find interaction partners for these RTNLBs, we used Arabidopsis plants stably expressing  
145 RTNLB3-YFP or RTNLB6-YFP fusion proteins under a 35S promoter to perform co-  
146 immunoprecipitations using GFP-Trap®\_A beads (Chromotek). For this approach whole  
147 seedling protein extracts were incubated with agarose slurry linked to anti-GFP camelid  
148 antibodies. This antibody is capable of binding the YFP-tag on the reticulon proteins. The  
149 RTNLB bait, along with the proteins that bind/interact with the RTNLB, were pelleted by slow  
150 centrifugation. The resulting proteins in this pellet were identified using mass spectrometry  
151 (nanoLC-ESI-MS/MS, Thermo Orbitrap Fusion, Thermo Scientific) and the data analysed  
152 using Scaffold Proteome Software (version Scaffold\_4.4.1.1, Proteome Software Inc.,  
153 Portland, OR). As a control, proteins bound to the antibody in untransformed plants were also  
154 analysed by MS.

155

156 The MS data showed a high percentage of overlap between proteins identified with RTNLB3  
157 and RTNLB6 as baits (Figure 1). Of a total of 706 identified proteins only 93 (13%) or 85  
158 (12%) proteins were unique to RTNLB3 or RTNLB6, respectively (Table 1). Proteins that were  
159 also identified in the control samples (two independent sets of wildtype Arabidopsis plants)  
160 were subtracted from the list of proteins resulting in 146 potential interacting partners for either  
161 RTNLB3 or RTNLB6. Despite the removal of false positives, a high percentage of proteins in  
162 common was maintained for both reticulons: out of a total of 146 proteins, RTNLB3 had 135  
163 potential interaction candidates with only with only 11 proteins (8%) showing unique specificity

164 for RTNLB3. Similarly, 126 proteins were co-immunoprecipitated with RTNLB6, with 20  
165 proteins (17%) being unique to RTNLB6 (Table 1).

166  
167 These resulting protein candidates were ranked according to their 'percentage of the total  
168 spectra', which represents the number of spectra matching a specific protein (across all MS  
169 samples) as a percentage of the total number of spectra in the sample (Supplemental Table  
170 1). This ranking indicates the amount of a specific protein bound to the RTNLB-baits and can  
171 therefore be used as a measure of the reliability of each potential interaction.

172  
173 *FRET-FLIM analysis to validate immunoprecipitation data*

174 17 proteins from the list of 146 potential interacting proteins were subjected to further analysis  
175 to test for interactions *in vivo* using a different methodology (FRET-FLIM; Table 1, Table 2).

176 The choice of these 17 proteins was based on three main criteria:

177 i) Known or expected interacting partners of RTNLBs from published work. These were used  
178 as positive controls and included; RTNLB3 and RTNLB6 (RTNLB3 dimerization in Sparkes et  
179 al., 2010) and RHD3/RL2 (Lee et al., 2013);

180 ii) Proteins present in the PD proteome, listed in Table 2.

181 iii) A selection of low-abundance proteins distributed throughout the quantitative lists (see  
182 Table 2, Supplemental Table 1). These were used to test the hypothesis that proteins with low  
183 abundance in the GFP-Trap assays were likely to represent weak or false-positive  
184 interactions. For example, thioredoxin 3 (TRX3; Table 2) showed very low abundance relative  
185 to, for example, DWARF1 (DWF1).

186 iv) TCP-1/cpn60 chaperonin family protein (TCP1) was chosen as a further control as this  
187 protein was found in the proteome for RTNLB3 but not for RTNLB6 (Table 2, Supplemental  
188 Table 1).

189  
190 Förster resonance energy transfer (FRET; Förster, 1948) measured by donor-excited state  
191 fluorescence lifetime imaging (FLIM; Becker, 2012; Schoberer and Botchway, 2014) was used  
192 to confirm independently the interactions suggested by the GFP-Trap assays. FRET-FLIM  
193 measures the reduction in the lifetime of the GFP (donor) fluorescence when an acceptor  
194 fluorophore (mRFP) is within a distance of 1 to 10 nm, thus allowing FRET to occur and  
195 indicating a physical interaction between the two protein fusions (Osterrieder et al., 2009,  
196 Sparkes et al., 2010). In the FRET-FLIM assay, each of the above 17 proteins was expressed  
197 transiently as an mRFP fusion (acceptor) in tobacco leaf epidermal cells expressing either  
198 RTNLB3-GFP or RTNLB6-GFP as donors. At least two biological samples with a minimum of  
199 three technical replicates each were used for the statistical analysis.

200



201 Due to limitations in the speed of photon counting of the FLIM apparatus, measurements were  
202 taken from high expressing areas of ER regions with relatively low mobility, such as the ER  
203 associated with the nuclear envelope. This allowed more reliable measurements than the fast  
204 moving cortical ER (see Sparkes et al., 2010). Furthermore, to allow consistent and reliable  
205 measurements also proteins that usually localise to PD or PM were driven to the ER by  
206 protein overexpression. FRET-FLIM interactions are shown in Table 2. RTNLB3-GFP or  
207 RTNLB6-GFP expression without acceptor presence was used as a negative control while  
208 known self-interactions between the RTNLBs (e.g. RTNLB3 against RTNLB3) or with the  
209 second RTNLB (e.g. RTNLB3 against RTNLB6) were used as positive controls and to  
210 determine the value of fluorescence that could be considered as a significantly positive  
211 interaction. Figure 2 shows a comparison of such negative and positive controls:

212  
213 RTNLB3-GFP alone showed a fluorescence lifetime of  $2.47 \pm 0.05$  ns, and RTNLB6-GFP alone  
214 a lifetime of  $2.63 \pm 0.06$  ns. Excited state lifetimes determined for RTNLB-RTNLB homomeric  
215 and heteromeric interactions varied from 2.31 to 2.38 ns (Table 3) which is statistically  
216 significantly different to that of the GFP alone. Figure 2 shows the FRET-FLIM analysis steps  
217 for RTNLB6-GFP alone (Figure 2 A-D) as a negative control, and for RTNLB6-GFP interacting  
218 with RFP-RTNLB6 (Figure 2 E-I) as a positive control. Raw FRET-FLIM images are shown in  
219 Figure 2 A and E. This analysis takes into account the lifetime values of each pixel within the  
220 image visualised by a pseudo-coloured lifetime map (Figure 2 B and F). The graph shows the  
221 distribution of lifetimes within the image (Figure 2 C and G) with blue shades representing  
222 longer GFP fluorescence lifetimes than green ones. Decay curves (Figure 2 D and H) of a  
223 representative single pixel highlight an optimal single exponential fit, where Chi square ( $\chi^2$ )  
224 values from 0.9 to 1.2 were considered an excellent fit to the data points (binning factor of 2).  
225 Confocal pictures for the region of interest showing the GFP-construct in green and the  
226 mRFP-construct in red are shown in Figure 2 A (inset) and I. This specific example shows that  
227 RTNLB6 homodimerizes because the lifetime values for the GFP/mRFP fusion pair ( $2.38 \pm 0.01$   
228 ns, Table 3) are lower than those for the GFP fusion alone ( $2.63 \pm 0.06$  ns).

229  
230 Next, RTNLB3-GFP and RTNLB6-GFP were co-infiltrated independently with each of the 17  
231 chosen proteins and the resulting lifetimes measured (Table 3, Figure 3). Representative  
232 FRET-FLIM data are shown for each combination (Supplemental Figure S1). As mentioned  
233 above, ER regions with relatively low mobility, such as the ER associated with the nuclear  
234 envelope allow more reliable measurements (Sparkes et al., 2010). Therefore, to be  
235 comparable with other data the interactions between RTNLBs and PM-localised proteins such  
236 as remorins and PIP3 were also measured in this area. These PM-bound proteins are also  
237 normally detected as they transit through the ER in transient expression experiments.

238  
239 Among the RTNLB3 putative interactors, all proteins with the exceptions of FASCICLIN-like  
240 arabinogalactan protein 8 (FLA8), annexin 4 (ANNAT4) and thioredoxin 3 (TRX3) showed  
241 interaction. Significantly, these proteins were not present in the second MS dataset (Table 2),  
242 and their lack of interaction using FRET-FLIM confirmed that these were likely to be false  
243 positives. The results for TCP1 are also significant because TCP1 was pulled down by  
244 RTNLB3, but not by RTNLB6, and in the FRET-FLIM assays TCP1 interacted with RTNLB3  
245 but not with RTNLB6, confirming the results of the GFP-Trap data. (Table 3, Figure 3). To  
246 summarise, the proteomics data from one biological sample yielded less than 18% false-  
247 negatives in the chosen selection with the three false-negative proteins showing  
248 comparatively low peptide coverage.

249  
250 *Mass spectrometry confirmation of proteomics data*

251 For further confirmation of the data, the GFP-Immunoprecipitation and MS proteomics was  
252 repeated with an independent biological sample of RTNLB3-YFP and RTNLB6-YFP plants, as  
253 well as wild-type Arabidopsis and a stable Arabidopsis line expressing the ER-membrane  
254 marker calnexin (CXN) tagged with GFP. The ER-integral protein calnexin was used to detect  
255 false-positive interactions resulting from proteins binding to the fluorescent tag rather than the  
256 RTNLBs. Results from the wildtype and CXN immunoprecipitations were subtracted from the  
257 proteins pulled down with RTNLB3 or RTNLB6. This second dataset was then compared with  
258 data from the first experiment and only proteins present in both datasets compiled into a final  
259 list of interaction candidates (Table 4, Supplemental Table 2).

260 This resulted in 42 interaction candidates for RTNLB3 and 57 for RTNLB6. Proteins were  
261 again ranked according to the quantity of peptide present in the total spectra. Interestingly  
262 proteins that were identified by FRET-FLIM to be false-positives in the first MS run (FLA8,  
263 ANNAT4 and TRX3) were not present in the second MS dataset thereby confirming and  
264 validating the FRET-FLIM methodology (Table 2). The final list of interaction candidates  
265 comprises furthermore a high percentage of proteins localised or predicted to be localised to  
266 PD and ER (Figure 4).

267  
268  
269 **Discussion**

270  
271 *Validation of the proteomics approach*  
272 Immunoprecipitation using the camelid GFP-Trap system with two of the PD proteome  
273 reticulons, RTNLB3 and RTNLB6 (Fernandez-Calvino et al., 2011; Knox et al., 2015),  
274 identified a high percentage of PD-localised proteins and also proteins that are more widely

275 distributed over the ER and PM. These proteomics data were validated by *in vivo* testing with  
276 FRET-FLIM, and further rounds of immunoprecipitation using different controls confirmed the  
277 initial data and removed the few proteins that did not interact *in vivo*, indicating that these  
278 were most likely false positives. The following points are stressed:

279  
280 a) FRET-FLIM analysis corresponded with the second mass spec run: In the FRET-FLIM  
281 analysis for both RTNLB3 and RTNLB6 FLA8, ANNAT4 and TRX3 did not show a decreased  
282 fluorescence lifetime (Table 3, Figure 3). These proteins were absent from the second MS data  
283 set for both reticulons and were therefore most likely false-positives in the first MS run.  
284 Additionally FLA8, ANNAT4 and TRX3 showed low peptide abundances in the MS spectra  
285 (Table 2) indicating a higher rate of false positives in the lower ranges of abundance.

286  
287 b) TCP1 was pulled down with RTNLB3 but not with RTNLB6 (Table 3, Figure 3) and indeed  
288 FRET-FLIM analysis showed interaction of TCP1 with RTNLB3 but not RTNLB6 validating  
289 both the proteomics data and the FRET-FLIM approach as a confirmatory method.

290  
291 c) Selectivity of reticulon protein-protein interactions: Out of 21 Arabidopsis reticulons, some of  
292 which have been shown to interact previously (Sparkes et al., 2010), only RTNLB3, RTNLB6,  
293 RTNLB5 and RTNLB1 showed up as interactors in the immunoprecipitation analysis with the  
294 bait PD reticulons RTNLB3 and RTNLB6 (Table 4, Supplemental Table 2). RTNLB5 is 84%  
295 identical at the amino acid level with RTNLB6 and therefore difficult to distinguish. However,  
296 the MS analysis revealed peptides unique to RTNLB5 and not RTNLB6, indicating that  
297 RTNLB5 was indeed detected. The role of this potential RTNLB5 interaction is unclear as  
298 RTNLB5 is mainly expressed in pollen (Arabidopsis eFP Browser, Winter et al., 2007) and  
299 involved in the karrikin response (Nelson et al., 2010). Thus, it is likely that RTNLB3 and  
300 RTNLB6 interact with each other in PD and are involved in the generation of the extremely  
301 fine ER-derived desmotubule (Knox et al., 2015). RTNLB1 is ubiquitously expressed in  
302 different tissues and developmental stages (Arabidopsis eFP Browser, Winter et al., 2007).  
303 Interestingly, it has been shown that a Serine-rich region in the N-terminal tail of RTNLB1, and  
304 also RTNLB2, interacts with the FLAGELLIN-SENSITIVE2 (FLS2) receptor (Lee et al., 2011).  
305 The double mutant *rtnlb1/rtnlb2*, as well as an RTNLB1-overexpressor, displayed reduced  
306 FLS2-dependent signalling and enhanced susceptibility to pathogen attacks (Lee et al., 2011).  
307 RTNLB1 and RTNLB2 may regulate FLS2-transport to the plasma membrane. FLS2 is  
308 localised at the plasma membrane, but also within PD (Monaghan and Zipfel, 2012), and may  
309 mediate the *flg22*-induced closure of PD (Faulkner et al., 2013).

310

311 d) Preference for PD and ER-localisation of the interaction candidates: The  
312 immunoprecipitation experiments identified several proteins present in the PD proteome,  
313 suggesting that RTNLB3 and RTNLB6 may be part of a protein complex within PD. We also  
314 found a number of PM-specific proteins that interacted with RTNLB3 and RTNLB6. Some of  
315 these proteins have a role in anchoring the ER to the PM. For example, SYTA is prevalent at  
316 ER-PM contact points in both animal cells (Giordano et al., 2013; Lin et al., 2014) and also in  
317 plant cells (Schapire et al., 2008; Yamazaki et al., 2010; Lewis and Lazarowitz, 2010;  
318 Uchiyama et al., 2014). Perez-Sancho et al. (2015) have suggested that SYTA on the PM may  
319 link the PM to the cortical ER conferring mechanotolerance at these points. However, they did  
320 not identify the interacting ER protein. Our current work suggests that RTNLBs on the cortical  
321 ER may perform such a linking function through a direct interaction with SYTA on the PM.

322  
323 Another protein prevalent at ER-PM contact sites is VAP27 (Wang et al., 2014), also identified  
324 here as an interacting partner of RTNLB3 and RTNLB6. Recent studies suggest that a unique  
325 complex of proteins resides at such ER-PM contacts. VAP27 can bind microtubules and  
326 RTNLBs (current study), and also NET3c (Wang et al., 2014), a protein that links the actin  
327 cytoskeleton to the ER contacts. This protein complex may perform unique functions in  
328 anchoring and signalling between ER and PM (Wang et al., 2014). We suggest that the same  
329 complex may also function to anchor the desmotubule to the PM within or at the neck of the  
330 PD, perhaps explaining their prevalence in the PD proteome. SYTA is a  $\text{Ca}^{2+}$ -sensitive  
331 contractile protein (Yamazaki et al., 2010) that in the contracted form reduces the distance  
332 between adjacent membrane bi-layers to about 5 nm (Lin et al., 2014). PD closure is acutely  
333 sensitive to elevated  $\text{Ca}^{2+}$  levels (Tucker and Boss, 1996) and SYTA therefore emerges as a  
334 potential candidate for forcing the desmotubule and PM together upon  $\text{Ca}^{2+}$  influx.  
335 Significantly, like RTNLB3 and RTNLB6, SYTA appears in developing primary PD during cell-  
336 plate formation (Schapire et al., 2008) and remains associated with the entrances of mature PD (Schapire et  
337 al., 2008).

338  
339 A number of the PD proteins that we found to interact with RTNLB3 and RTNLB6 are also the  
340 targets of viral MPs. These include SYTA (Lewis and Lazarowitz; Uchiyama et al., 2014),  
341 VAP27 (Carette et al., 2002), and the remorin proteins, remorin 1.2 and remorin 1.3 (Borner et  
342 al., 2005; Marin et al., 2012). A recent study (Levy et al., 2015) demonstrated that SYTA forms  
343 ER-PM junctions that are specifically recruited to PD during virus movement. Thus, proteins  
344 associated with the ER-PM contacts may be the specific targets of MPs during cell-cell  
345 movement. The association of these proteins with PD may provide a mechanism for targeting  
346 and concentrating viral genomes assembled on the actin-ER network and subsequently  
347 recruited to the entrances of PD (Tilsner et al., 2013; Levy et al., 2015). The PM intrinsic

348 protein, PIP3, functions as an aquaporin and is induced by salt-stress (Hachez et al., 2014a).  
349 The correct delivery of PIP3 to the PM involves specific interactions with two syntaxin proteins,  
350 SYP61 and SYP121 (Hachez et al., 2014b). PIP3 is also present in the PD proteome  
351 (Fernandez-Calvino et al., 2011), and via an interaction with RTNLBs may provide an  
352 additional link between the desmotubule and PM. TCP1 was pulled down only with RTNLB3  
353 but not RTNLB6 and interacted only with RTNLB3 in FRET-FLIM assays. The TCP1 protein is  
354 part of a chaperonin complex involved in transcription factor trafficking through PD (Xu et al.,  
355 2011). One protein of this complex, CCT8, was shown recently to be required for Knotted1  
356 trafficking through PD and is a target of the viral MP of TMV (Fichtenbauer et al., 2012). It  
357 appears that the entire chaperonin complex may be recruited for cell-cell trafficking (Xu et al.,  
358 2011). Conceivably, RTNLB3 provides a means of linking this complex to PD for the cell-cell  
359 movement of transcription factors.

360  
361 In addition to proteins present at ER-PM contacts, our data reveal a number of PD proteins  
362 associated with lipid-rich domains in plants (Tapken and Murphy, 2015). This finding is in  
363 agreement with the view that PD are rich in lipid components (Naulin et al., 2014; Grison et al.,  
364 2015) and may function as unique lipid-rafts (Mongrand et al., 2010), perhaps involved in  
365 receptor-mediated signalling (Faulkner, 2013). The PM within PD is rich in sterols and  
366 sphingolipids relative to the general PM (Naulin et al., 2015). The PD-localised protein sterol  
367 methyltransferase SMT1 (AT5G13710) controls cholesterol levels (Diener et al., 2000), while  
368 the remorin proteins that interact with RTNLB3 and RTNLB6 are components of lipid rafts  
369 (Mongrand et al., 2010). Remorin 1.3 (REM1.3, AT2G45820) has been localised to PD *in*  
370 *planta* (Raffaele et al., 2009) and is differentially phosphorylated upon contact with bacterial  
371 elicitors. It may function as a scaffold protein in plant innate immunity (Benschop et al., 2007;  
372 Jarsch and Ott, 2011). The tomato REM1.3 is required for the restriction of potato virus X  
373 (PVX) trafficking (Perraki et al., 2012), while the potato Remorin1.3 affects the ability of the  
374 Triple Gene Block 1 (TGBp1) MP of PVX and other viral MPs to increase PD permeability  
375 (Perraki et al., 2014). Several remorins, including Arabidopsis REM1.3, form non-amyloid  
376 filamentous structures of 5.7 to 8.0 nm (Bariola et al., 2004; Marin et al., 2012). These  
377 remorins could be linked with the cytoskeleton in superstructures to maintain cell integrity, or  
378 act as scaffold proteins for signalling and defence mechanisms (Bariola et al., 2004), a  
379 process that might occur in combination with the structural RTNLB proteins.

380  
381 *Additional interacting proteins*  
382 DWARF1 (DWF1, AT3G19820) is a Ca<sup>2+</sup>-dependent calmodulin-binding protein involved in  
383 the conversion of the early brassinosteroid precursor 24-methylenecholesterol to campesterol.  
384 As brassinosteroids affect cellular elongation *dwf1* mutants display a dwarf phenotype due to

385 reduced cell expansion. Superroot 2 (SUR2, AT4G31500), catalyses the conversion of indole-  
386 3-acetaldoxime to indole-3-thiohydroximate in indole glucosinolate biosynthesis (Barlier et al.,  
387 200; Bak et al., 2001) and was found here to interact with both RTNLB3 and RTNLB6. The  
388 biologically active degradation products of glucosinolates are formed under tissue disruption  
389 and are well known as the characteristic flavour compounds in mustard or cabbage (reviewed  
390 in Glawischnig et al., 2003). This could potentially link RTNLBs with defence mechanisms.  
391 DEFECTIVE GLYCOSYLATION (DGL1, AT5G66680) is a subunit of the ER  
392 oligosaccharyltransferase complex (Lerouxel et al., 2005). This protein complex is responsible  
393 for the transfer of N-linked glycan precursors onto Asn residues of candidate proteins in the  
394 ER. N-glycan synthesis pathways contribute to plant development as well as defence. The  
395 mutant *dgl1-1* displays developmental defects including reduced cell elongation and  
396 differentiation defects together with changes in the non-cellulosic matrix polysaccharides  
397 (Lerouxel et al., 2005).

398

399

#### 400 **Conclusions**

401

402 Our combined experimental approach of using sensitive pulldown assays coupled with FRET-  
403 FLIM provides a robust means of identifying functional interactions for reticulon proteins. The  
404 primary MS dataset was validated using FRET-FLIM and showed that more than 80% of the  
405 candidate proteins were indeed interacting with the reticulons. The intermediate dataset was  
406 confirmed by a second set of proteomics data for both reticulons and confirmed both the  
407 proteomics as well as in particular the FRET-FLIM analysis indicating a high confidence for  
408 the final protein interactome.

409

410 Using two RTNLB proteins as bait, we have highlighted a significant number of PD proteins  
411 that interact with RTNLB3 and RTNLB6. We identified predominantly proteins associated with  
412 ER-PM contacts, proteins resident in lipid rafts and proteins that interact with viral MPs. These  
413 interaction studies will form the basis for future research aimed at unravelling the PD  
414 interactome. It will be interesting to determine which of these interactions are significant in  
415 regulating PD functions, such as the gating response that occurs during viral infection (Oparka  
416 et al., 1997).

417 **Materials & Methods**

418  
419 *Immunoprecipitation (GFP-Trap®\_A beads)*

420 Plant material for immunoprecipitation with the GFP-Trap®\_A beads (Chromotek, Martinsried,  
421 Germany) was prepared according to the company's protocol with slight modifications.

422 In brief, approximately 5 g of whole seedling plant material grown for 2 weeks on MS plates  
423 were ground in liquid nitrogen and in lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl; 0.5  
424 mM EDTA, 0.5% NP-40, 1mM PMSF, protease inhibitor). The extracts were incubated on ice  
425 for 30 min and then centrifuged at 10,000g for 10 min at 4°C. The supernatant (about 2-3 ml)  
426 was poured into fresh tubes via 2 layers of muslin cloth.

427 The GFP-Trap®\_A beads were equilibrated in 500µl dilution buffer (10 mM Tris-HCl pH 7.5,  
428 150 mM NaCl, 0.5 mM EDTA) and centrifuged at 2,500 g for 2 min. The supernatant is being  
429 discarded and this wash is repeated twice.

430 100 µl of the washed beads were added to the plant extract and the mixture was shaken on  
431 ice for 2 hours. After this, tubes were centrifuged at 2,500 g for 2 min at 4°C, the supernatant  
432 discarded and the resulting agarose pellet was washed twice with dilution buffer.

433  
434 *Mass spectrometry (nanoLC-ESI-MS/MS) analysis*

435 Reversed phase chromatography was used to separate tryptic peptides prior to MS analysis.  
436 Two columns were utilised, an Acclaim PepMap µ-precolumn cartridge 300 µm i.d. x 5 mm 5  
437 µm 100 Å and an Acclaim PepMap RSLC 75 µm x 50 cm 2 µm 100 Å (Thermo Scientific). The  
438 columns were installed on an Ultimate 3000 RSLCnano system (Dionex). Mobile phase buffer  
439 A was composed of 0.1% aqueous formic acid and mobile phase B was composed of  
440 acetonitrile containing 0.1% formic acid. Samples were loaded onto the µ-precolumn  
441 equilibrated in 2% aqueous acetonitrile containing 0.1% trifluoroacetic acid for 8 min at 10 µL  
442 min<sup>-1</sup> after which peptides were eluted onto the analytical column by increasing the mobile  
443 phase B concentration from 3% B to 35% over 87 min then to 90% B over 5 min, followed by a  
444 4 min wash at 90% B and a 15 min re-equilibration at 3% B.

445 Eluting peptides were converted to gas-phase ions by means of electrospray ionization and  
446 analysed on a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo Scientific). Survey scans of  
447 peptide precursors from 350 to 1500 *m/z* were performed at 120K resolution (at 200 *m/z*) with  
448 a  $4 \times 10^5$  ion count target. Tandem MS was performed by isolation at 1.6 Th using the  
449 quadrupole, HCD fragmentation with normalized collision energy of 35, and rapid scan MS  
450 analysis in the ion trap. The MS<sup>2</sup> ion count target was set to  $10^4$  and the max injection time  
451 was 200 ms. Precursors with charge state 2–7 were selected and sampled for MS<sup>2</sup>. The  
452 dynamic exclusion duration was set to 45 s with a 10 ppm tolerance around the selected

453 precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument  
454 was run in top speed mode with 3 s cycles.

455

#### 456 *Mass spectrometry data analysis*

457 Raw data was processed using MSConvert in ProteoWizard Toolkit (version 3.0.5759,  
458 Kessner et al., 2008). MS<sup>2</sup> spectra were searched with Mascot engine (Matrix Science,  
459 version 2.4.1) Mascot was set up to search the ArabidopsisTAIR10 database (version  
460 20101214, 35508 entries) assuming the digestion enzyme trypsin. Mascot was searched with  
461 a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 20 PPM.  
462 Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Oxidation of  
463 methionine was specified in Mascot as a variable modification.

464 Scaffold (version Scaffold\_4.4.1.1, Proteome Software Inc., Portland, OR) was used to  
465 validate MS/MS based peptide and protein identifications. Peptide identifications were  
466 accepted if they could be established at greater than 95.0% probability by the Scaffold Local  
467 FDR algorithm. Protein identifications were accepted if they could be established at greater  
468 than 99.0% probability and contained at least two identified peptides. Protein probabilities  
469 were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that  
470 contained similar peptides and could not be differentiated based on MS/MS analysis alone  
471 were grouped to satisfy the principles of parsimony.

472

#### 473 *Cloning of expression plasmids*

474 Primers were obtained from MWG Biotech. Q5 high-fidelity DNA polymerase (New England  
475 Biolabs) was used for all polymerase chain reaction reactions. Vectors containing the genes of  
476 interest from the proteomics dataset were obtained from NASC (Scholl et al., 2000). Genes of  
477 interest were cloned into the modified binary vectors pB7FWG2,0 or pB7WGR2,0 clone  
478 providing expression from *Agrobacterium* T-DNA, using the cauliflower mosaic virus 35S  
479 promoter upstream of coding fusions to green fluorescent protein (GFP) or red fluorescent  
480 protein (RFP), respectively (Karimi et al., 2005).

481

#### 482 *Tobacco plant material and transient expression in tobacco leaves*

483 For *Agrobacterium*-mediated transient expression, 5-week-old tobacco (*Nicotiana tabacum*  
484 SR1 cv Petit Havana) plants grown in the greenhouse were used. Transient expression was  
485 induced and detected according to Sparkes et al. (2006). In brief, each expression vector was  
486 introduced into *Agrobacterium* strain GV3101 by heat shock. Transformants were inoculated  
487 into 5 ml of YEB medium (5 g/l beef extract, 1 g/l yeast extract, 5 g/l sucrose and 0.5 g/l of  
488 MgSO<sub>4</sub> · 7H<sub>2</sub>O) supplemented with the antibiotics for the vector and rifampicin to select for  
489 agrobacteria. After overnight shaking at 25°C, 1 ml of the bacterial culture was pelleted by



490 centrifugation at 2,500×g for 5 min at room temperature. The pellet was washed twice with 1  
491 ml of infiltration medium (50 mM MES, 2 mM Na<sub>3</sub>PO<sub>4</sub> · 12H<sub>2</sub>O, 0.1 mM acetosyringone and 5  
492 mg/ml glucose) and then resuspended in 1 ml of infiltration buffer. The suspension was diluted  
493 to a final OD<sub>600</sub> of 0.1 and gently pressed through the stomata on the lower epidermal surface  
494 using a 1 ml syringe. Transformed plants then were incubated under normal growth conditions  
495 for 48 to 72 h. Images were taken using a Zeiss LSM510 Meta laser scanning confocal  
496 microscope with a 63x oil immersion objective. For imaging of GFP/RFP combinations,  
497 samples were excited using 488 and 543 nm laser lines in multi-track mode with line  
498 switching. Images were edited using the LSM510 image browser.

499

#### 500 *FRET-FLIM data acquisition*

501 Epidermal samples of infiltrated tobacco leaves were excised, and FRET-FLIM data capture  
502 was performed according to Osterrieder et al. (2009) and Schoberer and Botchway (2014)  
503 using a two-photon microscope at the Central Laser Facility of the Rutherford Appleton  
504 Laboratory. In brief, a two-photon microscope built around a Nikon TE2000-U inverted  
505 microscope was used with a modified Nikon EC2 confocal scanning system to allow for  
506 multiphoton FLIM, Botchway et al. (2015). Laser light at a wavelength of 920 nm was  
507 produced by a mode-locked titanium sapphire laser (Mira; Coherent Lasers), producing 200-fs  
508 pulses at 76 MHz, pumped by a solid-state continuous wave 532-nm laser (Verdi V18;  
509 Coherent Laser). The laser beam was focused to a diffraction-limited spot through a water  
510 immersion objective (Nikon VC x60, numerical aperture of 1.2) to illuminate specimens at the  
511 microscope stage. Fluorescence emission was collected without descanning, bypassing the  
512 scanning system, and passed through a BG39 (Comar) filter to block the near infrared laser  
513 light. Line, frame, and pixel clock signals were generated and synchronized with an external  
514 detector in form of a fast microchannel plate photomultiplier tube (MCP-PMT; Hamamatsu  
515 R3809U). Linking these via a time-correlated single-photon-counting PC module SPC830  
516 (Becker and Hickl) generated the raw FLIM data. Prior to FLIM data collection, the GFP and  
517 mRFP expression levels in the plant samples within the region of interest were confirmed  
518 using a Nikon EC2 confocal microscope with excitation at 488 and 543 nm, respectively. A  
519 633-nm interference filter was used to significantly minimize the contaminating effect of  
520 chlorophyll autofluorescence emission that would otherwise obscure the mRFP emission as  
521 well as that of GFP. Data were analyzed by obtaining excited state lifetime values of a region  
522 of interest on the nucleus, and calculations were made using the SPCImage analysis software  
523 version 5.1 (Becker and Hickl). The distribution of lifetime values within the ROI were  
524 generated and displayed as a curve. Only values that had a  $\chi^2$  between 0.9 and 1.4 were  
525 taken. The median lifetime value and minimum and maximum values for a quarter of the

526 median lifetime values from the curve were taken to generate the range of lifetimes per  
527 sample.

528 At least three nuclei from at least three independent biological samples per protein-protein  
529 combination were analyzed, and the average of the ranges taken.

530

531

### 532 **Accession Numbers**

533 Sequence data for genes in this article can be found in GenBank/EMBL databases using the  
534 following accession numbers: RTNLB3, At1g64090 and RTNLB6, At3g61560. All access  
535 numbers from the proteomic analysis can be found in the corresponding tables.

536

537

### 538 **Author Contributions**

539 V.K, K.O, and C.H designed the research. V.K and S.S performed the research. V.K, S.B, S.S,  
540 K.O and C.H analysed the data. All authors contributed to the writing of the paper.

541

542 **Tables**

543

544 **Table 1:** Analysis steps and number of proteins derived from mass spec analysis.

<u>Analysis Step</u>	<u>N° of proteins</u>
Total (RTNLB3/RTNLB6)	706 (613/621)
Minus WT control total (RTNLB3/RTNLB6)	146 (135/126)
Proteins subjected to FRET-FLIM analysis	17

545

546

547 **Table 2:** Flowchart of the 17 proteins tested by FRET-FLIM with protein description (column 1)  
 548 and accession number (column 2) indicating their % abundance in the total spectrum for  
 549 RTNLB3 (column 3) or RTNLB6 (column 4), respectively. Proteins present in the PD proteome  
 550 (Fernandez-Calvino et al., 2011) are marked with an asterisk. Positive (+) or negative (-)  
 551 results for interaction in the FRET-FLIM analysis with either RTNLB3 (column 5) or RTNLB6  
 552 (column 6) are shown. The re-appearance of the corresponding protein in the second MS run  
 553 is shown (RTNLB3-2 and RTNLB6-2, column 7 and 8).

Proteins	Accession No	% in total spectra		FRET-FLIM		present in 2nd MS run	
		RTNLB3-1	RTNLB6 -1	RTNLB3-1	RTNLB6 -1	RTNLB3-2	RTNLB6 -2
Reticular like protein RTNLB3 *	AT1G64090.1	0.042%	0.036%	+	+	+	+
Reticulon family protein RTNLB6 *	AT3G61560.1	0.019%	0.170%	+	+	+	+
SYTA, SYT1   synaptotagmin A *	AT2G20990.1	0.032%	0.081%	+	+	+	+
Root hair defective 3 GTP-binding protein (RHD3)	AT3G13870.1	0.019%	0.052%	+	+	+	+
DWF1, DIM, EVE1, DIM1, CBB1   cell elongation protein	AT3G19820.1	0.023%	0.036%	+	+	+	+
DGL1   dolichyl-diphosphooligosaccharide-protein glycosyltransferase *	AT5G66680.1	0.013%	0.023%	+	+	+	+
ATB5-B, B5 #3, ATCB5-D, CB5-D   cytochrome B5 isoform D	AT5G48810.1	0.013%	0.019%	+	+	+	+
SMT1, CPH   sterol methyltransferase 1 *	AT5G13710.1	0.003%	0.013%	+	+	+	+
CYP83B1, SUR2, RNT1, RED1, ATR4   cytochrome P450, family 83	AT4G31500.1	0.010%	0.013%	+	+	+	+
PIP3, PIP3A, PIP2;7, SIMIP   plasma membrane intrinsic protein 3 *	AT4G35100.1	0.010%	0.013%	+	+	+	+
Remorin family protein REM1.3 *	AT2G45820.1	0.010%	0.010%	+	+	+	+
Remorin family protein REM1.2 *	AT3G61260.1	0.013%	0.010%	+	+	+	+
FLA8, AGP8   FASCICLIN-like arabinogalactan protein 8 *	AT2G45470.1	0.007%	0.010%	-	-	-	-
VAP27-1, VAP, (AT)VAP, VAP27   vesicle associated protein	AT3G60600.1	0.007%	0.007%	+	+	+	+
ANNAT4   annexin 4 *	AT2G38750.1	0.010%	0.007%	-	-	-	-
ATTRX3, ATH3, ATTRXH3, TRXH3, TRX3   thioredoxin 3 *	AT5G42980.1	0.007%	0.003%	-	-	-	-
TCP-1/cpn60 chaperonin family protein *	AT3G03960.1	0.013%	0.000%	+	-	+	-

554

555

556

557 **Table 3:** Fluorescent lifetimes in FRET-FLIM analysis. Donor and acceptor protein constructs  
 558 are indicated together with the average fluorescent lifetime in ns for the donor fluorophore and  
 559 the standard deviation for each combination. It was previously shown that a reduction in  
 560 excited state lifetime of 200 ps is indicative to energy transfer (Stubbs et al., 2005). For each  
 561 combination at least two biological samples with a minimum of three technical replicates were  
 562 used for the statistical analysis.

Donor (GFP)	Acceptor (mRFP)	Average [ns]	Std error	Donor (GFP)	Acceptor (mRFP)	Average [ns]	Std error		
RTNLB3	+	(-)	2.51	0.05	RTNLB6	+	(-)	2.63	0.06
RTNLB3	+	RTNLB3	2.28	0.01	RTNLB6	+	RTNLB3	2.37	0.04
RTNLB3	+	RTNLB6	2.31	0.01	RTNLB6	+	RTNLB6	2.38	0.01
RTNLB3	+	SYTA	2.29	0.04	RTNLB6	+	SYTA	2.46	0.02
RTNLB3	+	RHD3	2.30	0.01	RTNLB6	+	RHD3	2.34	0.01
RTNLB3	+	DWF1	2.28	0.01	RTNLB6	+	DWF1	2.32	0.01
RTNLB3	+	DGL1	2.37	0.01	RTNLB6	+	DGL1	2.37	0.07
RTNLB3	+	Cyb5D	2.30	0.01	RTNLB6	+	Cyb5D	2.47	0.02
RTNLB3	+	SMT1	2.29	0.03	RTNLB6	+	SMT1	2.36	0.07
RTNLB3	+	SUR2	2.28	0.06	RTNLB6	+	SUR2	2.34	0.11
RTNLB3	+	PIP3	2.37	0.02	RTNLB6	+	PIP3	2.35	0.04
RTNLB3	+	REM1.3	2.33	0.02	RTNLB6	+	REM1.3	2.44	0.05
RTNLB3	+	REM1.2	2.31	0.01	RTNLB6	+	REM1.2	2.48	0.01
RTNLB3	+	FLA8	2.53	0.09	RTNLB6	+	FLA8	2.67	0.07
RTNLB3	+	Vap27	2.33	0.03	RTNLB6	+	Vap27	2.33	0.04
RTNLB3	+	ANNAT4	2.50	0.03	RTNLB6	+	ANNAT4	2.66	0.03
RTNLB3	+	TRX3	2.48	0.03	RTNLB6	+	TRX3	2.62	0.06
RTNLB3	+	TCP1	2.32	0.01	RTNLB6	+	TCP1	2.60	0.02

563

564

565 **Table 4:** List of interacting proteins for RTNLB3 (top) and RTNLB6 (bottom), respectively,  
566 present in both MS datasets. The protein name, accession number and molecular weight  
567 (MW) are given. The percentage of the total spectra in both MS datasets (MS run1 and 2) is  
568 given as well as known or predicted subcellular localisation for plasmodesmata (PD, yellow),  
569 ER (blue), plasma membrane (PM, green) or cell plate (purple). More detail for the interacting  
570 proteins can be found in Supplemental Table 2.

RTNLB3: interacting proteins	Accession number	MW	MS run1	MS run2	Subcellular localisation			
					RTN3-1	RTN3-2	PD	ER
SYTA, NTMC2TYPE1.1, ATSYTA, NTMC2T1.1, SYT1 synaptotagmin A	AT2G20990.1	62 kDa	0.066%	0.032%	+			+
RTNLB3 Reticulan like protein B3	AT1G64090.2	31 kDa	0.058%	0.039%	+	+		
RTNLB3 Reticulan like protein B3	AT1G64090.1	29 kDa	0.042%	0.036%	+	+		
SYTA synaptotagmin A, SYT1	AT2G20990.3	66 kDa	0.032%	0.081%	+			+
ATC4H, C4H, CYP73A5, REF3 cinnamate-4-hydroxylase	AT2G30490.1	58 kDa	0.029%	0.049%	+	+		
ERD4 Early-responsive to dehydration stress protein (ERD4)	AT1G30360.1	82 kDa	0.026%	0.013%	+			+
DWF1, DIM, EVE1, DIM1, CBB1 cell elongation protein	AT3G19820.1	65 kDa	0.023%	0.036%	+	+		
Reticulon family protein RTNLB6	AT3G61560.1	29 kDa	0.019%	0.170%	+	+		
RHD3 Root hair defective 3 GTP-binding protein	AT3G13870.1	89 kDa	0.019%	0.052%		+		
ATB5-A, B5 #2, ATCB5-E, CB5-E cytochrome B5 isoform E	AT5G53560.1	15 kDa	0.019%	0.026%		+		
LACS4 AMP-dependent synthetase and ligase family protein	AT4G23850.1	75 kDa	0.016%	0.019%				+
DGL1 dolichyl-diphosphooligosaccharide-protein glycosyltransferase	AT5G66680.1	49 kDa	0.013%	0.023%	+	+		+
CYP71B7 cytochrome P450, family 71 subfamily B, polypeptide 7	AT1G13110.1	57 kDa	0.013%	0.019%				+
ATB5-B, B5 #3, ATCB5-D, CB5-D cytochrome B5 isoform D	AT5G48810.1	15 kDa	0.013%	0.019%		+		
Remorin family protein	AT3G61260.1	23 kDa	0.013%	0.010%	+			+
ADL1, ADL1A, AG68, DRP1A, RSW9, DL1 dynamin-like protein	AT5G42080.1	68 kDa	0.013%	0.010%				+
TCP-1/cpn60 chaperonin family protein	AT3G03960.1	59 kDa	0.013%	0.010%	+			
ATRA11A, ATRABA2C, ATRAB-A2C, RAB-A2C	AT3G46830.1	24 kDa	0.010%	0.026%				+
AT3G11560.1 catalytics	AT5G11560.1	109 kDa	0.010%	0.016%				+
CYP83B1, SUR2, RNT1, RED1, ATR4 cytochrome P450, family 83	AT4G31500.1	57 kDa	0.010%	0.013%		+		
PIP3, PIP3A, PIP2.7, SIMIP plasma membrane intrinsic protein 3	AT4G35100.1	30 kDa	0.010%	0.013%	+			+
ATCBR, CBR1, CBR NADH:cytochrome B5 reductase 1	AT5G17770.1	31 kDa	0.010%	0.013%		+		+
Remorin family protein	AT2G45820.1	21 kDa	0.010%	0.010%	+			+
unknown protein, protein family UPF0121	AT3G02420.1	40 kDa	0.010%	0.010%	+	+		
SOUL-1 AthBP2/ SOUL heme-binding family protein	AT2G37970.1	25 kDa	0.010%	0.007%				
SMT2, CVP1, FRL1 sterol methyltransferase 2	AT1G20330.1	40 kDa	0.007%	0.023%		+		
BT1, RTNLB1 VIRB2-interacting protein 1	AT4G23630.1	31 kDa	0.007%	0.019%		+		
ATJ3, ATJ DNAJ homologue 3	AT3G44110.1	46 kDa	0.007%	0.013%	+			
FAH1, CYP84A1 ferulic acid 5-hydroxylase 1	AT4G36220.1	59 kDa	0.007%	0.010%		+		
VAP27-1, VAP, (AT)VAP, VAP27 vesicle associated protein	AT3G60600.1	28 kDa	0.007%	0.003%		+		
SHD, HSP90.7, AtHsp90.7, AtHsp90-7 Chaperone protein htpG family protein	AT4G24190.1	94 kDa	0.007%	0.003%		+		
Ribosomal protein S8e family protein	AT5G20290.1	25 kDa	0.007%	0.003%		+		
NTMC2TYPE4, NTMC2T4 Calcium-dependent lipid-binding family protein	AT3G61050.1	55 kDa	0.003%	0.029%		+		+
Reticulon family protein RTNLB5	AT2G46170.1	29 kDa	0.003%	0.023%		+		
RHD4 Phosphoinositide phosphatase family protein	AT3G51460.1	68 kDa	0.003%	0.016%	+	+		+
GPAT8, AtGPAT8 glycerol-3-phosphate acyltransferase 8	AT4G00400.1	56 kDa	0.003%	0.016%		+		
Endomembrane protein 70 protein family	AT5G25100.1	74 kDa	0.003%	0.016%				+
SMT1, CPH sterol methyltransferase 1	AT5G13710.1	38 kDa	0.003%	0.013%	+	+		
RAB11, ATRABA1B, RABA1b RAB GTPase homolog A1B	AT1G16920.1	24 kDa	0.003%	0.007%				+
Ribophorin I	AT2G01720.1	52 kDa	0.003%	0.007%		+		+
Endomembrane protein 70 protein family	AT2G01970.1	68 kDa	0.003%	0.007%				+
PHOT1, NPH1, JK224, RPT1 phototropin 1	AT3G45780.1	112 kDa	0.003%	0.007%				+

571

RTNLB6: interacting proteins	Accession number	MW	MS run1	MS run2	Subcellular localisation			
					PD	ER	PM	Cell plate
Reticulon family protein RTNLB6	AT3G61560.1	29 kDa	0.019%	0.170%	+	+		
SYTA, NTMC2TYPE1.1, ATSYTA, NTMC2T1.1, SYT1 synaptotagmin A	AT2G20990.1	62 kDa	0.032%	0.081%	+		+	
SYTA synaptotagmin A, SYT1	AT2G20990.3	66 kDa	0.032%	0.081%	+	+		
RHD3 Root hair defective 3 GTP-binding protein	AT3G13870.1	89 kDa	0.019%	0.052%		+		
ATC4H, C4H, CYP73A5, REF3 cinnamate-4-hydroxylase	AT2G30490.1	58 kDa	0.029%	0.049%	+	+		
FUNCTIONS IN: molecular_function unknown	AT2G32240.1	?	0.052%	0.039%			+	
RTNLB3 Reticulan like protein B3	AT1G64090.1	29 kDa	0.042%	0.036%	+	+		
DWF1, DIM, EVE1, DIM1, CBB1 cell elongation protein	AT3G19820.1	65 kDa	0.023%	0.036%		+	+	
CCD1, ATCCD1, ATNCED1, NCED1 carotenoid cleavage dioxygenase 1	AT3G63520.1	61 kDa	0.023%	0.029%	+		+	
NTMC2TYPE4, NTMC2T4 Calcium-dependent lipid-binding family protein	AT3G61050.1	55 kDa	0.003%	0.029%		+	+	
ALDH3F1 aldehyde dehydrogenase 3F1	AT4G36250.1	54 kDa	0.000%	0.029%		+		
DGL1 dolichyl-diphosphooligosaccharide-protein glycosyltransferase	AT5G66680.1	49 kDa	0.013%	0.023%	+	+	+	
SMT2, CVP1, FRL1 sterol methyltransferase 2	AT1G20330.1	40 kDa	0.007%	0.023%		+		
Reticulon family protein RTNLB3	AT2G46170.1	29 kDa	0.003%	0.023%		+		
LACS4 AMP-dependent synthetase and ligase family protein	AT4G23850.1	75 kDa	0.016%	0.019%			+	
CYP71B7 cytochrome P450, family 71 subfamily B, polypeptide 7	AT1G13110.1	57 kDa	0.013%	0.019%			+	
ATB5-B, B5 #3, ATCB5-D, CB5-D cytochrome B5 isoform D	AT5G48810.1	15 kDa	0.013%	0.019%		+		
BT11, RTNLB1 VIRB2-interacting protein 1	AT4G23630.1	31 kDa	0.007%	0.019%		+		
Eukaryotic aspartyl protease family protein	AT1G03220.1	46 kDa	0.032%	0.016%	+		+	
MO1 monooxygenase 1	AT4G15760.1	47 kDa	0.023%	0.016%		+		
APX3 ascorbate peroxidase 3	AT4G35000.1	32 kDa	0.019%	0.016%		+		
UCC2 uclacyanin 2	AT2G44790.1	20 kDa	0.016%	0.016%	+		+	
catalytics	AT5G11560.1	109 kDa	0.010%	0.016%		+	+	
RHD4 Phosphoinositide phosphatase family protein	AT3G51460.1	68 kDa	0.003%	0.016%	+	+	+	
Endomembrane protein 70 protein family	AT5G25100.1	74 kDa	0.003%	0.016%			+	
ERD4 Early-responsive to dehydration stress protein (ERD4)	AT1G30360.1	82 kDa	0.026%	0.013%	+		+	
CYP83B1, SUR2, RNT1, RED1, ATR4 cytochrome P450, family 83	AT4G31500.1	57 kDa	0.010%	0.013%		+		
PIP3, PIP3A, PIP2;7, SIMIP plasma membrane intrinsic protein 3	AT4G35100.1	30 kDa	0.010%	0.013%	+		+	
CYP71B6 cytochrome p450 71b6	AT2G24180.1	57 kDa	0.007%	0.013%		+	+	
Carbohydrate-binding-like fold	AT3G62360.1	133 kDa	0.007%	0.013%		+	+	
ALDH22A1 aldehyde dehydrogenase 22A1	AT3G66658.2	66 kDa	0.007%	0.013%		+		
SMT1, CPH sterol methyltransferase 1	AT5G13710.1	38 kDa	0.003%	0.013%	+	+		
Remorin family protein	AT3G61260.1	23 kDa	0.013%	0.010%	+		+	
Remorin family protein	AT2G45820.1	21 kDa	0.010%	0.010%	+		+	
unknown protein, protein family UPF0121	AT3G02420.1	40 kDa	0.010%	0.010%	+	+		
Protein of unknown function DUF2359, transmembrane	AT1G70770.1	67 kDa	0.007%	0.010%		+	+	
FAH1, CYP84A1 ferulic acid 5-hydroxylase 1	AT4G36620.1	59 kDa	0.007%	0.010%		+		
ATPDIL5-2, ATPDI8, PDI8, PDIL5-2 PDI-like 5-2	AT1G35620.1	50 kDa	0.003%	0.010%	+	+		
Endomembrane protein 70 protein family	AT4G12650.1	74 kDa	0.003%	0.010%	+			
Saccharopine dehydrogenase	AT5G39410.1	50 kDa	0.003%	0.010%			+	
VAP27-1, VAP, (AT)VAP, VAP27 vesicle associated protein	AT3G60600.1	28 kDa	0.007%	0.007%		+		
STL2P, ATSEC12 SEC12P-like 2 protein	AT2G01470.1	43 kDa	0.010%	0.007%		+		
ALDH3H1, ALDH4 aldehyde dehydrogenase 3H1	AT1G44170.1	53 kDa	0.007%	0.007%	+	+		
ATRA11C, ATRABA2A, ATRAB-A2A, RAB-A2A,	AT1G09630.1	24 kDa	0.003%	0.007%			+	+
RAB11, ATRABA1B, RABA1b RAB GTPase homolog A1B	AT1G16920.1	24 kDa	0.003%	0.007%			+	
Endomembrane protein 70 protein family	AT2G01970.1	68 kDa	0.003%	0.007%			+	
Protein of unknown function (DUF3754)	AT3G19340.1	57 kDa	0.003%	0.007%			+	
PHOT1, NPH1, JK224, RPT1 phototropin 1	AT3G45780.1	112 kDa	0.003%	0.007%			+	
Leucine-rich repeat protein kinase family protein	AT5G49760.1	105 kDa	0.003%	0.007%			+	
Oligosaccharyltransferase complex/magnesium transporter family protein	AT1G61790.1	39 kDa	0.000%	0.007%		+	+	
Calcium-dependent phosphotriesterase superfamily protein	AT3G57030.1	41 kDa	0.000%	0.007%		+	+	
SQS1, ERG9 squalene synthase 1	AT4G34640.1	47 kDa	0.000%	0.007%		+	+	
ATPLC2, PLC2 phospholipase C 2	AT3G08510.1	66 kDa	0.010%	0.003%			+	
Clathrin light chain protein	AT2G20760.1	37 kDa	0.007%	0.003%			+	
ATMIN7, BEN1 HOPM interactor 7	AT3G43300.1	195 kDa	0.007%	0.003%			+	
SHD, HSP90.7, AtHsp90.7, AtHsp90-7 Chaperone protein htpG	AT4G24190.1	94 kDa	0.007%	0.003%		+		
ATBAG7, BAG7 BCL-2-associated athanogene 7	AT5G62390.1	52 kDa	0.007%	0.003%		+		

572

573

574 **Figure Legends**

575

576 **Figure 1**

577 Quantitative scatterplot for overlay and distribution of candidate interaction proteins for  
578 RTNLB3 and RTNLB6 (Scaffold\_4.4.1.1 Proteome Software). Each protein is plotted as a  
579 point on a two dimensional scatterplot with the X-axis showing a normalized spectral count for  
580 proteins binding to RTLB3 and the Y-axis for RTNLB6. The Scaffold software shows a line  
581 with a slope of 1 on the graph. Therefore proteins with similar abundances in both co-  
582 immunoprecipitation assays will plot as points near this line. Proteins that plot outside the  
583 indicated dashed lines on the plot are more than two standard deviations away from being the  
584 same in both co-immunoprecipitation. These proteins are considered to be differentially  
585 expressed.

586

587 **Figure 2**

588 FRET-FLIM analysis of RTNLB6 without an interaction partner (**A-D**) or RTNLB6 dimerization  
589 (**E-I**). Images A and E display the raw FRET-FLIM data. In **B** and **F** pseudo-coloured lifetime  
590 maps show the lifetime values for each point within the region of interest while the distribution  
591 of lifetimes across the entire image is shown in **C** and **G** with blue shades representing longer  
592 GFP-fluorescence lifetimes than green ones. Images **D** and **H** display representative decay  
593 curves of a single point with an optimal single exponential fit, where Chi square ( $\chi^2$ ) values  
594 from 0.9 to 1.2 were considered an excellent fit to the data points (binning factor of 2. Images  
595 **A** (inset) and **I** are the respective confocal images for the analysis showing the GFP-construct  
596 in green and the mRFP-construct in red. This example of FRET-FLIM analysis shows that  
597 RTNLB6 homodimerizes because the lifetime values for the GFP/mRFP fusion pair (image H,  
598  $2.38 \pm 0.01$  ns) are lower than those for the GFP fusion alone (image D,  $2.63 \pm 0.06$  ns). White  
599 bar =  $5\mu\text{m}$ .

600

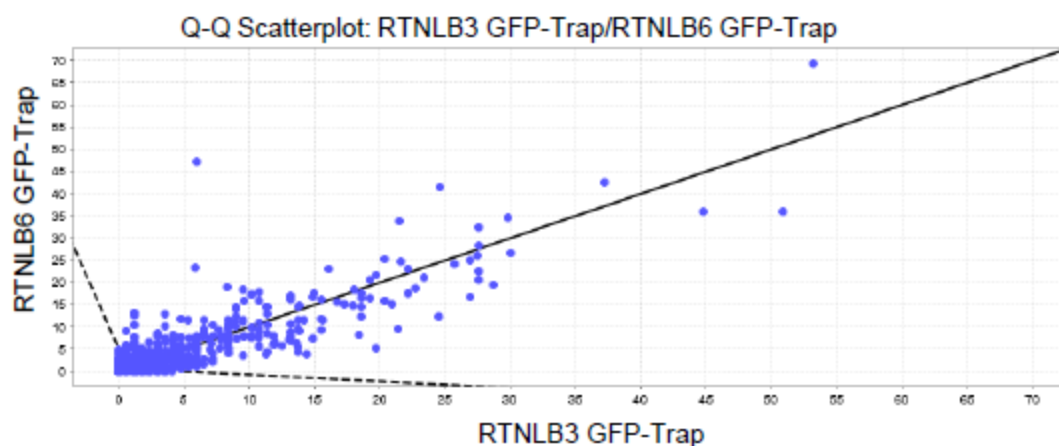
601 **Figure 3**

602 Fluorescent lifetimes in FRET-FLIM interactions. The bar graph represents average  
603 fluorescent lifetimes [ns] and the corresponding standard deviations for the GFP-donors  
604 RTNLB3 and RTNLB6, respectively. The data show 17 candidate interaction proteins (blue  
605 bars) compared to RTNLB3-GFP or RTNLB6-GFP without interaction partners (grey bars).  
606 Lifetimes significantly lower than those of RTNLB3-GFP or RTNLB6-GFP alone (left hand side  
607 of the red line) indicate protein-protein interactions.

608

609 **Figure 4**

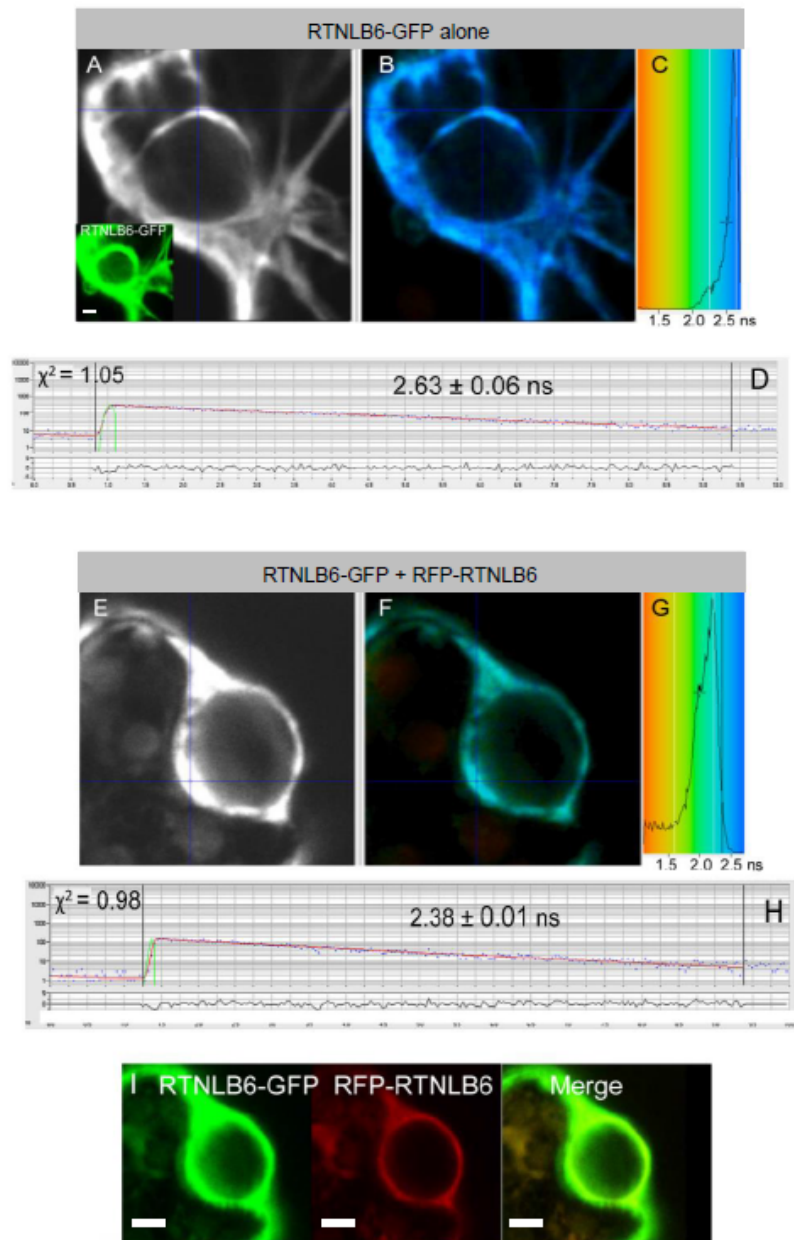
610 Quantitative distribution (%) of predicted or known subcellular localisations for RTNLB3 and  
611 RTNLB6 interaction candidates validated by two MS datasets and FRET-FLIM.



**Figure 1**

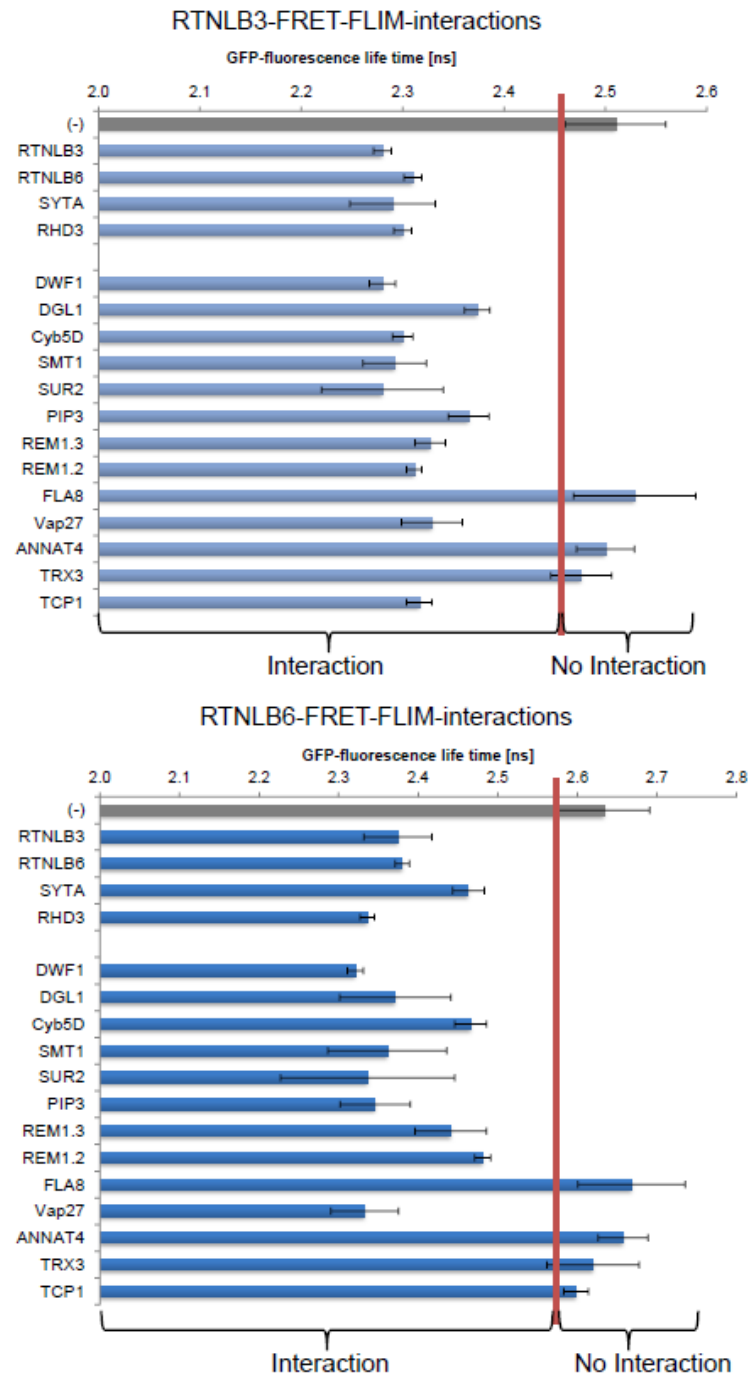
Quantitative scatterplot for overlay and distribution of candidate interaction proteins for RTNLB3 and RTNLB6 (Scaffold\_4.4.1.1 Proteome Software). Each protein is plotted as a point on a two dimensional scatterplot with the X-axis showing a normalized spectral count for proteins binding to RTNLB3 and the Y-axis for RTNLB6. The Scaffold software shows a line with a slope of 1 on the graph. Therefore proteins with similar abundances in both co-immunoprecipitation assays will plot as points near this line. Proteins that plot outside the indicated dashed lines on the plot are more than two standard deviations away from being the same in both co-immunoprecipitation. These proteins are considered to be differentially expressed.





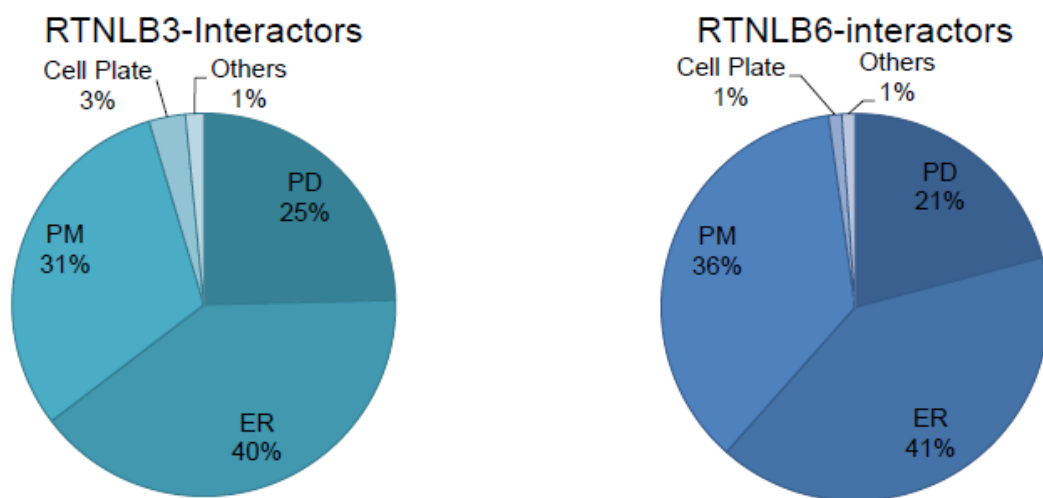
**Figure 2**

FRET-FLIM analysis of RTNLB6 without an interaction partner (A-D) or RTNLB6 dimerization (E-I). Images A and E display the raw FRET-FLIM data. In B and F pseudo-coloured lifetime maps show the lifetime values for each point within the region of interest while the distribution of lifetimes across the entire image is shown in C and G with blue shades representing longer GFP-fluorescence lifetimes than green ones. Images D and H display representative decay curves of a single point with an optimal single exponential fit, where Chi square ( $\chi^2$ ) values from 0.9 to 1.2 were considered an excellent fit to the data points (binning factor of 2). Images A (inset) and I are the respective confocal images for the analysis showing the GFP-construct in green and the mRFP-construct in red. This example of FRET-FLIM analysis shows that RTNLB6 homodimerizes because the lifetime values for the GFP/mRFP fusion pair (image H,  $2.38 \pm 0.01$  ns) are lower than those for the GFP fusion alone (image D,  $2.63 \pm 0.06$  ns). White bar =  $5\mu\text{m}$ .



**Figure 3**

Fluorescent lifetimes in FRET-FLIM interactions. The bar graph represents average fluorescent lifetimes [ns] and the corresponding standard deviations for the GFP-donors RTNLB3 and RTNLB6, respectively. The data show 17 candidate interaction proteins (blue bars) compared to RTNLB3-GFP or RTNLB6-GFP without interaction partners (grey bars). Lifetimes significantly lower than those of RTNLB3-GFP or RTNLB6-GFP alone (left hand side of the red line) indicate protein-protein interactions.



**Figure 4**  
Quantitative distribution (%) of predicted or known subcellular localisations for RTNLB3 and RTNLB6 interaction candidates validated by two MS datasets and FRET-FLIM.

615

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