Investigating Functional Motifs within the N- Terminal Domain of Novel Actin Binding Proteins NET1A and NET2A

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Abstract

The NET proteins are a novel family of actin binding proteins that all share a homologous n-terminal actin binding domain (ABD). The aims of this project were to probe potential functional domains within this ABD by creating mutants of NET1A and NET2A as representative family members. Mutants were designed with the aim of disrupting f-actin binding. To identify possible sites for mutation, the ABDs of the NET family were analysed to look for residues that were homologous to all members except NET3B, which has been shown not to bind actin. Once potential residues were identified, point mutants were designed and cloned alongside an ABD deleted construct. The clones were then expressed in a dominant negative approach using transient expression techniques.

Infiltrations of NET1A mutants into *Nicotiana benthamiana* leaves showed changes in the localisation and behaviour of the protein relative to wild type. These included a potential association between NET1A and the ER, identification of a potential single residue in the ABD that contacts f-actin and whose mutation reduces NET1A's f-actin affinity and the disruption to the association between both the ER and f-actin, and the plasma membrane. Results from these infiltrations have led to the development of a model that suggests potential functions for discrete domains within NET1A. This model will be useful for directing future experiments that aim to identify proteins that NET family interact with and perhaps identify common functions for these novel actin binding proteins.

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Thanks go to everyone who has worked in the Hussey laboratory during the last year, for creating such an enjoyable, relaxed environment to work in and offering technical advice and tips for experimental work. Thanks in particular go to Dr Michael Deeks. His knowledge in experimental design has helped me immensely through the cloning stages of the project, and I am hugely grateful for the hours he has spent proof reading this thesis and for putting together the model for NET1A function based on group discussions between Dr Michael Deeks, Dr Tim Hawkins and myself. Particular thanks also go to Dr Tim Hawkins for his patience and help with the confocal microscopes, which has been fundamental for my success in this Imaging MSc.

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Chapter 1: Introduction

1.1. Overview

The cytoskeleton is a fibrous protein network found within the cytoplasm of plant and animal cells. It has a multitude of different functions within cells. These include: establishing cell polarity, defining cell shape, controlling the process of cell division, organelle organisation and facilitating inter- and intra- cellular transport. The plant cell cytoskeleton has two major components: microtubules are composed of tubulin dimers and microfilaments consisting of a protein named actin. Unlike an animal's anatomical skeleton, the cellular cytoskeleton is a highly dynamic structure. The constituent subunits are assembled into filaments that are able to grow, shrink, reorganise and travel around the cell in response to cell signalling processes. The cytoskeleton interacts with a host of accessory proteins that enable its diverse array of functions and flexibility and organise it through the cell in time and space. The wide variety of cytoskeleton associated proteins enable the relatively simple filament to become the basis of vitally important cellular functions (Lodish et al., 2007).

1.2. Structure of the Actin Cytoskeleton

The microfilament component of the plant cytoskeleton is composed of a protein called actin, found in two forms in the cell. G-actin is a soluble, globular monomer and f-actin is a filamentous polymer. Each actin molecule has an adenosine nucleotide binding cleft. The hydrolysis state of the nucleotide influences the assembly of the filamentous cytoskeleton. Under the correct physiological conditions, adenosine-5'-triphosphate (ATP)-associated monomers spontaneously dimerise and then form trimers. The trimers are unstable but provide a nucleus onto which filament assembly can occur. Elongation of the filament occurs via addition of actin monomers onto the nucleus in the same orientation. This results in a filament with an intrinsic polarity (Blanchoin et al., 2010). The polarity is described by a (+) end where polymerisation is favoured and a (-) end where depolymerisation is most persistent. An actin filament is a helical polymer containing two parallel microfilaments wound around each other to produce a right-handed helix with a diameter of between 7-9nm.

1.3. The Basis of Actin Dynamics

The actin cytoskeleton is a highly dynamic structure. In a eukaryotic cell the assembly and disassembly of the filamentous network is regulated by a wide variety of actin binding proteins (ABPs) on a temporal and spatial basis. At the most basic level, the growth and aging of the actin filament is defined by the hydrolysis of the adenosine nucleotide and the following release of the terminal phosphate group. Soon after incorporation into the filament, the ATP nucleotide is hydrolysed to ADP+Pi and then slowly dissociates the terminal phosphate group as new ATP-bound actin monomers are added to the + end of the filament. In its ADP-bound state, the actin molecule becomes unstable in the filament and therefore the minus end of the filament, where all actin is in an ADP-bound form, is more prone to depolymerisation. In this way, ATP hydrolysis provides the driving force for actin dynamics and gives the filaments their intrinsic polarity (Blanchoin et al., 2010). The turnover of actin filaments, defined by a net growth at the (+) end and a net loss at the –end, is known as 'treadmilling'. This process is illustrated in figure 1.

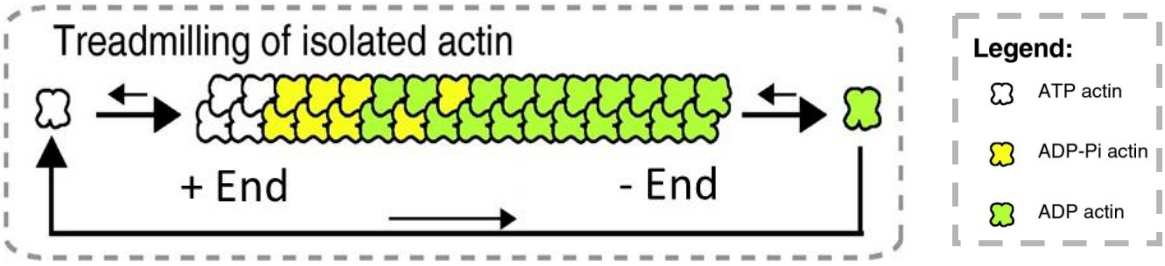


Figure 1: Treadmilling within an actin filament (adapted from Blanchoin et al., 2010)

The dynamic nature of the filaments allows them to be spatially and temporally organised into different arrays within the cell. This is achieved *in vivo* by an interaction with a multitude of actin-binding proteins (ABPs) that also facilitate the cytoskeleton's wide variety of functions. ABPs will be discussed in more depth in section 1.5.

During interphase there are three forms of actin array: the nuclear array, the cortical array and the cytoplasmic strands. These are illustrated in figure 2.

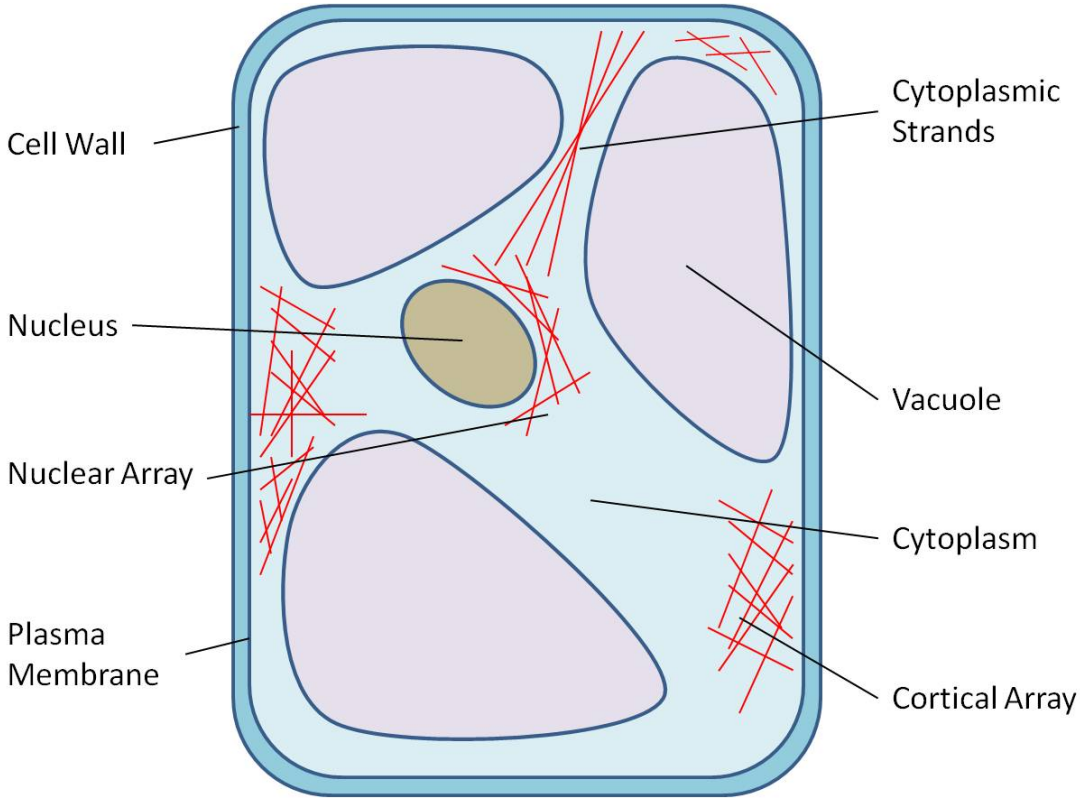


Figure 2: The actin arrays found in an interphase plant cell.

The actin cytoskeleton also forms functional arrays during mitosis alongside the microtubule (MT) component of the cytoskeleton. At the start of prophase the actin filaments align with MTs in the pre-prophase band (Kost et al., 1999). During anaphase, actin forms a basket array around the MT mitotic spindle and during cytokinesis, actin again aligns with MTs in the phragmoplast to define the plane of cell division (Sylvester, 2000). The phragmoplast array disassembles as the newly forming cell plate fuses with the plasma membrane to complete cell division (Wasteneys & Galway, 2003).

1.4. Function of the Actin Cytoskeleton

The actin cytoskeleton is a highly dynamic structure which coordinates important biological processes such as cell growth, polarity and morphology. Specific functions include intra-cellular transport of organelles around the cytosol, positioning the plane of cell division during mitosis, construction of new cell walls and responding to both inter- and intra- cell signalling (McCurdy et al., 2001).

Plant cell growth and expansion is an important function for the actin cytoskeleton. In anisotropically growing cells, such as leaf pavement cells, expansion is not restricted to a specific plane but occurs in defined but diffuse areas. The actin cytoskeleton guides growth material contained within vesicles to the correct section of the cell wall. It does this in conjunction with myosin motor proteins that use filamentous actin cables as tracks that guide cytoplasmic streaming to the cell cortex. In contrast to animal cells, which are capable of moving within their environment relative to each other, plant cells are non-motile. As a result, variety in tissue shape is created by regulating the orientation of the new cell wall that is laid down at cytokinesis and the axis of cell expansion during interphase. The actin cytoskeleton is fundamental in defining these variables and thus plays an important role in determining tissue structure and morphology (Hussey et al., 2006). It has also been suggested that fine f-actin arrays at the cell cortex define the area of cell surface where expansion is taking place (Ketelaar et al., 2003).

Growth in tip-growing cells occurs in a polarised manner at one end of an elongating cell. Examples of this form of growth in plants include root hairs and pollen tubes. In this type of cell actin is responsible for mediating the transport of golgi-derived growth material down the length of the cell to the site of exocytosis at the cell tip (Miller et al., 1999). It is also thought the site of growth is marked by increased filamentous actin instability (Ketelaar et al., 2003).

1.5. Actin Binding Proteins

The actin binding proteins (ABPs) are a large and diverse group of proteins which associate with the actin cytoskeleton to ensure its correct formation, function and organisation. They control the spatial and temporal distribution of actin throughout the cell. The ABPs are important because they coordinate all the biologically vital roles of the actin cytoskeleton. Research to identify and classify new ABPs is essential to understand the functions of the actin cytoskeleton.

1.5.1. Actin Binding Proteins that Regulate Actin Dynamics and Filament Reorganisation

The actin cytoskeleton is a highly dynamic structure but this feature is only made possible by the complex mixture of proteins that associate and interact with it. Functions of these ABPs include controlling what proportion of the g-actin monomers in the cell are available to become incorporated into filaments, severing f-actin to increase the rate of depolymerisation and stabilising actin filaments. The most highly

characterised plant ABP is profilin. In the presence of capped filament ends, it acts to sequester g-actin in the cytosol to prevent its incorporation into actin filaments and thus inhibits polymerisation. In contrast, when actin filament ends are uncapped, the profilin-actin complex can add to the (+) end of filaments to allow assembly (Drobak et al., 2004). The particular activity of profilin is determined by the concentration and ratio of free g-actin to f-actin in the cytoplasm. High concentrations of profilin triggers depolymerisation of the actin cytoskeleton. Profilin is found concentrated at the tips of growing root hairs and pollen tubes and the protein regulates tip growth by altering the amount of free g-actin available for incorporation into filaments (Wasteneys & Galway, 2003).

Formin acts as an actin nucleator to create dimers and trimers of G-actin onto which polymerisation can occur (as discussed in section 1.2). This is necessary because actin seeds are very unstable *in vivo* thus formin stabilises the nuclei so that polymerisation can be allowed to begin. Once polymerisation has been initiated, g-actin molecules are able to add on to the (+) end of the filament spontaneously (Deeks et al, 2002). An additional function of formin is to stimulate extension of actin filaments from the (+) end. To achieve this, the protein binds to the (+) end of the filament and acts to inhibit actin depolymerisation and protect the filament end from other ABPs that terminate end growth (Hussey et al, 2006).

Capping proteins stabilise the ends of filaments to prevent depolymerisation and reduce the elongation rate. They influence filament dynamics because their addition to the ends of filaments reduces the number of filament ends that are available for addition or loss of subunits of actin. In this way they reduce the dynamic nature of the cytoskeleton array where they are localised (McCurdy et al., 2001).

1.5.2. Actin Binding Proteins that Mediate Transport Activity

Secondly there are ABPs named myosins that use actin filaments and bundles as tracks to transport organelles and Golgi-derived vesicles around the cellular environment. Through an interaction with these motor proteins the actin cytoskeleton is able to control exocytosis, growth, shape and the polarity of the cell. This occurs via vesicle mediated transport of substances such as new cell wall material, enzymes, receptors or signalling molecules to particular areas of the cell (Wasteneys & Galway, 2003). They use energy derived from ATP hydrolysis to translocate along actin filaments. It is suspected that they are regulated by Ca^{2+} signalling (Malho et al., 2006). In this way the myosin motor protein can transport its cargo down the length of a pollen tube or root hair cell until the vesicle draws near to the Ca^{2+} rich environment

found at the tip. At this point further transport is inhibited, vesicles are unloaded from the myosin transport protein and their contents can be used for cell growth (Vidali & Hepler, 2001).

1.5.3. Actin Binding Proteins that Organise Filaments into Higher Order Structures

The third group of ABPs organise actin filaments into higher-order structures to create arrays with diverse functionality. These proteins connect adjacent filaments in different ways: cross-linking parallel bundles into tightly packed cables, arranging more loosely associated filaments into complex networks, or anchoring the actin filamentous array to organelles within the cell.

Actin bundles consist of many actin filaments aligned together to create a strong cable. Fimbrin is a monomeric cross-linking protein. It has two actin binding domains which join two adjacent filaments to give rise to sturdy actin cables that are used for cytoplasmic streaming (Wasteneys & Galway, 2003). Villins also bundle actin filaments but they do this specifically so that all component filaments in the bundle are in the same polar orientation. This is important for actin bundles that are used as transport tracks as some myosins move along filaments in a specific direction. In addition, bundling of filaments by villin protects the filaments from the depolymerisation activity of other ABPs (Hussey et al., 2006).

There are a wide variety of ABPs (e.g. α-actinin, dystrophin, filamin, and spectrin) that organise filaments into arrays with varying conformations in different parts of the cell and at different stages of the cell cycle. Most of these have one actin binding domain and therefore form homodimers so they can interact with multiple filaments and connect them together (figure 3 demonstrates the function of some of these proteins).

Many ABPs function at the plasma membrane or just below the cell surface in the cortical region. Here they can associate with cortical actin arrays and regulate the cytoskeleton's interaction with the plasma membrane, the interface between the internal and external environment. The plasma membrane is thus an important platform for signalling which facilitates communication to and from the cell. The actin cytoskeleton is a target for such signals and its localisation directly under the membrane surface enables it to respond quickly to the external environment.

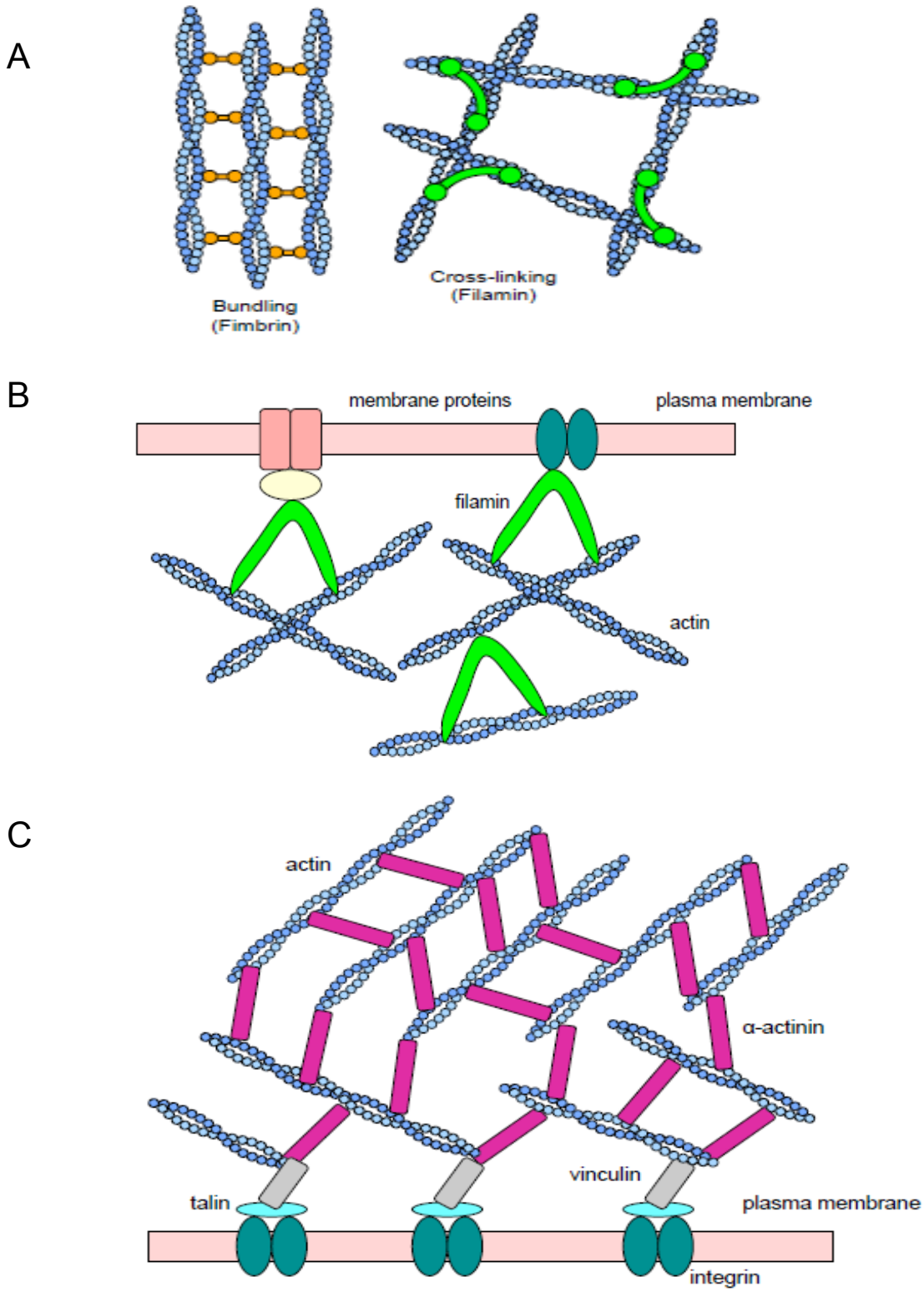


Figure 3: ABPs that organise actin into higher order structures. A. Fimbrin is a monomeric bundling protein that creates sturdy actin cables. B. Filamin is a monomeric cross-linking protein that creates arrays at the plasma membrane and can anchor the cytoskeleton to membrane associated proteins. C. α-actinin arranges a lattice like array that lies below the plasma membrane in the cell cortex (Calcutt, 2009),

Many signal receptors, kinases, and enzymes are localised to the plasma membrane and concentrated at sites such as lipid rafts where it is hypothesised that signalling molecules accumulate and interact with actin.

1.6. Role for the Actin Cytoskeleton in Plant Cell Signalling.

Plants are non-motile organisms. In order to survive they must be able to perceive changes in the external environment, communicate these changes to cells throughout their tissues and respond by altering their growth and development. In plants, communication occurs via signals such as growth regulators, ions, sugars, and amino acids (Gilroy & Trewavas, 2001). As discussed above, the actin cytoskeleton has primary functions maintaining cell shape and structure. However, recent research has demonstrated that the cytoskeleton and the proteins that interact with it participate in cell signalling events. The cytoskeleton can reorganise in response to environmentally influenced processes such as cell division, cell elongation, wounding and pollen development (Kovar et al., 2001). In addition, it is capable of transducing signal information to other organelles via associated proteins. Extracellular signals are transmitted into the cell via ABPs which respond to concentration fluctuations of second messengers. The ABPs respond by stimulating changes to actin filament organisation and dynamics (Franklin-Tong, 1999), (Wasteneys & Galway, 2003). In addition to ABPs there are a variety of other signalling molecules that have downstream effects on the actin cytoskeleton.

1.6.1. ROPs

ROPs (rho of plants) are GTPases that are signalling molecules that have been shown to affect the organisation of the actin cytoskeleton in response to both intra- and extra-cellular stimuli. They localise to sites of tip growth, developing cell plates and cross walls - all areas where actin is known to function (Hussey et al., 2006). When ROPs are over-expressed or constitutively active there are resulting changes in the actin cytoskeleton that cause defects in cell growth (Jones et al., 2002), (Molendijk et al., 2001).

1.6.2. Ca^{2+}

Ca^{2+} is another signalling molecule affecting the actin cytoskeleton. It has roles regulating cytoplasmic streaming, exocytosis and the pollen self-incompatibility (SI) response (Franklin-Tong, 1999). It can independently trigger cytoskeleton reorganisation by activating Ca^{2+} dependant ABPs that alter f-actin dynamics

(Wasteneys & Galway, 2003). The important cellular process, exocytosis, is triggered by Ca^{2+} and its control is intimately linked to cytoplasmic streaming of vesicles to areas of cell wall where growth is occurring. It has been suggested that Ca^{2+} regulates vesicle-membrane fusion at growth sites (Clark et al., 2001)

1.6.3. Phosphoinositides

Animal cell plasma membranes are cholesterol rich and contain microdomains or 'rafts' in which sterols and sphingolipids accumulate. These rafts are points of accumulation of receptors, kinases, proteins and signalling molecules. It is thought that these sterol and sphingolipid enriched microdomains also exist in the plasma membrane of plant cells but there is much greater structural diversity of the components which may have a functional significance (Laloi et al., 2007). One family of molecules that are found in these lipid rafts are phosphoinositides. Along with their derivatives, they are key components of many signal transduction pathways in plant cells. Phosphatidylinositol 4,5-bisphosphate (PIP2) is a member of this family that has been shown to influence the actin cytoskeleton through complex signalling cascades. As discussed in section 1.5.1, profilin is an ABP that influences actin dynamics. One function of PIP2 is to inhibit the function of profilin (Kovar et al., 2001). Since PIP2 is localised to membranes, this would suggest that PIP2 is particularly important for controlling actin assembly at the plasma membrane. Binding of profilin to PIP2 prevents the ABP from binding to g-actin to inhibit f-actin polymerisation. PIP2 is part of a signalling cascade that can respond to intra- and extra-cellular signals to affect the cytoskeleton and could thus act to prevent profilin binding to actin until a signalling event is initiated.

Phosphatidylinositol 4-phosphate (PI4P) is a precursor to PIP2 and an additional signalling molecule found at the plasma membrane that controls cytoskeleton organisation. Levels of PI4P are regulated by kinases and phosphatases, for example, sac1 phosphotinositide (PI) phosphatase. Sac1 mutant studies (Foti et al., 2001) show that sac1 PI phosphatases are vital for a wide range of cellular functions. It is localised to ER membranes but, by a mechanism that is unknown, regulates PI4P levels at the plasma membrane. It has been proposed that there are proteins that link sac1 on the ER (Endoplasmic Reticulum) membrane to its downstream signalling targets at the plasma membrane such as PI4P (Stefan et al., 2011).

1.7. ABP Function and Regulation in Specific Structures and Tissues

The diverse pool of ABPs present in plant cells have their activity regulated spatially and temporally. There are various ways this is achieved so that a particular ABP is active in the correct plant tissue, at a specific location in a cell at the appropriate point in time of the plant's development or stage of the cell cycle (depending on how the protein is temporally regulated). In spite of the large numbers of ABPs, there are some that have different effects on the actin cytoskeleton according to where in the cell they are localised, how they interact with other proteins/signalling molecules or what tissue they are present in.

ABPs are differentially localised within cells depending on where their function is required. This can be mediated through a localisation domain (such as a transmembrane domain) or localisation signal that targets the protein to a particular organelle or sub-cellular structures. For example, *A. thaliana* formin homology5 is anchored in the cell membrane of pollen tubes via a trans-membrane domain (Cheung et al., 2010). Once a protein is localised to a particular organelle or structure is can perform a function relevant to its environmental context. The presence of other ABPs can influence what functions an individual protein performs. For example, AtADF1 acts exclusively as an actin depolymerising protein in the presence of the barbed-end-capping protein gelsolin, releasing g-actin into the cytoplasm. However when gelsolin is not present in the environment the net result of ADF activity is enhanced polymerisation as the released g-actin is recharged with ATP and able to be added onto free filament ends (Carlier & Pantaloni, 1997).

Protein function can be mediated by the cellular environment such as the presence of second messengers such as Ca^{2+}, or changes in pH. ADF/cofilin binds f-actin at acidic pH but severs filaments at alkaline pH; myosin activity is inactivated by Ca^{2+} and is not longer able to bind actin (Vidali et al., 2001). Alternatively, an ABP can have a variety of different binding partners, differentially localised in space or time, which confer different functions on the protein. These binding partners can be associated with large protein complexes to recruit an ABP to a site, or alternatively expressed at a particular stage in the cell cycle to recruit an ABP to a particular mitotic array. For example, profilin can bind g-actin to prevent actin nucleation, or stimulate their addition onto free filament ends. However, when associated with a binding partner PIP2 at the membranes profilin is prevented from binding g-actin and filament polymerisation is prevented. Interactions with binding partners could also mean that a protein present in a pool in the cytoplasm, either carrying out a particular

function or being inactive, could be recruited to a binding partner when its expression was stimulated, perhaps as a response to stress, and have a particular function initiated.

ABP expression can also be differentiated by tissue-type specific promoters. Thus, for example, ABPs that have a function specific to pollen are expressed under the activity of a pollen specific promoter. This can be applied to any plant tissue such as roots, flowers, or leaves. It is not only the ABPs whose expression can be tissue specific. The *Arabidopsis* genome has eight different genes encoding isoforms of actin (Gilliland et al., 2003). These are expressed in different tissues, for example ACT7 is expressed in young vegetative tissue and ACT3 is expressed in reproductive tissue such as ovules and young meristematic tissue (Kandasamy et al., 2001). The fact that small differences found in the different actin sequences are maintained through evolution, suggests that they have functional significance. The different isoforms of actin interact differently with ABPs, thus an ABP that is expressed in all cells throughout a plant can have unique functions in specific tissue types depending on the actin isoform expressed there or which of its binding partners are present.

The ABPs that are under investigation in this thesis have a very specific expression. One is expressed in roots, where it associates with plasmodesmata, and the other in the developing pollen tube. The following sections will describe in detail the features of plasmodesmata and pollen tubes, their dependence on the actin cytoskeleton and how their development and function is regulated by the activity of ABPs.

1.8. Plasmodesmata

Plant tissues have distinct but organised structures, the growth and development of which is tightly controlled by co-ordinated communication at an intra- and inter-cellular level. Within individual cells, communication is achieved via complex cell signalling pathways involving diverse receptors, enzymes, second messengers, hormones and organelles, some of which were discussed in section 1.6 above.

PIN proteins are a family of integral membrane proteins that coordinate communication about developmental processes. The proteins have a polar localisation within the roots i.e. they are found within the plasma membrane on a particular face of the cell wall. They are responsible for directing and controlling the movement of the plant hormone auxin within the root. This hormone is important for developmental processes such as vascular differentiation, organ development and gravitropic growth (Blakeslee et al., 2005).

In addition to communication via hormones such as PIN proteins, plant cells are unique amongst eukaryotic cells in having plasmodesmata. Plant cells differ from animal cells by having a thick cell wall surrounding the plasma membrane. Whilst this is important for the structural support of the organism, it also acts as a physical barrier and communication-block between two adjacent cells. One way plants have evolved to solve this problem is the formation of plasmodesmata in the cell wall by incomplete cytokinesis (Lucas et al., 2009). Plasmodesmata are cylindrical channels in the plasma membrane where there is a close association between the plasma membrane and the endoplasmic reticulum (ER). These two membrane systems form a narrow rod called a desmotubule as they pass through the pore (Tilsner et al., 2011). The basic structure of a plasmodesmata can be seen in figure 4.

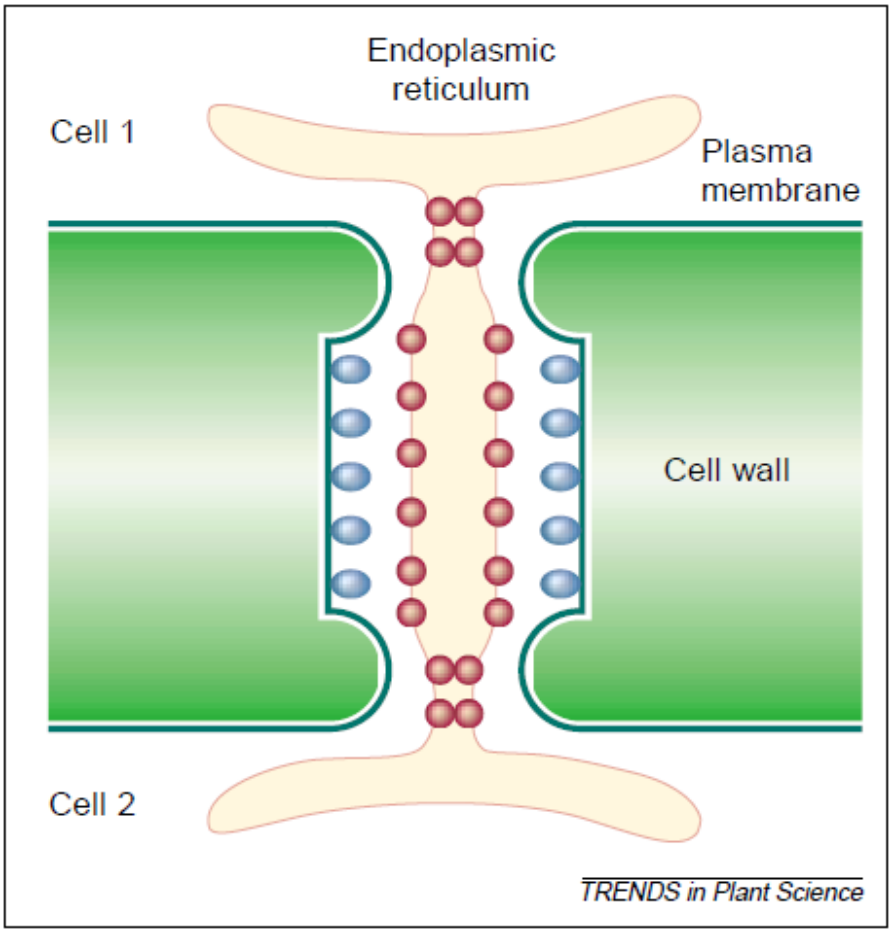


Figure 4: Simplified structure of a plasmodesmata. The ER and cytoplasm are continuous between the two cells. The red and blue spheres represent plasmodesmata-associated proteins that regulate the channel and help intercellular transport (Aaziz et al., 2001)

1.8.1. Structure and Function of Plasmodesmata

Plasmodesmata allow free diffusion of small molecules below a size exclusion limit, whilst larger macromolecules are selectively trafficked between cells. The diameter of the pore is regulated to control its permeability to different substances (Aaziz et al., 2001). Only very recently has progress been made understanding the structure of the plasmodesmal pore. This has proven difficult in the past because the protein complexes are deeply embedded in the cell walls. This has meant that their composite proteins are not easily accessible for immuno-staining or fluorescence imaging. Attempts to isolate individual components using precipitation have been unsuccessful as the extracts tend to be contaminated with cell membrane fractions. Despite these difficulties it has recently been possible to identify the proteome of plasmodesmata using nano-liquid chromatography and an ion-trap tandem mass spectrometer (Fernandez-Calvino et al., 2011). Using these techniques researchers were able to identify 1341 proteins that constituted and associated with plasmodesmata. Examples of proteins identified include actin, myosin, callose synthase, calreticulin, remorin, and various phosphatases and receptor-like kinases. The identification of these proteins, receptors and signalling molecules at the plasmodesmata go to show that plasmodesmata represent a domain within the plasma membrane that is rich in receptor functions. The presence of enzymes involved in callose synthesis and degradation suggests there is an additional biological role for callose deposition in plasmodesmata structure and function (Faulkner & Maule, 2011). Before the proteome analysis it was thought that deposition of callose was a response of the cell to stress and acted to block inter-cell trafficking (Botha & Cross, 2000).

1.8.2. Role of Actin at the Plasmodesmata

It is known that plasmodesmata are closely associated with the actin cytoskeleton. Immuno-based techniques have demonstrated that actin (Blackman & Overall, 1998) and ABPs such as myosins (Reichelt et al., 1999), the ARP2/3 complex (Kelleher et al., 1995) and tropomyosin (Faulkner et al., 2009) are associated with the pores. This association has been confirmed by the recent identification of the plasmodesmal proteome (Fernandez-Calvino et al., 2011). Experiments to date have established that f-actin contributes to regulation of pore dilation (Ding et al., 1996) and passes through the entire length of the channel (White et al., 1994). It is not clear however, the mechanism by which actin regulates pore dilation; or whether the actin filaments passing through the pore help to regulate its dilation or if the actin is directly involved

in inter-cellular transport of molecules. Furthermore, recent experiments have studied plasmodesmata using a movement protein (MP) from tobacco mosaic virus (TMV) which is known to spread between cells via the pores. Use of TMV has shown that the actin cytoskeleton is required to target proteins to the pore for inter-cellular transport (Chen et al., 2005), (Wright et al., 2007).

1.9. Pollen

The pollen grain is the vehicle that is released from the stamen of most flowering plants and contains the male gamete. It develops via a tightly controlled series of cell divisions. Sperm cells are carried to the embryonic sac in the ovary via the germination and growth of a pollen tube that extends down the pistil (McCormick, 2004). Figure 5 shows the plant reproductive tissues and the course that the pollen tube takes delivering the male gamete to the ovule. The actin cytoskeleton is fundamental for pollen tube growth and cell expansion.

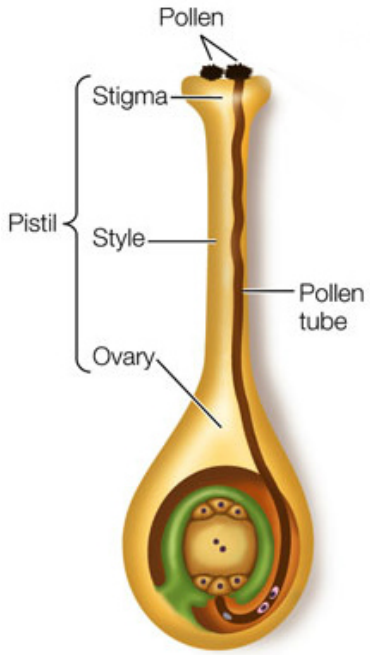


Figure 5: Diagrammatic representation of the route that the pollen tube takes. Its role is to transport the male gamete to the ovary at the base of the pistil (Savada et al., 2008).

1.9.1. Structure of the Pollen Tube

The pollen tube is a uni-cellular, highly polarised structure. Growth is restricted to a small area at the apex and is a characteristic example of tip growth. At the tube apex, secretory vesicles deliver plasma membrane and cell wall material that enables tube

extension (Cai et al., 2000). The pollen tube can be described by different zones along its length which have unique characteristics (shown in figure 6).

1.9.2. Actin Structures and Functions in the Pollen Tube

Growth of the pollen tube is facilitated by the actin cytoskeleton. F-actin is organised into different structural arrays throughout the tube that reflect its function at that particular point. These are visually represented in figure 6. Thick bundles of actin are aligned longitudinally down the shank of the pollen tube and interact with the mechano-chemical enzyme, myosin, to facilitate movement of organelles and vesicles toward the pollen tube tip. (Raudaskoski et al., 2001). In the sub-apical region of the tube, microscopic imaging of the actin cytoskeleton has revealed a dynamic structure described as a collar, ring or funnel in different species of plant. This array contains individual f-actin filaments in contrast to the previously mentioned bundles (Ren & Ziang, 2007). In addition, new actin-binding probes have revealed a highly dynamic, fine and filamentous actin network at the tip of pollen tubes (Vidali et al., 2009).

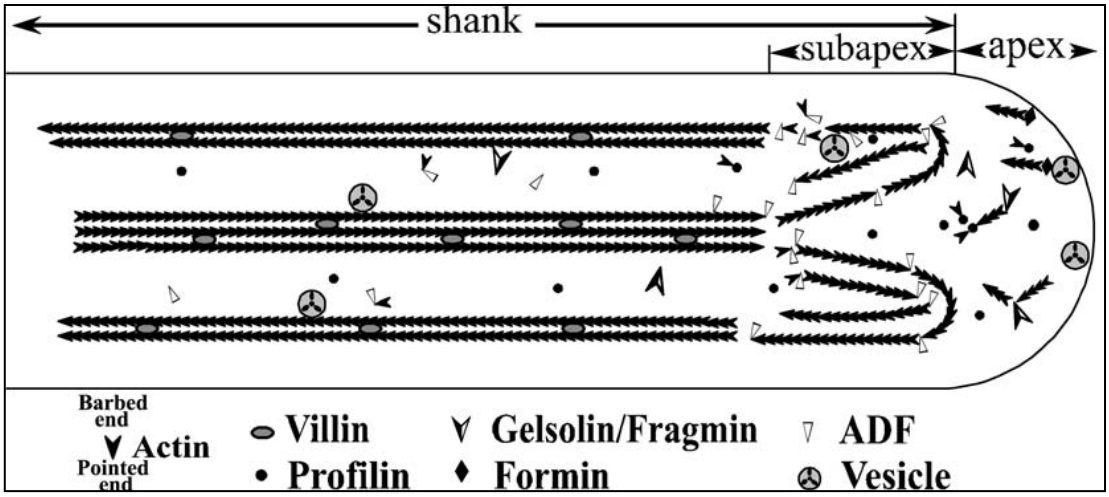


Figure 6: Different regions of the pollen tube and localisation of a number of proteins shown to interact with actin (Ren & Ziang, 2007).

Cytoplasmic streaming and vesicle delivery at the tube tip are regulated by several signalling pathways that interact to maintain cell polarity and growth. These signals are generally transmitted to the cytoskeleton via ABPs which control f-actin dynamics to alter the structural arrays and regulate filament turnover (Fu, 2010).

1.9.4. Signalling to the Actin Cytoskeleton in Pollen Tubes

Many of the signalling pathways discussed in section 1.6 can be evidenced in the pollen tube. The tip of the pollen tube has a [Ca^{2+}] gradient signals to ABPs. The vesicle trafficking activity of myosin and actin bundling activity of villin are both suppressed by high Ca^{2+} concentrations and the pollen tube tip (Malho et al., 2006), (Vidali & Hepler, 2001) causing the deposition of vesicles containing new cell membrane material necessary for growth (Fu, 2010).

In a similar way, a pH gradient exists that resulting in the acidification of the apex and alkalinisation of the sub-apical region (Vidali & Hepler, 2001). The ABPs ADF/cofilin and AIP1 cause depolymerisation of f-actin in a pH dependant manner to increase actin turnover (Ren & Ziang, 2007). The alkaline band in the sub-apical region of the tube provides an environment where ADF and AIP1 are activated to release g-actin and small actin seeds from actin filaments toward the tip where they can re-polymerise to enable tube elongation (Allwood et al., 2002).

The self-incompatability (SI) response is a genetically controlled, actin dependant process that occurs along the shank of the pollen tube to prevent self-fertilisation (Staiger et al., 2010). The response causes programmed cell death of the pollen tube by actin filament depolymerisation. Depolymerised actin accumulates at the pollen tube membrane in a punctate localisation to create large actin foci. This is enabled by the activity of cyclase associated protein (CAP) and ADF, ABPs that increase actin turnover (Poulter et al., 2010).

As discussed in section 1.8.1, deposition of callose at plasmodesmata is a response to cellular stress that blocks the pore to prevent potential translocation by viruses and pathogens. Localisation of callose at leaf epidermal cell membrane shows a punctate co-localisation with plasmodesmata (Faulkner et al., 2009). Recent research into the distribution of callose in pollen tubes has identified the enzyme callose synthase that is active at the plasma membrane of pollen tubes in response to stress. It has a similar localisation pattern to that of callose at plasmodesmata: punctate foci at the plasma membrane of pollen tubes (Cai et al., 2011). Pollen tubes have not been shown to have plasmodesmata embedded in the cell wall and the function of these punctate foci is currently unknown.

1.10. The NET Family of Plant Specific Actin Binding Proteins.

The ABPs are important proteins in the cell because they coordinate all the biologically important roles of the actin cytoskeleton. Research to identify and classify new ABPs is essential to understand the functions of the actin cytoskeleton.

1.10.1. Identification of NET1A – A Novel, Plant Specific ABP.

There are currently very few plant specific ABPs with functional domains that are different to those found in non-plant cells. However, the NET family of proteins has a common functional domain that is unique to plants. The founding member of the group, NET1A was recently identified in a screen of a partial cDNA library extracted from *Arabidopsis thaliana* root tissue. The screen was undertaken in the laboratory of Professor Karl Oparka with the aim of using a high-throughput technique to find the localisation of unknown expressed proteins (Escobar et al., 2003). The screen involved fusing GFP to partial cDNAs and expressing them in *Nicotiana benthamiana* leaf epidermal cells. Laser scanning confocal microscopy was used to identify GFP fusion proteins with interesting localisation patterns. Oparka's screen was designed to identify novel proteins that associate with the plasmodesmata but many additional proteins were also identified with a variety of sub-cellular localisations. The results of this screen are available via the Protein Localisation (ProtLoc) database.

One of the fusion proteins identified in the screen decorated an extensive filament system in *N. benthamiana* – the actin cytoskeleton. Further cloning of this fusion protein found that the sequence encoded the n-terminal region of a novel protein. Bioinformatic analysis of this protein led to the identification of two features of interest: coiled-coil domains along its length and a 'KIP1' domain at the n-terminus.

1.10.2. Identification of the NET Family of Proteins

KIP1 (kinase interacting protein 1) was identified in a yeast two-hybrid screen as a pollen specific protein that showed a strong association with the kinase domain of a receptor-like kinase of petunia (*Petunia inflate)* called PRK1 (Skirpan et al., 2001). The kinase-interacting ability of KIP1 with PRK1 occurs through a central domain but, rather confusingly, the n-terminal domain of KIP1 is called the 'KIP1 domain'. This is the domain that was identified in NET1A.

Once the structure of NET1A was established, a BLAST search was conducted to determine whether there were any other *A. thaliana* proteins with

homologous sequences. This search identified 12 new proteins that were described as being ‘kinase interacting protein-related’ and all shared a homologous n-terminal region, the KIP1 domain (Calcutt, 2009). This group of 13 proteins were classified as the NET family.

Initial localisation studies of the isolated n-terminal functional domain of almost all the proteins in the family showed decoration of the actin cytoskeleton. This protein family was named the NET family as the filamentous localisation throughout the cell had a ‘net’ like structure. It was classified as a novel family of plant specific ABPs defined by a homologous n-terminal actin binding domain (ABD). Figure 7 shows the NET protein family with their homologous ABD and other c-terminal functional domains that define smaller groups of proteins within the family.

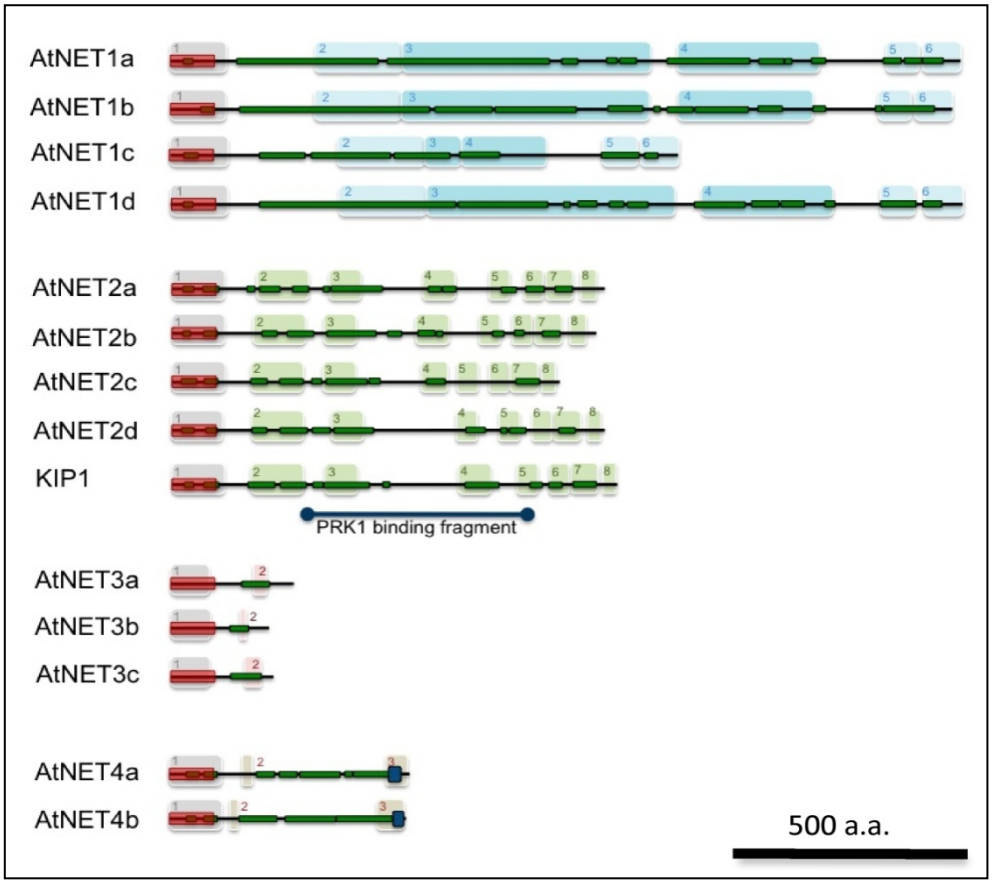


Figure 7: The NET Family of ABPs. Proteins are classified into smaller groups based on the characteristics of their central and c-terminal domains. The left hand red domain is the homologous ABD. The green domains are predicted coiled-coil regions. The numbered boxes represent domains that are conserved within a particular sub-family between dicot and monocot plants.

Whilst all the members of the NET family have the homologous n-terminal ABD, the c-terminal regions of these proteins are varied. This suggests that the cellular processes the proteins are involved in and/or the sub-cellular localisation of different family members could differ considerably. The KIP1 protein from petunia, with the KIP1 domain it shares in common with the NET proteins is shown on figure 7 with the central domain through which it interacts with the cytosolic region of PRK1. KIP1 is a pollen specific protein thus it is not surprising the domains that are downstream of the ABD are most similar to those group two proteins that are also pollen specific.

1.10.2. NET1A and NET2A: what is known to date

The NET proteins were only recently identified so have not yet been fully characterised. Initial work by Dr J. Calcutt (2009) on NET1A has studied its localisation which is shown to be at the plasma membrane of cross-walls in cells in the stele cell files of roots. It is associated with cortical actin arrays and perhaps provides a link between actin in the cell cortex and the plasma membrane. Since NET1A does not have a transmembrane domain this link is most likely mediated through an interaction with another integral membrane protein. NET1A is appropriate for this sort of structural function as analysis of the protein has shown the c-terminus has large coiled-coil domains. Proteins with coiled-coils usually interact with other cellular components, either acting as anchoring, cross-linking or scaffolding proteins (Rose et al., 2005).

Large coiled-coil proteins have been known to be multi-functional. Thus the protein could have both structural and signalling activities (Burkhard et al., 2001). Its localisation at the plasma membrane and physical link to the actin cytoskeleton means that it is well placed to enable the transduction of environmental or developmental signals from the extra-cellular environment. Initial images suggested a punctate localisation at the cross-walls similar to the distribution of plasmodesmata. Use of aniline blue (a callose stain) has confirmed an association between the NET1A punctate foci and plasmodesmatal pores (Hussey and colleagues, unpublished data). This would provide further support for a hypothesis that the protein is involved in signalling or has cross-linking functions as the plasmodesmata are complexes where it is known that cell membrane systems and the actin cytoskeleton come together (Tilsner et al., 2011).

NET2A has a pollen specific expression but has been less extensively studied to date. It interacts with the actin cytoskeleton and in a superficially similar way to NET1A, forms punctate foci. These are localised to the pollen tube cell membrane

along the shank of the tube and excluded from the tip region. It is yet unknown what these punctate structures might represent as there are no plasmodesmatal-like protein complexes known in pollen tubes. Research by Hussey and colleagues (unpublished data) has shown that the punctate foci appear to align with the actin cytoskeleton as 'beads on a string' although co-localisation in live pollen tubes has not yet been possible.

1.11. Strategy

This thesis sets out to probe key features of the ABD that is characteristic of the NET family of proteins. NET1A and NET2A were used as representative members of the family. NET1A has been most highly characterised so far and thus had the largest knowledge base on which to build. Although not as much is known about NET2A, its expression in pollen means that it had a potentially simple method of transient expression (DNA coated-gold particle bombardment) and a straightforward method of assessing a phenotype. Pollen tube growth is an easy process to characterise, measure and identify abnormal developmental patterns.

There are various approaches that can be taken to investigate the function of the NET1A and NET2A genes. A gene knockdown or knockout reduces or eliminates the function of a gene, respectively, by altering the DNA sequence or deleting a large portion of the gene. Alternatively, a dominant-negative approach can be used to target a particular gene function. It involves over-expressing a mutant gene so that the mutant protein is produced in large quantities in the cell relative to the wild type (WT) protein that is expressed at normal levels. This results in the function of the WT gene being eliminated because, although the gene has a particular function inactivated by the mutation, it is still able to associate with the WT protein's binding partners. This blocks the functional protein from making its native associations so it is unable to carry out its function. This project utilised the dominant-negative approach. The sub-groups of proteins within the NET family show functional redundancy. This means that using the knockdown or knockout approach would not produce a mutant phenotype because the other proteins within the group compensate for the function lost by the mutated protein.

This project has created two types of construct for each protein. The first was a sub-clone with the ABD deleted so only the central and c-terminal domains were expressed. The second construct had the full length protein expressed but with point mutations at particular residues within the ABD. Both of these approaches set out to disrupt the protein's association with the actin cytoskeleton whilst maintaining other

protein functions. The ABD deletion was the most direct method of disrupting the protein's interaction with actin. However, the n-terminal region was also likely to include residues that have functional significance for the protein other than actin binding. Thus, the point mutations within the ABD represent more subtle attempts to disrupt ABD whilst leaving any other functions intact. The details of the point mutations and the rationale for choosing which residues to target will be discussed in section 3.1.1.

Over-expression of the mutated constructs aimed to elucidate a change in the localisation of the protein in transient expression systems or bring about a mutant phenotype in the stable expression lines. The aim of the dominant negative approach was to displace the wild-type proteins from their normal site of action and replace them with the mutated gene. This would be brought about as the mutant construct would be expressed at a very high level under the control of either the 35S (NET1A) or *LAT52* (NET2A) promoters and thus it would be able to out-compete the WT protein.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Plant Materials

Nicotiana benthamiana leaves were used to transiently express the mutant constructs to gain results for the localisation of the ABD mutations. In addition *N. benthamiana* were used to test the alcohol inducible pALC construct. *Nicotiana tabaccum* pollen was used for transient expression of NET2A constructs via pollen bombardment with DNA coated gold particles. *Arabidopsis thaliana,* columbia (Col-0) ecotype, was used to produce stably transformed lines of the ABD mutated constructs of both NET1A and NET2A.

2.1.2. Bacterial Strains

Agrobacteruim tumefaciens GV3101 strain was used to transiently express constructs in *N. benthamiana* leaves as it expressed at a high level using the leaf infiltration protocol described by Wroblewski et al (2005) and also for creating stably transformed lines via the plant dipping method (Clough & Bent, 1998). *Escherichia coli* strain DH5α (Invitrogen) and strain DB3.1 (Invitrogen) were used for Gateway molecular cloning reactions and for transient expression of constructs in *N. tabaccum* pollen.

2.1.3. Vectors and Constructs

Table 1: Vectors Used in the Cloning, Transient Expression and Stable Expression of Constructs. A list of all constructs cloned during the project can be found in appendix 1.

Vector Name	Application	Size (bp)	Resistance Cassette (s)	Constructs used for	Source
pDONR 207	Gateway Entry Vector	5585	Gentamicin (bacteria)	NET1A and NET2A	Invitrogen
pDONR Zeo	Gateway Entry Vector	4291	Zeocin (bacteria)	NET1A and NET2A	Invitrogen
pENTR3C	Gateway Entry Vector	3765	Kanamycin (bacteria)	NET1A Cloned by Dr J. Calcutt	Invitrogen
pMDC107	Destination vector for c-terminal GPF fusions under endogenous promoter	11744	Kanamycin (bacteria) Hygromycin (plants)	NET2A (Cloned by Laura Bell) mutants for dipping	University of Zurich via ABRC
pMDC83	Destination vector for c-terminal GFP fusion for expression under 2x35S promoter	12513	Kanamycin (bacteria) Hygromycin (plants)	NET1A (Cloned by Dr J. Calcutt)	Invitrogen

pH7FWG2	Destination vector for c-terminal GFP fusion for expression under 35S promoter	12267	Spectinomycin (bacteria) Hygromycin (plants)	NET1A for dipping and infiltrations	VIB/Gent
pH7CWG52	Destination vector for c-terminal CFP fusion for expression in pollen under *LAT52* promoter	11774	Spectinomycin (bacteria) Hygromycin (plants)	NET2A WT and mutants for dipping	VIB/Gent and Abigail Waters
pH7WGC52	Control plasmid for pollen, expressing CFP under *LAT52* promoter	11776	Spectinomycin (bacteria) Hygromycin (plants)	Control for CFP dipped NET2A plants	VIB/Gent and Abigail Waters
p52GWC7	Destination vector for pollen expressing CFP fusion to c-terminus	6183	Ampicillin (bacteria)	NET2A WT and mutants for bombardment	VIB/Gent and Abigail Waters
p52CGW7	Control plasmid for pollen, expressing CFP under *LAT52* promoter	6185	Ampicillin (bacteria)	Control of CFP pollen bombardment	VIB/Gent and Abigail Waters
pH7CWG2	Destination vector for c-terminal CFP fusion for expression in *N. benth*	12267	Spectinomycin	NET1A constructs for infiltration	VIB/Gent
pH7RWG2	Destination vector for c-terminal RFP fusion for expression in *N. benth*	12226	Spectinomycin	Lifeact actin marker	VIB/Gent

2.2 Molecular Biology Methods

2.2.1. Transformation of Chemically-Competent Cells by Heat Shock

2.2.1.1. *E. coli* Strains

Approximately 100ng of plasmid DNA was added to a pre-thawed, 200µl aliquot of cells and left on ice to incubate for 25-30 minutes. DNA and cells were placed in a 42^{o}C heat block for 40 seconds and then returned to ice to rest for two minutes. 500µl of SOC media (20g/l tryptone, 5g/l yeast extract, 0.5g/l NaCl, 0.2g/l KCl, 1g/l $MgCl_2$, 2.5g/l $MgSO_4$, 0.2M glucose, adjusted to pH7 with NaOH) were added to the cells and they were incubated on a shaker for one hour at 37^{o}C to allow the cells to recover. The cells were then centrifuged at 3000xg for three minutes and resuspended in approximately 50µl of liquid LB media (10g/l tryptone, 10g/l NaCl, 5g/l yeast extract, pH7). The resuspended cells were spread onto plates containing solid LB medium (2% microagar) and the necessary antibiotic for selection. Plates were incubated at 37^{o}C for 14-16 hours to allow colonies to grow. Single colonies were used to inoculate a 5ml overnight culture of SOB media (20g/l tryptone, 5g/l yeast extract, 0.5g/l NaCl, 0.2g/l KCl, 1g/l $MgCl_2$, 2.5g/l $MgSO_4$, adjusted to pH7 with NaOH) containing the same antibiotics used in the solid agar plates. Cultures were incubated on a shaker at 37^{o}C for 12-16 hours.

2.2.1.2. *A. tumefaciens* GV3101 Strain

Approximately 100ng of plasmid DNA was added to a pre-thawed, 200µl aliquot of *A. tumefaciens* cells on ice. The cells were frozen in liquid nitrogen for five minutes and then defrosted in a 37^{o}C heat block for five minutes. 600µl of YEB medium (5g/l meat extract, 1g/l yeast extract, 5g/l sucrose, 0.5g/l $Mg_2SO_4.7H_2O$, pH 7) were added to the cells which were then incubated on a shaker at 30^{o}C for three hours to allow the cells to recover. Recovered cells were centrifuged at 3000xg for three minutes and then resuspended in approximately 50µl of YEB liquid media. Resuspended cells were spread onto plates made with solid YEB medium (20g/l microagar) containing 25µg/ml rifampicin, 25µg/ml gentamicin and the necessary antibiotic for selection. Plates were incubated at 30^{o}C for two days. Single colonies were used to inoculate 5ml overnight cultures of liquid YEB media containing the same antibiotics as required in the plates. These were incubated on a shaker at 30^{o}C for 12-16 hours.

2.2.2. Plasmid DNA Purification

Plasmid DNA was purified from bacterial overnight culture using the Promega wizard plus SV miniprep DNA purification system according to manufacturer's instructions. Briefly, 5ml of overnight culture was pelleted by centrifugation at 5000xg for five minutes and the culture medium discarded. The cells were resuspended and lysed. Next, any active bacterial proteins were inactivated by a five minute incubation at RT (room temperature) in alkaline protease solution. Cell debris was separated from the DNA-containing solution by a ten minute centrifugation and the cleared lysate was transferred to a spin column containing a DNA binding membrane. The DNA was washed three times and then eluted in 100µl nuclease free water and stored at -20^{o}C.
To prepare DNA for coating gold particles for pollen bombardment a Qiagen endotoxin free maxi prep kit was used to purify highly concentrated DNA according to manufacturer's instructions. Briefly, 250ml of LB media was inoculated with a few colonies of DH5α transformed with the construct. The culture was shaken at 37^{o}C overnight. The culture was spun down by centrifugation at 6000xg for 15 minutes at 4^{o}C. The pelleted cells were resuspended, lysed and the cell debris precipitated. The debris was extracted using the QIAfilter cartridge and the DNA washed using the QIAGEN-tip system. Eluted DNA was precipitated using isopropanol and the DNA was pelleted by centrifugation at >15,000xg for 30 minutes. The DNA pellet was then washed with endotoxin-free 70% EtOH and re-pelleted by centrifugation at >15,000g for a further 10 minutes. Finally the pellet was air dried for approximately 30 minutes and resuspended in 100µl of endotoxin-free TE buffer.

2.2.3. Restriction Digests

Restriction digests were used as a diagnostic tool to determine whether the desired protein fragment had been successfully cloned into the Gateway entry vector or expression vector. The digestion mix was made up with 10µl of DNA (purified according to 2.2.2. above), 1µl of the appropriate restriction enzyme(s), 2µl of appropriate buffer and made up to 20µl with sterilised distilled water. The digestion mixture was then incubated at 37^{o}C for 2 hours. Digested fragments were separated using agarose gel electrophoresis (see 2.2.4. below).

2.2.4. Agarose Gel Electrophoresis

Agarose gels were prepared by dissolving 1% (w/v) agarose low EEO (Melford) in 1xTAE buffer (40mM tris acetate, 1mM EDTA) by heating. 5µl of ethidium bromide solution (0.3mg/ml) were added per 200ml of 1xTAE. The mixture was then poured into trays containing well-combs and left to set for 30 minutes. For the electrophoresis the solidified gel was placed in an electrophoresis tank containing 1xTAE buffer. The DNA samples were loaded into the wells using 5x DNA loading buffer (Bioline) and 5µl of Bioline hyperladder 1 as a DNA marker. Gels were run at 135V for 20-30 minutes and then gels were imaged under ultraviolet illumination using the Syngene InGenius gel documentation system.

2.2.5. Polymerase Chain Reaction to Amplify a Fragment of Interest

Reactions were done in a 50µl volume containing 10ng/µl DNA, 0.5µl Phusion (Finnzyme) DNA polymerase enzyme, 10µl HF Phusion 5x buffer, 1.5µl of 10pM forward primer, 1.5µl of 10pM reverse primer, 1µl dNTPs (consisting of 10mM of dATP, dCTP, dGTP and dTTP) and made up to 50µl with sdH_2O. The standard PCR program in table 2 was used to amplify NET1A and NET2A without the actin binding domain. To try to optimise the PCR reaction, a series of annealing temperatures were used in a gradient block in the PCR machine. In addition, the ‘hot start’ method was used whereby the phusion enzyme was added to the PCR mix once it had been placed in the gradient block and heated up to 98^{o}C. This was done to reduce the occurrence of primer dimers which was a problem when amplifying NET1A without the actin binding domain.

Table 2: Standard PCR Program Used.

Stage	Temp (°C)	Time (sec)	
Initial Denature	98	30	
Denature	98	10	These steps are repeated in a cycle approx. 30 times.
Annealing	60*	30	
Extension	72	180**	
Final Extension	72	600	
Hold	4	∞	

* Annealing temperature is variable, dependant on the primers used
**Extension time is based upon the length of the DNA fragment to be amplified – approx 1kb/minute

The primer sequences used for PCR fragment amplifications can be found in appendix 2.

2.2.6. Mutagenesis of ABDs

The mutagenesis reactions were done using the QuikChange lightening site-directed mutagenesis kit from Agilent. In brief, NET1A in pENTR3C and NET2A in pDONR207 were used as the dsDNA template. The specially designed primers (see appendix 2) contained the desired mutation and the forward and reverse primers together resulted in the synthesis of new plasmid dsDNA containing the mutation. The mutagenesis primers were designed using the Agilent website (http://www.genomics.agilent.com/). The sample and control reactions were set up as described in the protocol on ice and then put in a thermal cycler for the following program:

Table 3: Thermal Cycler Program for the Mutagenesis Reactions.

Segment	Cycles	Temp. (°C)	Time
1	1	95	2 min
2	18	95	20 sec
		60	10 sec
		68	30 sec/kb of plasmid*
3	1	68	5 min

*An extension time of 4.5 minutes was used for all reactions.

After the thermal cycling, *dpn1* restriction enzyme was added to destroy any non-mutated plasmid DNA. 2µl of *dpn1*-treated DNA from each reaction were transformed into ultracompetent cells and then spread onto LB plates containing the correct antibiotic for selection.

2.2.7. Gateway Cloning Technology

Gateway cloning was used as a quick and efficient method of transferring DNA sequences into multiple types of plasmid. It relies on a set of recombination sequences termed 'att sites'.

2.2.7.1. BP Reaction

The BP reaction involves inserting the PCR fragment or DNA sequence that is flanked by Gateway *attB* sites into a donor vector containing *attP* sites. An enzyme mix called BP clonase catalyses this reaction to produce a Gateway entry clone containing the gene of interest. The reaction was set up to include 150ng of the entry vector (pDONR 207, pDONR Zeo or pENTR 3C), 1µl of 10x TE Buffer (1M Tris (pH7.5), 0.5M EDTA (pH8.0)), 200ng of PCR fragment and made up to 8µl with dsH_2O. 2µl of BP clonase enzyme mix were added and the mixture was incubated at 25^oC for one hour. After the incubation, 1µl of proteinase K was added and the mixture was incubated for 10 minutes at 37^oC. 2µl of the completed reaction was transformed into DH5α cells and selected by antibiotic resistance for the entry clone (see table 1) according to section 2.2.1.1. The entry clone was then purified from bacteria (section 2.2.2) and a restriction digest was carried out (section 2.2.3) to confirm the presence of the PCR fragment in the entry vector.

2.2.7.2. LR Reaction

The LR reaction involves transferring the gene of interest flanked by *attL* sites from the Gateway entry clone produced in the BP reaction to the destination vector (the types of vector used are shown in table 1) containing *attR* sites. An enzyme mix called LR clonase catalyses the reaction. The reaction is made up of 150ng of entry clone, 1µl 10xTE Buffer, 150ng of destination clone and made up to 8µl with dsH_2O. 2µl of LR clonase enzyme mix were added and the mixture was incubated at 25^oC for one hour. After the incubation, 1µl of proteinase K was added and the mixture incubated for 10 minutes at 37^oC. 2µl of the completed reaction were then transformed into DH5α cells and selected by antibiotic resistance for the expression clone (see table 1). The amplified DNA was then purified from bacteria (section 2.2.1.1) and a restriction digest (section 2.2.3) carried out. The expression clone was

then sent for DNA sequencing to confirm that the correct DNA sequence was accurately cloned into the correct destination vector. For sequencing primers see appendix 3.

2.2.8. Transient Expression of NET1A Constructs in *N. benthamiana* Leaves by Infiltration

N. benthamiana was used for transient expression of NET1A constructs. The protocol was based on Voinnet et al (2003). Firstly 2µl of the expression clone were transformed into GV3101 cells as described in section 2.2.1.2. Overnight cultures of both P19 (a gene silencing suppressor) and the expression clone were pelleted for four minutes at 3000xg and the pellet resuspended in 2ml of infiltration buffer (10mM MES pH 6.5, 10mM $MgCl_2.6H_2O$). This centrifugation and resuspension was repeated three times with the final resuspension volume being 1ml of infiltration buffer. 1µl of 200µM acetosyringene stock was added to the final cell resuspensions. The optical density of each construct was determined (at a wavelength of 600nm) using a spectrophotometer and the cell suspension for infiltration was assembled such that the relative optical density proportion of cells was 0.5 for P19, 0.4 for the expression clone, and 0.1 split equally between the actin/ER markers used. The *A. tumefaciens* cells were sucked up using a 1ml syringe without a needle and injected into the underside of *N. benthamiana* leaves through three scalpel slits. The infiltrated plants were left for three days for protein expression to occur and then sections of the leaf were cut out, mounted on microscope slides in distilled water and imaged using the Leica SP5 LSCM.

2.2.9. Transient Expression of NET2A Constructs in *N.tabaccum* Pollen.

The NET2A constructs were transiently expressed in pollen tubes by bombarding ungerminated pollen grains, collected from *N. tabaccum*, with gold particles coated in maxi-prepped DNA. DNA-coated gold particle cartridge preparation was adapted from Kost et al (1998). Each cartridge contained 0.5mg of gold and the cartridges were made up in batches of ten per construct. The gold particles had a diameter of 1µm and were aliquoted from a 50mg/ml stock solution and washed three times with dH_2O to remove the glycerol. 100µl of 0.05M freshly made spermidine stock was added to the gold and vortexed before the plasmid DNA was added to the gold at a concentration of 1µg DNA per cartridge. The mixture was vortexed again. While vortexing, 100µl of 1M $CaCl_2$ was added dropwise, then the mixture was left for ten minutes to precipitate. The gold particle solution was pelleted at 2300xg for 15

seconds and washed three times with 1ml of 100% ethanol before finally being suspended in 0.5mg/ml PVP. The gold suspension was loaded onto gold-coat tubing (Bio-Rad) using the Bio-Rad cartridge kit and the individual cartridges cut up using Bio-Rad tubing cutter. Prepared bullets were stored in a parafilm-sealed container at -20^{o}C until they were used.

For the transformation, pollen from 1-2 flowers of *N. tabaccum* were collected and suspended in 50µl of tobacco pollen tube medium (1mM $CaCl_2$, 1mM KCl, 0.8mM $MgSO_4$, 1.6mM H_3BO_3, 30µM $CuSO_4$, 0.03% casein acid-hydrolysate, 5% w/v sucrose, 12.5% w/v PEG-6000, 0.3% MES, adjusted to pH5.9 with KOH or HCl and filter sterilised (Kost et al., 1998)). The pollen suspension was pipetted onto filter paper and bombarded with the DNA-coated gold particles using the Helios gene gun (protocol adapted from Sommer et al. 2008). The pollen grains were immediately transferred to solid pollen tube medium (0.25% phytagel) on microscope slides and covered in a small amount of liquid medium. The slides were incubated, inside a petri dish, in the dark at 24^{o}C for three hours to allow the pollen to germinate. Once germinated, the pollen tubes were imaged using the Leica SP5 LSCM.

2.2.10. Stable Gene Expression in *A. thaliana* using the Floral Dipping Method.

This protocol was modified from Clough and Bent (1998). *A. thaliana* plants (columbia ecotype) were grown for 3-4 weeks until they were about 10-15cm tall and had open flowers. *A. tumefaciens* GV3101 strain was used for all transformations containing the expression vector to be transformed. The *A. tumefaciens* was transformed according to section 2.2.1.2. Overnight cultures were grown for 48 hours at 30^{o}C in 200ml of YEB media containing rifampicin, gentamicin and the correct antibiotic for selection of the vector being used. The culture was transferred to conical-bottom 50ml falcon tubes and centrifuged at 2000xg for 20 minutes to pellet the *A. tumefaciens.* Cells were resuspended in 1litre of 5% sucrose solution also containing 0.05% silwett L-77. The *A. thaliana* plants were dipped individually in the solution for 10-15 seconds and then placed in a sealed bag to maintain humidity and stored in a shaded environment overnight. The next day the dipped plants were taken out of the sealed bag and grown under normal growth room conditions (see section 2.4.2). The dipping process was repeated seven days later using freshly cultured bacteria. After the second dipping the plants were left to set seed and dry out in the greenhouse for 2-4 weeks, with each plant self-contained in an aracon to prevent cross contamination of seeds. After seeds were collected they were left to mature for 2 weeks at RT.

The transformed seeds were sterilised with 6% sodium hypochlorite solution and germinated on ½ MS (half-strength Murashige and Skoog (MS) basal salt mixture

2.15g/L corrected to pH5.7 with KOH and 0.8% plant agar) plates with timentin (50mg/ml) and the correct antibiotic for transformant selection (hygromycin at 40mg/ml). The seeds that germinated and developed into seedlings were transferred to soil and grown up as the first generation of stably transformed plants.

2.3. Laser Scanning Confocal Microscopy

To visualise GFP- or CFP- tagged constructs, the proteins were transiently expressed in *N. benthamiana* leaf epidermal cells using agrobacterium-mediated infiltration (section 2.2.8). Imaging was done three days after infiltration by mounting an excised leaf section from near the infiltration wound site in dH_2O. It was placed under a cover slip and the abaxial surface of the leaf imaged.

To visualise CFP-tagged constructs in pollen tubes, the proteins were transiently expressed using the DNA-coated gold particle bombardment system (section 2.2.9). Bombarded pollen grains were mounted on a slide with solid pollen tube medium under a cover slip.

Most of the imaging was done on a Leica SP5 LSCM although for some imaging a Zeiss 510 LSCM was used instead. The objective used was a x40 oil immersion lens. Table 4 below shows the properties of the lasers and excitation and emission spectra used.

Table 4: Excitation and Emission Spectra used to Image Different Fluorescent Proteins

Fluorescent protein	**CFP**	**GFP**	**RFP**
Excitation wavelength	405nm	488nm	543nm
Emission wavelengths detected	450-480nm	505-530nm	560-650nm

Where two different fluorescent tags were imaged at the same time, a sequential scan system was used after each line of laser scanning.

NET1A constructs were initially expressed with a GFP tag. To investigate a relationship between the constructs and the ER and actin cytoskeleton, fluorescent markers were used. To visualise the actin cytoskeleton, lifeact (Reidl et al., 2008) tagged with RFP and FABD2 tagged with GFP were expressed. To visualise the ER an HDEL marker was used tagged with RFP or CFP. To increase flexibility with which markers could be used to co-localise, the NET1A constructs were cloned into CFP destination clones.

Visualisation of NET2A constructs was done using a CFP tag. The only actin marker expressed under the pollen specific *LAT52* promoter was Lifeact::CFP which was not suitable for investigating a co-localisation between the construct and actin. To try to show a co-localisation, FABD2::GFP expressed under the 35S promoter was used in the pollen bombardment.

2.4. Plant Growth Conditions

2.4.1. *Nicotiana benthamiana* and *Nicotiana tabaccum*

Seeds were sprinkled directly onto tray substrat (Klasmann) in small pots. The soil was pre-treated with intercept insecticide according to manufacturer's directions. The pot was covered with cling film to maintain a humid environment until the seeds had germinated and seedling had grown to about 1.5cm tall. The pots were kept in a greenhouse with a temperature of 20± 2^{o}C during the day and 15 ± 2^{o}C at night. The light cycle consisted of a 16 hour day and 8hour night. After approximately three weeks seedlings were transferred to individual 10cm pots.

2.4.2. *Arabidopsis thaliana*

Plants grown for dipping were non-sterile. A pinch of *A. thaliana* ecotype Col-0 seeds (Lehle, Texas) were sprinkled onto intercept-treated tray substrat (Klasmann) soil in one cell of a seed tray. The cell was covered with cling film and placed in a fridge for three days to synchronise germination. The seeds were then moved to a Sanyo growth cabinet (21^{o}C during a 16 hour day and 18^{o}C during an 8 hour night) to germinate. Once the seedlings had developed 4-6 true leaves they were transferred to individual cells in seed trays containing intercept treated soil. They were left to grow in the Sanyo growth cabinet and cut back twice once they had bolted, to increase the number of inflorescences.

A. thaliana seeds from dipped plants were grown under sterile conditions. Seeds were surface sterilised for 10 minutes in a 6% sodium hypochlorite solution. To remove the bleach seeds were washed three times in sterile, autoclaved water in a flow cabinet. Seeds were spread onto petri dishes containing ½ MS media. First generation transformants were grown on small round petri dishes in a horizontal orientation. Plants required for imaging pollen were transferred to tray substrat (Klasmann) in seed trays after approximately 12 days, covered with ventilated plastic lids to maintain humidity until the seedlings were properly established, and kept in a Sanyo growth cabinet.

2.4.3. *Arabidopsis thaliana* Seed Collection

Dipped *A. thaliana* plants were individually place in clear plastic bags. This kept seeds from individual plants isolated and prevented loss of any seeds released from the siliques. Once the plants had been left to develop siliques and senesce, seeds were collected.

Seeds were collected from transformed plants using the ARACON container system. As soon as the rosette of the plant was fully developed an ARACON base was placed over the plant into the soil and an ARACON tube enclosing any stems that had already developed. As the plant further developed, new stems grew up into the ARACON tube.

Collected seeds were stored in micropore-sealed petri dishes for 2 weeks to dry out and then transferred to 1.5ml eppendorf tubes.

Chapter 3: Results

3.1. Creation of Mutated Constructs

3.1.1 Rationalisation for Mutant Design

The 96 residue ABD is the highly conserved functional domain common to all members of the NET family. Previous work by Dr J. Calcutt (2009) has shown that NET1A interacts with actin through this n-terminal domain. When the isolated ABD of NET1A was expressed in *N. benthamiana* leaves the localisation was filamentous. Further, when the actin cytoskeleton was disrupted with the drugs cytochalasin D and latrunculin B, the localisation of the ABD was no longer filamentous but the peptide formed aggregates that accumulated in the cytoplasm. The region defined as containing the f-actin binding domain may also contain motifs with alternative functions. This means it is likely that this region is responsible for other protein functions and it would be informative to investigate, firstly what these could be, and secondly what parts of the unique domain are directly responsible for the actin interaction.

The aim of mutating NET1A and NET2A was to remove their ability to interact with the actin cytoskeleton. Any resulting phenotype or change to the localisation of the proteins would provide an insight into the function of the protein and the purpose of its f-actin interaction. The most straight forward and sure way of disrupting f-actin binding was to remove the entire n-terminal ABD, leaving just the protein's central and c-terminal domains to be expressed. To achieve this, both NET1A and NET2A were sub-cloned by PCR using the method described in section 2.2.5. The sequencing primers (see appendix 3) incorporated a new start codon with a Kozak consensus sequence so that the target start codon was prioritised for translation initiation To rule of the presence of another domain within the protein that interacts with f-actin outside the ABD, it was necessary to demonstrate that the ABD deleted construct did not bind f-actin.

The size of the ABD has been defined by the extent of the highly homologous region between different members of the NET family. It has not yet been determined precisely the minimal number of residues needed for the f-actin interaction to occur. A functional domain of 96 residues is relatively large. Within this region it is likely that smaller groups of residues contribute different functions for the protein; perhaps

interacting with other cellular components or providing a structural role. Deleting the ABD in its entirety would also disrupt these other functions.

The additional point mutation approach was taken to disrupt f-actin binding whilst keeping intact the majority of the n-terminal domain, and any non actin-binding functions it may have. In order to identify residues involved in actin binding, the dysfunctional and least homologous actin binding domain of NET3B (highlighted by the red box in figure 8 below) was considered. It was predicted that the root cause of the lack of actin binding was particular NET3B residues that were different to the corresponding residues in ABDs of all other members of the NET family that were able to bind actin. Examples of these residues are highlighted in figure 8 by black boxes. Changes to individual residues or particular motifs within the ABD could result in deficient actin binding through the loss of a residue that contacts actin or by causing changes to protein secondary or tertiary structure which could alter its activity. Once potential residues (those that were homologous between all NET proteins but altered in NET3B) had been identified the structure of the ABDs were analysed using an online program called “NetSurfP ver. 1.1 - Protein Surface Accessibility and Secondary Structure Predictions” found at http://expasy.org/tools/. This was to confirm that the residues raised as potential candidates for mutation were found at the surface of the protein and thus were capable of a direct f-actin interaction.

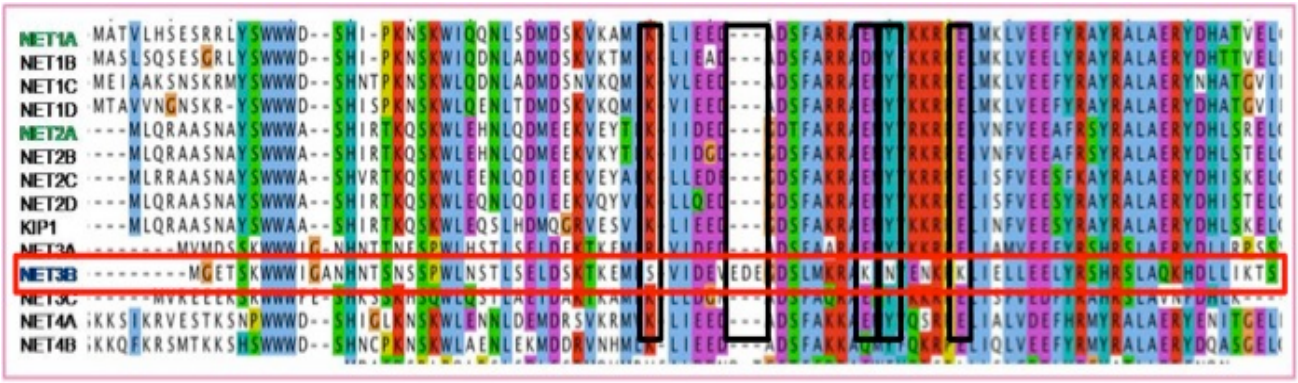


Figure 8: Amino acid sequences of the n-terminal ABD of the NET proteins. This alignment was used to identify residues or regions of sequence (black vertical boxes) that were homologous between those proteins shown to bind actin but different in NET3B (sequence highlighted by horizontal red box) which has been shown not to bind actin. (Dr T. Hawkins, University of Durham, UK).

Following this analysis, residue 66 of NET1A and residue 53 of NET2A were chosen to be mutated. This involved changing a charged glutamic acid residue to a neutral glycine. In NET3B the residue at this location is a lysine which has a long positively

charged side group. This is in contrast to the NET family members that do bind f-actin which have a negatively charged glutamic acid residue. Perhaps this reversal of charge is what causes the disruption to f-actin binding of the protein. Glycine was used as the amino acid to mutate to for a variety of reasons. Firstly it meant only one base needed to be changed and the resulting codon had a strong bias in *A. thaliana* so there was a high proportion of tRNA for its translation. Secondly, this mutation would neutralise the charge of the wild-type residue so that it can no longer interact with oppositely charged residues in the surrounding environment. Thirdly, mutation to glycine would increase flexibility within the protein structure in which the glutamic acid would usually confer rigidity. Neutralising the charge and reducing the rigidity would result in maximum disruption to the protein's usual state and thus give the most chance of producing a mutant phenotype. For the purpose of this MSc thesis, this mutation will be referred to as E66G (in relation to NET1A) and E53G (in relation to NET2A). The numeric value refers to the position of the amino acid residue from the start of the coding sequence and the letters refer to the one letter amino acid alphabet. The location of this mutation in the context of the whole ABD is demonstrated in figure 9.

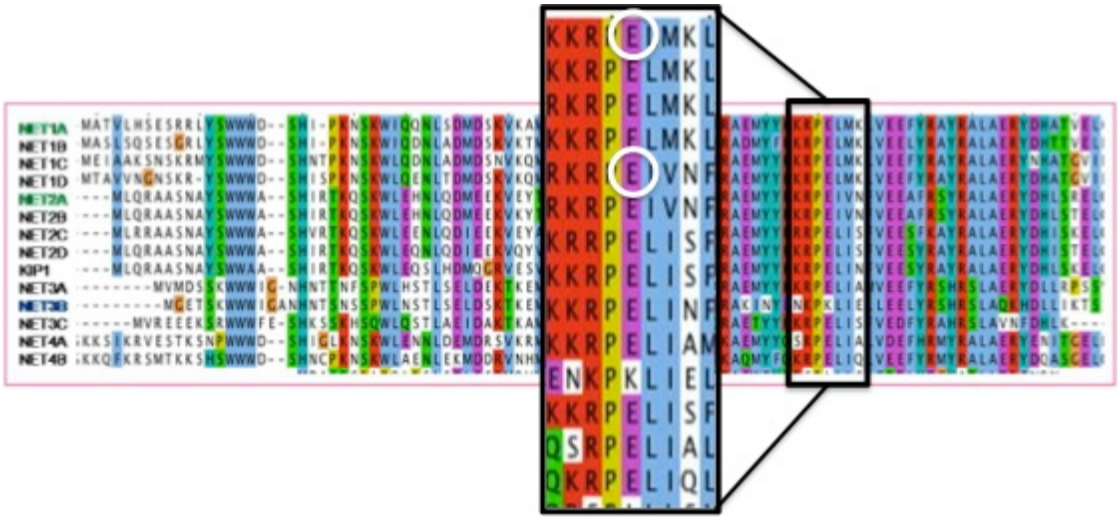


Figure 9: The glutamic acid residues highlighted by the white circles are the specific amino acids that were mutated to glycine to create the E66G and E53G mutations.

The second mutation that arose from analysing the sequence homology of the NET protein ABDs involves an insertion of three glycine residues "GGA GGA GGA" into the NET1A and NET2A coding sequence at the location where NET3B has an extra three residues (EDE: glutamic acid, aspartic acid, glutamic acid) compared to all other members of the NET family. This region is shown in figure 10. This mutation was trying to determine whether the additional spacing in NET3B is what interferes with f-

actin binding ability. It is likely that the three residue insert in NET3B could affect the tertiary structure as the additional residues create an extension to the length of the protein in that area. Additionally, the insert is made up of acidic residues which are negatively charged at physiological pH. These types of amino acid are often exposed at the protein's surface and interact with oppositely charged residues in adjacent proteins, a feature that could play a role in NET3B function. Glycine was selected as the amino acid to mutate to because it was a convenient choice for the mutagenesis kit in terms of the DNA sequence of the residues to insert and it was consistent with the residues used to create the other mutant constructs described in this section. For the purpose of this MSc thesis, this mutation will be referred to as ins3G.

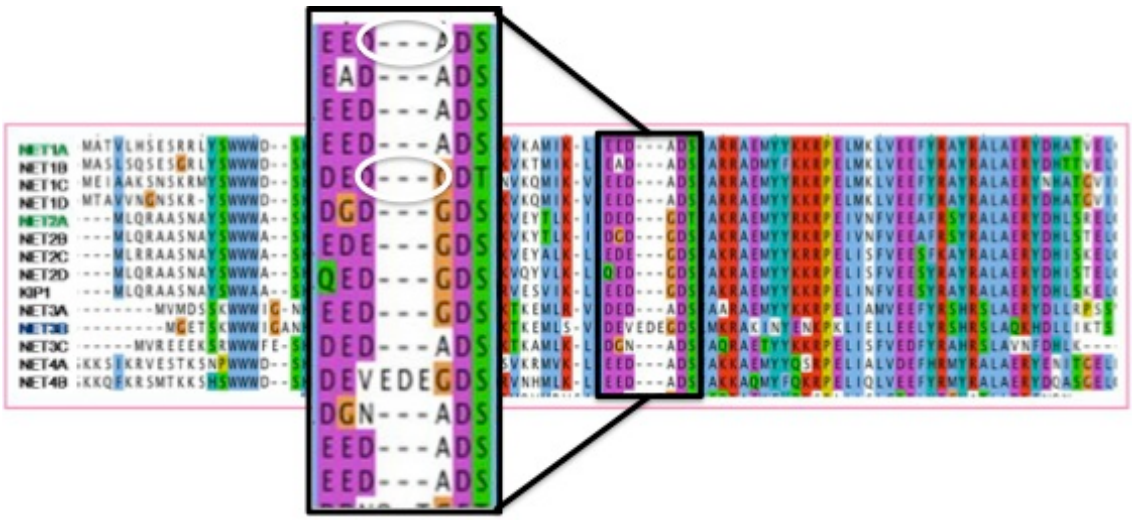


Figure 10: Location of the three glycine residue inserts in the NET1A and NET2A ABDs (white circles) in the context of the whole ABD. This insertion created the ins3G mutants.

The final mutation was not primarily designed to disrupt f-actin binding as the targeted motif is common between all members of the NET family and is identified in figure 11. The motif in question consists of three adjacent tryptophan residues. This motif very rare in plants and thus an interesting target for mutagenesis to try to establish its function. The three tryptophan residues (TGG TGG TGG) were mutated to three glycines (GGG GGA GGG). The middle glycine used a different conformation of bases to try to prevent slipping during translation. This can happen when there is a long string of the same base in the reading frame. Glycine was the chosen amino acid to mutate to (in contrast to alanine, often a more common choice) because of the minimal number of nucleotide bases needed to be changed to alter the amino acid. This was more appropriate for the mutagenesis kit used in the experiment (see section 2.2.6). Glycine is a neutral and very small amino acid. This contrasted well

with tryptophan, one of the larger amino acids with an indole functional group and a very hydrophobic side chain. Dr T. Hawkins (Durham University, UK) carried out tertiary structural analysis on the ABDs of the NET family, the results of which suggested that the triple tryptophan motif could create a kink in the protein structure. Taking the three tryptophans, with their three large, rigid side chains and replacing them with three glycine residues which are very small would increase the flexibility of that region of the protein. This aimed to maximise the contrast between the WT and mutant constructs to disrupt normal protein function as much as possible, thus triggering a strong mutant phenotype. For the purposes of this MSc thesis, this mutation will be referred to as 3W-3G.

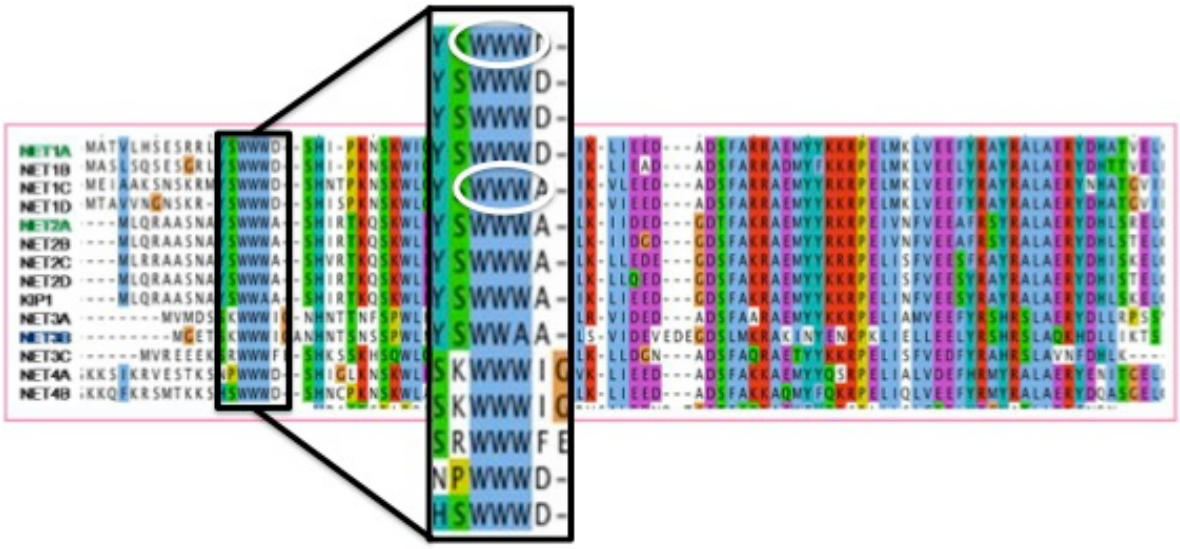


Figure 11: Location of the triple tryptophan motifs in NET1A and NET2A (white circles) that are mutated to three glycine residues. This mutation created the 3W to 3G mutants.

3.1.2. Mutagenesis Reactions

The original clone of NET1A used in this project had been amplified without its native promoter by Dr J. Calcutt (2009). This could easily be used as the template in the mutagenesis reactions and expressed under the 35S over expression promoter. The NET2A clone from Laura Bell still had the native promoter attached. Attempts to amplify the protein without the promoter were not successful, so when the protein was cloned into the *LAT52* (constitutive promoter expressed in pollen) containing vectors it was hoped that the enhancer elements of the *LAT52* promoter would augment the expression from the native promoter.

The QuickChange lightening mutagenesis kit (Agilent) was successfully used to introduce the 3W to 3G mutation and the E66G/E53G mutations into both NET1A

and NET2A clones. Unfortunately the ins3G mutagenesis was not successful. Due to time constraints on the project it was decided that the ins3G mutation would be left after two unsuccessful attempts. In addition, both proteins were successfully amplified without their ABDs using PCR. Thus the wild type (WT) versions of NET1A and NET2A (to be used as a control) and an additional three mutated constructs were suitable for transformation and expression as described in section 1.11.

3.2. Stable Expression of Mutants in *A.thaliana*

All successfully cloned mutants were transformed into *A. thaliana* to create plant lines stably expressing the mutant protein (as described in section 2.2.10). NET2A –ABD, E53G and 3W-3G were all expressed in a CFP *LAT52* vector and NET1A –ABD, E66G and 3W-3G were tagged with GFP under the expression of the 35S promoter. In addition, a stable line was created expressing a CFP empty vector as a control for the NET2A constructs. The stable line expressing a GFP vector as a control of the NET1A constructs had already been created by Dr J. Calcutt (2009). After antibiotic selection of seeds from dipped plants (section 2.2.10) a number of potential transformants were identified. Currently the selected T0 generation are maturing. Due to time constraints in the project it was not possible to image or analyse the stable lines to check for appropriate expression or to try to identify a mutant phenotype.

3.3. Transient Expression of NET2A Constructs in *N. tabaccum* Pollen

Despite numerous attempts throughout the year to achieve the transient expression of WT and mutated NET2A constructs in *N. tabaccum* pollen, this was unsuccessful. The only small success with the pollen bombardment was managing to express the CFP only control (the empty vector p52CGW7) and the f-actin marker CFP lifeact. In the sample of pollen grains bombarded there were only a very small number of transformed tubes. Figures 12 and 13 show examples of pollen tubes expressing these constructs.

Figure 12 shows the CFP only control which was used to identify any effect on pollen tube growth that resulted from over-expressing a protein under the *LAT52* promoter. The presence of CFP in a pollen tube is abnormal. Thus expression of the empty vector containing CFP was to control for any mutant phenotype resulting from the presence of the fluorescent protein tag which was also present in the WT and mutant constructs. Further, over-expression of any protein can stress a cell so in addition to the CFP only control, the WT protein was planned as a control to identify

any phenotype resulting from over-expression of a large protein distinct from the phenotype resulting from the dominant negative.

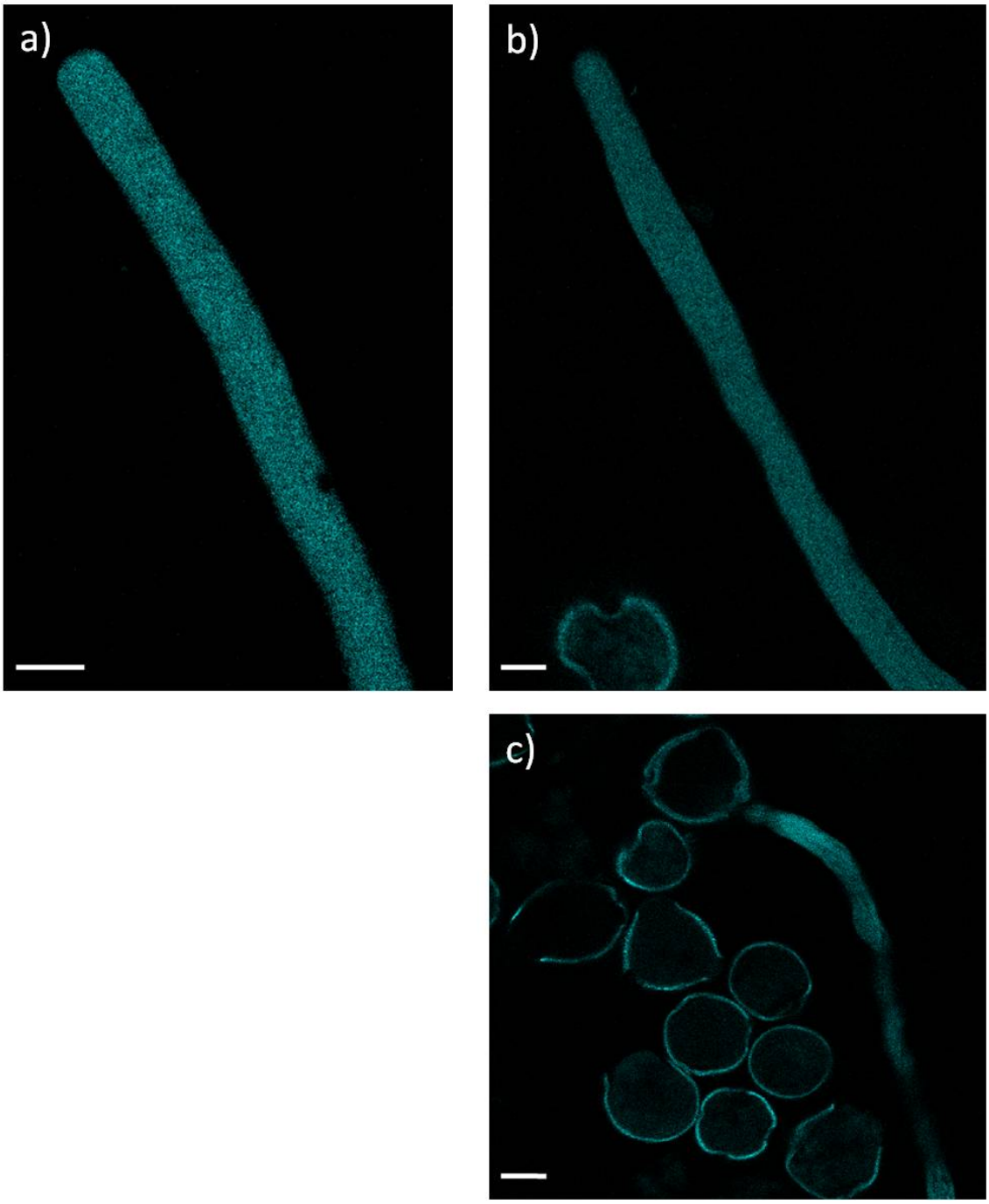


Figure 12: *N. tabaccum* pollen tubes expressing the p52CGW7 empty vector as a CFP only control. The CFP has a cytosolic localisation.
Scale bars: 10µm = **a)** and **b)**, 15µm = **c)**

Figure 12 shows pollen tubes expressing CFP lifeact. This is a small actin marker (17 amino acid residues) which would be relatively efficient to transcribe in the pollen tube. The images show actin cables down the length of the pollen tube shank in the cortical region of the tube. It is apparent that lifeact does not show actin at the tip of the pollen tube. This actin marker would have been used to demonstrate co-localisation between the actin cytoskeleton and NET2A foci in the pollen tube cortex that seem to align along some sort of filament like “beads on a string” (Hussey and colleagues, unpublished data). Although to achieve co-localisation, the constructs would have needed to be cloned into a plasmid with a different fluorescent tag once the bombardment technique had been shown to work. Figure 14 shows non-transformed pollen grains and tubes taken with similar microscope settings as the images produced in figures 12 and 13. It demonstrates that, whilst the pollen grain shows auto-fluorescence, when not transformed the pollen tubes don’t fluoresce. Therefore the protein localisations shown in figures 12 and 13 are true and not an artefact.

Despite the images produced in figures 12 and 13, the transformation efficiency of these constructs was very low despite their relatively small size. The empty plasmid was only 6185bp and the lifeact construct 4785bp. When a large insert, such as the NET2A coding sequence (5213bp), was cloned into the vector the transformation rate was further decreased to a point where the technique was not efficient enough to result in any transformed pollen tubes in a typical sample of pollen grains. There were a number of possible reasons for this decrease in efficiency. Firstly, when expression plasmids contained large inserts of DNA it was much less efficient for the DNA to become successfully coated on the gold particles when making the bullets for pollen bombardment. This would mean there was less DNA arriving at the pollen grains to be transformed. Alternatively, the larger the coding sequence that needed to be transcribed, the less efficient this process was. For the expressed protein to be visualised during imaging, not only did the coding sequence of the protein need to be successfully transcribed but also the CFP tag so the construct could be visualised.

Various attempts were made to increase the efficiency of the transient expression in pollen because this technique had been successfully used by Hussey and colleagues (unpublished data) to visualise full-length NET2A in the past. These included: purifying DNA for coating the bullets using a Qiagen endotoxin free maxi prep kit to obtain DNA that was highly concentrated; leaving pollen grains to germinate in a 24°C incubator as the ambient temperature in the laboratory was reduced during the winter, and using pollen from *N. tabaccum* plants of different ages.

This technique would benefit from further trouble-shooting to try to identify what conditions changed to decrease the efficiency of the transformation. New plants should be grown up from seed, all reagents used in the various solutions and media should be checked again, and varying the amounts/ concentrations of DNA used to coat the bullets should be done determine if there is an optimum.

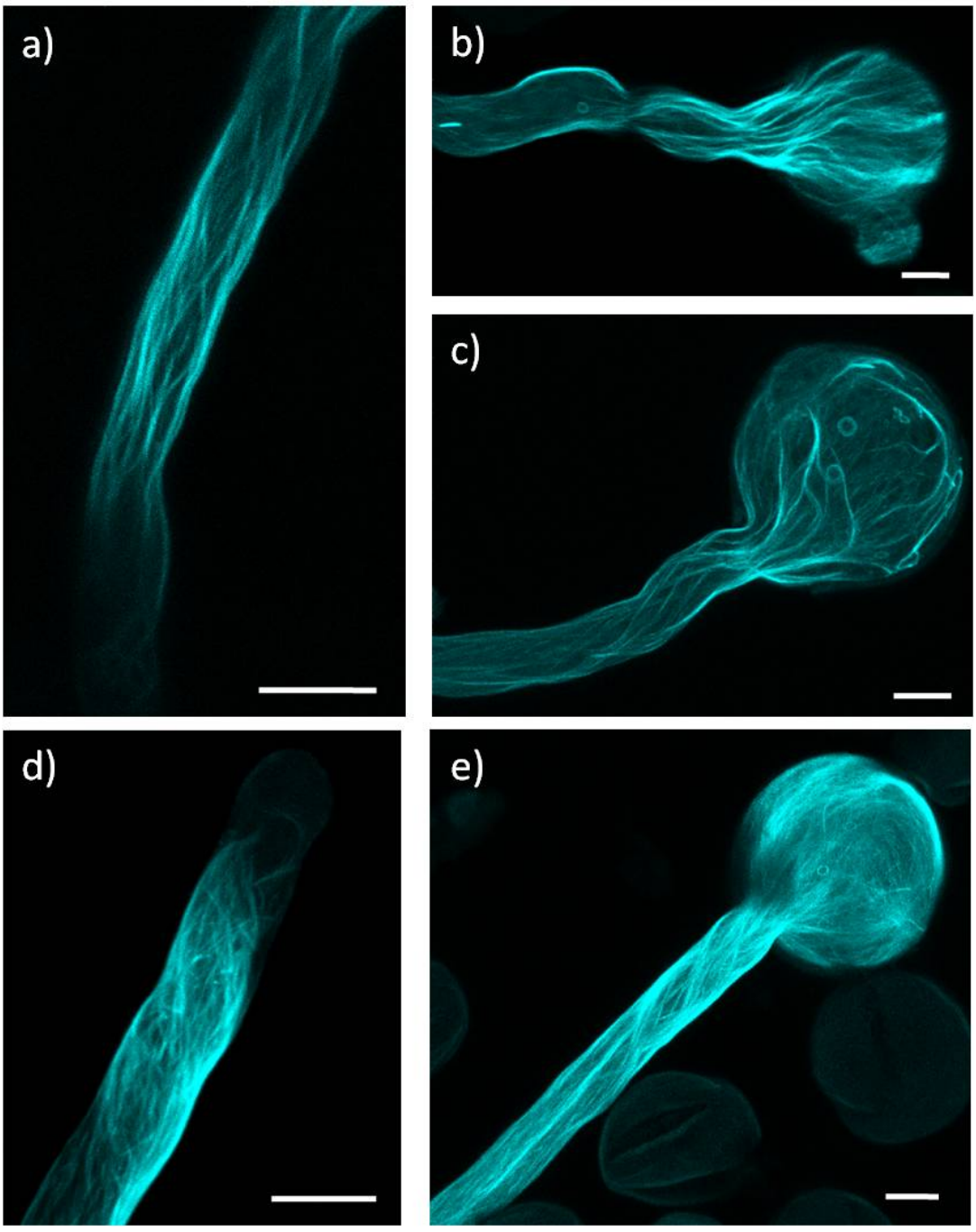


Figure 13: *N. tabaccum* pollen expressing lifeact::CFP. Lifeact decorates the actin cables that pass along the shank of the pollen tube. Scale bars = 10µm.

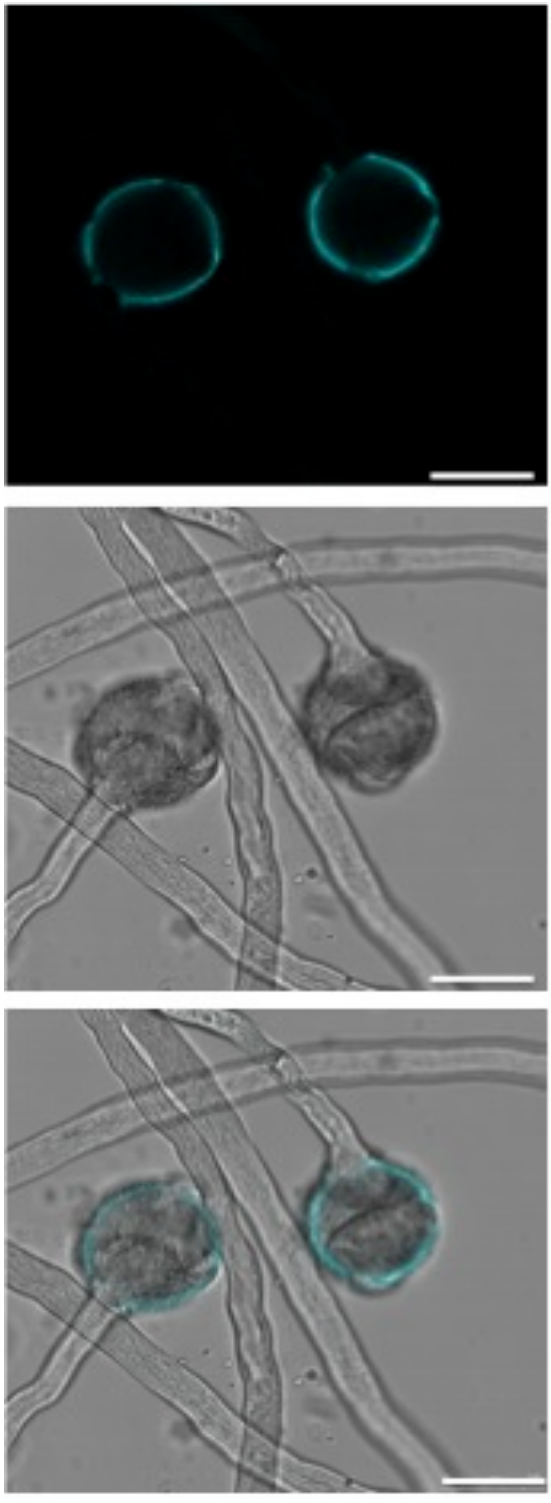


Figure 14: Non-transformed pollen tubes from *N. tabaccum* showing auto fluorescence in the pollen grain but no fluorescence in the pollen tube. Scale bar = 25µm

3.4. Transient Expression of NET1A Constructs in *N. benthamiana* Leaves

To get an initial idea of the localisation of the NET1A WT and mutant constructs they were independently infiltrated (as described in section 2.2.8) into *N. benthamiana* leaves tagged to GFP. Figure 15 shows their localisations in the cortex of the leaf epidermal cells.

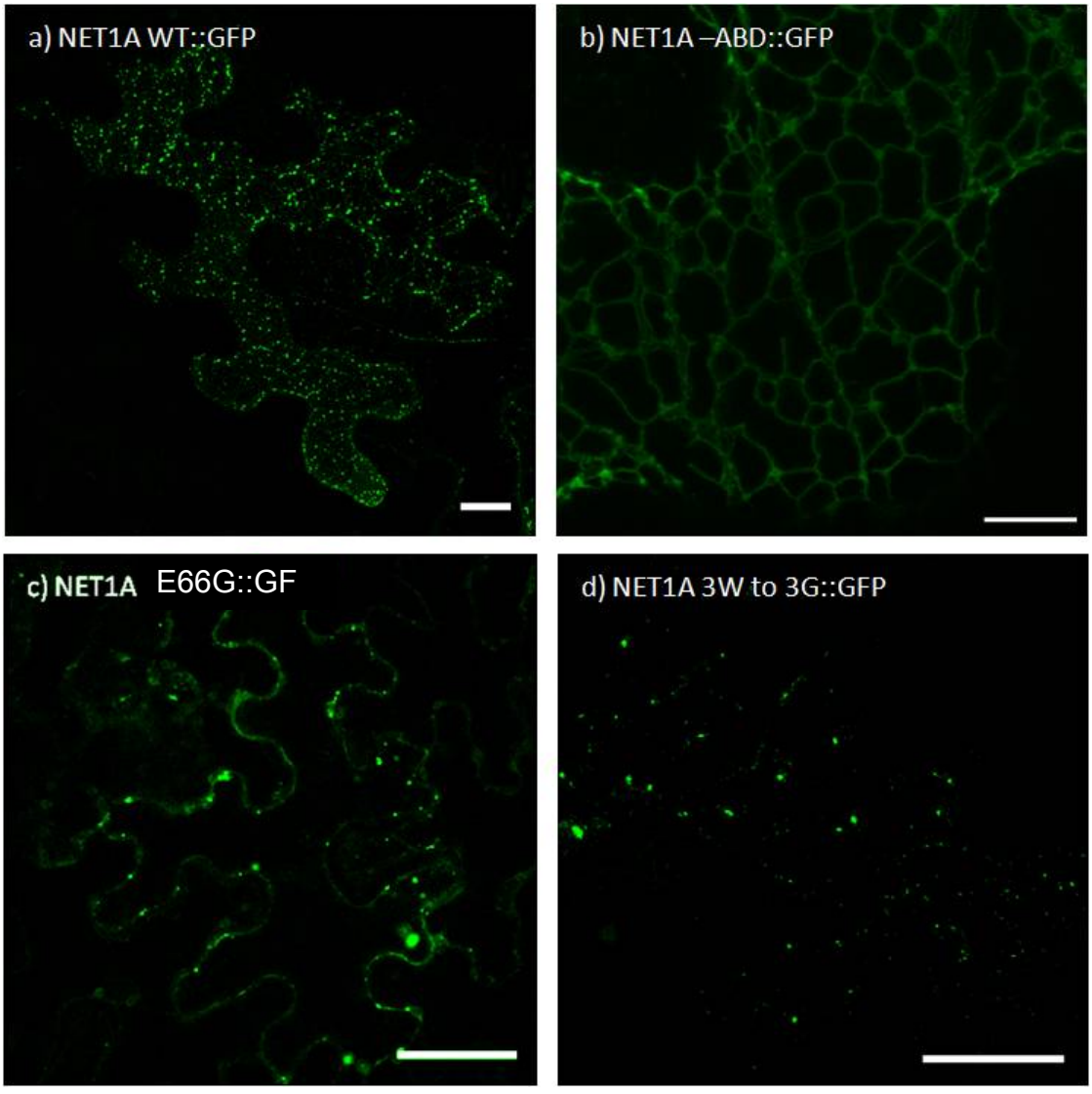


Figure 15: Localisation of GFP tagged NET1A constructs. The WT was expressed in pMDC83 with 2x35S promoter, the mutant constructs expressed in the pH7FWG2 plasmid with a single 35S. **a)** WT NET1A has a punctate localisation. The individual focus appeared to align along filaments **b)** –ABD mutation has an ER-like distribution **c)** E66G mutation appears punctate, although first impressions suggest the foci do not appear to align along filaments as the WT do **d)** 3W to 3G mutation has a punctate localisation similar to WT and seems to align along filaments. Constructs were transiently expressed using Agrobacterium-mediated expression system in *N. benthamiana*.
Scale bar = 25μm (except **b)** =10μm)

These results raised points of interest for further investigation. The localisation of the full length WT protein (figure 15a) confirmed findings by Dr J. Calcutt (2009) that showed the protein to have a punctate localisation along the length of filaments. Dr J. Calcutt demonstrated that this filament system was the actin cytoskeleton. This was achieved using actin-disrupting drugs and showing a co-localisation between the isolated n-terminal region of NET1A and the actin marker GFP-FABD2. However, an association between full-length NET1A and the actin cytoskeleton has not yet been confirmed. This will be investigated further in section 3.4.1, using co-localisation between the full length protein and an actin marker.

The localisation of NET1A –ABD (figure 15b) suggested a potential interaction between NET1A and the ER membrane system. This interaction could be mediated through a coiled-coil domain in the mid- or c-terminal regions of the protein or an ER binding domain distinct from the n-terminal ABD. This relationship was investigated further using co-localisation between the –ABD mutant and ER and actin markers. It could suggest a role for the protein interacting with the actin cytoskeleton and ER at the plasma membrane. Because of the interaction between this mutant and the ER the other constructs were looked at with an ER marker. These results are shown in section 3.4.2

The 3W to 3G mutation showed a superficially similar localisation pattern to the WT protein (figure 15c). Its distribution is punctate and the foci seem to align along filaments as the WT foci do. Since this mutation did not set out to disrupt f-actin binding, a drastic change in protein localisation was not expected. To investigate the function of the triple tryptophan domain further the properties of the punctate foci were studied. The size and mobility of the punctate foci can be analysed to identify any differences to the WT. In addition actin and ER markers were used in co-localisation experiments to determine if the dots were still co-localised with f-actin. Results of these experiments are shown in section 3.4.3 below.

Finally, the E66G mutation also showed a punctate localisation. However, in contrast to the WT which seems to align along filaments, the foci in the E66G mutant seemed to be much more random. They no longer appeared to co-align with filamentous structures in the cytoplasm and in the cell cortex. If this mutant was no longer co-aligned with filaments it could have meant that f-actin binding was successfully disrupted by this mutation and the project strategy had been successful. To investigate this, the mutant was co-expressed with an actin marker to confirm the lack of association, the results of which can be seen in section 3.4.4.

Figure 16 shows non-transformed control images of the abaxial side of an *N. benthamiana* leaf. Images shown are taken using the three standard setting used in

the presentation of localisation experiments shown below as described in table 4. Figure 16 demonstrates that a non-transformed leaf has only a small amount of auto fluorescence surrounding the stomata, therefore the fluorescence seen in figures 17 to 29 are due to target protein localisation and not auto fluorescence or an artefact of the imaging technique.

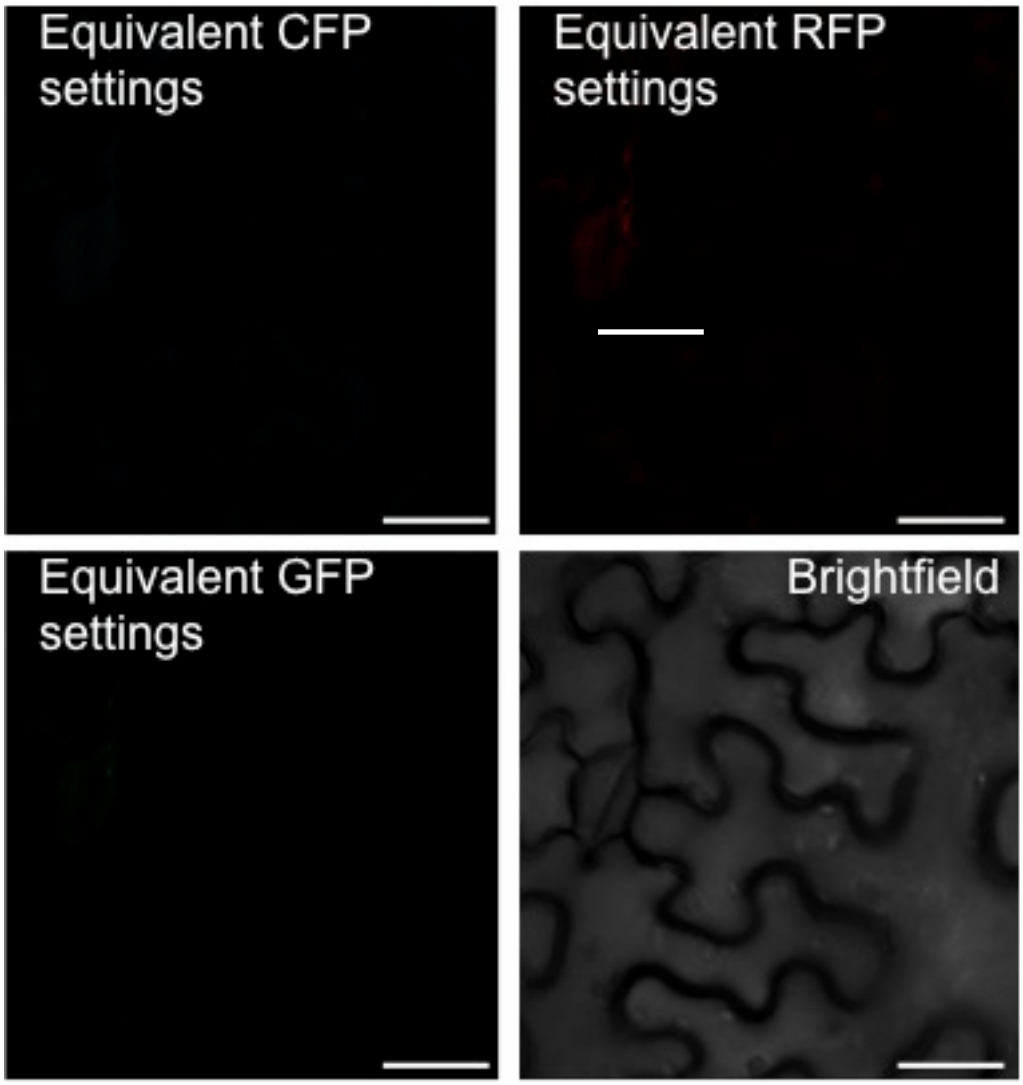


Figure 16: Non-transformed *N. benthamiana* leaf pavement cells showing no fluorescence. The same settings were used as for studying the localisation of fluorescent proteins as detailed in table 4. Scale bar = 25μm

The results presented in figures 15 to 29 showed variable replicability. Expression of fluorescently tagged mutant proteins on their own showed consistent localisation patterns but when the mutants were co-expressed with actin or ER marker replicability decreased. The health of the cells often appeared to be negatively affected by the over-expression of ER markers and the presence of actin markers

often caused filament bundling. As a result it was often difficult to find healthy cells that were expressing both the fluorescently tagged mutant protein and the marker. Cells that did meet these conditions showed mutant protein localisations and dynamics as demonstrated in the figures below from which interesting hypotheses can be made. If in future experiments steps were taken to reduce the expression levels of the actin and ER markers to improve the health of the cells. This would improve the reproducibility of the experiments and help confirm the observations that were made.

3.4.1. WT NET1A

The NET1A WT construct has a punctate localisation in the cortex of the infiltrated epidermal cells. It is also found with a punctate distribution on the trans-vacuolar strands. This localisation is demonstrated in figure 15a) and figure 17a). It had previously been demonstrated that the isolated n-terminal domain of NET1A (the ABD) associates with the actin cytoskeleton using co-localisation and actin disrupting drugs. However an association between the actin cytoskeleton and the full length NET1A protein had not been previously shown (Calcutt, 2009).

Figure 17c) shows that the punctate foci representing full length NET1A tagged to GFP co-localise to actin filaments in the epidermal cells of *N. benthamiana* leaves. This association was seen both in the cortex of the cell and along trans-vacuole strands. The NET1A foci have been shown to associate with plasmodesmata by co-localisation of the protein with a plasmodesmatal marker, aniline blue (Hussey group, unpublished data). The association with actin shown in figure 17c) suggests that NET1A could provide a link between the plasmodesmata and the actin cytoskeleton.

Expression of NET1A –ABD alone produced a protein localisation similar to the ER when the ABD was removed (figure 15b). This suggested a potential association between NET1A and the ER through a domain that is independent of the ABD. This led to an investigation of the interaction between the WT and mutant NET1A constructs and the ER. Figure 18 shows the co-localisation between NET1A and the ER. Although the NET1A dots still appear in lines, the merged image shows that the foci are also co-aligned with strands of ER throughout the cortex. This result suggests that NET1A could interact with both the actin cytoskeleton and the ER membrane system at punctate points at the plasma membrane.

Multiple attempts were made to achieve a triple expression of a NET1A construct (tagged with GFP), HDEL marker (tagged with CFP) and actin marker

(tagged with RFP). This would have helped determine if the suggested interactions between NET1A and the ER and NET1A and actin were happening at the same place, i.e. the actin cytoskeleton and ER network were brought together where NET1A was located. However, whilst the triple infiltrations resulted in expression of all three constructs within a leaf, they were never expressed together in the same cell to an extent that they could be imaged. It seemed that over-expressing that extent of foreign protein often meant the cell showed signs of stress, or perhaps competition between the constructs for transcription factors in any individual cell was too great to enable expression of all three together.

3.4.2. NET1A –ABD

Expressing the full length NET1A with only the n-terminal 96 residues deleted had not previously been studied. Initial expression of the mutant suggested that it was possible that the protein localised to the ER (as shown in figure 15b). This association was investigated using fluorescently tagged HDEL (a known ER marker). Figure 19 shows expression of NET1A –ABD::GFP and HDEL::CFP individually in two adjacent cells. This demonstrates that the similar localisation pattern is real and not an artefact of bleed-through (CFP emission detected by the GFP channel of the LSCM). The co-localisation of the NET1A –ABD mutant to the ER is confirmed in figure 20 a-c), which shows a complete overlap between the mutant construct and the ER marker, confirming that NET1A –ABD localises to the ER. This co-localisation has been quantified using Pearson's correlation coefficient and the CoLocaliser Pro software. Figure 20d) shows the area chosen for the quantification. This area has a mixture of different types of ER (fine tubules and thicker, flattened areas of membrane). Figure 20e) represents the co-localising pixels. Each data point on the graph represents a pixel pair from the two channels that co-localise. Points that are positioned toward the x-axis represent those where the two levels are co-localising but the level of the GFP channel is brighter and vice versa for the y-axis. The Pearson's correlation coefficient for the two channels is 0.6865 and the results show that 79.56% of the pixels pairs from the two images are co-localising. This quantification confirms the co-localisation between the ER and the NET1A –ABD mutant. The wide spread of points on the scatter graph arises due to differences in the levels between the red and green channel rather than the pixel pair not co-localising.

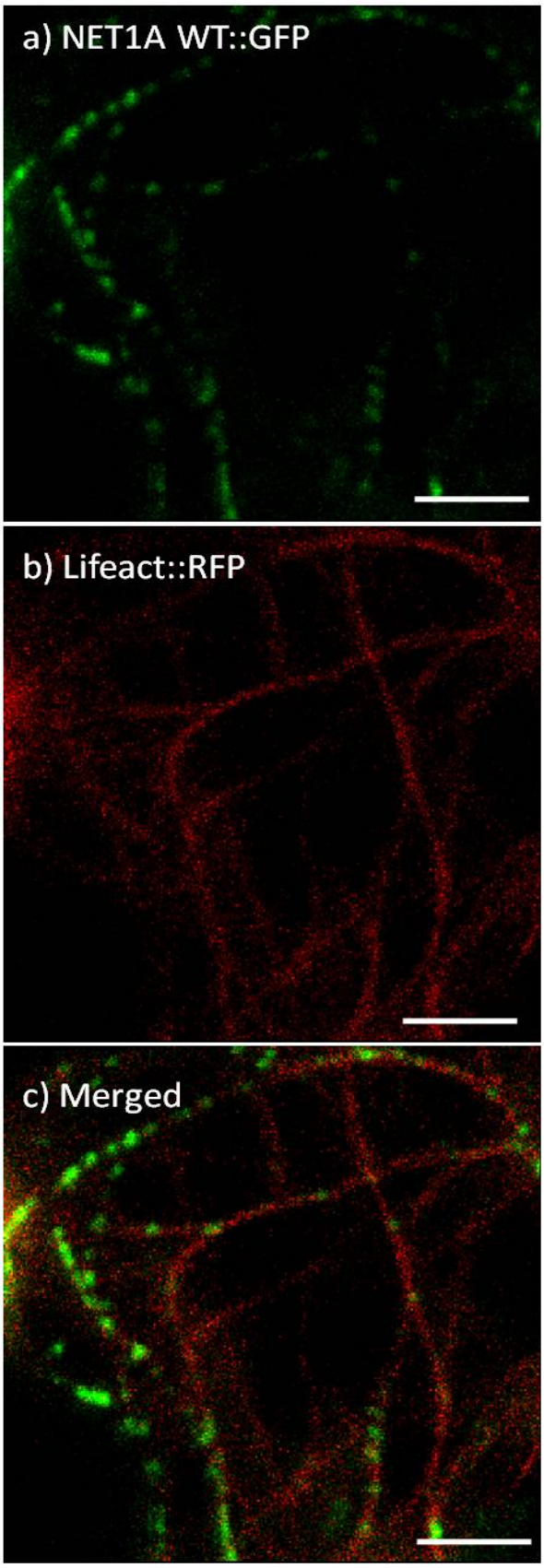


Figure 17: Expression of WT NET1A::GFP **a)** and the actin marker lifeact::RFP **b)** in the same cell **c)** shows the WT NET1A dots localise to actin filaments.
Scale bar = 5μm.

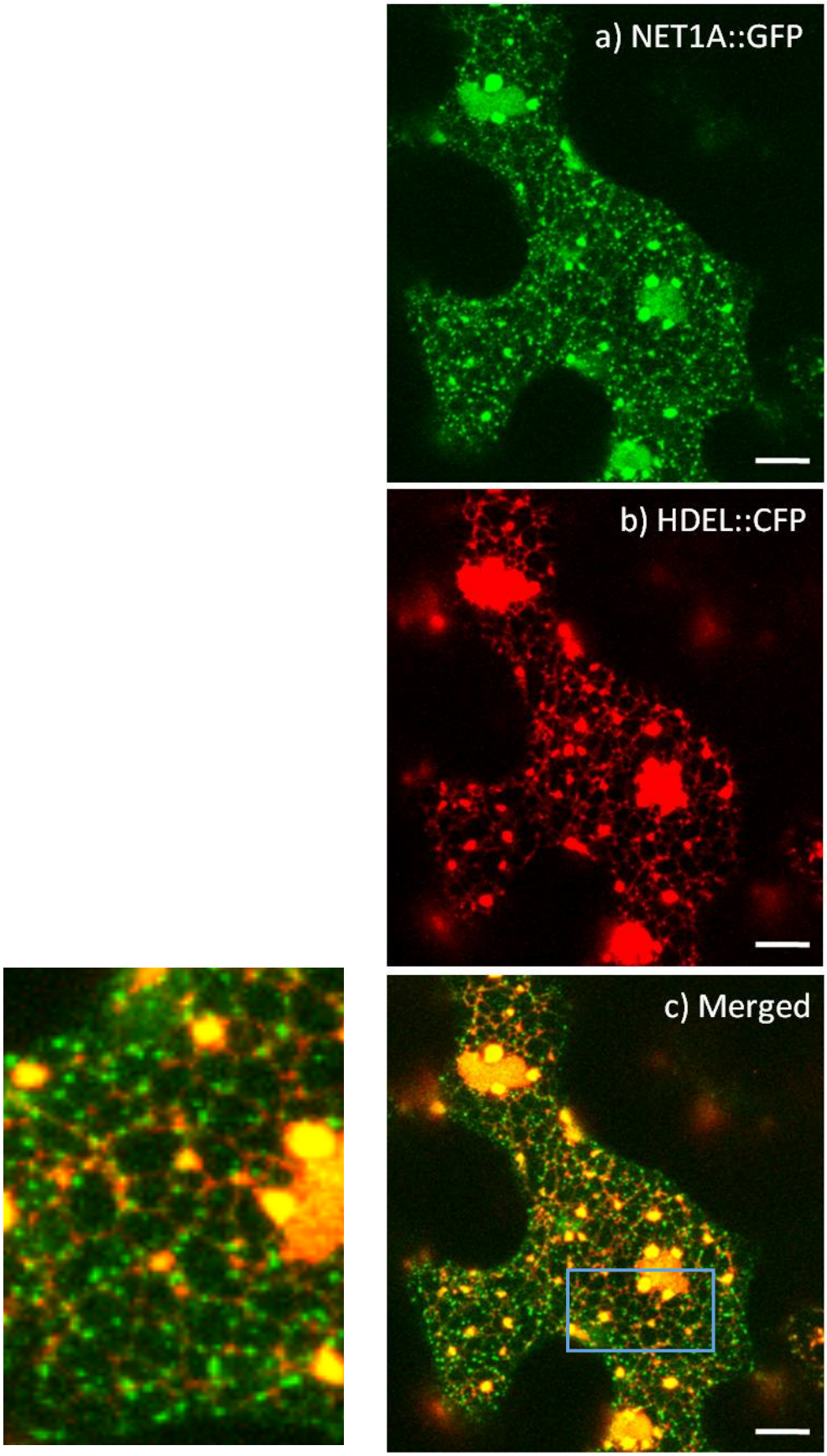


Figure 18: Expression of WT NET1A::GFP **a)** and the ER marker HDEL::CFP **b)**. The NET1A WT punctate dots seem to align along filaments but when showing co-localisation with the HDEL marker **c)** a close relationship between the protein and ER is also apparent. **d)** The area defined by the square in **c)** is magnified so the relationship between protein and the ER is more apparent. Scale bar = 10μm

An association between NET1A and the ER could be due to the protein defaulting to its ER binding partner in the absence of the ABD. Alternatively, the removal of the ABD could cause the protein to misfold and thus become trapped in the ER. This will be considered in the discussion.

The localisation of this mutation is also not punctate as seen in the other three mutants (figure 15). This could be a result of misfolding so the protein is not exported from the ER and thus never forms punctate foci. Alternatively, the domain of the protein that results in the punctae could be found within the ABD. Thus when this domain is deleted, the protein loses its ability to form punctate foci.

3.4.3. NET1A E66G

NET1A E66G has a punctate localisation in the cell cortex that, at a first look (figure 15c) appears similar to WT. It was therefore beneficial to study its localisation alongside actin and ER marker to investigate what its associations were.

Figures 21, 22 and 23 show the association between the E66G mutant and the actin cytoskeleton using lifeact::RFP as an actin marker. It is clear that, unlike the co-alignment between NET1A WT and actin (figure 17), the punctate foci of NET1A E66G do not lie on actin filaments. This suggests that mutating the charged glutamic acid residue to neutral Glycine in the ABD disrupted the protein's ability to bind F-actin. It is interesting to note, however, that the foci are generally located directly adjacent to an actin filament whilst distinctly not being localised on top of it. Perhaps there are other components in the cell that are forcing the mutant protein against the filament despite its inability to bind.

The association between the E66G mutant and the ER has been harder to determine. Figure 24 is inconclusive as to whether the protein co-localises with the ER. There is some evidence that there is a close association between the two but the image suggests that the protein might be excluded from the ER because in some cases the protein seems to be localised in the gaps between the ER membranes. The ER in these cells has a granular appearance which suggests the cell is not very healthy. This could affect the interaction between the protein and the ER if the ER is not functioning properly, thus these images should be analysed with caution.

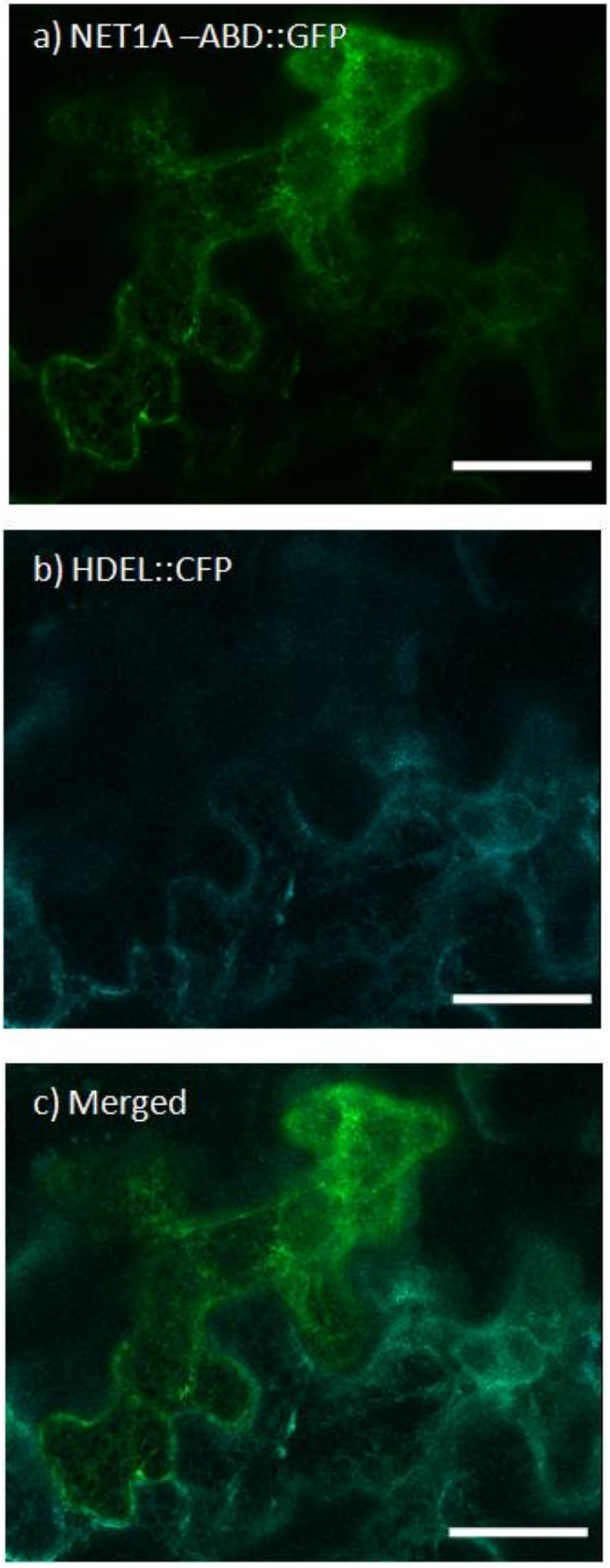


Figure 19: Localisation of NET1A–ABD ::GFP **a)** and the ER marker, HDEL::CFP **b)**. The merged image **c)** proves that the similar localisation pattern is not a function of bleed-through of the fluorescent proteins as the two constructs are expressed in different cells. Scale bar = 25µm

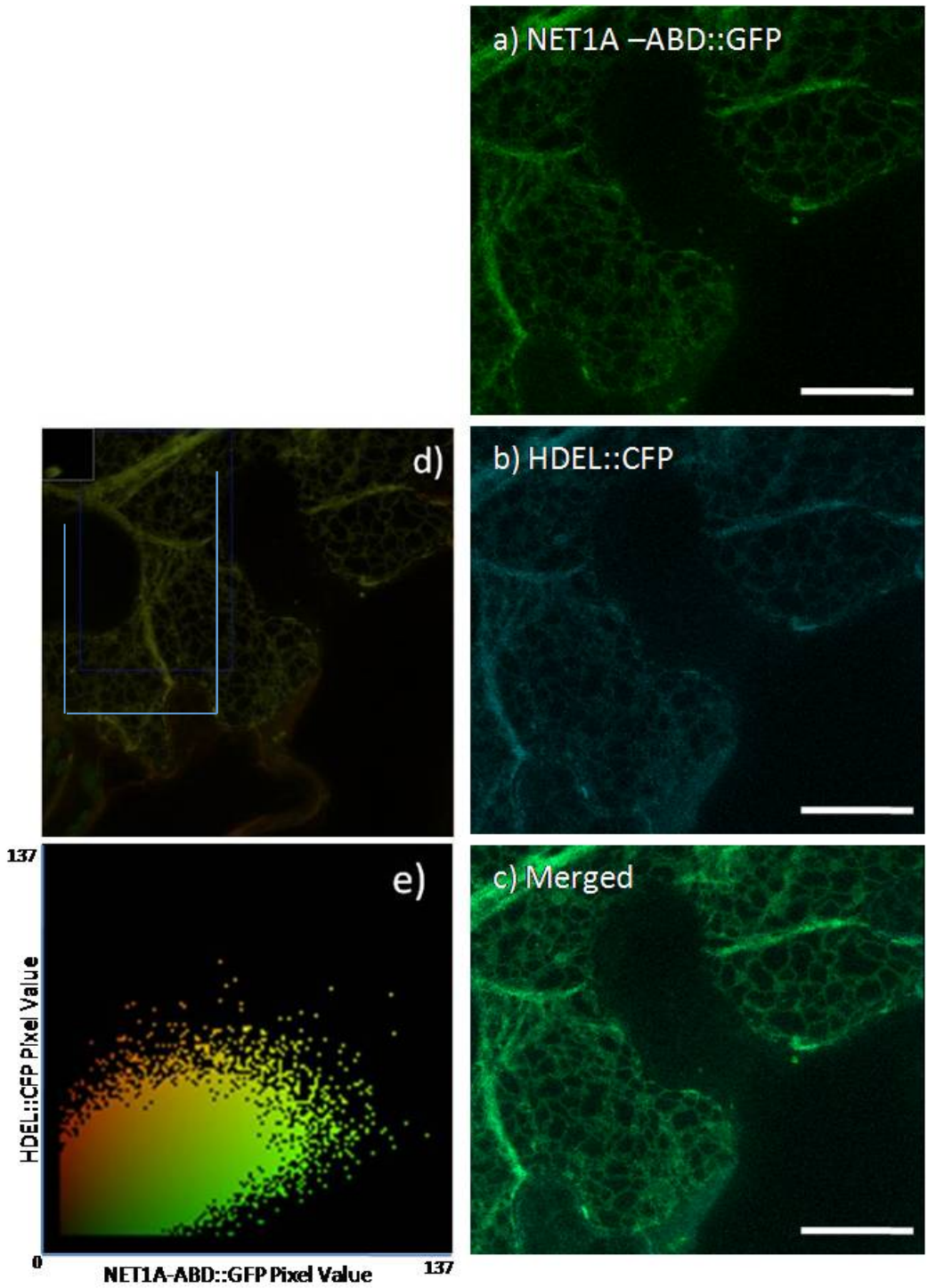


Figure 20: Expression of NET1A –ABD::GFP **a)** and the ER marker HDEL::CFP **b)** shows co-localisation when they are expressed in the same cell **c)**. **d)** Blue box represents the area used to quantify co-localisation using Pearsons correlation coefficient. The inset at the top right of figure **d)** is the scattergram **e). e)** represents the co-localisation of pixels. The graph is cropped to represent levels up to 137 and the thresholds were set to level 6 for the NET1A –ABD green channel and level 4 for CFP HDEL represented by the red channel. These thresholds were used to eliminate cytosolic noise from the calculation. Scale bar = 10µm.

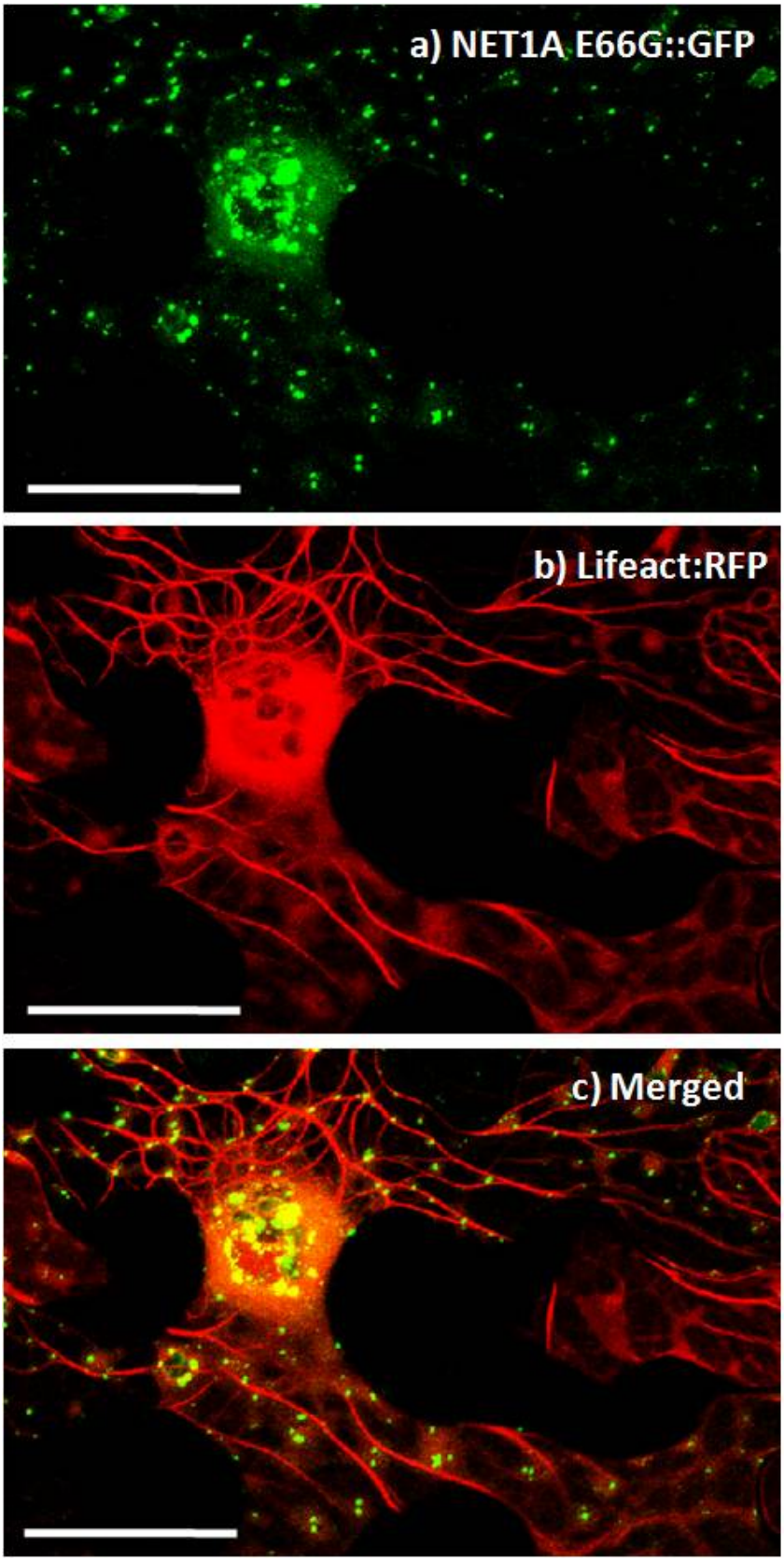


Figure 21: Expression of NET1A E66G::GFP **a)** and Lifeact::RFP **b)** in the same cell **c)** shows that the association between the E66G mutation and actin filaments is questionable. Scale bar = 50µm

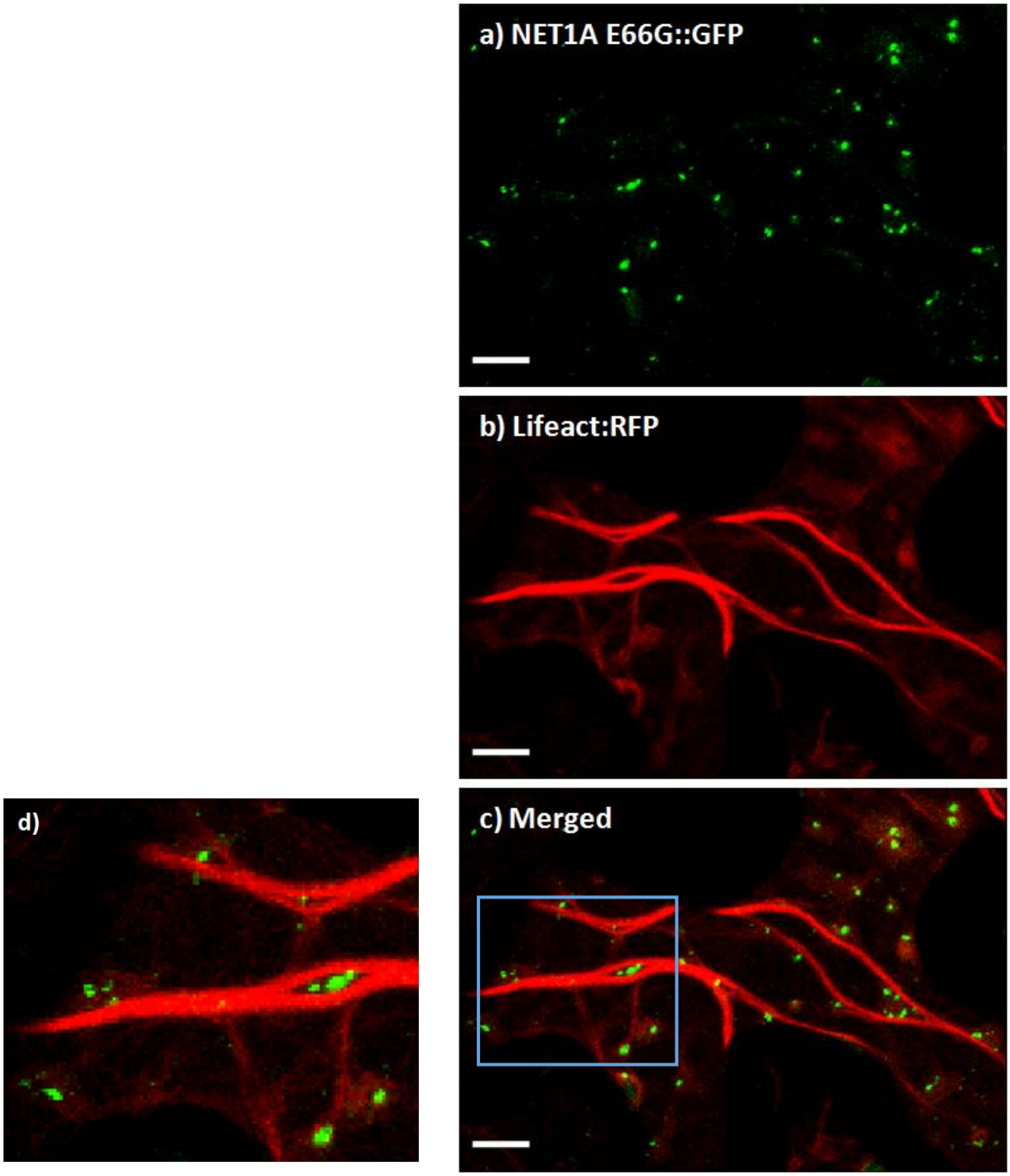


Figure 22: Expression of NET1A E66G::GFP **a)** and lifeact::RFP **b)** in the same cell **c)** shows that the E66G mutated construct seems to be localised alongside the actin filaments without aligning directly on top of them as can be seen in figure 17. **d)** Magnification of the area of **c)** marked by the box.
Scale bar = 10µm

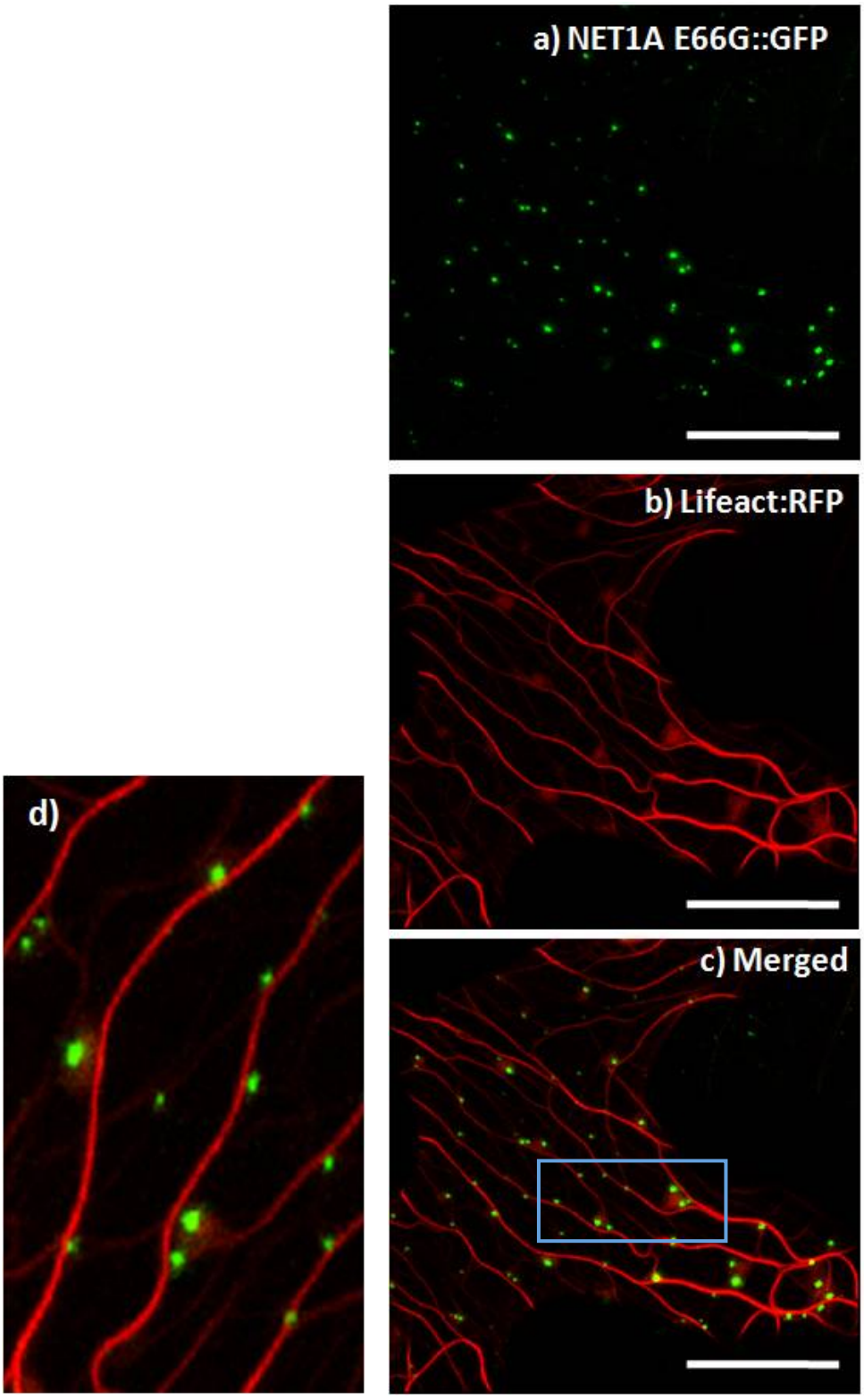


Figure 23: Expression of NET1A E66G::GFP **a)** and lifeact::RFP **b)** in the same cell **c)** shows that the E66G mutated construct seems to be localised alongside the actin filaments without aligning directly on top of them as can be seen in figure 17. **d)** Magnification and rotation of the area of **c)** marked with a square.
Scale bar = 25µm

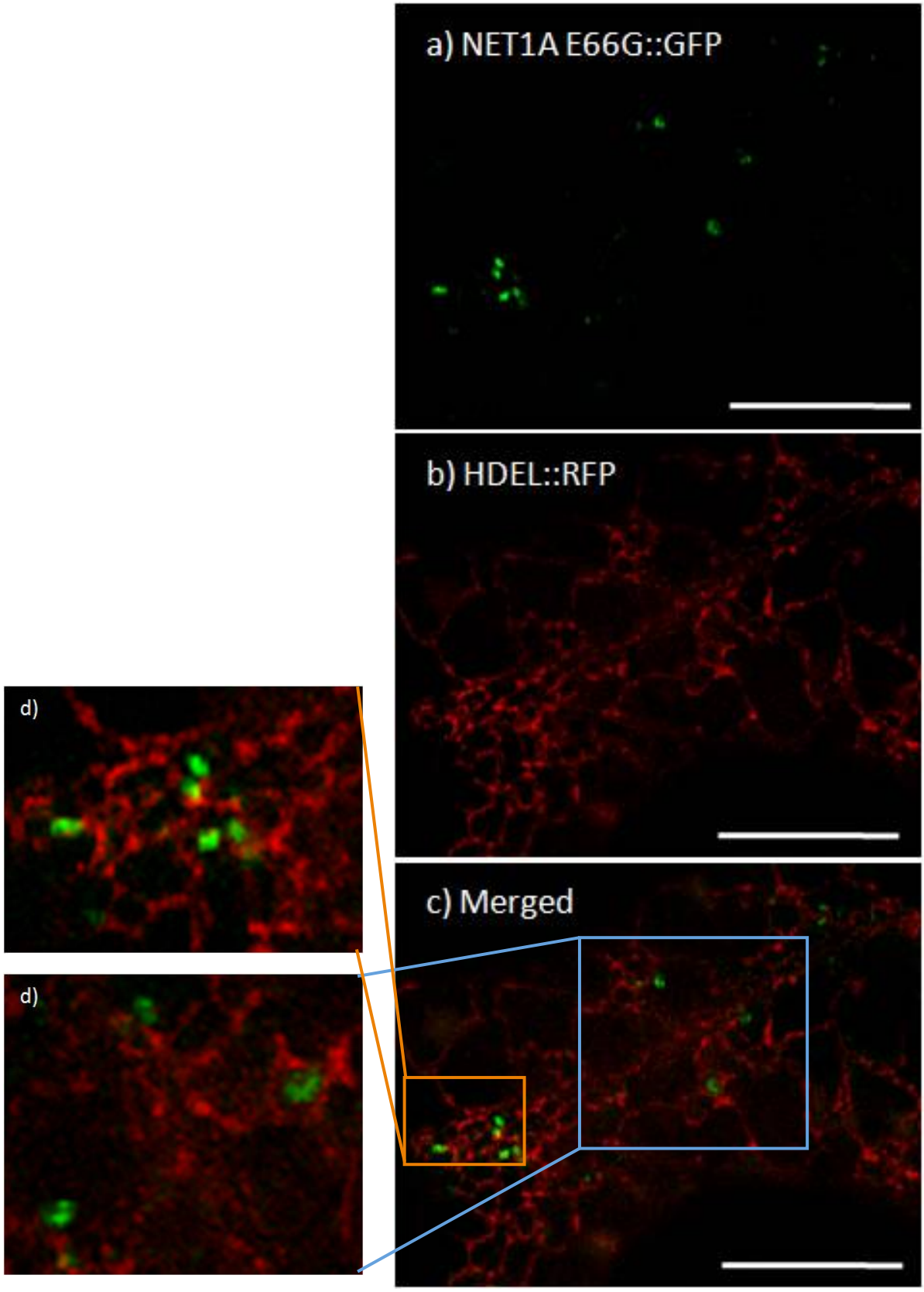


Figure 24: Expression of NET1A E66G::GFP **a)** and the ER marker HDEL::RFP **b)** in the same cell **c)** shows the localisation between the NET1A E66G mutant and the ER. **d)** The areas defined by the squares in **c)** are magnified. Scale bar = 10µm.

3.4.4. NET1A 3W to 3G

The initial studies of the NET1A 3W to 3G mutation (figure 15d) show that the mutated protein foci align along filaments and thus are likely to still associate with the actin cytoskeleton. This association has been confirmed by showing co-localisation with the actin marker, lifeact in figure 25. The association is particularly distinct along trans-vacuolar strands.

Whilst carrying out imaging it was noticeable that the NET1A 3W to 3G foci were much more motile in the cell particularly those associated with trans-vacuolar strands. Using time-lapse imaging it was possible to visualise foci aligned on f-actin moving through the cytoplasm. This phenomenon was also apparent when taking z-stacks of cells as the individual sections making up the stack were taken sequentially over a period of approximately six minutes. This movement is portrayed in montages of time series (figures 26 and 27). Following the highlighted foci of protein in figure 26, in the direction of the arrows, it appears that mutant protein foci and actin filament are sliding through the cytoplasm. This conclusion is reached because the relative distances between other foci remains constant thus they are not moving independently along the actin filament but in association with the filament. This is quantitatively shown by the overlap between the scatter points representing both foci on the graph in figure 26. The close alignment of both the data sets along a straight line demonstrates that the foci and actin cable are moving at a constant speed. The mean speed through the time series is 0.055μm/s for the lower dot and 0.051μm/s for the upper dot. Using a student t-test, the probability of 0.233 is greater than the threshold value of 0.05 so it can be determined that the mean speeds of the dots aren't significantly different. However, to confirm that they were significantly similar, more data sets would need to be analysed.

In addition to the increased mobility of protein and actin bundles in the trans-vacuole strands, the fine cortical filaments also seem less anchored to the plasma membrane and appear to 'wave' in the cortex of the cell. This is apparent in figure 26 where the filament marked with a ★ appears in and out of the focal plane and moves to different parts of the image.

The second time series shown in figure 27 also shows the actin filament sliding with mutant protein foci. The graph in the figure shows the movement of two protein foci with the actin. Whilst both foci seem to initially move at the same speed, their proximity, and possibly an affinity for each other, causes interference to the otherwise constant movement demonstrated in a similar situation in the graph in figure 26. It seems the upper focus is pulled back toward the lower one before they form an association and continue their movement in contact with each other. Their

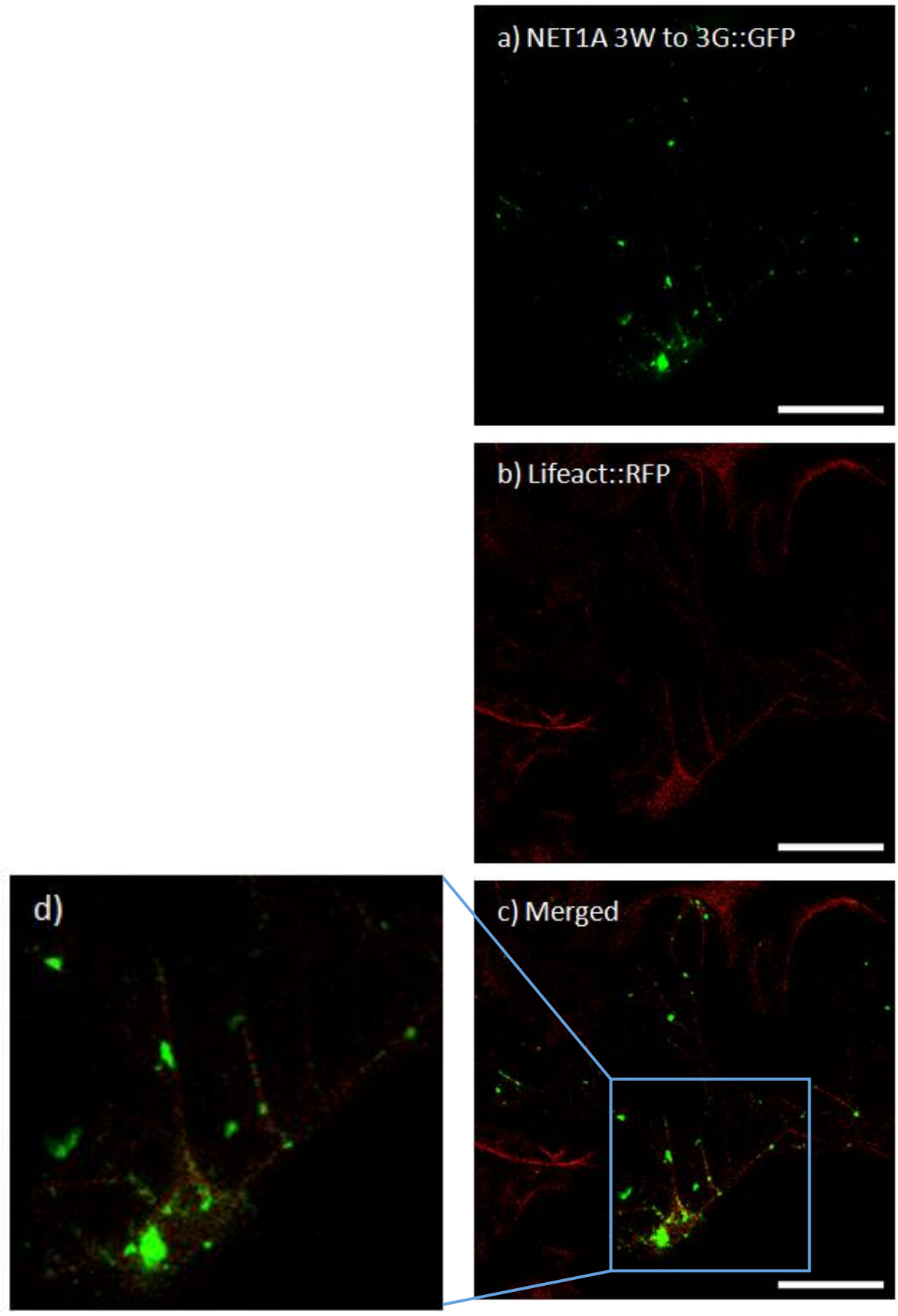


Figure 25: Expression of NET1A 3W to 3G::GFP **a)** and the actin marker, lifeact::RFP **b)** in the same cell **c)** shows the mutated NET1A dots align along actin filaments **d)** The area defined by the square in **c)** is magnified. Scale bar = 25µm.

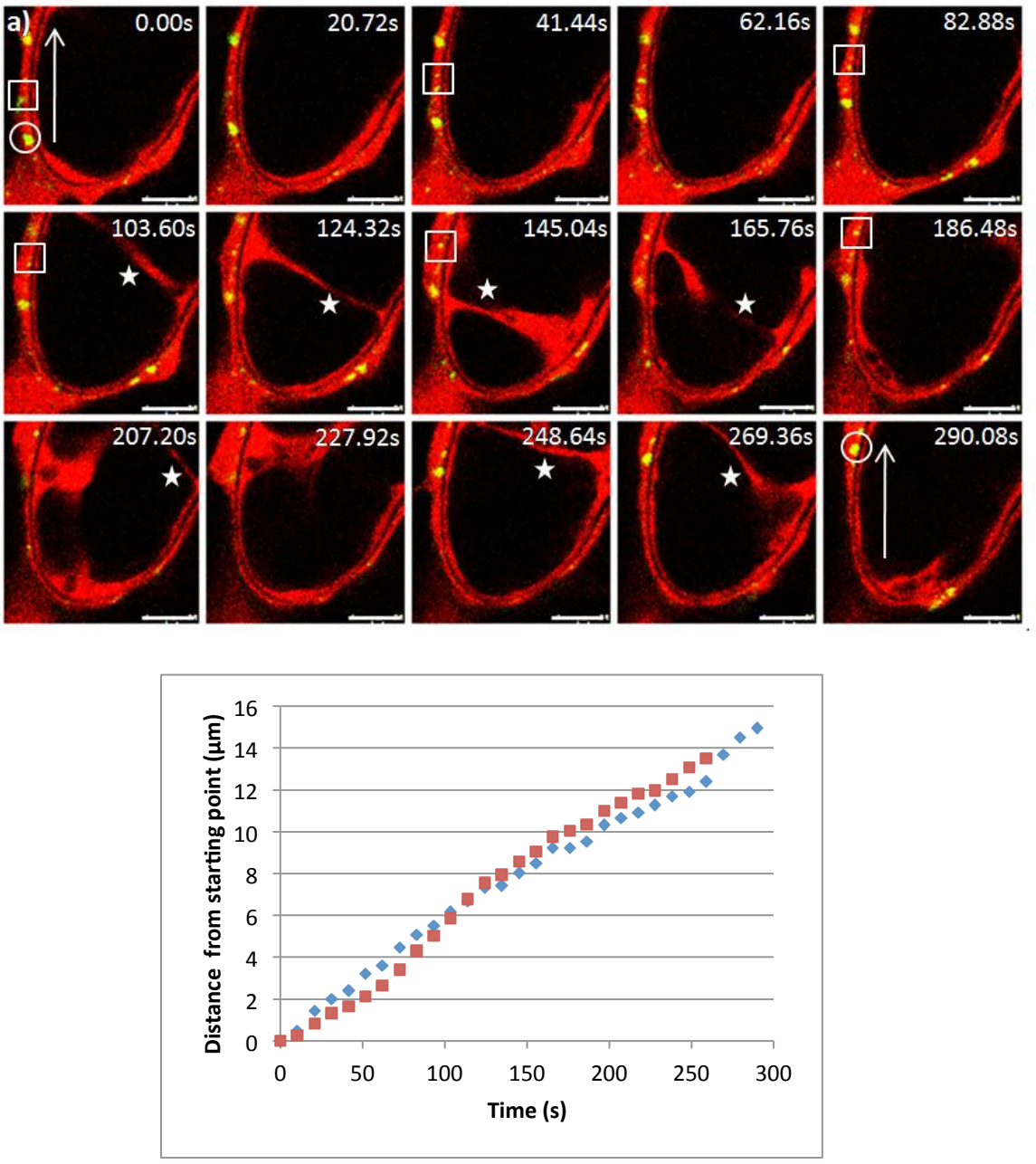
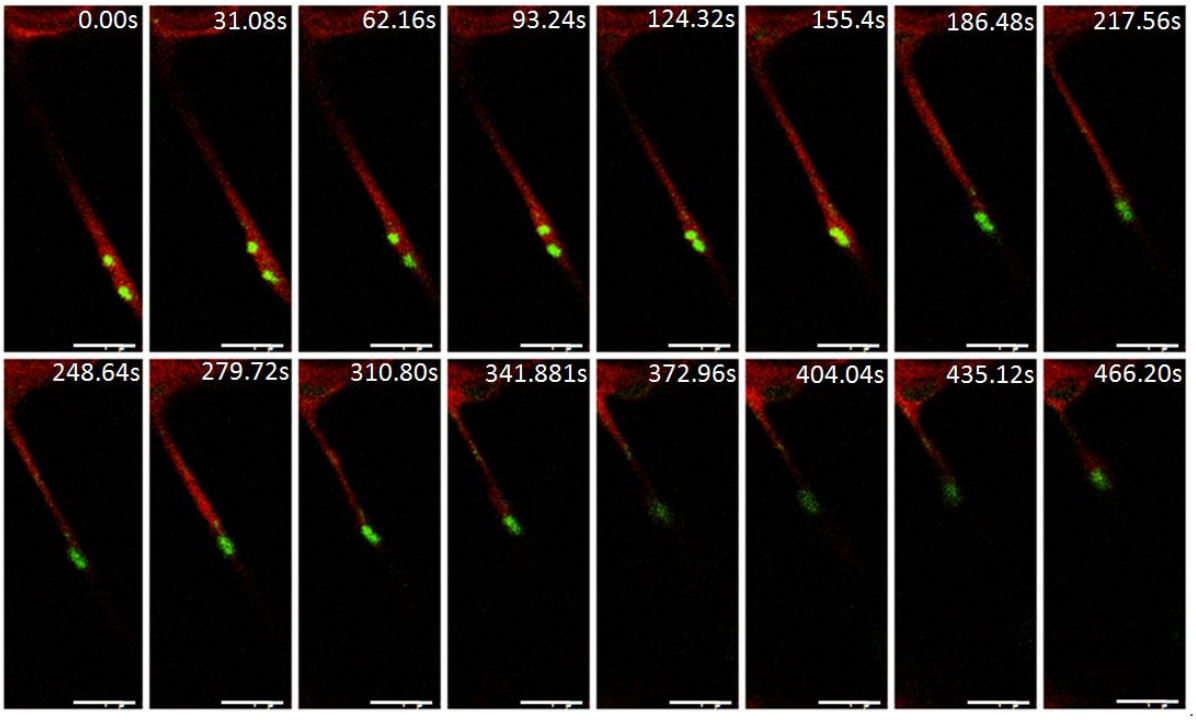


Figure 26: **a)** A montage showing the mobility of the NET1A 3W to 3G::GFP mutation. The protein foci seem to be anchored on actin cables that are sliding through the cytosol. The actin is marked using lifeact::RFP. The circle and square highlight two protein foci that can be seen to slide through the image on an actin bundle. The star highlights the appearance of an actin filament that has increased mobility in the cell cortex. The montage is formed from alternative images from a time series. Scale bar = 7.5µm. **b)** Graph plots the movement of the protein foci marked by the circle (blue points) and square (red line) through the time series. Distance is measured relative to their starting position.



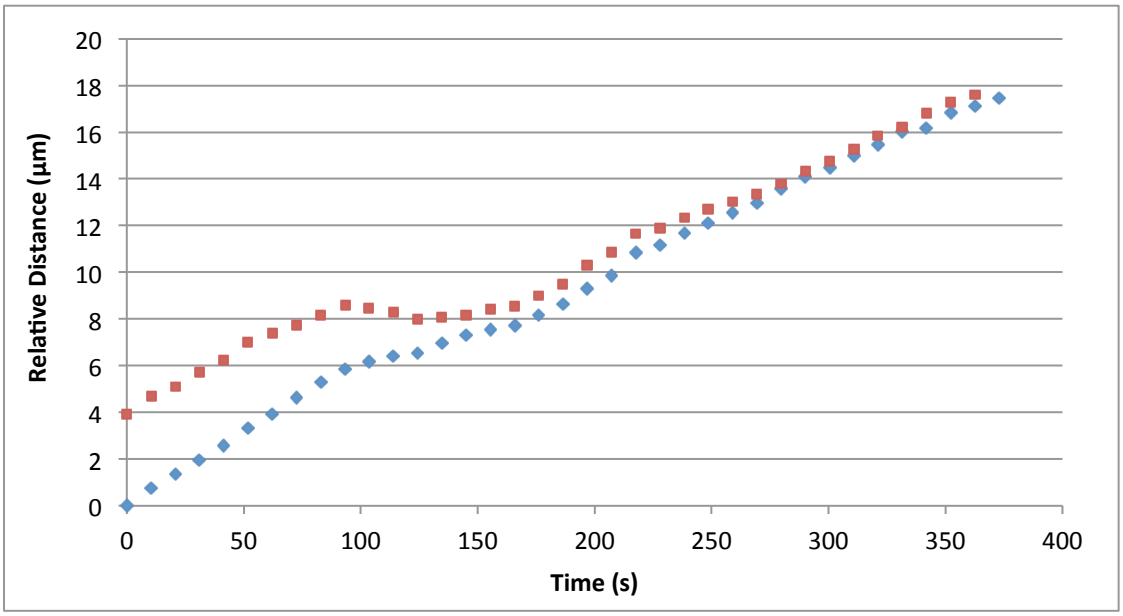


Figure 27: Montage shows the mobility of NET1A 3W to 3G::GFP mutation moving with actin cables over time. The actin is marked using lifeact::RFP. The montage is formed from every third image of a time series. Scale bar = 7.5µm. The graph shows the movement of the two protein foci present on the actin cable in the image. The blue points represent the lower focus and the red points represent the upper focus. Distances are measured relative to the starting position of the lower focus.

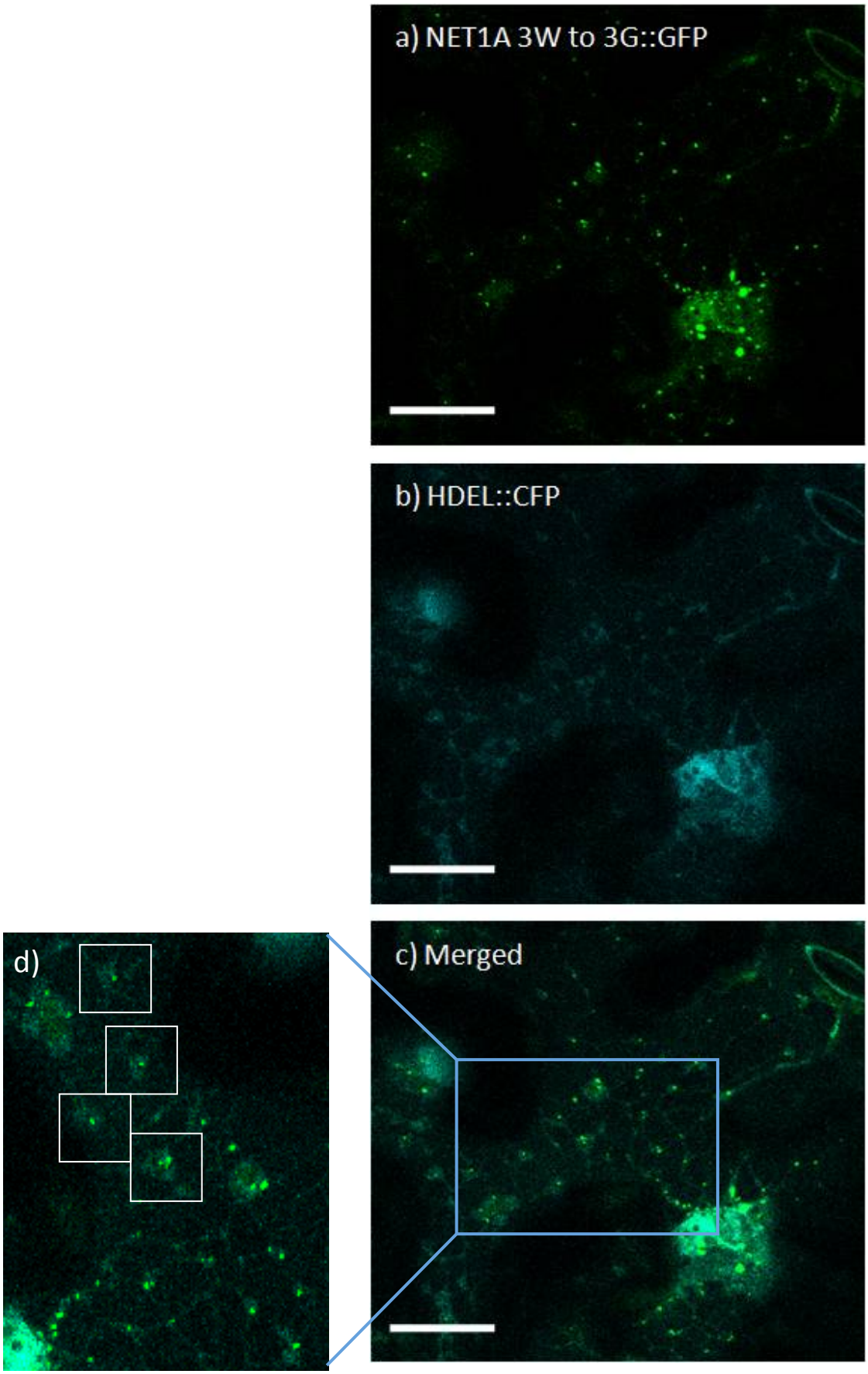


Figure 28: Expression of NET1A 3W-3G::GFP **a)** and the ER marker HDEL::CFP **b)** in the same cell **c)** show that the mutant NET1A dots seem to localise to the ER cisternae **d)** The area defined by the square in **c)** is magnified. Scale bar = 25µm.

speed, as demonstrated by the gradient of the line represented by the scatter points then evens out. This suggests that although the proteins are associated with f-actin they also have an affinity for each other that is able to briefly overcome the f-actin association.

The NET1A 3W to 3G mutant also interacts with the ER as shown in figure 28. The punctate foci localise to the ER cisternae (as highlighted in boxes in figure 28). These could represent stable anchor points where the more motile parts of the ER are attached to the plasma membrane or coincide with plasmodesmata.

This evidence shows that the unusual triple tryptophan motif in the ABD of the NET proteins could be involved in anchoring the actin filaments to the plasma membrane. The loss of this function in the mutant could result in filaments associated with protein, moving through the cytoplasm and waving around in the cortex. The mutant's association with the ER cisternae might occur because, due to a reduced ability to anchor in the plasma membrane, the ER cisternae represent a stable domain where the mutant protein can accumulate. The ideas mentioned above will be discussed in more detail in section 4.6.

Chapter 4: Discussion

The aim of this project was to create mutants of NET1A and NET2A in which the ability of the protein to bind to f-actin was disrupted and to probe potential functional domains within the ABD. To achieve this, a domain deletion and point mutations were made to the ABD and these constructs were expressed to induce a dominant-negative phenotype. The mutants were stably transformed into *Arabidopsis thaliana* but due to time constraints it was not possible to analyse these for a mutant phenotype or to study the localisation of the mutant proteins *in situ*. It was planned that the NET2A WT and mutant constructs would be transiently expressed in *Nicotiana tabaccum* pollen via bombardment but it was not possible to obtain results for this as the transformation efficiency was low. The technique worked for the smaller constructs (lifeact::CFP and CFP only control) but not the NET2A protein insert. The NET1A constructs were transiently expressed by agrobacterium-mediated infiltration into *Nicotiana benthamiana* leaves. Using this technique it was possible to visualise how the mutations of the ABD affected the protein's localisation. Using fluorescent markers for actin and the ER it was possible to investigate the co-localisation between the WT and mutant proteins and these cellular networks. The results of these experiments will be discussed below.

4.1. Full Length WT NET1A Associates with the Actin Cytoskeleton

Results in figure 16 demonstrate for the first time that full length NET1A co-localises to actin filaments. Previously the isolated n-terminal domain had been shown to localise to actin filaments and the full-length protein had been shown to align in dots, possibly on a filamentous template. Because co-localisation with an actin marker had not been carried out, it could only be hypothesised that these filaments were the actin cytoskeleton (Calcutt, 2009). Recently, aniline blue staining has been used to confirm the co-localisation of NET1A and plasmodesmata in the cell files in the roots of seedlings (Hussey and colleagues, unpublished data). Confirming that NET1A localises to the actin cytoskeleton makes it likely that it functions as a bridge, tethering the actin cytoskeleton to the plasmodesmata. Since NET1A has not been shown to have a transmembrane domain (Calcutt, 2009), this bridge is likely to function through a single binding partner of NET1A, or perhaps a larger protein complex, that is anchored at the plasmodesmata.

There is a lot of evidence in the primary literature that the actin cytoskeleton associates with plasmodesmata. It has been shown to form an array surrounding the neck of the pore which enables regulation of its dilation to restrict or enable molecules

of varying sizes to pass between cells (Ding et al., 1996). In addition, a model of plasmodesmata structure shows actin passing through the pore (Overall & Blackman, 1996). It has not been determined whether this is for structural or transport purposes but there are myosins associated with this array that physically link the f-actin to the plasma membrane (O'Parka, 2004).

Until recently little was known about the structure of plasmodesmata and what its constituent proteins were. With the recent publication of the proteomic analysis, some of the proteins that are found localised to the pores have been identified. These include myosin VIII, centrin, remorin, calreticulin and PDLP1. (Fernandez-Calvino et al., 2011) Proteins, such as these, that are enriched at the plasmodesmata, are potential binding partners for NET1A. PDLP1 is a possible candidate and meets these criteria. Figure 29 shows the recently identified plasmodesmata-localised proteins and a model suggesting how they are arranged in the pores.

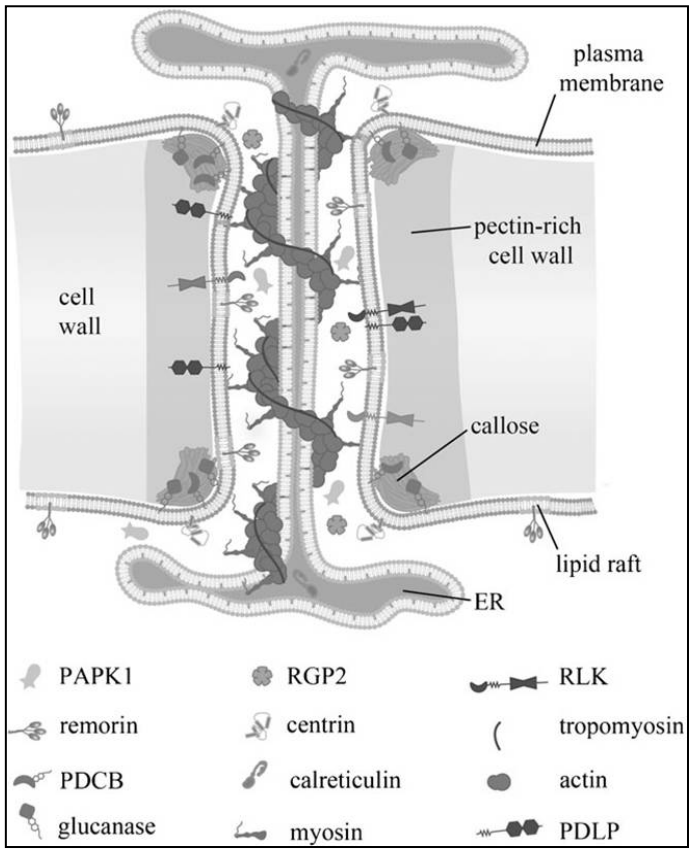


Figure 29: A new model for the structure of plasmodesmata, showing the proteins that are localised there and potential interactions between proteins and the ER and actin networks (Faulkner & Maule, 2011).

4.2. The -ABD Mutant Suggests NET1A has the Potential to Link F-Actin to the ER.

The mutation involving a deletion of the entire ABD of NET1A prevented the protein from associating with the actin cytoskeleton. It had previously been shown that this was the domain through which the interaction with f-actin occurred (Calcutt, 2009) and this result suggests the lack of a secondary ABD in the c-terminus of the protein. It was interesting to discover that the –ABD mutant co-localised with the ER marker HDEL (figure 20). This result, which has been confirmed using Pearson's correlation coefficient, could suggest a potential interaction between the WT protein and the ER. When the domain through which NET1A binds f-actin was lost, it is possible that the protein defaulted to another binding partner that coats the surface of the ER. Previous evidence supports this interaction. Dr J. Calcutt (2009) showed that a GFP fusion to the mid-section of NET1A had an ER localisation whilst the GFP fusion to a fragment of the NET1A c-terminus, did not. The localisation of the c-terminus was mostly cytoplasmic and nuclear. This evidence points toward an interaction between NET1A and the ER mediated through a binding domain found within the mid section of the protein between amino acids 961-1164 (the domain defined by Dr J. Calcutt's NET1Amid2 clone (2009)). Structural analysis of this region showed the presence of coiled-coil domains. These are structural motifs that are often involved in protein-protein interactions (Rose et al., 2005). Thus, NET1A could be associating with a binding partner on the outer surface of the ER through and interaction between coiled-coils.

An alternative explanation for the localisation between the –ABD mutation and the ER is that the mutant protein is misfolded and thus retained in the ER. Misfolding can occur when proteins are mutated and are unable to form their normal tertiary structure. It is unlikely however that a misfolded protein would be diffuse throughout the ER as seen by the localisation of NET1A –ABD. Research investigating the ER retention and misfolding of the rhodopsin protein (Chen et al. 2011) shows that ER retention of truncated rhodopsin proteins result in the formation of aggregates. These arise as the mutant becomes associated with chaperones such as BiP and calnexin which act to prevent the export of misfolded protein from the ER. No such aggregates are apparent in the distribution of NET1A-ABD in the ER which supports the hypothesis that the construct has a domain within the mid-region that confers an ER association.

The ER is a highly dynamic web-like network within cells that is continually undergoing growth and rearrangement. An association between actin filaments and

the ER has been well established and can be confirmed by studying electron micrographs that show deformation of the ER membrane at the point where it meets actin filaments (Staehelin, 1997). The ER is composed of a network of membrane tubules which come together and flatten at small cisternae which are thought to represent points where the ER network is anchored at the plasma membrane. These ER-plasma membrane anchor points have shown to be resistant to plasmolysis and centrifugation experiments (Staehelin, 1997). It is known that the actin cytoskeleton provides the tracks along which the ER tubules extend and this association is thought to be mediated by myosin motor proteins, particularly class XI (Sparkes et al., 2009). Interactions between actin, myosin and the ER determine the structure of the ER network (Ueda et al., 2010). With an association between the ER and the actin cytoskeleton so well characterised, NET1A could be presented as a potential candidate protein for linking these two cellular networks together. It has an n-terminal ABD through which it binds f-actin and, as results discussed above from this project and by Dr J. Calcutt (2009) suggest, it interacts with the ER through a domain in the mid-region of the protein.

Further experiments are needed to rule out the ER association of NET1A-ABD being due to a misfolding response. One method for exploring this would be to extract soluble and membrane fractions from transformed plant tissue. This would result in the formation of ER membrane micelles containing the protein construct. Addition of proteinase K to the micelles would cause digestion of any protein on the surface of the ER micelles but any protein that was trapped within the ER lumen would be protected. If the NET1A –ABD construct was digested it would prove that it is binding to the outside of the ER through an interaction with an ER localised protein. However, if the construct was protected from digestion it would suggest that it was trapped inside the lumen of the ER, potentially as a result of misfolding.

An alternative method that could be used to investigate the NET proteins' interaction with the ER is bimolecular fluorescence complementation (BiFC). This involves splitting a molecule of a fluorescent protein, such as YFP, into two fragments. When the two halves of the fluorophore are brought together, via an interaction between the proteins they are attached to, fluorescence would be produced (Kerppola, 2006). For the purpose of investigating the NET1A-ER interaction, one half of the YFP molecule would be attached to the NET1A-ABD construct and the other half attached to either HDEL (ER localisation sequence known to be held within the ER lumen) or a cytosolic protein. The combination that produced fluorescence would tell us whether the –ABD mutant was trapped within the ER or associating with the outside of the membrane.

4.3. WT NET1A Co-Aligns with ER

As discussed in section 4.1, it is thought that NET1A may also associate with the ER network through a coiled-coil domain in the mid section of the protein. Evidence shown in figure 20, supports this theory by demonstrating WT NET1A punctae aligning along ER tubules. Whilst this could represent an affinity between ER and NET1A, an alternative explanation could be that the protein is forced into an ER localisation by its affinity for actin filaments which co-align with ER tubules (Ueda et al., 2010). actin-disrupting drugs (such as latrunculin B and cytochalsin D) could be used to determine if the ER association is direct. If the apparent ER association is an artefact of the overlap between actin and ER, fragmentation of the actin cytoskeleton would also disrupt the distribution of the punctate NET1A. If however, the protein was also anchored to the ER and is positioned at junctions where the ER and the plasma membrane meet, the protein would show some resistance to drug treatment. The drug studies could be carried out in the presence of an ER marker with the NET1A construct so that any disruption to protein localisation that did occur could be analysed in the context of the ER co-alignment. Using an ER marker would show if the protein still localised to the ER once the actin cytoskeleton was fragmented. Dr J. Calcutt (2009) used actin-disrupting drugs to treat roots expressing NET1A localised with antibodies. Whilst there was a certain amount of disruption to NET1A localisation, some of the protein did appear to remain at the cross-walls. This suggests some resistance of the protein toward actin-disrupting drugs. Since the magnification and resolution of images presented by Dr J. Calcutt (2009) were not high enough to distinguish the punctate distribution of the protein, there would be much to gain by carrying out further drug studies.

The drug treatments discussed above would help confirm the association between NET1A and the ER that is demonstrated in figure 20. Multiple attempts were made throughout the project to express a protein construct alongside markers for both ER and actin in the same cell. This was never successful. On the rare occasion that all three constructs were expressed together, cell death occurred due to over-expression. This meant that meaningful results could not be obtained. Mostly the NET1A construct and one or other of the markers were expressed together. The aim of the triple expression was to determine if the NET1A WT punctae that aligned on actin filaments (as demonstrated in figure 17) were coinciding with ER at the same point.

The ER network is continuous between adjacent cells and passes through the plasmodesmata. It comes together with the actin cytoskeleton to form a narrow rod

called a desmotubule (Tilsner et al., 2011). Since there is such a close association between the ER and actin cytoskeleton at plasmodesmata it is feasible to speculate that there is a protein that links them together. If it can be shown that the point where the NET1A WT foci associates with actin filaments is also where an interaction with the ER network is occurring, NET1A could be a candidate protein for bringing these two structures together at the plasmodesmata.

4.4. The E66G Mutation Potentially Disrupts F-Actin Binding

The results discussed in section 3.4.3 demonstrate that this project was successful in creating a mutant of NET1A that had its f-actin binding ability disrupted *in vivo*. This was achieved by mutating a single charged residue within the f-actin binding domain to a neutral glycine residue. Initial bioinformatic analysis of the ABD (discussed in section 3.1.1.) suggested that this residue had the potential for providing a link between the protein and the actin cytoskeleton. It was a charged residue, exposed at the surface of the protein and was replaced by an oppositely charged residue in NET3B, a NET family member that was thought not to bind f-actin. Recent observations of NET3B now suggest this protein has a weak but detectable affinity for f-actin *in vivo*. Successful mutagenesis, cloning and transient expression of this construct in *N. benthamiana* has shown that mutation of this single residue is sufficient to disrupt the ability of the protein to bind f-actin. This is shown in figures 21, 22 and 23, where the protein foci can be seen to localise adjacent to the actin filaments but distinctly excluded from them.

From figures 22 and 23 it seems like the protein is being held up against the filaments by some cellular component that is not shown by a marker, such as the microtubule cytoskeleton or the ER network. This is likely due to the dense cellular environment. For a LSCM to resolve two points they must be 250nm apart. So the fact that, by eye, there is a clear distinction between the protein and the filament, at the scale of the cell means this is an obvious separation. In figure 17, showing the co-localisation between the WT NET1A and the actin cytoskeleton, the association is obvious because the punctate foci align directly over the filaments. Despite the credible difference between the WT and the E66G mutant, there are some dots that do not align with filaments in WT images and dots that do align with filaments in mutant images. To investigate the significance of the lack of f-actin binding in cells expressing mutant constructs, quantitative analysis should be done by taking more images of both constructs and counting dots that lie on and off filaments in both samples.

An alternative approach to determine if the f-actin interaction had been disrupted would have been to express an isolated ABD containing the mutation. This would have made it much easier to distinguish whether f-actin binding was occurring. If the mutated domain was able to bind f-actin the localisation would have been filamentous but if not its localisation would be cytosolic. Because of time limitations in the project it was decided that all transient expression experiments would be done with the full length WT and mutated clones.

Results from imaging suggest that the glutamic acid residue in the PELM motif is a good candidate for a contact point with the actin cytoskeleton. To publish these findings it would be necessary to quantify this interaction using an *in vitro* actin binding assay so its actin affinity could be compared to the WT protein and other mutants. To achieve this, purified construct would be mixed in a test tube with actin. Following ultra-centrifugation the pellet would contain any protein that was able to bind actin and any protein deficient in actin binding would be found in the supernatant. This ratio could be quantified using fluorimetry to determine a dissociation constant between the protein construct and its actin ligand (Preuss et al., 2004).

The E66G construct has the potential to be an invaluable tool for investigating the function of f-actin binding in NET1A. When expressed in stable *A. thaliana* lines under the expression of a constitutive promoter it will have a clean dominant negative effect. The f-actin binding ability has been disrupted by mutating a single residue and the remainder of the ABD is intact so other functional domains within the ABD should be preserved. Taking the dominant negative approach would mean that all the binding partners of the WT protein would become blocked with over-expressed mutant protein. This would disrupt the link between the WT protein and the actin cytoskeleton. Stable lines expressing this dominant negative mutant could then be analysed for any phenotype resulting from un-coupling the NET1A-actin link. This would suggest a function for the cytoskeletal interaction. In addition, the roots of seedlings (where the protein is natively expressed) could be studied to determine if there was any change in localisation of the protein at the cross walls. Expressing the protein in *N. benthamiana* leaf epidermal cells is an alien system for NET1A. In this environment, a binding partner of the protein may not be expressed which could affect the protein's distribution in a different way that would be observed in a stable line. During the project all constructs, including E66G, were dipped to create stable lines and there were potential transformants following seed selection. There was not time, however, for these transformants to develop and set seed so that the T1 generation were available for analysis. Once these lines are ready it would be useful to cross them with stable lines expressing ER and actin markers. This would mean the WT

and mutant's co-localisation with these structures could be investigated in the protein's natural environment rather than using the artificial system of *N. benthamiana*.

As discussed in section 3.1.1, in this project it was decided to mutate the glutamic acid residue in the PELM motif to glycine. If in future, different mutations were designed and cloned to further probe the properties of the NET family's ABD it would be interesting to try mutating this residue from an acidic to a basic one such as lysine. Instead of neutralising the charge, as the mutation to glycine did, this would result in a charge reversal. This situation would better reflect the properties of NET3B in which the glutamic acid is replaced by lysine and results in f-actin binding being disrupted. Introducing this mutation would have the added benefit of maintaining the solubility of the protein which could have been affected by the mutation to glycine.

A useful step toward further understanding the NET family of proteins would be to determine the tertiary structure of NET1A using protein crystallography. Having an understanding of the structure of the protein and the position of the ABD and other functional/structural domains relative to each other would help determine the role of the protein. Identification of this single glutamic acid residue that is a likely contact point with the actin cytoskeleton would be useful information when calculating the tertiary structure as it is highly likely to be found on the outer surface of the protein when it is folded up. The triple tryptophan motif would be an interesting aspect of the tertiary structure to interpret. Since it is a residue sequence that is unique to plants and is predicted to introduce a kink into the secondary structure it would be fascinating to try to establish what properties it confers on the protein.

4.5. An Association Between the E66G Mutant and the ER is Undetermined.

Imaging results investigating the interaction between the E66G mutant and the ER have been inconclusive. Although those images presented in figure 24 demonstrate a close alignment between the construct and the ER, it is hard to infer whether the two are co-aligned. It could be predicted that the E66G mutant would bind the ER through the coiled-coil domains in the mid section of the protein. However, it seems that the protein foci could be excluded from the ER membrane in figure 24, and even sometimes appear wedged in the gaps between the tubules. Since the foci seen in figure 24c) appear green, this would suggest that there was not a co-alignment because if the protein was aligning with the ER tubules the foci would appear yellow. To reach a more reliable conclusion it would be necessary to obtain further images at

higher resolution. The ER in these images has a granular appearance which can be a sign of cell death, therefore the results of the co-localisation should be analysed with caution. The conclusion that it is possible to draw from these results is that the E66G mutant does not co-align with the ER as defined by the marker HDEL. The ER has a complex structure and is made up of 14 discrete domains including the nuclear pore domain, protein forming domain, actin binding domain, plasma membrane anchoring domain and plasmodesmata (Staehelin, 1997). There are a number of different ER markers such as HDEL (used in the present project), calnexin and calreticulin. These all have slightly different localisations in the ER and thus will highlight different discrete domains expressed as an ER marker. It could be that if the E66G mutant was co-expressed with a different marker a co-alignment with the ER would be shown. Whilst a co-alignment between the mutant and HDEL would support an ER localisation, the lack of co-localisation is not sufficient to rule this out.

Carrying out drug studies, as described in section 4.4, on the E66G mutation would help confirm or rule out an ER association. Since the mutant does not associate with f-actin it should not be affected by the actin disrupting drugs. Whilst the WT protein may show some disruption to its localisation following drug treatment because of its f-actin binding ability, if the mutant is attached to the ER then it should remain anchored in place.

4.6. Mutating the Triple Tryptophan Domain Alters the Behaviour of NET1A Punctae

Evidence presented in figure 25 confirms that the 3W to 3G mutation is still able to bind f-actin. This was expected as the mutation was not designed to disrupt f-actin binding but rather probe the function of the rare triple Tryptophan motif. It has been proposed in the discussion above that the function of NET1A is to attach the ER membrane system and cortical actin filaments to plasmodesmata at the plasma membrane. The evidence collected from imaging the 3W to 3G mutation shows that whilst the ER and actin associations still occur, the filaments in the cell seem much more motile which suggests a role for the triple tryptophan motif in stabilising the location of NET1A punctate.

Figure 25 shows the localisation of the protein along actin cables on the trans-vacuolar strands. This seems to be more prevalent than in the WT, but perhaps a lack of interaction with the plasma membrane has allowed more of the protein to be pulled away from the cortex and localise to the more structurally stable actin cables as opposed to fine actin filaments found in the cortical array. Despite this, when imaging

the cortex of the cell with an ER marker, there is a population of mutant protein foci found there. As shown in figure 28, in the cell cortex, the mutant seems to associate with the ER but specifically to the ER cisternae. To confirm the significance of this localisation it would be helpful to undertake a quantitative analysis by studying a large number of images to determine what proportion of the foci were found at the ER cisternae as opposed to tubular membrane regions of the ER or free in the cytosol. This would help establish the statistical significance of this localisation.

As discussed in section 3.3.1, it is thought that the unusual triple tryptophan domain would introduce a rigid kink into the protein structure. Interestingly the region of the NET ABDs that has the least homology between sub-groups of the NET family are the residues between the start of the coding sequence and the triple tryptophan domain. These are represented in figure 30.

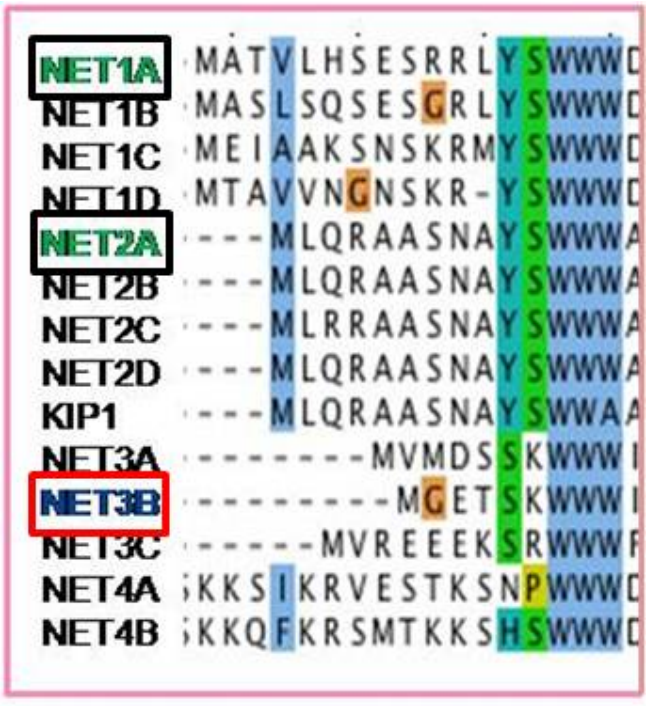


Figure 30: The coding sequence n-terminal to the triple tryptophan domain of the NET protein family (bioinformatic analysis by Dr T. Hawkins, Durham University, UK).

In figure 30 it is clear that the region of the ABD that is n-terminal to the triple tryptophan domain does not have the same homology across the family sub groups as the rest of the ABD shows (figure 8). There are however, clear similarities within the different sub-groups. In the NET2s this region is highly homologous; the NET3s are all noticeably shorter than the other groups and NET1s are all the same length and contain residues with similar properties in the same position. It is known that the different sub-groups of NET proteins localise to different types of membrane in the

cell. Considering this, it is reasonable to suggest that this n-terminal region could be responsible for targeting the proteins to a particular membrane site. To test this theory it would be interesting to swap this small n-terminal domain of NET1A that could target the protein to the plasma membrane, with that of another NET which localises to the vacuole membrane or ER membrane. If this managed to switch the localisation of the proteins, it would suggest the role of membrane targeting for the n-terminal region. The predicted kink in the structure caused by the triple tryptophan domain could facilitate this membrane targeting by introducing a rigid turn in the protein structure that exposes the membrane targeting sequence. This could enable the ABD of the protein to bind f-actin at the PELM motif whilst simultaneously allowing the n-terminus to interact with a membrane protein.

The changes to the localisation in the 3W to 3G mutant are potentially due to the exchange of the three tryptophans, which were large, rigid residues that created a kink in protein sequence, with glycines. The mutated motif would be much more flexible because the glycine residues are very small. The results shown in section 3.4.4 show the mutant still able to bind f-actin and the ER. The protein function that has been affected seems to be the ability to anchor these networks to the plasma membrane resulting in increased mobility of actin filaments and cables. This could be because the membrane targeting region at the n-terminus of the protein is no longer positioned in the right conformation or orientation because of the disruption to the structural kink that arranges it for membrane binding.

4.7. The ABD of NET1A is Necessary but not Sufficient for a Punctate Distribution

A punctate distribution at cellular membranes is a common feature of the NET proteins. It was interesting to see that the –ABD mutant had lost this punctate nature where as the WT and other mutants retained this localisation pattern (figure 15). This would suggest that there is some motif within the NET1A ABD that is necessary to confer the dotty localisation. However, it is known from research by Dr J. Calcutt (2009) that the n-terminal domain is not sufficient to achieve a dotty localisation. Dr J. Calcutt (2009) expressed NET1A split into four different isolated domains along its length. These showed various different localisations (cytosol, ER, actin filaments) but none of these were punctate (Calcutt, 2009). The dottiness is only seen when the protein is expressed as a whole. This suggests that it is a function of the tertiary or quaternary structure of the protein. Perhaps the protein needs to fold up in the correct

fashion or multiple copies of the folded protein are arranged together in a particular way to create the foci.

There is evidence to suggest that there is a specific domain within the ABD that defines dottiness because, in the images presented in chapter 3, the size and definition of the dots is changeable. The size of the punctae vary between cells and constructs, and in some images the protein seems to accumulate in large aggregates. The E66G mutation involved changing a charged residue to a neutral one. This would decrease the solubility of the protein as the glutamic acid residue is thought to be exposed on the protein's surface. Although tryptophan is not a charged residue, the mutation of the triple tryptophan motif (three very large side chains) to three glycine residues (no side chain) could alter the secondary structure of the protein, affect protein folding, and thus bury charged residues that would usually be exposed at the surface. This decrease in solubility could encourage proteins to aggregate, a reaction that would be further encouraged by the large quantity of recombinant protein being translated by the cell.

4.8. Conclusion

Experiments from imaging the different mutants and the WT constructs alongside ER and actin markers has led to the development of the model in figure 31, which identifies discrete functional domains through which NET1A could potentially interact with the ER, f-actin and the plasma membrane.

The different mutants that were designed and successfully cloned each provided an interesting insight into potential functions for the residues that were being mutated or the function of the truncated protein (in the case of the –ABD mutant). Imaging of the –ABD mutant confirmed that NET1A did not have another domain outside of the n-terminal 96 residues through which it binds f-actin, In addition it showed the mutant co-localising with the ER. Evidence provided in research by Dr J. Calcutt (2009) suggested that the ER-NET1A association was mediated through a coiled-coil domain within the mid-section of the WT protein. This result led to the use of an ER marker in addition to the actin marker to be used for co-localisation studies in the transient expression of the WT NET1A and mutants. Expression of the WT protein showed protein foci aligning with both the ER and f-actin networks. This was the first time that an association between full length NET1A and the actin cytoskeleton had been confirmed and the link to the ER raised the possibility that NET1A was involved in linking both these networks to the plasmodesmata where they are known to associate. The E66G mutation showed a reduced affinity for f-actin. This would

need to be confirmed and quantified using an *in vitro* actin binding assay, but images show that the protein foci are no longer aligned on the actin filament but seem to be positioned alongside. This would suggest the E66 residue as a candidate for contacting actin filaments. Finally, results from the 3W to 3G mutation show the actin cytoskeleton being much more mobile in the cell, which could suggest a function for this residue motif being involved in the mechanism for anchoring or targeting the protein to the plasma membrane.

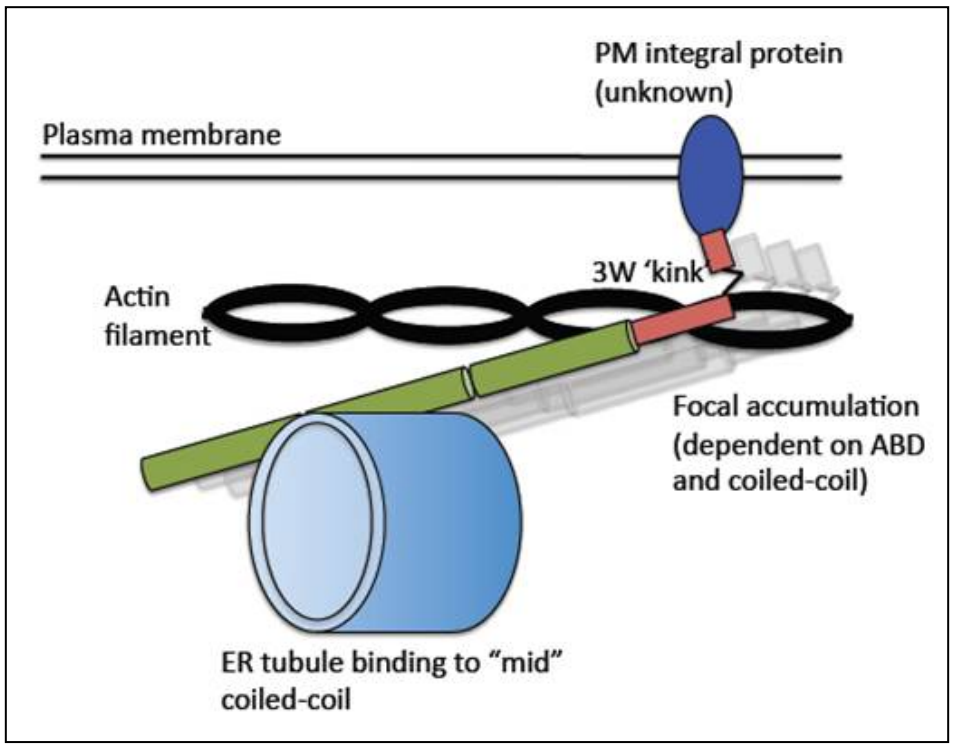


Figure 31: A model suggesting discrete domains through which NET1A interacts with the actin cytoskeleton, the ER network and an integral membrane protein at plasmodesmata at the plasma membrane.

The model represented in figure 31 is a good platform from which to design future experiments to test the hypotheses put forward about the functions of different domains within NET1A. The evidence presented above suggests that NET1A interacts with the ER network. From research carried out by Dr J. Calcutt (2009) it can be hypothesised that this is through a coiled-coil domain in the mid region of the protein. NET1A's interaction with actin could potentially be mediated through the E66 residue in the ABD as this has been highlighted as a potential contact point with actin filaments. Structural analysis carried out by Dr T. Hawkins (Durham University, UK) has suggested that the triple tryptophan motif in the ABD creates a kink in the protein secondary structure which, research in this project has suggested, could hold the n-

terminus of the protein in a position in which it could contact a protein embedded in the plasma membrane, potentially at the plasmodesmata. Finally, it has been suggested that the small number of residues n-terminal to the triple tryptophan motif could be involved in targeting NET family members to their cellular membrane locations. This hypothesis has not been explored in this thesis but would be interesting to investigate in future work. Furthermore, once the technique for transient expression in pollen can be used successfully again, it would be interesting to look at NET2A mutant and WT localisation alongside both actin and ER markers and see what similarities can be drawn between the model presented in figure 31 and the domain functions and protein interactions of NET2A in pollen tubes.

Appendix 1: NET1A and NET2A Constructs Cloned

Vector	Insert	Purpose
Entry Clones:		
pDONR Zeo	NET1A –ABD	To LR into destination vectors
pENTR3C	NET1A E66G	To LR into destination vectors
pENTR3C	NET1A 3W to 3G	To LR into destination vectors
pDONR207	NET2A E53G	To LR into destination vectors
pDONR207	NET2A 3W to 3G	To LR into destination vectors
pDONR207	NET2A -ABD	To LR into destination vectors
Destination Clones:		
pH7FWG2	NET1A E66G	GFP transient and stable expression construct
pH7FWG2	NET1A 3W to 3G	GFP transient and stable expression construct
pH7FWG2	NET1A -ABD	GFP transient and stable expression construct
pH7CWG2	NET1A E66G	CFP transient expression construct
pH7CWG2	NET1A 3W to 3G	CFP transient expression construct
pH7CWG2	NET1A -ABD	CFP transient expression construct
p52GWC7	NET2A WT	CFP bombardment construct
p52GWC7	NET2A E53G	CFP bombardment construct
p52GWC7	NET2A 3W to 3G	CFP bombardment construct
p52GWC7	NET2A -ABD	CFP bombardment construct
pH7CWG52	NET2A WT	CFP construct for stable expression
pMDC107	NET2A E53G	GFP construct for stable expression

pMDC107	NET2A 3W to 3G	GFP construct for stable expression
pH7CWG52	NET2A -ABD	CFP construct for stable expression
pH7RWG52	NET2A -ABD	RFP construct for stable expression

Appendix 2: Primer Sequences for mutagenesis:

	Forward	**Reverse**
NET1A minus ABD	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCTAAAACCATGGCTGAGGCGTTCCCTAA	For c-terminal FP fusion: GGGGACCACTTTGTACAAGAAAGCTGGGTCCTCTTCATTACCGGAAGAT For N -terminal FP Fusion: GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACTCTTCATTACCGGAAGAT
NET2A minus ABD	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCTCGTACAATAGCCACAGCTTTCCCTGAACAT	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTCAGGGAGCTTCCCAGGTGGCCTAC
NET1A E66G	GTATTACAAGAAACGTCCAGGGCTGATGAAACTTGTGGAAG	CTTCCACAAGTTTCATCAGCCCTGGACGTTTCTTGTAATAC
NET2A E53G	GTATTACCGTAAAAGACCAGGGATTGTTAATTTCGTGGAGG	CCTCCACGAAATTAACAATCCCTGGTCTTTTACGGTAATAC
NET1A Insert 3G	ATGATTAAACTCATCGAAGAAGATGGAGGAGGAGCAGATTCTTTTGCAAGAAGGG	CCCTTCTTGCAAAAGAATCTGCTCCTCCTCCATCTTCTTCGATGAGTTTAATCAT
NET2A Insert 3G	TACTCTTAAGATTATAGATGAAGATGGAGGAGGAGGAGACACTTTTGCTAAAAGAGC	GCTCTTTTAGCAAAAGTGTCTCCTCCTCCTCCATCTTCATCTATAATCTTAAGAGTA
NET1A 3W to 3G	GTCCAGGCGGTTATACTCCGGGGGAGGGGATAGTCACATTCCCAA	TTGGGAATGTGACTATCCCCTCCCCCGGAGTATAACCGCCTGGAC
NET2A 3W to 3G	GAGAGCAGCGAGCAATGCTTATTCAGGGGGAGGGGCAAGCCACAT	ATGTGGCTTGCCCCTCCCCCTGAATAAGCATTGCTCGCTGCTCTC

Appendix 3: Sequencing Primers:

pDONR207 FOR	TCGCGTTAACGCTAGCATGGATCT
pDONR207 REV	GTAACATCAGAGATTTTGAGACAC
pENTR3C FOR	GTTACTTAAGCTCGGGCCCC
pENTR3C REV	GAGACACGGGCCAGAGCTGC
M13 FOR	GTAAAACGACGGCCAGT
M13 REV	AACAGCTATGACCATG
NET1A MID 1-2F	GGTTTGCGTCTTCGGTACCAGC
NET1A MID 1-1R	GTGAAGTTCTGGCTGGAGCTGC
NET1A MID 1-3F	CAGTCCCAAGAGGAACAAAAGG
NET1A MID 1-4F	GCTCTTACTGAGAAGCTGCGTG
NET1A MID 1-5F	CCACTGAGAAGCAAGAGCGTGC
NET1A C-2F	GAGCTGCAGGAGAAAAGCAATG
NET1A C-1R	CAGCTGTCCGTGAAGTCTTGC
NET1A C-3F	GGGAAGCTGAGAAGACGAAGC
NET1A C-2R	CTCGGGTGAAATCAGACAGCC
NET1A C-4F	CGCAGAAGCAGGAATCCCTCTG
210GEN2439	GCTTCAAAGCGCAAACCG
210GEN3013	TCTATTGAAGAAGCCGAG
210GEN3616	TTGAAGTCAGTCTCAACGGA
210GEN4194	GTCAACTGAGGTTAGGGT

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