NETWORKED3B (NET3B): a novel protein involved in the actin cytoskeleton-endoplasmic reticulum interaction.

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Highlight

NET3B, a member of the NETWORKED superfamily, is involved in the ER-actin cytoskeleton interaction in plant cells. Enhanced ER-actin association is observed in cells expressing GFP tagged NET3B at high levels.

Abstract

In plants, the endoplasmic reticulum (ER) moves in an actin cytoskeleton dependent fashion. However, little is known about proteins that link the ER membrane and the actin cytoskeleton. Here, we have identified a novel ER and actin cytoskeleton associated protein *in vivo*, NET3B, which belongs to a super-family of plant specific actin binding proteins (the NETWORKED family). NET3B associates with the actin cytoskeleton *in vivo* through an N-terminal actin binding (NAB) domain that is well characterised for other members of the NET family. However, a three amino acid insertion, VED, in the NAB domain of NET3B, appears to lower the ability of the NET3B to localise to the actin cytoskeleton compared to NET1A, the founding member of the NET family. The C-terminal domain of NET3B links the protein to the ER. Over-expression of NET3B enhanced the association between ER and the actin cytoskeleton, and the extent of the association is dependent on the amount of NET3B available. By essentially 'gluing' the ER to the actin network, another effect was to reduce membrane ER diffusion. In conclusion, our results revealed that NET3B modulates ER and actin cytoskeleton interactions in higher plants.

Key words:

Endoplasmic reticulum, Actin cytoskeleton, NET super-family, Endomembrane system, *N. benthamiana*

Introduction

The remodelling of the endoplasmic reticulum (ER) in plants is mainly dependent on the actin cytoskeleton (Boevink *et al.,* 1998; Sparkes *et al.,* 2009). Recent studies have also suggested a role for microtubules in the slow movement of the ER (Hamada *et al.,* 2014). Although several studies have indicated that certain actin regulatory proteins (such as the Scar/Wave complexes, capping proteins) are localised to the ER membrane (Zhang *et al.,* 2013; Jimenez-Lopez *et al.,* 2014; Wang *et al.,* 2016a), only members of the myosin XI family have been shown to modulate the ER network so far (Sparkes *et al.,* 2009; Yokota *et al.,* 2011). ER dynamics, organelle movement, actin organization and plant development are affected by knocking out myosin expression or by over-expressing myosin dominant negative constructs (Sparkes *et al.,* 2008; Griffing *et al.,* 2014; Peremyslov *et al.,* 2010). Several independent studies have demonstrated that myosin XI-K is enriched in the ER fraction (Ueda *et al.,* 2010), and localized to motile membrane puncta (Peremyslov *et al.,* 2012).

The ER can be divided into two morphological classes: tubular ER and cisternal ER, but the transition between these two types of ER membrane is poorly understood. One major structural component of the ER is the reticulon protein family, a family of integral membrane proteins that are able to induce the formation of ER tubules (Tolley *et al.,* 2008; Sparkes *et al.,* 2010). They consist of four transmembrane domains in a 'W-shaped' organization, and localise predominantly to ER membrane where there is high membrane curvature (Knox *et al.,* 2015; Kriechbaumer *et al.,* 2015; Breeze *et al.,* 2016). The tubular structure of ER is also maintained by a dynamin-like GTPase, atlastin, which is required for network formation and homotypic ER membrane fusion (Hu *et al.,* 2009). The plant homologue of atlastin is RHD3 (Root Hair Defective 3) and there are several similar proteins called RHD3-like proteins (Chen *et al.,* 2011; Zheng *et al.,* 2004). RHD3 interacts with reticulons and ER morphology and Golgi movement are affected when dominant negative constructs are expressed in cells (Lee *et al.,* 2013).

In addition, the structure of cortical ER is also maintained by ER/PM contact sites (EPCS; Wang *et al.,* 2016b). Depletion of ER-PM tethering proteins (such as Scs2, extended-synaptotagmins or Ist2) affects the formation of ECPS and the structure of the cortical ER

network (Siao *et al.,* 2016; Fernández-Busnadiego *et al.,* 2015; Kralt *et al.,* 2015). The EPCS in plants integrates a complex of proteins with the actin and microtubule networks (Wang *et al.,* 2014). This protein complex includes both VAP27 (a homologue of Scs2; Wang *et al.,* 2016c) and NET3C, which localise to the ER membrane and plasma membrane. NET3C belongs to a plant specific actin binding protein superfamily, the NET family (Deeks *et al.,* 2012). NET3C binds to the actin cytoskeleton through its N-terminal NAB (NET Actin Binding) domain and is recruited to the membrane by its C-terminal sequence (Wang *et al.,* 2014). This special feature suggests that NET proteins are 'membrane-actin cytoskeleton adapters' that link membranes to the actin cytoskeleton (Wang and Hussey, 2015).

In this paper we demonstrate that NET3B localises to the actin cytoskeleton and the ER membrane *in vivo* as an adapter between ER and actin. We show that the NAB domain of NET3B is unique amongst the NET superfamily in that it has an insertion of three amino-acids (Val-Glu-Asp; Hawkins *et al.,* 2014) that affects its ability to co-localise with the actin cytoskeleton *in vivo*. The C-terminal domain is responsible for NET3B's interaction with the ER. Overexpressing NET3B increases the association of the ER with the actin cytoskeleton confirming its ER and actin cytoskeleton linking capability. However, T-DNA mutants of NET3B, *net2b-1* and *net2b-2*, show no significant morphological phenotype. Finally, we propose that NET3B mediates the ER and actin cytoskeleton interaction *in vivo*, a mechanism that is so far specific to higher plants.

Materials and Methods

Molecular biology

The primers used in the vector constructions are listed in Supplementary table 1. The NET3B (At4g03153) full-length cDNA was amplified from CDS cDNA template (TAIR) with gene specific primers. The domain deletion mutants of NET3B were generated by over-lapping PCR with appropriate primers (Table S1). Fluorescent protein fusions of NET3B (full length or deletion) were generated using Gateway recombination (Invitrogen) into GFP/RFP destination vectors (pMDC83 derivatives). The NET3B promoter::GUS construct was made

by fusing 2Kb of sequence up-stream of the coding sequence of NET3B to the GUS reporter coding sequence (Table S2).

Plant growth and transformation

The transformation and growth of *Arabidopsis* and *N. benthamiana* were performed as described in Wang et al., 2014. The two NET3B SALK T-DNA insertion lines were ordered from NASC. Homozygosity was confirmed by PCR using gene specific primers, and a T-DNA specific primer. Arabidopsis wild type (col-0) and *net3b-2* lines were transformed with RFP-HDEL by floral-dipping. Tissues from Arabidopsis lines expressing NET3B::GUS were incubated in GUS staining solution (100 mM phosphate buffer, 10 mM EDTA, 0.1% (v/v) Triton X-100, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), three hours at 37 degree. Before imaging, samples were decoloured by washing with 70% ethanol over-night.

Confocal microscopy

Live cell imaging was carried out using a Leica SP5 laser scanning confocal microscope with a 63x oil immersion lens. For GFP/YFP combination, GFP was excited at 458nm and detected at 470-510nm; YFP was excited at 514nm and detected at 550-580nm. For CFP/GFP/RFP combinations, these were excited at 405nm, 488nm and 543nm and detected at 450-490nm, 510-550nm and 590-650nm respectively. FRAP experiments were performed as described in Wang et al., 2011. All images presented here are representative of at least three independent experiments.

Immunofluorescence

For making the polyclonal antibody of NET3B, DNA corresponding to amino-acid residues 157-215 was cloned into pGAT4 plasmid, which incorporates an N-terminal His tag into the expressed protein. The recombinant proteins were generated in *E.coli* (Rosseta 2, Novagen) and purified using nickel agarose beads (Qiagen). Polyclonal antibodies were raised in mice as described (Deeks *et al.,* 2012). The specificity of the antiserum was tested on a western blot of a total protein extract from two week old Arabidopsis seedlings. Immunofluorescence with freeze shattering was performed as described (Zhang et al., 2013). Antibodies were diluted and used at 1:100 for NET3B and 1:500 for BIP2 (Agrisera), followed

by secondary antibody incubation with TRITC-conjugated anti-mouse IgG and FICT-conjugated anti-rabbit (Jackson ImmunoResearch).

High speed centrifugation and Microsomal isolation

N. benthamiana leaves expressing NET3B-GFP were used for total microsomal fraction isolation; approximately 0.1g of leaf tissue was homogenized in 12% (w/v) sucrose buffer containing Tris-HCl (50mM, pH7.6), NaCl (100mM) and EDTA (5mM). Ultra-centrifugation was performed at 55000rpm using a Beckman TLA-100 rotor for 60min. Both total microsome pellet and supernatant were mixed with SDS buffer and fractionated by SDS-PAGE and subsequently subjected to western blotting. For immunoblotting, primary antibodies of anti-GFP (Abcam, 1:1000), anti-BIP2 (Agrisera, 1:1000) and anti-actin (C4, Millipore, 1:500) were used. HRP-conjugated secondary antibody and ECL reagent (GE Heathcare) were used for developing the membrane.

Result and discussion

NET3B links the ER membrane and the actin cytoskeleton

Arabidopsis NET3B cDNA was fused in frame with GFP at its C-terminus (Fig.1a) and transiently expressed in *N. benthamiana* leaves using the infiltration method (Sparkes *et al.,* 2006). At expression levels achieved up to 30 hours after infiltration (low expression), NET3B-GFP labelled F-actin associated punctae (Fig.1b), producing the 'beads-on-a-string' pattern that is characteristic of members of the NET family (Deeks *et al.,* 2012). At expression levels over 48hrs (high expression) the chimeric protein labelled the entire filamentous network that is also co-labelled with YFP-actin-cb (Rocchetti *et al.,* 2014), indicating that NET3B associates with actin cytoskeleton *in vivo* like other members of the NET family (Fig.1c).

For actin cytoskeleton markers (e.g. Lifeact), treatment of the cells with the actin depolymerising drug Latrunculin B causes the marker to become cytosolic (Fig.1d, inset). However, NET3B-GFP became ER localised when the actin cytoskeletons were

depolymerised, indicating that NET3B-GFP has ER binding capability (Fig.1d). Interestingly, ER morphology is significantly altered by high expression of NET3B-GFP. Under normal conditions, when GFP-Lifeact is used as a single marker in the leaf cells, the labelled actin cables and the ER network were generally intertwined with each other (Fig.1f); little co-localization was seen. Whilst overexpressing NET3B-GFP, however, the ER network mostly overlaid the actin cytoskeleton and clear co-localization was observed (Fig.1e). Whereas a normal wild type ER network is formed by membrane cisternae interlinking with tubular structures, little ER cisternae were identified under these conditions and some of the ER network followed the track and organization of the NET3B-GFP labelled actin cytoskeleton (Fig.1e). When actin is removed in the presence of NET3B-GFP, the morphology of the ER network was restored, suggesting that it had lost the constraint of any potential NET3B-actin interaction (Fig.1d). Expressing full length NET3B without a fluorescent protein tag produced a similar effect to NET3B-GFP on the ER, indicating that the GFP tag is not significantly affecting its activity (Fig.1e, inset).

The effect that NET3B has on ER morphology at the different expression levels is better illustrated in triple expressing NET3B-GFP, YFP-Actin-Cb and RFP-HDEL cells (Fig.2a-b). Note the strong ER-actin co-alignment observed when the level of NET3B-GFP is high whereas NET3B-GFP labels puncta that co-align with the actin cytoskeleton at the lower expression levels (as in Fig.1a).

We have studied the localization of endogenous NET3B in Arabidopsis cotyledon cells using a NET3B polyclonal antibody raised in mice. This antiserum detects a single band on ID gel immunoblots of Arabidopsis protein extracts (Fig.2c). In Arabidopsis cotyledon cells, endogenous NET3B localised to puncta, and these puncta co-localise with both the actin cytoskeleton (Fig.2d) and the ER membrane (Fig.2e). This result is in agreement with the localisation of NET3B-GFP expressed in *N. benthamiana* leaves at the lower expression levels (Fig.1a, Fig.2b). Taken together, these data indicate that NET3B localises to specific ER domains that associate with the actin cytoskeleton. When the NET3B-GFP expression level is high in the *N. benthamiana* leaf epidermal cells, NET3B-GFP localises throughout the ER membrane and as a result appears to enhance the ER-Actin association and this is likely to be because there is more NET3B available to mediate this interaction.

NET3B has two functional domains

NET3B-GFP is not observed on the nuclear envelope (NE) which is continuous with the ER network. The NE harbours many ER proteins and the lack of NET3B-GFP at the NE indicates that its membrane association is specific (Fig.3a). Two distinct domains are found in the NET3B sequence, namely an N-terminal NET Actin Binding domain (NAB) and a C-terminal coil-coiled domain. Direct association between the NAB domain from NET1 and NET4 and F-actin has been well demonstrated in our previous studies (Deeks et al., 2012). Unfortunately, due to the insolubility of NET3B and its NAB domain it has not been possible to verify direct binding *in vitro* but its association with the actin cytoskeleton is supported by its localisation *in vivo* (Fig.1b-c).

Domain deletion mutants of NET3B cDNA were made and fused in frame to GFP (Fig.1a) and used to transiently transform *N.benthamiana* leaf epidermal cells. NET3BΔNAB-GFP localized to the ER network as well as the nuclear envelope, which suggests that the NET3B C-terminus including the coil-coiled sequence is involved in the ER interaction (Fig.3b), whereas the N-terminal NAB domain co-localises with the actin cytoskeleton (Fig.3c). When NET3B-NAB-GFP was expressed in the leaf epidermal cells, strong cytoplasmic background fluorescence was observed, suggesting that its localisation to the actin cytoskeleton is not as efficient as the full length NET3B-GFP (discussed later in Fig.4). This phenomenon is not observed when using the NAB domains of other NET proteins in similar experiments (Deeks *et al.,* 2012). This could indicate that the co-localisation of the NAB domain of NET3B with the actin cytoskeleton is lower than other NET proteins, and that the ER association of NET3B is important for its actin cytoskeleton localisation. ER cisternae were also formed in the presence of NET3B NAB-GFP, with less tubular ER structure observed compared to that in wild type cells (Fig.3c, inset).

NET3B without the coiled-coil domain, NET3BΔCCD-GFP localizes to numerous puncta that co-align with the actin cytoskeleton (Fig.3d). However, this deletion mutant is unable to interact with the ER membrane as little co-alignment between the ER and the chimeric protein is seen (Fig.3c, inset). Moreover, the morphology of the ER network is disrupted (with more ER aggregates) in the presence of NET3BΔCCD-GFP suggesting that this deletion mutant interferes with the normal function of the endogenous protein, a dominant negative

effect. Alterations in ER morphology are observed when expressing these NET3B deletion mutants. This could be because the interaction between NET3B and other ER structural proteins is disturbed, causing the ER phenotype.

Bearing these data in mind, we can propose a model for the association of NET3B with the ER and the actin cytoskeleton. This model proposes that NET3B behaves as a linker protein; it associates with the actin cytoskeleton through the N-terminal NAB domain, and to the ER membrane through its C-terminus (which requires the coiled-coil domain). This is supported by the facts that (1) *In planta* immuno-localisation studies in Arabidopsis have shown that NET3B localizes to specific ER domains, where it is also able to associate with actin and (2), when overexpressed in *N.benthamiana* the NET3B-GFP appears to distribute across the whole actin and ER network resulting in the observed enhanced ER/actin association (3) when actin is disrupted the NET3B-GFP localizes to the ER (Fig.3e).

NET3B has a unique NAB domain.

When full length NET3B-GFP is co-expressed in *N.benthamiana* epidermal cells with the NAB domain from NET1A (NET1A-NAB-RFP), the NET3B-GFP distributed to the ER surface and to the cytoplasm but no labelling of the actin cytoskeleton was observed (Fig.4a). This phenomenon is very similar to the effect of LatB treatment on NET3B-GFP expressing cells (Fig.1d). These data suggest that the NET1A-NAB, which is known to bind directly to F-actin, interferes with the association of NET3B with the actin cytoskeleton suggesting that they both interact with the actin cytoskeleton at the same site but that NET1A-NAB has a higher affinity for this site and is therefore more competitive.

The conserved N-terminal NAB domain is a characteristic feature for the NET superfamily of proteins. However the NAB domain of NET3B is unique in having an insertion of three amino acids, namely Valine, Glutamic acid and Aspartic acid (VED, Fig. 4b). The 3D structures of the NAB domain in NET1A, NET3B and NET3BΔVED were analysed *in silico* (Phyre2). Interestingly, the predicted structure of NET3B NAB is significantly different from the NET1A NAB because a beta-turn is induced by the VED insertion (Fig.4c-d). However, by removing the VED (NET3BΔVED), the structure of the NET3B NAB reverted to a structure similar to the NAB of NET1A (Fig.4e). With this in mind, the NAB domain of NET3B construct was made without the VED motif (NET3B NABΔVED-GFP) and expressed in parallel with NET3B NAB-

GFP under the same conditions. The cytoplasmic background of NET3B NAB is much stronger than NET3B NABΔVED, as suggested by the actin filament:cytoplasmic signal intensity ratio (Fig.4f-h). These data indicate that the actin associating capability of NET3B is enhanced by removing the VED motif from the wild-type NET3B NAB domain. Also, the NET3BΔVED-GFP mutant protein is still able to link the actin and ER networks which is expected as the C-terminus of NET3B is unaffected and is responsible for its ER localisation (Fig.S1a-b).

NET3B-GFP overexpression restricts membrane diffusion.

We have used high speed centrifugation to determine whether NET3B is a peripheral membrane protein. The data show that NET3B-GFP is found in the microsomal fraction and also in the supernatant after centrifugation (Fig.5a). BIP2 is a protein localized to the ER lumen and this was used as a control to show that this was only found in the microsomal fraction, the pellet (Fig.5a). Endogenous actin is only found in the supernatant as it is not able to sediment at this speed. As NET3B-GFP was found in both the pellet, the microsomal fraction, as well as in the supernatant, this suggests that NET3B is an ER peripheral membrane protein, and its association with the ER is likely to be through either interaction with other ER membrane proteins or directly with membrane lipids.

High levels of NET3B-GFP expression changes the structure of the ER by reducing the amount of membrane cisternae (Fig.1e). In order to determine whether the expression of NET3B-RFP effected membrane diffusion we performed Fluorescence Recovery After Photobleaching (FRAP) on the ER protein calnexin in the presence or absence of NET3B-RFP. In the presence of NET3B-RFP the recovery of CXN-GFP in the photobleached region was much slower than in its absence and a large immobile fraction was also observed (Fig.5b-d). These data indicate that the NET3B/ER interaction can affect ER membrane diffusion.

Characterization of NET3B T-DNA mutants, *net3b-1* and *net3b-2*

The expression pattern of NET3B was studied using the GUS reporter gene system. The NET3B promoter (2KB upstream of the translation start codon) was fused in frame with the GUS reporter cDNA and the construct used to transform Arabidopsis. GUS was observed to

be expressed in various tissues including pollen, embryos, roots and the leaf vasculature (Fig. 6a). A high level of NET3B promoter activity was also observed in the meristematic cells of the root tip.

We have identified two homozygous NET3B T-DNA mutant lines and the T-DNA insertion is in the promoter of *net3b-1* and in the second exon of *net3b-2* (Fig.6b). As assessed by western blotting with anti-NET3B antibody, the protein level of NET3B is knocked-down in *net3b-1* whereas it is knocked-out in *net3b-2.* The specificity of NET3B antibody is also confirmed, as no other protein band is detected in the knock-out mutant (Fig. 6c-d). Both mutants exhibit normal growth and development, and have no significant morphological defects when compared to wild type. The ER organization in the *net3b-2* mutant was further analyzed by stably expressing RFP-HDEL in the mutant; no significant phenotype on ER morphology can be observed (Fig.6e). It is known that multiple proteins are required for actin-myosin based ER movement and that the overall structure of the ER network is not affected significantly when the actin cytoskeleton is removed chemically. Therefore, depleting one protein i.e. NET3B may not be sufficient to produce significant defects in ER morphology.

In conclusion, we have shown that the NET3B protein is a novel membrane adaptor protein, which links membranes to the actin cytoskeleton. NET3B associates directly with the ER and co-localises with the actin cytoskeleton. NET3B may also be involved in dictating ER morphology and dynamics as overexpression affects both the structure of the ER and diffusion within the ER membrane. Furthermore, NAB domain of NET3B contains a unique three amino acid, VED, insertion, which reduces its ability to associate with actin cytoskeleton *in vivo* compared to another member of the NET family, NET1A, which has been shown to bind F-actin directly. How NET3B can modulate ER structure remains to be determined but it is possible that NET3B works in conjunction with other ER localized actin regulatory/motor proteins. For example, the dynamics of the ER network in plants is modulated by the myosin XI family which contain a motor domain used for driving actin-based movement. It is possible that NET3B provides an anchorage thereby facilitating the process of myosin-based ER movement. Taken together; our study suggests that NET3B is a novel protein involved in actin cytoskeleton based ER modelling.

Figure Legends

Figure 1. NET3B-GFP co-localises with both the actin cytoskeleton and the ER in transformed *N. bentahamiana* leaf epidermal cells.

N. Benthamiana leaf epidermal cells transiently transfected with fluorescent protein constructs either singly or in combination with other construct(s) as shown in each panel. **(a)** Graphical illustration of the construction of the NET3B fusions. **(b)** 3D maximum projection of NET3B-GFP expressing cells with low expression. NET3B-GFP labels numerous actin cytoskeleton associated puncta producing the typical 'beads-on-a-string' localisation characteristic of the NET family proteins. **(c)** NET3B-GFP/YFP-Actin-Cb expressing cells. NET3B-GFP localises to filamentous structures that are also labelled with the actin marker. **(d)** NET3B-GFP redistributes to the ER membrane when the actin cytoskeleton is depolymerised by LatB. The inset picture shows that GFP-Lifeact becomes cytoplasmic after Lat B treatment. **(e)** NET3B-GFP/RFP-HDEL expressing cells. The expression of NET3B-GFP enhances the association between the ER and the actin cytoskeleton. Consequently, the morphology of the RFP-HDEL labelled ER network has become more aligned with the actin network. The inset picture shows that full length NET3B without a tag is also able to enhance the actin cytoskeleton-ER association. **(f)** GFP-Lifeact/RFP-HDEL expressing cells. The actin and ER networks exhibit little co-alignment in contrast to (e) (Scale bar = 10 μm).

Figure 2. Endogenous Arabidopsis NET3B co-localises with the actin and ER networks.

(a-b) NET3B-GFP (magenta) co-expressed with YFP-Actin-Cb (green) and RFP-HDEL (red) at different time points after infiltration into *N. benthamiana leaves*. The same detection setting were used for imaging GFP. A strong ER-actin cytoskeleton co-alignment was seen in (a) but not in (b) and the NET3B puncta seen in (b) co-align with the actin cytoskeleton (inset) and are associated with ER membrane **(c)** Western blot of a total Arabidopsis seedling protein extract probed with a polyclonal NET3B antibody (anti-NET3B) showing a clear band at around 25 kDa. **(d)** Immunofluorescence images of cotyledon cells of stable Arabidopsis FABD2-GFP actin marker lines using anti-NET3B (TRITC, red) and anti-GFP antibodies (FITC, green). Endogenous NET3B was detected in both the cytoplasm and as punctate structures,

which co-aligned with the actin network. **(e)** Immunofluorescence images showing that the anti-NET3B labelled puncta also associated with the ER network which is labelled with anti-BIP2 (FITC, green; Scale bar = 10 μm).

Figure 3. NET3B colocalises with the actin and ER networks through its N-terminal NAB domain and a C-terminal ER binding domain, respectively

Domain deletion mutants of NET3B were fused in frame to GFP and transiently expressed with RFP-HDEL in *N. benthamiana* leaf epidermal cells. **(a)** 3D projection of cells expressing NET3B-GFP and H2B-YFP (nuclear marker). Note: Unlike most ER membrane proteins, NET3B-RFP does not label the nuclear envelope. **(b)** NET3BΔNAB-GFP/RFP-HDEL co-expressing cells. NET3BΔNAB localises to the ER network and also localises to the nuclear envelope (inset). **(c)** NET3B NAB-GFP expressing cells. NET3B NAB-GFP labels the actin cytoskeleton and a strong cytoplasmic background is also observed. Under these conditions, the ER network becomes more cisternae-like when compared to cells expressing RFP-HDEL (inset). **(d)** NET3BΔCCD-GFP expressing cells. NET3BΔCCD-GFP labels the actin cytoskeleton, but in addition numerous puncta are also formed that co-align with the actin network. The ER network is disrupted, and its association with NET3BΔCCD-GFP labeled actin cytoskeleton is much reduced. **(e)** Proposed model of the NET3B-ER interaction: NET3B associates with the ER and the actin cytoskeleton at specific membrane foci (1); NET3B over-expression brings the actin cytoskeleton and the ER network closer together by increasing the number of sites of interaction (2); NET3B becomes ER localised when the actin cytoskeleton is removed (3; Scale bar = 10μm).

Figure 4. NET3B has an insertion in the NAB domain that reduces its ability to associate with the actin cytoskeleton *in vivo*.

(a) *N. benthamiana* leaf epidermal cells expressing NET3B-GFP/NET1A NAB-RFP/CFP-HDEL**.** NET3B-GFP is not able to associate with the actin cytoskeleton in the presence of NET1A NAB-RFP. Note: the actin cytoskeleton is only labelled with NET1A NAB-RFP and NET3B-GFP becomes ER localised as determined by its co-localisation with CFP-HDEL. **(b)** Sequence

alignment of the NAB domain from NET1A, NET2A, NET3A, NET3B and NET3C. The three amino acid, VED, insertion is a unique feature of NET3B. **(c-d)** Predicted 3D structures of the NAB domains analysed using the Phyre2 program. Significant differences are seen in the 3D structure of NET1A NAB and NET3B NAB. The arrow indicates the approximate position of the VED insertion. **(e)** Once the VED motif is removed from NET3B NAB, its predicted 3D structure is very similar to NET1A. **(f-g)** *N. benthamiana* leaf epidermal cells expressing the NET3B NAB-GFP and the NET3B NAB-GFP with the VED motif deleted. A stronger cytoplasmic signal is seen in NET3B NAB-GFP expressing cells, **(h)** This result is further quantified by determining the ratio between the fluorescence intensities of the cytoplasm and the actin cytoskeleton (scale bar = 10µm).

Figure 5. NET3B-GFP overexpression restricts membrane diffusion.

(a) Western blots of high speed (100,000g) ultracentrifuge pellets and supernatants from *N. benthamiana* leaf extracts expressing NET3B-GFP. (i) NET3B-GFP is detected in the pellet (which harbours the ER membrane microsome fraction) and supernatant by anti-GFP. Anti-actin detects a band only in the supernatant; (ii) BIP2, an ER protein is found only in the pellet as detected using anti-BIP2. Anti-actin detects a band only in the supernatant. Note: NET3B-GFP has the characteristics of a peripheral membrane protein. **(b-c)** Representative images of photobleached ER membrane imaged for CXN-GFP in the presence of NET3B-RFP. (**d**) FRAP analysis of CXN-GFP in the CXN-GFP/NET3B-RFP expressing cells compared to singly expressing CXN-GFP cells Note: The movement of ER membrane as depicted by the recovery of CXN-GFP fluorescence is much retarded in the presence of NET3B-RFP. This is reflected in a prolonged $T_{1/2}$ and reduced Rmax (Scale bar = 10µm).

Figure 6. NET3B knock-out mutants, *net3b-1* and *net3b-2*, exhibit no significant change on ER networks.

(a) NET3B::GUS expression in stably transformed Arabidopsis lines. GUS staining was seen in seedlings (1), developing seeds (2-3), anther/pollen (4) and pollen tube (5). **(b)** Illustration of the position of the T-DNA insertions in *net3b-1* and *net3b-2* lines. **(c)** Western

blot of Arabidopsis flower total protein extracts from Col-0, *net3b-1* and *net3b-2* probed with anti-NET3B. Note: NET3B is absent from *net3b-2* and is a knock-out mutant, whereas the expression of NET3B in *net3b-1* is significantly knocked-down**. (d)** The amido black stain indicates equal protein loading. **(e)** Both wild type and *net3b-2* Arabidopsis are transformed with RFP-HDEL. Note: the ER organisation in these plants (hypocotyl epidermal cells) looks similar and no obvious defect is observed (scale bar = 10µm).

Supplementary legends

Figure S1. NET3B has an insertion in the NAB domain that reduces its actin associating ability but not its ER binding ability.

(a) NET3B without the VED motif is fused in frame to GFP. In NET3BΔVED-GFP/ NET1A NAB-RFP co-expressing cells, interestingly, actin cytoskeleton co-labelling with both proteins are observed. **(b)** NET3BΔVED-GFP and RFP-HDEL coexpressing cells. NET3BΔVED is still able to associate with the ER membrane (scale bar = 10µm).

Movie S1. NET3B-GFP (green) co-expressed with an ER marker, RFP-HDEL (red). Enhanced association between the actin cytoskeleton and ER network is seen, and the two structures are moving together.

Supplementary Table 1. List of Primers used in this study.

Supplementary Table 2. List of constructs generated in this study.

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Supplementary Table 1. List of Primers used in this study.

Name of Primer	Sequence (5'-3')
NET3B-F	gggg aca agt ttg tac aaa aaa gca ggc ttc ccg cca ATG GGT GAG ACA TCA AAA TGG TG
NET3B-R	gggg acc ac ttt gta caa gaa agc tgg gtc AAA CGA AAA CAT TAT GAG AAA ATA GTA AC
NET3B NAB-R	gggg aca agt ttg tac aaa aaa gca ggc ttc ccg cca GAT CAA GAG ATC ATG CTT CTG
NET3B NAB-F	Same to NET3B-F
NET3BΔCCD-F	CAA ATT GTT GAA TTT GAC GAT GGT GTT TGC TTT TGC TTC CAA TTC A
NET3BΔCCD-R	TGA ATT GGA AGC AAA AGC AAA C ACC ATC GTC AAA TTC AAC AAT TTG
NET3BΔNAB-F	gggg aca agt ttg tac aaa aaa gca ggc ttc ccg cca ATG AAAACCTCT TCTCTGAATTCGG
NET3BΔNAB-R	Same to NET3B-R
NET3B ab-F	gggg aca agt ttg tac aaa aaa gca ggc ttc ccg cca ATG GTG ACT CGG TTG CTT GCA A
NET3B ab-R	Same to NET3B-R
NET3BΔVED-F	CTCATCAATCACGCTCAGC GAAGGAGATTCCTTGATGAAAC
NET3BΔVED-R	GTTTCATCAAGGAATCTCCTTC CTCATCAATCACGCTCAGC
NET3B Promoter-F	gggg aca agt ttg tac aaa aaa gca ggc ttc ccg cca ATA TCT CAA TTA TGA ATT AAT TCA ATA TAT
NET3B Promoter-R	gggg acc ac ttt gta caa gaa agc tgg gtc CGT CTT CAC TTG TAA TTT TGC AC

Supplementary Table 2. List of constructs generated in this study.

Name of Construct	Expression Vector	Cloning Primers
NET3B-GFP	pMDC83-GFP	NET3B-F + NET3B-R
NET3B-RFP	pMDC83-RFP	NET3B-F + NET3B-R
NET3B NAB-GFP	pMDC83-GFP	NET3B-NAB-F + NET3B-NAB-R
NET3BΔNAB-GFP	pMDC83-GFP	NET3BΔNAB-F + NET3B-R
NET3BΔCCD-GFP	pMDC83-GFP	NET3BΔCCD-F + NET3BΔCCD-R
NET3BΔVED-GFP	pMDC83-GFP	NET3BΔVED-F + NET3BΔVED-R
NET3B-GUS	pBI101-GUS	NET3B Promoter-F + NET3B Promoter-R
NET3B antigen	pGAT4-His	NET3B ab-F + NET3B ab-R