Investigating the recruitment of centrosomal proteins to the nuclear envelope during myogenesis

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by

Caroline Shak BSc
Department of Molecular and Cell Biology
University of Leicester

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Declaration

The accompanying thesis submitted for the degree of Doctor of Philosophy, entitled “Investigating the recruitment of centrosomal proteins to the nuclear envelope during myogenesis” is based on work conducted by the author in the Department of Molecular and Cell Biology at the University of Leicester mainly during the period between September 2016 and June 2019. All work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other University.

Signed:

Date:

Department of Molecular and Cell Biology
University of Leicester
Lancaster Road
Leicester
LE1 9HN

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Caroline Shak

Abstract

Several stages of nuclear movement and positioning occur during the differentiation of myoblasts into myotubes and myofibres, allowing nuclei to be spread along the cell length and periphery. In a developing myotube, the nuclear envelope (NE) spanning linker of nucleoskeleton to cytoskeleton (LINC) complex recruits centrosomal proteins such as PCM1, pericentrin and AKAP450 to form the nuclear microtubule organising centre (nMTOC). Microtubules nucleated from the nMTOC are used by motor proteins to position myonuclei, failure of which leads to myonuclear clustering and muscle disease. This study explored how the LINC complex component nesprin-1 acts as a centrosomal protein receptor, and how centrosomal proteins relocate to the NE. The muscle-specific isoform nesprin-1α2 recruited PCM1 and AKAP450 to the NE in nesprin-1 null myotubes, through its N-terminal region comprising a 31 residue isoform-specific sequence, 3 spectrin repeats and the adaptive domain. PCM1 residues 1-331 localised to the NE and interacted with nesprin-1α2. In contrast, residues 302-573 localised to the centriolar satellites, suggesting the mode of PCM1 binding, and potentially, function at the two sites is different. GFP-nesprin-1α2 expression in non-myotube cells was not sufficient to recruit PCM1 or pericentrin to the NE. Yet, forced tethering of PCM1 residues 1-1089 to the NE appeared to be able to weakly recruit pericentrin to the NE. This suggests that upon myogenesis, myogenic events such as phosphorylation, mediate PCM1 transfer to the NE, where it readily functions as a scaffolding protein. In nesprin-1 null myotubes, NE-tethered PCM1 appeared to recruit AKAP450 to the NE. However, depletion of PCM1 in myotubes only partially reduced the NE localisation of pericentrin, whereas AKAP450 was unaffected. This suggests that at the NE, PCM1 functions as part of a larger protein scaffold to recruit pericentrin and AKAP450 to the NE. This study starts to delineate nMTOC formation at the nesprin-1α2 interface.

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Contents

Abbreviations

AA	Amino acid
AD	Adaptive domain
AKAP450	A-kinase anchor protein 450
ANC1	Abnormal nuclear anchorage 1
APS	Ammonium persulfate
BSA	Bovine serum albumin
CDK5RAP2	CDK5 Regulatory Subunit Associated Protein 2
Cep	Centrosomal protein
CH	Calponin homology
Co-IP	Co-immunoprecipitation
CO2	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
dH2O	Distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
E. Coli	Escherichia coli
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
FBS	Fetal bovine serum
FL	Full-length
g	Gram
GCP	Gamma-tubulin complex proteins
γ-TuRC	Gamma-tubulin ring complex
γ-TuSC	Gamma-tubulin small complex
GFP	Green fluorescent protein
h	Hour
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
INM	Inner nuclear membrane

KASH	Klarsicht, ANC-1, Syne-1 homology
Kb	Kilobase
kDa	Kilodalton
KHC	Kinesin heavy chain
KLC	Kinesin light chain
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LINC	Linker of nucleoskeleton and cytoskeleton
M	Molar
MAP	Microtubule associated protein
mg	Microgram
mg	Milligram
min	Minutes
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MSP-300	Muscle-specific protein-300
MTOC	Microtubule organising centre
NE	Nuclear envelope
NE	Nuclear envelope
Nesprin	Nuclear envelope spectrin repeat proteins
Nesprin-1G	Nesprin-1giant
Nesprin-1α2	Nesprin-1alpha2
NPC	Nuclear pore complex
ng	Nanogram
NLS	Nuclear localisation signal
nMTOC	Nuclear microtubule organising centre
NP-40	Nonident P-40
OMN	Outer nuclear membrane
PACT	Pericentrin-AKAP450 centrosomal targeting
PBS	Phosphate-buffered saline
PCM	Pericentriolar material
PCM1	Pericentriolar Material 1

PCNT	Pericentrin
PCR	Polymerase chain reaction
PIC	Protease inhibitor cocktail
PLK1	Polo-like kinase 1
PLK4	Polo-like kinase 4
POI	Protein of interest
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROI	Region of interest
RPM	Revolutions per minute
SEM	Standard error of the mean
SR	Spectrin repeat
SUN	Sad1, Unc-84 homology
Syne	Synaptic nuclear envelope
TAN	Transmembrane actin-associated nuclear
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylethylenediamine
VSMC	Vascular smooth muscle cells
v/v	Volume per volume ratio
WT	Wildtype
µl	Microlitre
µM	Millimolar

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Chapter 1
Introduction

1.1 Skeletal muscle

Skeletal muscle is the largest tissue of the body, and accounts for around 40% of total body weight (Frontera and Ochala, 2015). Attached to bone by tendons, it is required for voluntary movement of the body and the maintenance of posture. The generation of skeletal muscle, a process called myogenesis, mainly occurs during embryogenesis, whereas muscle satellite cells are maintained in adult muscle for muscle regeneration in the case of injury (Bentzinger *et al.,* 2012; Frontera and Ochala, 2015). Skeletal muscle diseases lead to the wasting of muscle tissue, impaired movement and decreased quality of life. Thus, it is important to understand how myogenesis occurs for the formation of healthy and functional skeletal muscle.

1.1.1 Skeletal muscle structure

Skeletal muscle is composed of parallel bundles of fascicles, which are surrounded by a layer of connective tissue called the perimysium (Fig. 1.1). Fascicles, in turn are composed of longitudinally arranged muscle cells called myofibres, which are each surrounded by the basal lamina (Relaix and Zammit, 2012).

The neuromuscular junction (NMJ) is a synapse between a motor neuron and a myofibre. This is where information is transmitted to innervate a myofibre for contraction. The myofibre cell membrane, known as the sarcolemma, penetrates into the cell through extensions called transverse-tubules (T-tubules), allowing electric impulses to travel from the cell surface to the cell interior (Relaix and Zammit, 2012). Thread-like myofibrils run through the length of a myofibre, and are composed of thick filaments of myosin and thin filaments of actin. These overlap to form the sarcomere, the contractile unit of muscle (Relaix and Zammit, 2012). The organisation of the filaments is what gives muscle its striated appearance. For muscle contraction, the thick and thin filaments slide over each other to shorten the sarcomere (Herzog *et al.,* 2015). Mitochondria in the myofibre provide the cell with energy, in the form of ATP, for contraction.

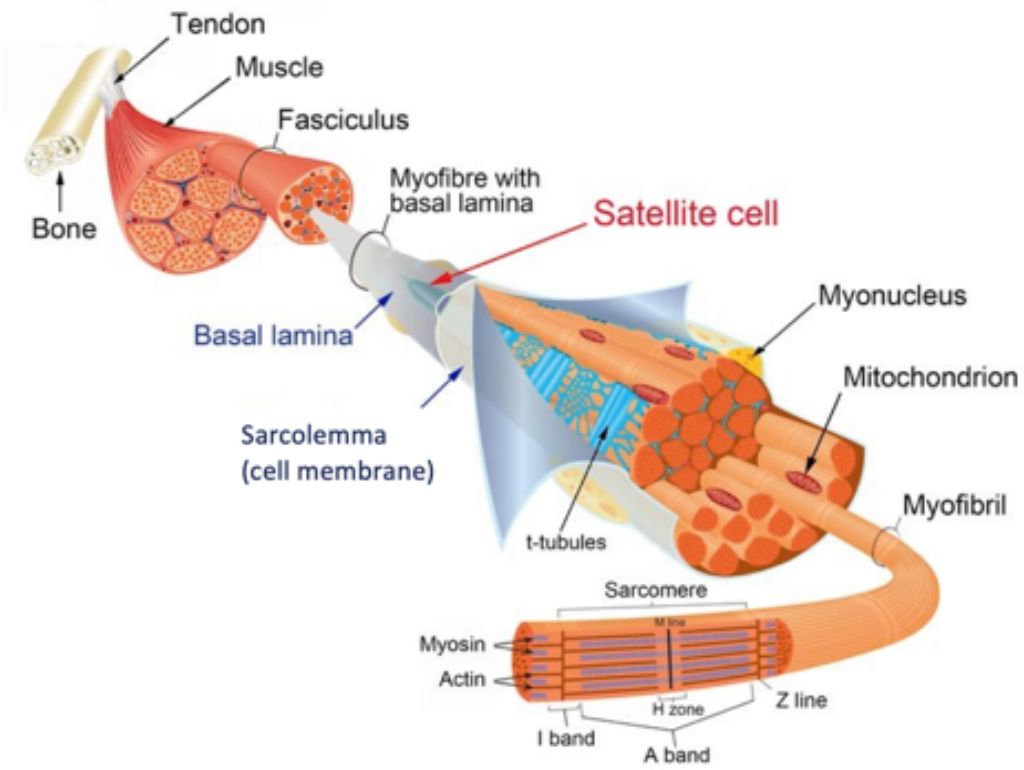


Figure 1.1 Skeletal muscle structure

Skeletal muscle contains bundles of fascicles, which in turn are formed by individual myofibres. The myofibre cell membrane, the sarcolemma, protrudes into the cell as transverse tubules (t-tubules), allowing electric impulses to travel in the cell to mediate contraction. Thread like myofibrils contain contractile sarcomere units, which are powered to contract by ATP, generated by mitochondria. Myofibres are multinucleated, and myonuclei are anchored under the sarcolemma. A reservoir of satellite cells sit between the basal lamina and sarcolemma, and are activated to undergo myogenesis upon muscle injury. From Relaix and Zammit, 2012.

1.1.2 Nuclear positioning in a myofibre

In contrast to most other mammalian cells, myofibres are multinucleated, and nuclei are arranged at the cell periphery, under the sarcolemma. Nuclei are evenly distributed along the myofibre, apart from under the NMJ, where the synaptic nuclei are clustered (Folker and Baylies, 2013). There are a number of reasons for the need to spread non-synaptic nuclei along the periphery of a muscle cell. Firstly, the peripheral positioning offers protection against contractile forces exerted at the centre of the cell, which would be damaging to the nucleus (Folker and Baylies, 2013). Secondly, each nucleus is responsible for the synthesis of proteins for its local environment (Pavlath *et al.,* 1989; Frontera and Ochala, 2015). If myonuclei were not spread out, protein transport over long distances would be required and responses to stimuli would be slow. Finally, as the position of nuclei determines the localisation of other cell structures, the spreading of myonuclei is required for structures to be distributed along the whole cell length. For instance, sarcomeres are assembled near myonuclei, whether nuclear positioning is normal or impaired (Auld and Folker, 2016).

1.1.3 Myogenesis

1.1.3.1 Overview

A myofibre is a syncytium, formed by the fusion of mono-nucleated precursor cells during muscle cell differentiation (Fig. 1.2). Myogenesis primarily occurs during embryonic development and is mediated by transcription factors. Expression of the transcription factors PAX3 and PAX7 promote myogenic precursors in the somite to undergo determination into myoblasts (Beaudry *et al.,* 2016). MyoD and Myf5 promote myoblasts to exit the cell cycle and become committed to differentiation, and fuse to form multinucleated myotubes (Beaudry *et al.,* 2016). Maturation of myotubes into myofibres is promoted by myogenin and MRF4, which allows the transcription of genes encoding muscle-specific proteins

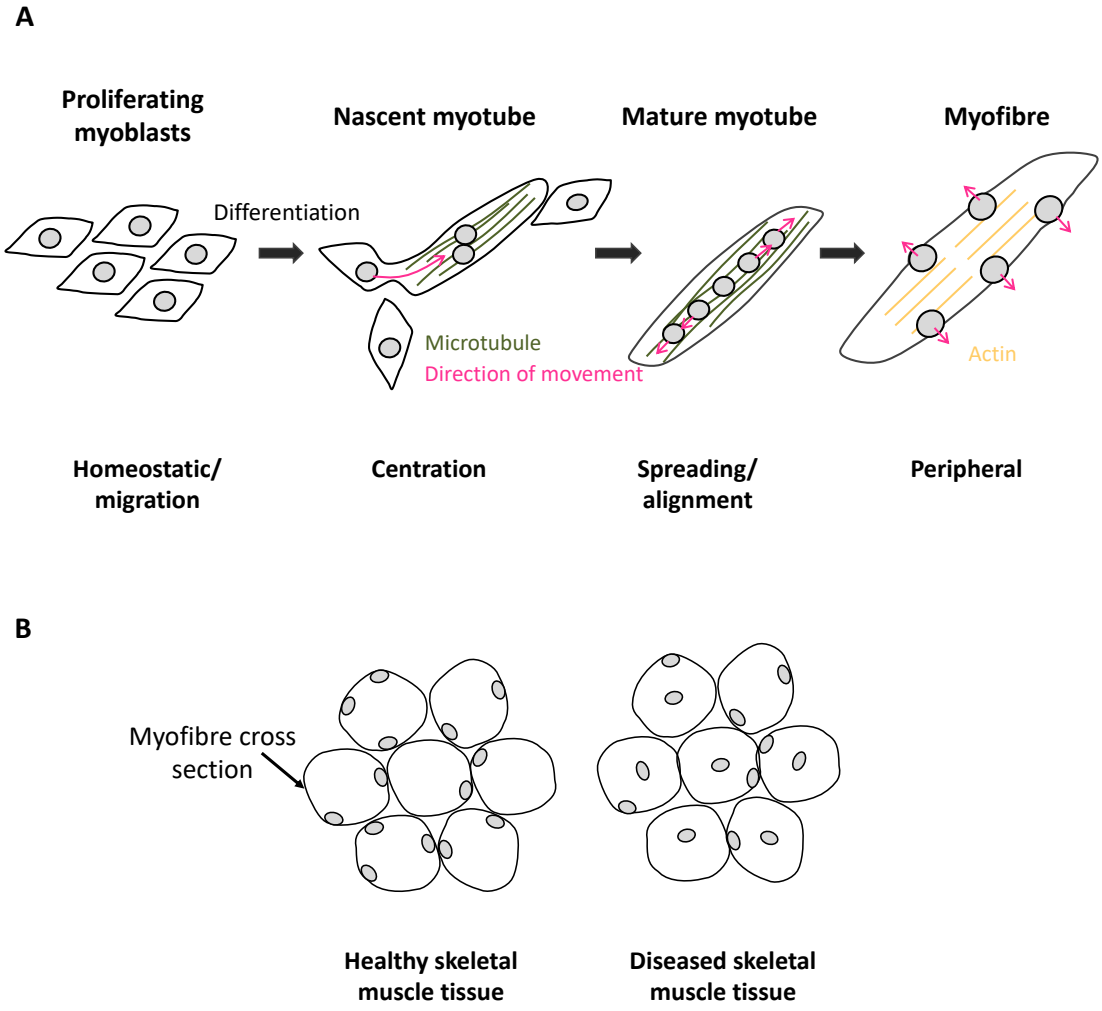


Figure 1.2 Nuclear positioning occurs during myogenesis and is required for skeletal muscle function

(A) Upon differentiation, myoblasts fuse with neighbouring cells. When it fuses with a nascent myotube, microtubules and the minus-end directed motor protein dynein position the newly incorporated nucleus to the cell centre. Next, microtubules and plus-end directed kinesins align and spread nuclei along the myotube length. As the cell matures into a myofibre, nuclei are moved to the cell periphery in an actin-dependent mechanism. Only non-synaptic nuclei are shown. (B) A cross-section of muscle shows bundles of myofibres with myonuclei at the cell periphery. Instead, centrally positioned nuclei are observed in tissues from muscle disease patients.

(Beaudry *et al.,* 2016; Burattini *et al.,* 2004). After muscle formation, remaining progenitor cells enter quiescence and localise between the basal lamina and sarcolemma as satellite cells. In adult, these cells are activated and undergo myogenesis when regeneration and repair of muscle upon injury is required (Bentzinger *et al.,* 2012; Frontera and Ochala, 2015).

1.1.3.2 Nuclear positioning throughout myogenesis

The movement of a nucleus is mediated by the cytoskeleton. Microtubule-mediated nuclear movement can be exerted through forces at the microtubule organising centre (MTOC), or via transport by microtubule-motor proteins kinesin and dynein. Actin-mediated rearward nuclear movement may by driven by an actin retrograde flow (Chang *et al.,* 2015b). Intermediate filaments, play a smaller role in nuclear positioning and have been less studied. Nuclear positioning in a myofibre is achieved through a series of controlled nuclear movements during myogenesis which are initially mediated by microtubules, and later, actin.

Upon signals for myoblasts to commit to differentiate, there are two stages of nuclear movement which are microtubule-dependent (Fig. 1.2). Firstly, a new nucleus entering a nascent myotube is driven to the centre of the cell by the minus-end directed microtubule motor protein dynein (Cadot *et al.,* 2012). Secondly, marking the last stage of microtubule-dependent nuclear movements, nuclei are aligned and spread out equidistant from each other along the length of the myotube by the plus-end directed microtubule protein kinesin-1 (Roman and Gomes, 2017). The myotube now matures into a myofibre where nuclei move to the cell periphery by an actin-dependent mechanism and become anchored under the sarcolemma (Roman and Gomes, 2017; Cadot *et al.,* 2015). During adult muscle repair, newly incorporated nuclei, as in myotube development, first move to the cell centre before distribution to the cell periphery (Blaveri *et al.,* 1999).

1.1.3.3 Impaired myonuclear positioning

Presence of centrally positioned nuclei in histological samples from muscular dystrophy patients is common (Meinke *et al.,* 2014; Folker and Baylies, 2013; Mattioli *et al.,* 2018) (Fig 1.2), and was considered to reflect muscle repair where newly incorporated nuclei are taken to the cell centre. However it is now known that nuclear mispositioning itself can be a cause, rather than a consequence of muscle dysfunction. This was shown by artificially creating muscle disease in *Drosophila melanogaster (D. melanogaster),* by mutating Ens, a protein required for myonuclear positioning. Ens is the *D. melanogaster* orthologue of microtubule-associating protein 7 (MAP7), which binds kinesin-1 to mediate microtubule-dependent nuclear positioning. Upon mutating Ens, myonuclei from larvae became unable to position in a forming myotube, and resulted muscle function becomes impaired (Metzger *et al.,* 2012).

Further details of how nuclear positioning is mediated during myogenesis is vital to understand why nuclei are at the centre of diseased myofibres. It has been known for many years that nuclear positioning is controlled by the cytoskeleton, but the mechanism of how the cytoskeleton connects to the nucleus was not known until identification of the linker of nucleoskeleton and cytoskeleton (LINC) complex, a protein complex which spans the nuclear envelope (NE).

1.2 Nuclear envelope

The nuclear envelope (NE) surrounds the nucleus to separate nuclear contents from the cytoplasm. It consists of the inner nuclear membrane (INM) and outer nuclear membrane (OMN) which is continuous with the endoplasmic reticulum (Watson, 1955). The membranes are separated by a 30-50 nm perinuclear space (PNS) (Cain and Starr, 2015). Protein complexes bridge the NE to physically connect the nucleus and cytoplasm (Fig. 1.3). There are two such protein complexes: transmembrane nuclear pore complexes (NPCs) (section 1.3) and linker of nucleoskeleton and cytoskeleton (LINC) complexes (section 1.4).

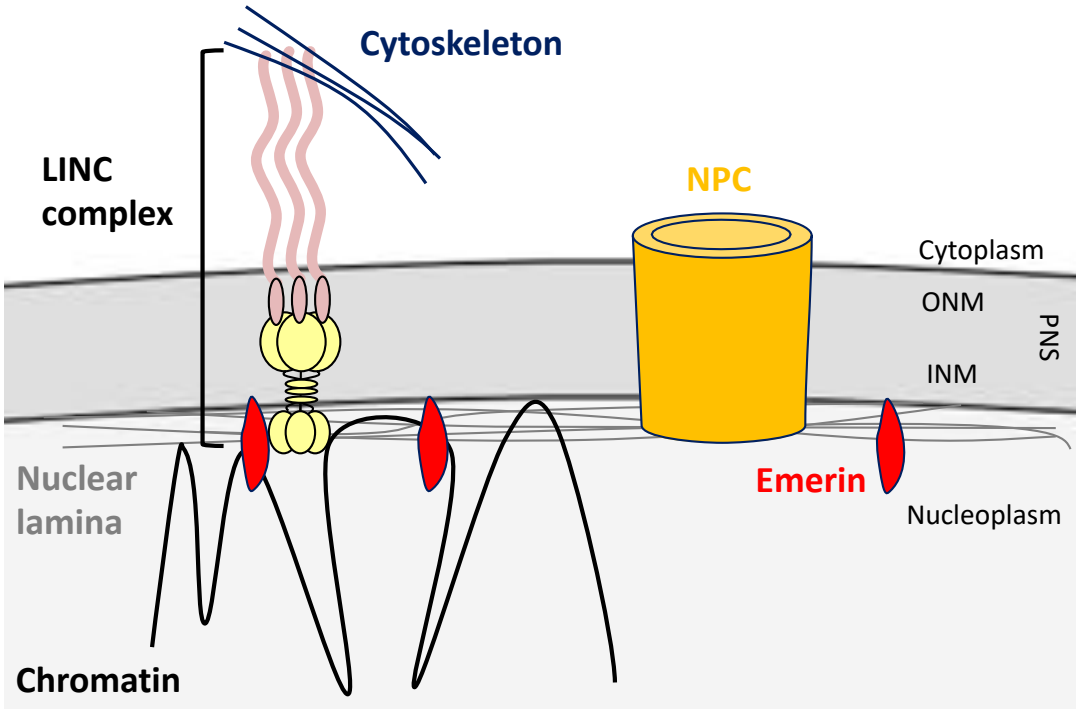


Figure 1.3 The nuclear envelope (NE) contains membrane spanning proteins to connect the nucleoplasm and cytoplasm

The NE is a double membrane consisting of the outer nuclear membrane (ONM) and the inner nuclear membrane (INM) separated by the perinuclear space (PNS). The nuclear lamina resides under the INM to provide rigidity and is composed of lamins and associated proteins such as emerin. The NE contains two integral transmembrane protein complexes. Linker of nucleoskeleton and cytoskeleton (LINC) complexes form a physical bridge across the NE and bind nucleoplasmic elements such as the nuclear lamina and chromatin, and cytoplasmic elements such as the cytoskeleton at the cytoplasm. Nuclear pore complexes (NPCs) allow controlled movement of materials such as proteins and mRNA in and out of the nucleus.

The nuclear lamina is a fibrillar network which underlies the INM. It is a meshwork of intermediate filament proteins: A type lamins consisting of lamin A and C, B type lamins consisting of lamin B1 and B2, and their associated proteins (Gruenbaum *et al.,* 2005). There are many INM proteins. One such protein is emerin, an integral membrane protein of the INM which is tethered to the NE by interaction with lamins (Sakaki *et al.,* 2001). The nuclear lamina is involved in maintaining nuclear structure and providing mechanical stability to the nucleus (Kim *et al.,* 2017). It also interacts with chromatin to maintain and control its organisation (Ranade *et al.,* 2019), and functions in the elongation phase of DNA replication by maintaining the localisation of the elongation polymerase cofactor PCNA (Spann *et al.,* 1997).

Components of the nuclear lamina are mutated in laminopathies, a range of diseases including muscle dystrophy, lipodystrophy and premature-aging syndromes (Oldenburg and Collas, 2016). Early genetic screens identified *EMD,* coding for emerin, and *LMNA,* coding for A-type lamins, as genes mutated in muscular diseases (Bione *et al.,* 1994; Bonne *et al.,* 1999). Common to lamins and emerin are their association with other NE proteins, creating new protein candidates to be studied in muscle disease. Indeed, three *LMNA* mutations identified separately from different patients were found to displace proteins of the NPC (section 1.3) and LINC complex (section 1.4) from the NE (Dialynas *et al.,* 2012).

1.3 Nuclear pore complex

The double membrane of the nucleus creates a largely impermeable barrier (Paine *et al.,* 1975) and a system is required to control transportation of materials through the NE. Nuclear pore complexes (NPCs) span the NE and regulate the flow of molecules across the NE (Fig. 1.3). They are embedded in the NE and are composed of up to 100 proteins collectively called nucleoporins (Görlich and Kutay, 1999). The transfer of materials in and out of the nucleus is required in many cellular processes. In mammalians cells, genetic information is contained in the nucleus where transcription occurs. mRNA is exported out into the cytoplasm for protein synthesis, and proteins such as DNA and RNA polymerases and

histones must be transported inside the nucleus for function (Görlich and Kutay, 1999). Other proteins shuttle in and out of the nucleus. Small molecules up to 40 kDa are able freely move through the NPC by passive diffusion whereas larger proteins require facilitation.

Large cargoes which require transport into the nucleus contain a nuclear localisation signal (NLS), a stretch of sequence rich in basic residues (Lange *et al.,* 2007). NLS-containing proteins bind directly to importin-β1 or indirectly through a ternary complex with importin-α1, to be transported through a NPC into the nucleus, where RanGTP is more abundant. The cargo is released from the complex when RanGTP binds to importin-β1. RanGTP-bound importin returns to the cytosol where hydrolysis and release of RanGDP allows importin to bind its next cargo (Cautain *et al.,* 2015).

Proteins requiring nuclear export contain a hydrophobic nuclear export sequence (NES) (Xu *et al.,* 2012). NES containing-proteins bind exportins together with RanGTP for movement through the NPC outside of the nucleus. The cargo is released from the complex when RanGTP becomes hydrolysed into RanGDP. Exportin returns inside the nucleus to bind its next cargo (Cautain *et al.,* 2015).

Mislocalisation of nucleoporins from the NE of myofibers has been observed only in *LMNA*-associated muscle diseases and coincides with the simultaneous mislocalisation of SUN proteins (Dialynas *et al.,* 2012). With the lack of further reports that associate NPCs with muscle disease, the mislocalisation of nucleoporins from the NE may be an effect of lamin dysfunction, but not itself contribute toward muscle diseases.

1.4 Linker of nucleoskeleton and cytoskeleton complex

The nucleus is physically linked with the cytoskeleton, by the linker of nucleoskeleton and cytoskeleton (LINC) complex, a protein bridge which spans the NE and binds both nucleoskeletal and cytoskeletal proteins (Chang *et al.,* 2015b) (Fig. 1.3). In mammals, it is composed of two components: Sad1p/UNC-84 (SUN)

proteins embedded in the inner nuclear membrane, and Klarsicht/Anc/Syne homology (KASH) domain proteins embedded in the outer nuclear membrane (Fig. 1.4). (section 1.4.3) (Sosa *et al.,* 2012).

The LINC complex is conserved between mammals, *Caenorhabditis elegans (C. elegans)*, yeast and plant cells, and is involved in a vast number of roles such as nuclear positioning, force transmission, maintaining nuclear rigidity and mechanotransduction (Tapley and Starr, 2013), all functions which are particularly important in a muscle cell. There is some redundancy between different KASH domain and SUN proteins, however there are some specific functions which are carried out by specific SUN and KASH domain isoforms, through their capabilities to bind different cellular components (Fig. 1.4).

1.4.1 SUN proteins

SUN proteins form the nucleoplasmic facing element of the LINC complex and are anchored to the INM through a central transmembrane domain. They contain a conserved C-terminal SUN domain which interacts with and tethers KASH domain proteins at the perinuclear space (PNS) (Sosa *et al.,* 2012; Stewart-Hutchinson *et al.,* 2008). A coiled-coil region forms an elastic extendable linker across the PNS and the N-terminal region is located within the nucleoplasm (Hodzic *et al.,* 2004). Variations within the N-terminal region mediate direct and indirect binding to different components in the nucleus. Through its N-terminal region, SUN1/2 interact with the nuclear lamina proteins emerin (Haque *et al.,* 2010) and lamin A (Haque *et al.,* 2006). Lamin depletion reduces SUN1/2 protein localisation to the NE (Crisp *et al.,* 2006; Haque *et al.,* 2006), showing lamins play a role in anchoring SUN proteins to the NE.

To date, five SUN proteins have been identified. SUN3, SUN4 and SUN5 are sperm-specific and are involved in germ cell formation (Göb *et al.,* 2010) whereas SUN1 and SUN2 are widely expressed.

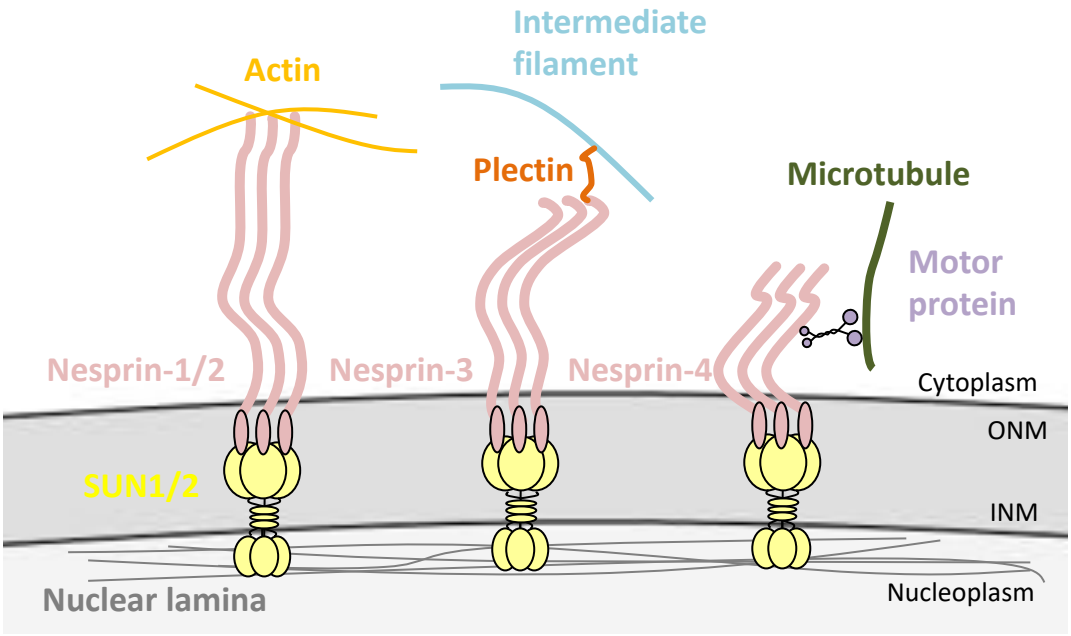


Figure 1.4 Different mammalian LINC complex components form different connections between the cytoskeleton and nucleoplasm

A LINC complex is composed of a SUN homotrimer directly binding to three KASH domain proteins. The N-terminal end of SUN proteins face the nucleoplasm to bind nucleoskeletal elements. SUN1/2 expression is ubiquitous and perform most major LINC complex functions. Nesprins-1, 2, 3 and 4 are mammalian KASH domain proteins. The N-terminal of nesprin proteins extend into the cytoplasm and bind to various cytoskeletal elements. Nesprin-1/2 are giant proteins and bind actin. Nesprin-3 interacts with plectin to indirectly bind intermediate filaments. Nesprin-4 binds motor proteins to indirectly bind microtubules.

1.4.1.1. Splice variants

The expression of SUN protein splice variants gives rise to further LINC complex functions. Multiple SUN1 splice variants have been identified at the mRNA level across different tissues (Crisp *et al.,* 2006; Göb *et al.,* 2010). Sun1η is testis-specific, expressed during spermiogenesis and the only SUN protein isoform expressed at the postmeiotic stage (Göb *et al.,* 2010). This may be because the function of Sun1η LINC complexes are germ cell-specific whereas SUN2 LINC complexes are not required (Göb *et al.,* 2010). There is evidence of SUN1 splice variants in myotubes that are not in myoblasts. Six SUN1 isoforms were detected in muscle tissue at the level of mRNA and upon sequencing, were found to differ at the N-terminal region where nucleoplasmic connections are made (Göb *et al.,* 2014). Although these splice variants have not been confirmed at the protein level, western blots of SUN1 in myoblasts and myotubes detect myotube-specific bands of lower molecular weight than full-length SUN1 (unpublished data from Shackleton lab). This supports the idea that smaller splice variants are expressed only upon myogenesis. Equally, the myotube-specific bands could reflect unknown post-translational modifications. Regardless, it is clear that there is a change in SUN1 at the protein level during myogenesis.

1.4.2 KASH domain proteins

KASH domain proteins form the cytoplasmic facing element of the LINC complex by a SUN-KASH interaction at the PNS (Crisp *et al.,* 2006). Many mammalian KASH domain proteins contain spectrin repeats (SRs), and are known as nuclear envelope spectrin repeat (nesprin) proteins. In mammals, five KASH domain proteins have been identified, four which are nesprins. The length of a nesprin protein is largely defined by the number of SRs in its structure (Fig. 1.5). A single SR is approximately 100 residues, which form three anti-parallel α-helices connected by loop regions (Autore *et al.,* 2013). SRs function as molecular spacers between protein domains, provide elastic properties and contain binding sites for interacting proteins (Djinovic-Carugo *et al.,* 2002; D'Alessandro *et al.,* 2015).

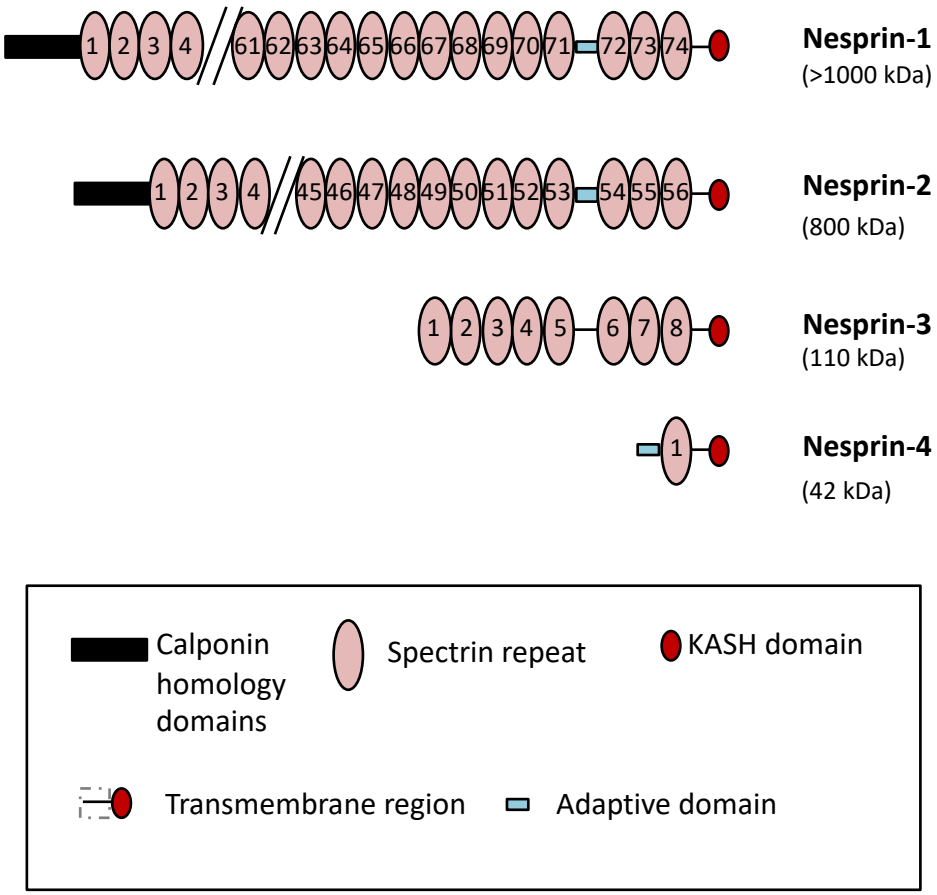


Figure 1.5 Schematic representation of mammalian nesprin proteins

There are four nesprin proteins in mammals. They vary in length by the number of spectrin repeats, and contain domains which bind to cytoskeletal proteins. Nesprin-1/2 contain 74 and 56 spectrin repeats, respectively, and N-terminal calponin homology domains for binding with actin. Nesprin-3 contains 8 SRs, with a plectin binding domain contained within the first SR, for binding to plectin to indirectly associate with intermediate filaments. Nesprin-4 contains one SR. Nesprin-1/2/4 contain an adaptive domain, which via a conserved LEWD motif, interacts with kinesin-1.

Aside from differing in length, nesprin proteins contain different domains to bind different elements (Fig. 1.5). Nesprin-1 and nesprin-2 are giant proteins and contain N-terminal calponin homology (CH) domains allowing direct binding to actin filaments, and an adaptive domain (AD) which stabilises SRs and contains a conserved kinesin-binding LEWD motif (Wilson and Holzbaur, 2015; Zhong *et al.,* 2010). Nesprin-3 contains a plectin binding domain to allow indirect binding to intermediate filaments. Nesprin-4 also contains the LEWD motif and binds the motor protein kinesin-1 subunit KIF5b (Rajgor and Shanahan, 2013; Roux *et al.,* 2009). KASH5 is germ-cell specific and contains a coiled-coil region and does not comprise any SRs. During meiosis, the KASH domain protein connects dynein at the cytoplasm, to chromosomes in the nucleus (Morimoto *et al.,* 2012; Horn *et al.,* 2013b).

Alternative initiation sites lead to a larger number of nesprin isoform possibilities that have been detected at the mRNA level, however, few of these have been confirmed at the protein level (Zhang *et al.,* 2001). The following discussion focuses on KASH-containing nesprins and their isoforms. It should be noted that the emergence of KASH-less isoforms in various subcellular localisations show that nesprins are involved in roles other than linking the nucleus to the cytoskeleton (Duong *et al.,* 2014).

1.4.2.1 Nesprin-1 isoforms

Nesprin-1 is encoded by *SYNE1*. It encodes up to 16 possible nesprin-1 KASH-containing isoforms, all N-terminal truncations of the full-length nesprin-1 (Rajgor and Shanahan, 2013). The nesprin-1 cDNA was first isolated from rat vascular smooth muscle cells as a differentiation marker candidate. The sequence was used to identify human nesprin-1, which was later isolated from the spleen (Zhang *et al.,* 2001). Northern blots and RT-PCR of nesprin-1 in various human tissues showed it was ubiquitously expressed, however transcripts differed between tissues suggesting that different nesprin-1 isoforms (Fig. 1.6) play tissue-specific roles

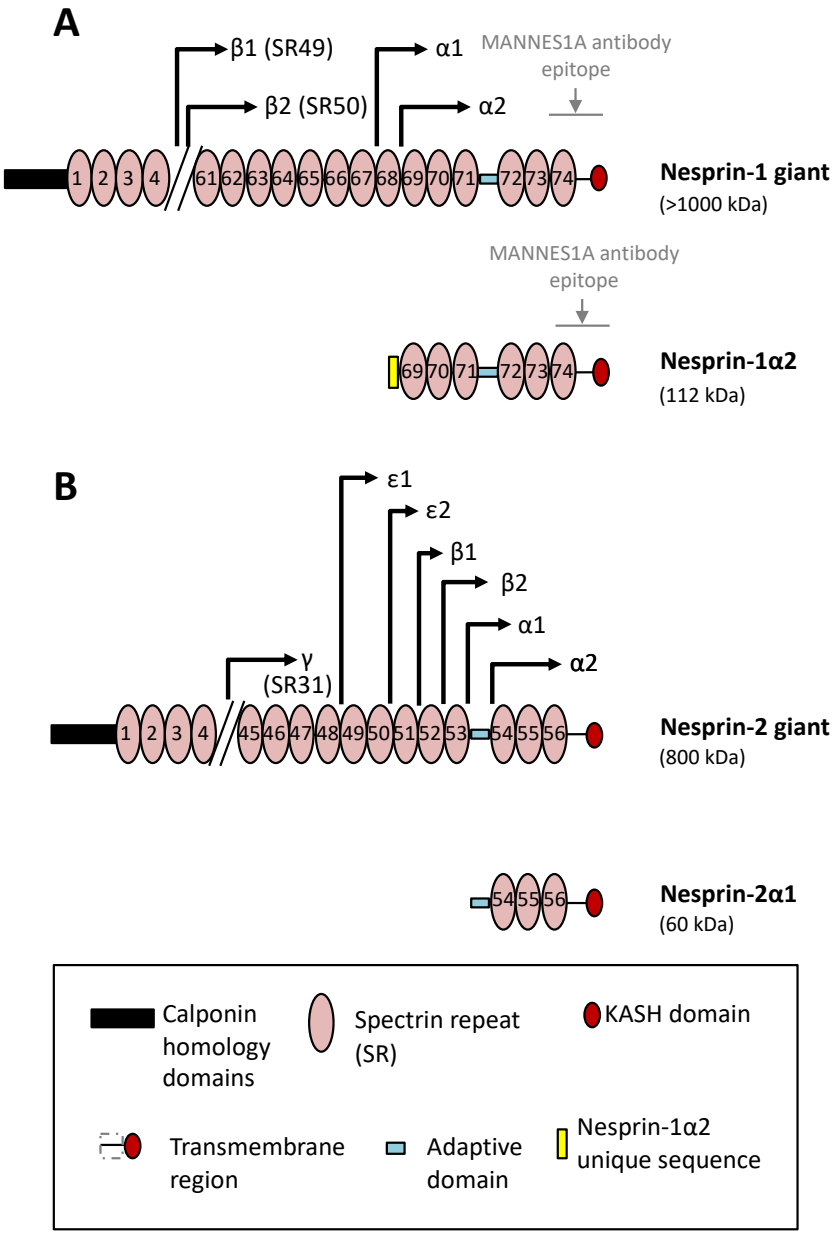


Figure 1.6 Schematic representation of nesprin-1 and nesprin-2 and muscle isoforms, and N-terminal start points of short isoforms

Schematic of nesprin-1G (A) and nesprin-2G (B), with N-terminal start points of shorter isoforms for which mRNA has been detected by Duong *et al.* (2014). Spectrin repeats from which isoforms are expressed are in brackets if not indicated by the arrow. Both giant proteins contains calponin homology domains at the N-terminus, spectrin repeats, an adaptive domain and a transmembrane KASH domain at the C-terminal end. In differentiating myoblasts, expression from alternative initiation sites results in the expression of N-terminal truncations of the proteins as indicated, the 112 kDa nesprin-1α2 and 60 kDa nesprin-2α1 isoform. The nesprin-1α2 isoform contains an isoform-specific 31 amino acid unique sequence. The region containing the epitope for the nesprin-1 antibody MANNES1A, used for immunofluorescence staining in this study, is indicated.

(Zhang *et al.,* 2001; Duong *et al.,* 2014). Table 1.1 summarises the nesprin-1 isoforms with the tissues in which they are expressed.

Nesprin-1giant (nesprin-1G) is the full-length protein with a molecular weight >1000 kDa. It encompasses two actin-binding N-terminal calponin homology (CH) domains and a C-terminal transmembrane KASH domain separated by a central rod region of 74 SRs with an adaptive domain (AD), an unstructured region between SR71 and SR72 (Fig. 1.4). Nesprin-1G mRNA is present in at least 20 tissues (Duong *et al.,* 2014). At the protein level, it can be detected by western blot with antibodies targeting the CH, central rod or C-terminal domain, with bands corresponding to the expected molecular weight in many cell types including myoblasts and myotubes (Holt *et al.,* 2016; Duong *et al.,* 2014).

Nesprin-1α1 is around 108 kDa and comprises the last 6 SRs, AD and KASH domain of nesprin-1G. It is detected at the mRNA level at low levels (Zhang *et al.,* 2001; Duong *et al.,* 2014). **Nesprin-1α2** is a muscle-specific 112 kDa protein that uses an alternative initiation site, resulting in the inclusion of an unique exon that encodes 31 unique residues at its N-terminus (Fig. 1.6), but is otherwise the same as nesprin-1α1 (Zhang *et al.,* 2001). In northern blots of multiple tissues, nesprin-1α2 was detected in heart, skeletal muscle and spleen tissue, whereas it was only detected by western blot in skeletal muscle and heart tissue (Zhang *et al.,* 2001; Duong *et al.,* 2014). Due to its smaller size it is possible that nesprin-1α2 may also reside at the INM. Indeed, it directly binds emerin and lamin A *in vitro*, suggesting roles other than regulating the cytoskeleton (Mislow *et al.,* 2002). Nesprin-1α1 and nesprin-1α2 are very similar and in early studies, were not distinguished and identified simply as nesprin-1α. The two isoforms have only been formally identified in humans, and is therefore still referred to nesprin-1α in mouse.

Nesprin-1β1 is around 380 kDa and is highly expressed in spleen, supported at both mRNA and protein level. **Nesprin-1β2** is around 320 kDa and is barely detected at the mRNA level, though is most abundant in vascular smooth muscle cells (VSMC) (Duong *et al.,* 2014).

Table 1.1 Nesprin-1/2 isoforms, tissue expression and expression levels

List of nesprin-1/2 isoforms and the tissues in which they are expressed as detected by RT-PCR, western blot or immunofluorescence microscopy. No tissues of expression are indicated where expression is low. Nesprin isoforms expressed in skeletal muscle are nesprin-1G, nesprin-1α2, nesprin-2G and nesprin-2α1. Nesprin-1 isoforms are predominantly expressed in myotubes during early myogenesis and replaced by nesprin-2 isoforms later in myogenesis

Nesprin	Isoform	Tissue(s)	Expression level	
Nesprin-1	1G	Ubiquitous	High High in immature and mature myofibres Higher in regenerating myofibres	High in myotubes Lower in myofibres
	α1		Very low	
	α2	Skeletal muscle, heart	High High in immature and regenerating myofibres Weak in mature myofibres	
	β1	Spleen	High	
	β2		Low	
Nesprin-2	2G	Ubiquitous	High	Low in myotubes Higher in myofibres
	α1	Skeletal muscle, heart	High	
	α2	Kidney	High	
	β1		Very low	
	β2		Low	
	ε1	Embryonic cells	High	
	ε2	Heart, brain	High	
	γ		Very low	

Black = mRNA and protein evidence (Zhang *et al.*, 2001, Duong *et al.*, 2014)
Grey = mRNA evidence only (Duong *et al.*, 2014)
Immunofluorescence microscopy evidence (Randles *et al.*, 2010)
Immunofluorescence microscopy evidence (Holt *et al.*, 2016)

1.4.2.2 Nesprin-2 isoforms

Nesprin-2 was identified during a database search against nesprin-1, where a homology of 64% between the proteins was determined (Zhang *et al.,* 2001). Encoded by *SYNE2,* there are 12 possible nesprin-2 KASH-containing isoforms (Rajgor and Shanahan, 2013). All are N-terminal truncations of the full-length nesprin-2. As with nesprin-1, northern blots and RT-PCR of nesprin-2 showed transcript variations between tissues (Zhang *et al.,* 2001; Duong *et al.,* 2014) (Fig. 1.6). Table 1.1 summarises the nesprin-2 isoforms with the tissues in which they are expressed.

Nesprin-2giant (nesprin-2G) is 800 kDa and is the full-length isoform. It contains two CH domains at its N-terminus, a central domain containing 56 SRs, an AD between SR53 and SR54 and a C-terminal KASH domain (Fig. 1.4). Nesprin-2G mRNA is ubiquitous in tissues and the protein can be detected by western blot in various tissues, including skeletal muscle (Duong *et al.,* 2014; Zhang *et al.,* 2005).

Nesprin-2α1 is 60 kDa and contains the AD and SR54-56 of nesprin-2G. Its mRNA was detected in high levels only in heart and skeletal muscle, though it was only detected in skeletal muscle by western blot (Duong *et al.,* 2014; Zhang *et al.,* 2005) (Fig. 1.4). **Nesprin-2α2** is 47 kDa, lacks the AD of nesprin-2α1, and its mRNA was detected at low levels in kidney and other tissues, but not skeletal muscle (Duong *et al.,* 2014).

Nesprin-2ε1 is 112 kDa and its mRNA and protein are only detected in embryonic cells (Duong *et al.,* 2014). **Nesprin-2ε2** is 98 kDa and its mRNA and can be detected in a number of tissues including the heart , brain and skeletal muscle tissue, but can only be detected by western blot in heart and brain tissue (Duong *et al.,* 2014).

Nesprin-2γ is 377 kDa and its mRNA is expressed at low levels across tissues, including skeletal muscle but not detected by western blot (Duong *et al.,* 2014).

Nesprin-2β is 87 kDa and its mRNA is barely detectable in any tissue (Duong *et al.,* 2014).

1.4.2.3 Other KASH-containing proteins

Nesprin-3 is expressed at high levels in a wide range in tissues (Wilhelmsen *et al.,* 2005). There are two isoforms in mice, nesprin-3α which consists of a C-terminal KASH domain and 8 SRs, and nesprin-3β which lacks the first SR (Wilhelmsen *et al.,* 2005). The isoform-specific SR of nesprin-3α binds plectin to indirectly interact with intermediate filaments (Wilhelmsen *et al.,* 2005).

Nesprin-4 is mostly expressed in epithelial cells and consists of a C-terminal KASH domain, one SR and the AD (Roux *et al.,* 2009). The AD, like in nesprin-1/2, contains the kinesin-binding LEWD domain (Roux *et al.,* 2009; Wilson and Holzbaur, 2015; Zhou *et al.,* 2017).

KASH5 is only expressed in the germ cells of the testis and ovaries. It is composed of a C-terminal KASH domain and a cytoplasmic region, which instead of SRs, comprises a coiled-coil region and a N-terminal EF-hand domain (Horn *et al.,* 2013b).

1.4.3 SUN/KASH protein interaction

LINC complex formation requires direct binding of the C-terminal SUN domain of SUN proteins, and the C-terminal KASH domain of KASH domain proteins at the PNS. The crystal structure of the SUN2 SUN domain showed that SUN proteins oligomerise. Each SUN protomer binds two other SUN protomers to form a homotrimer with a three-fold symmetry (Zhou *et al.,* 2012). This was confirmed by another study which solved the structure of the SUN2-KASH1 and SUN2-KASH2 complex (Sosa *et al.,* 2012). Sequence alignment of SUN domains between human SUN1-5, and SUN proteins of different species showed that the residues involved in homotrimerization and binding to KASH proteins are conserved (Sosa *et al.,* 2012).

The KASH domain is also conserved, with pairwise identities of 64–79% between nesprin-1, 2 and 3 (Roux *et al.,* 2009; Sosa *et al.,* 2012). Five KASH residues differ between KASH1/2, but the binding interface of SUN2/KASH1 and SUN2/KASH2 is the same (Sosa *et al.,* 2012). Each interface between two SUN protomers provides a site for one KASH2 protein to bind by hydrogen bonds (Sosa *et al.,* 2012). The three-fold symmetry of the SUN homotrimer allows three KASH proteins to bind to form a hexameric LINC complex. Disulphide bonds between SUN and KASH residues stabilise the binding (Sosa *et al.,* 2012). Computer modelling showed that SUN1, but not SUN2, protomers may be able to bind other SUN1 trimers to form a higher ordered network of SUN1 at the NE (Jahed *et al.,* 2018). KASH proteins such as nesprin-1α2 can oligomerise (Mislow *et al.,* 2002), and it is unclear whether oligomerised or monomer KASH proteins bind the three sites in a SUN homotrimer. The ability for different nesprins to bind to one SUN homotrimer would give rise to many different LINC complexes.

In a cell free system, interactions between overexpressed SUN1/2 domains and nesprin-1/2/3 KASH domains were all detected by co-immunoprecipitation (Stewart-Hutchinson *et al.,* 2008), possibly due to the conservation between SUN and KASH domains. The question of whether and how interactions between particular SUN/KASH proteins is specified *in vivo*, remains unanswered. Interaction is not mediated by the glycosylation of SUN proteins (Stewart-Hutchinson *et al.,* 2008), though other post-translational modifications are possible. If LINC complex formation is indeed non-specific, it will still be partially regulated by the relative levels of SUN/KASH proteins and presence of cell-specific splice variants and isoforms in the cell.

1.4.4 LINC complex in non-skeletal muscle cells

The LINC complex is involved in diverse processes, including nuclear positioning, force transmission, maintaining nuclear rigidity and mechanotransduction (Tapley and Starr, 2013). SUN1/2 are ubiquitous and are generally redundant in their roles. Indeed, SUN1 or SUN2 knockout mice are viable, whereas SUN1/2 knockout mice exhibit postnatal lethality (Lei *et al.,* 2009). SUN proteins interact with lamins

and chromatin at INM, and such interactors can affect SUN protein localisation at the NE. *LMNA* mutations encoding lamins, can lead the mislocalisation or accumulation of SUN proteins at the NE (Dialynas *et al.,* 2012; Haque *et al.,* 2010; Chen *et al.,* 2012). In contrast, an emerin mutation associated with EDMD was shown to have reduced interaction with SUN1 (Haque *et al.,* 2010). As SUN proteins tether KASH domain proteins at the PNS to form the LINC complex, disrupting SUN proteins may in turn disrupt their KASH domain partner by affecting its function or localisation. Indeed, SUN1/2 double KO cells did not have KASH-containing proteins at the NE (Lei *et al.,* 2009).

Likewise, nesprin-1G and nesprin-2G are similar in structure and are redundant in many roles, such as NE organisation and migration in endothelial cells (King *et al.,* 2014). Like SUN1/2, nesprin-1 or nesprin-2 knockout mice are viable, whereas nesprin-1/2 double knockout mice exhibit postnatal lethality (Zhang *et al.*, 2009b). Some nesprin-1 mouse models were unable to survive; however this was thought to be due to the genetic background of the mouse line used (discussed in section 1.4.5.2). The roles of nesprin1/2 can be distinguished in some cells, but such roles are likely carried out by smaller isoforms.

1.4.4.1 Nuclear positioning and anchorage

The role of the LINC complex in nuclear positioning was first examined in the syncytial hypodermal cells of *C. elegans*, each of which contain greater than 100 nuclei, evenly spaced throughout the cytoplasm (Starr and Han, 2002). The SUN protein orthologue UNC-84, tethers the nesprin-1/2 orthologue nuclear anchorage protein 1 (ANC-1), to the NE (Starr and Han, 2002). UNC-84 mutants caused defects in the migration and anchorage of nuclei in hypodermal cells, which were able to freely move in the cytoplasm (Malone *et al.,* 1999). ANC-1 mutants also led to the loss of nuclear anchorage, as did overexpression of its actin-binding domain, showing the involvement of ANC-1 with actin in nuclear positioning (Starr and Han, 2002). The *D. melanogaster* nesprin-1/2 orthologue, muscle-specific protein-300 (MSP-300) also associates with actin (Volk, 1992), and regulates nuclear anchorage during oogenesis by regulating the actin cytoskeleton (Yu *et al.,* 2006).

Mammalian nesprin proteins were later found to be required for nuclear positioning in skeletal muscle cells (section 1.4.5).

Nuclear positioning of the outer hair cells (OHC) of the cochlea rely on a specific LINC complex composed of SUN1/nesprin-4. Both nesprin-4 and SUN1 null mice are viable at birth. However, the OHCs of the cochlea degrade as hearing matures, coinciding with the mispositioning of the cell nucleus from the cell base to the apical region (Horn *et al.,* 2013a). In agreement with this, mutations in the nesprin-4 gene, *SYNE4,* is associated with hearing loss in human (Horn *et al.,* 2013a).

SUN1/2 and nesprin-2 LINC complexes are involved in homeostatic mechanisms to keep a nucleus in position in fibroblasts and myoblasts. Upon centrifugation displacement of the cell nucleus, SUN1 and SUN2 are required for forward and backward re-centering, respectively, whereas nesprin-2 is required for both (Zhu *et al.,* 2017). The nesprin-2 N-terminal CH-domain containing region is responsible for actin-dependent rearward nuclear movement, whereas SR52-56 is responsible for forward movement through an unknown mechanism (Zhu *et al.,* 2017).

1.4.4.2 Cellular organisation

The LINC complex is required for the positioning of organelles other than the nucleus. ANC-1 was also found to be required for mitochondria positioning (Starr and Han, 2002). Later, nesprin-1 mutants were found disrupt the maintenance of the Golgi structure in mammalian epithelial cells (Gough *et al.,* 2003). SUN1/2 and nesprin-2 LINC complexes were found to be essential to connect the centrosome to the NE during neurogenesis and neuronal migration (Zhang *et al.*, 2009b), whereas in epithelial cells, nesprin-4 binds microtubules through kinesin-1 to position the centrosome and Golgi (Roux *et al.,* 2009).

The LINC complex also plays part in cytoskeletal organisation. Depletion of either actin-binding proteins nesprin-1G or nesprin-2G in endothelial cells led to altered actin distribution, together with defects in cell morphology and migration (King *et al.,* 2014). On the other hand, nesprin-3 interacts with plectin, which in turn binds

intermediate filaments (Wilhelmsen *et al.,* 2005). Overexpression of nesprin-3 caused accumulation of plectin at the NE, whereas ablation in zebrafish resulted in a less dense keratin filament network around the NE of the epidermal cells (Postel *et al.,* 2011). Plectin is also able to interact with actin and microtubules. Thus, although not experimentally validated, nesprin-3 has the potential to mediate interactions between all three cytoskeletal elements (Ketema *et al.,* 2007).

1.4.4.3 Homologous chromosome pairing

Homologous chromosome pairing occurs during meiosis for accurate segregation of chromosomes. This is mediated by a unique SUN1/KASH5 LINC complex (Ding *et al.,* 2007; Morimoto *et al.,* 2012). The EF-hand of KASH5 is thought to recruit the microtubule motor protein dynein to the ONM, whereas at the other end of the LINC complex, SUN1 associates with telomeres at the INM. The association of microtubules and chromosomes mediates chromosomal movements required for homologous pairing (Horn *et al.,* 2013b; Morimoto *et al.,* 2012). Indeed, KASH5 and SUN1 null mice are infertile (Horn *et al.,* 2013b; Ding *et al.,* 2007; Lei *et al.,* 2009). Telomeres of SUN1 null spermatocytes fail to attach to the NE, and cells ultimately undergo apoptosis (Ding *et al.,* 2007).

1.4.5 LINC complex in skeletal muscle cells

It is clear that the LINC complex is also involved in nuclear positioning of syncytial skeletal muscle cells, as mutations in nesprin-1/2 and SUN1/2 proteins have been associated with muscle diseases, where nuclear mispositioning is a common phenotype (Zhang *et al.*, 2007a; Meinke *et al.*, 2014). However, nuclear positioning during early myogenesis does not appear to be mediated by nesprin-1G/2G, but instead by the muscle-specific nesprin-1α2 isoform (Gimpel *et al.,* 2017; Stroud *et al.,* 2017).

1.4.5.1 Nesprin protein expression in myogenesis

Nesprin-1/2 expression levels vary during myogenesis. In culture, nesprin-1 expression increases upon myogenesis, and is abundant in myotubes (Holt *et al.,* 2016). In muscle, antibodies to nesprin-1 stain brighter in regenerating muscle fibres compared to surrounding mature fibres (Randles *et al.,* 2010). Conversely, nesprin-2 localisation to the NE is weak in cultured myotubes (Zhang *et al.,* 2005), and in muscle, stains brighter in mature fibres compared to regenerating fibres (Randles *et al.,* 2010; Espigat-Georger *et al.,* 2016). This shows nesprin-1 is required in early myogenesis in both cultured cells and adult muscle, perhaps for nuclear positioning in myotubes, whereas nesprin-2 is required later in mature myofibers, where it may hold nuclei under the sarcolemma (Randles *et al.,* 2010).

The change in nesprin-1/2 expression levels are likely due to the expression of muscle-specific isoforms. Nesprin-1G is expressed in cultured myoblasts and myotubes, whilst expression of nesprin-1α2 is only switched on upon induction of myogenesis (Espigat-Georger *et al.,* 2016; Gimpel *et al.,* 2017; Holt *et al.,* 2016). Western blots support this as nesprin-1G bands are seen in myoblast and myotube lysates, whereas nesprin-1α2 bands are only in myotube samples. Likewise, in immunofluorescence microscopy of muscle cells, antibodies specifically towards nesprin-1α2 only stains the NE upon the differentiation of myoblasts into myotubes (Holt *et al.,* 2016). Indeed, many *SYNE1* mutations within the region coding for nesprin-1α2 are associated with muscle disease, suggesting the dysfunction of this isoform is responsible for muscle disease (Zhang *et al.*, 2007a; Chen *et al.*, 2017; Zhou *et al.*, 2017, 2018). Instead, *SYNE1* mutations located at the N-terminal regions of nesprin-1 are more commonly associated with cerebellar ataxia (Gros-Louis *et al.,* 2007). The increase in nesprin-2 expression in mature myofibres is possibly due to the expression of the muscle-specific nesprin-2α1 (Zhang *et al.,* 2005). Table 1.1 summarises the expression level of nesprin-1 and nesprin-2 isoforms in myotubes and myofibres, along with supporting evidence.

Nesprin-3 is not required for nuclear positioning in skeletal muscle cells, as its expression is decreased upon myotube differentiation (Chen *et al.,* 2006), and

importantly, is not present at the NE of myotubes (Espigat-Georger *et al.* 2016). Indeed, nesprin-3 ablation does not affect nuclear positioning in embryonic skeletal muscle cells (Postel *et al.,* 2011). Nesprin-4 is only expressed in epithelial cells and is therefore not in muscle cells. Hence, nesprin-1α2 is likely the only nesprin protein responsible for nuclear positioning during early myogenesis.

1.4.5.2 LINC complex mouse models

The role of the LINC complex has been explored in mouse models to show the function of SUN1/2 and nesprin-1 in myonuclear positioning (Lei *et al.,* 2009; Zhang *et al.,* 2007b, Zhang *et al.,* 2007a; Puckelwartz *et al.,* 2009; Stroud *et al.,* 2017). In contrast to *in vitro* studies where myogenesis can only be studied in myoblasts and myotubes, *in vivo* studies allow the study of myofibres, and therefore the positioning of non-synaptic and synaptic myonuclei. Both SUN1 null ($SUN1^{-/-}$) and $SUN2^{-/-}$ mice myofibres showed reduced nesprin-1 staining at the NE, whereas SUN1/2 double-knockout myofibres displayed the complete loss of nesprin-1 at the NE. On the other hand, only $SUN1^{-/-}$ mice showed a decrease in the number of synaptic nuclei under the NMJ, whereas SUN1/2 double-knock out mice myofibres also exhibited significant mispositioning of non-synaptic nuclei (Lei *et al.,* 2009). This shows that SUN1/2 act redundantly for nesprin-1 anchorage and non-synaptic nuclear positioning, whereas SUN1 is required for synaptic nuclei positioning.

Several nesprin knockout mouse models have been generated to study their role in myonuclear positioning. Zhang *et al. (*2007b) generated nesprin-$1^{-/-}$ and nesprin-$2^{-/-}$ mice by inserting an early stop codon in the genes, to generate nesprins lacking the KASH domain and unable to localise to the NE. The non-synaptic nuclei became clustered, and synaptic nuclei failed to be anchored under the NMJ of nesprin-$1^{-/-}$, but not nesprin-$2^{-/-}$ mice, verifying that only nesprin-1 is responsible for nuclear positioning. These single knockout mice were viable and fertile, whereas nesprin-1/2 double-knockout mice died shortly after birth, showing redundancy of the proteins (Zhang *et al.*, 2007b). Another nesprin-$1^{-/-}$ mouse model, generated by deletion of the KASH domain-coding region, confirmed the

role of nesprin-1, as myonuclei were mispositioned, and the mice had muscle disease (Puckelwartz *et al.,* 2009). However, whereas the earlier nesprin-1$^{-/-}$ mouse model was viable (Zhang *et al.*, 2007b), the nesprin-1$^{-/-}$ mice in this study showed 50% perinatal lethality. In a third nesprin-1 mouse model, nesprin-1$^{-/-}$ mice were generated by deleting all nesprin-1 isoforms with C-terminal SRs (Zhang *et al.*, 2009a). Again, nuclear mispositioning was observed in myofibres, and this nesprin-1$^{-/-}$ mouse also showed postnatal lethality, along with growth retardation and increased variability in weight. The different phenotypes observed in different nesprin-1 mouse models was thought to be due to the use of different mouse lines, and the method by which the mouse models were generated (Zhang *et al.*, 2009a). However, all models consistently showed a requirement of nesprin-1 in myonuclear positioning.

These nesprin-1 mouse studies did not show whether it was the lack of nesprin-1G or nesprin-1α2 which was responsible for myonuclear mispositioning, since both isoforms were ablated. Stroud *et al.* (2017) showed for the first time, that nesprin-1α2, not nesprin-1G is required for healthy muscle development and function. They generated nesprin-1ΔCH$^{-/-}$ mice, in which all nesprin-1 isoforms with CH domains, including nesprin-1G are ablated, and nesprin-1α2$^{-/-}$ mice, where only the nesprin-1α2 isoform is ablated. Nesprin-1ΔCH$^{-/-}$ nuclear spreading in embryonic myofibres was not affected, and mice were phenotypically normal, and showed no muscle defects, even at eighteen months of age. In contrast, nesprin-1α2$^{-/-}$ mice embryo myofibres exhibited nuclear clustering. Furthermore, mice were smaller and appeared to have dysfunctional skeletal muscle. The survival rate of nesprin-1ΔCH$^{-/-}$ mice was higher than that of nesprin-1α2$^{-/-}$, at 40% and 12%, respectively, thought to be due to differences in the mouse line used. Importantly, this showed that nesprin-1α2, not nesprin-1G, is the nesprin-1 isoform which form LINC complexes vital for nuclear positioning in early skeletal muscle development.

1.4.5.3 Recruitment of centrosomal proteins to the NE by nesprin-1α2

In contrast with early studies which described the role of nesprin-1/2 in nuclear positioning to be by interaction with the actin cytoskeleton (Starr and Han, 2002), the role of nesprin-1 in myonuclear positioning is thought to be due to muscle-specific connections between proteins of the NE and cytoskeleton. During myogenesis, centrosome and motor proteins are recruited to the NE (Srsen *et al.,* 2009; Gimpel *et al.,* 2017; Wilson and Holzbaur, 2015, 2012; Espigat-Georger *et al.,* 2016; Fant *et al.,* 2009). Centrosome and motor proteins are not recruited to the NE in nesprin-1 null myotubes *in vitro*, which, instead display nuclear clustering (Gimpel *et al.,* 2017; Espigat-Georger *et al.,* 2016). Nuclear mispositioning is also observed in PCM1 or kinesin-1 null myotubes, showing it is the recruitment of these proteins which mediate nuclear positioning (Wilson and Holzbaur, 2015; Espigat-Georger *et al.,* 2016). Exogenous expression of nesprin-1α2 in nesprin-1 null myotubes rescues the recruitment of the centrosomal protein pericentrin to the NE (Gimpel *et al.,* 2017). Finally, the microtubule motor protein kinesin-1 component KIF5B is mislocalised from the NE of nesprin-1α2$^{-/-}$, but not nesprin-1ΔCH$^{-/-}$ mouse embryos (Stroud *et al.,* 2017). Altogether, this suggests that during myogenesis, nesprin-1α2 recruits centrosomal and motor proteins to the NE for myonuclear positioning. Centrosomal proteins are required for microtubule nucleation at the centrosome, and once recruited to the NE, mediate microtubule nucleation from the NE (Gimpel *et al.,* 2017; Srsen *et al.,* 2009; Zaal *et al.,* 2011). To understand how the recruitment of centrosomal and motor proteins to the NE mediates nuclear positioning, their roles and details of microtubule nucleation must be further explored.

1.5 Microtubules

Microtubules, together with actin and intermediate filaments, form the cytoskeleton. They are required for the movement of organelles and molecules within the cytosol (Zhu *et al.,* 2017; Caviston and Holzbaur, 2006), for cell

migration (Kaverina and Straube, 2011) and cell division (Forth and Kapoor, 2017).

1.5.1 Microtubule nucleation

Microtubules are stiff, hollow tube structures with a diameter of 25 nm, composed of α/β-tubulin heterodimers (Tuszynski *et al.,* 2003). Heterodimers polymerise in a head-to-tail fashion to form linear protofilaments (Fig. 1.7). Thirteen protofilaments bind laterally in the same orientation to form the hollow cylindrical tube structure (Tuszynski *et al.,* 2003). On one end, α-tubulin is exposed to form a slow-growing minus-end, whereas β-tubulin is exposed on the other end to form the fast-growing plus-end. High concentrations of α/β-tubulin heterodimers are sufficient to promote nucleation *in vitro* (Woodruff *et al.,* 2017), however, *in vivo*, it is often initiated by the γ-gamma tubulin ring complex (γ-TuRC) (section 1.6.2.1). Microtubules undergo constant polymerisation and depolymerisation (Kirschner and Mitchison, 1986), modulated by the binding of microtubule associated proteins (MAPs) to either ends of the microtubule, such as EB and CLASP proteins (Muroyama and Lechler, 2017). The constant switch between the two modes is known as dynamic instability and allows a cell to immediately respond to cues requiring reorganisation of the microtubule cytoskeleton. Microtubule-severing enzymes katanin, spastin, and fidgetin cut stable filaments for the amplification of microtubules, or unstable microtubules to promote their depolymerisation (McNally and Roll-Mecak, 2018).

1.5.2 Microtubule motor proteins

There are three superfamilies of motor proteins: myosins, kinesins and dyneins, all of which are powered by ATP hydrolysis. Myosins utilise the actin cytoskeleton for processes such as contraction, organelle positioning and vesicle transport (DePina and Langford, 1999). Kinesins and dyneins are microtubule-associated motor proteins which utilise microtubules to move cargoes such as organelles, vesicles

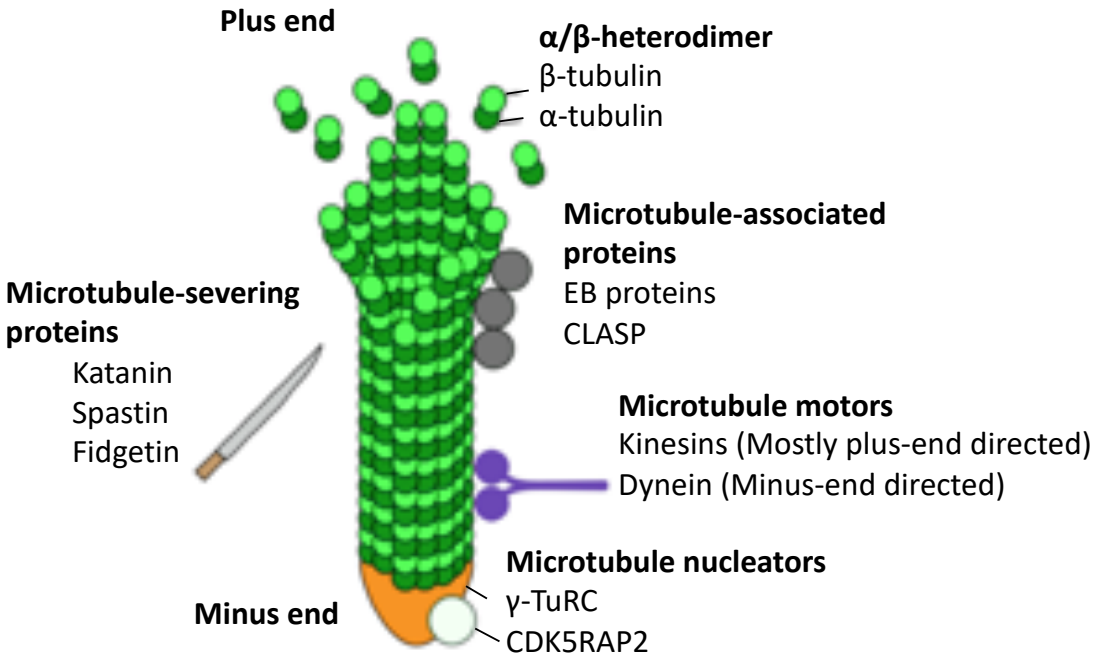


Figure 1.7 Microtubule nucleation and organisation

Microtubules are composed of α/β-heterodimers which interact in a head-to-tail fashion to form longitudinal protofilaments, which then bind laterally to form a hollow tube. The plus-end has has β-tubulin exposed and polymerisation is fast. The minus-end has α-tubulin exposed and polymerisation is slow. Microtubule nucleation is often stabilised and catalysed by a γ-TuRC which caps the minus-end. The γ-TuRC itself requires activation from CDK5RAP2. Microtubule-associated proteins bind to microtubules to regulate dynamic properties. Microtubule motor proteins carry and move cargo using microtubules as tracks, using its polarity to mediate directed movement. Microtubule-severing proteins cut microtubules for amplification or to promote depolymerisation. From Muroyama and Lechler, 2017.

and mRNA granules with the polarity of the microtubules mediating direction. Kinesins are generally plus-end directed motors whereas dyneins are minus end-directed (Klinman and Holzbaur, 2018).

Microtubule motor proteins are required for nuclear positioning in myotubes (Wilson and Holzbaur, 2015, 2012; Gache *et al.,* 2017). At least 19 motor proteins and motor protein subunits are required in early myogenesis for the centration and spreading of myonuclei (Gache *et al.,* 2017; Cadot *et al.,* 2012; Roman and Gomes, 2017). The role of each motor protein can be to modulate one or a combination of the following properties: the speed, time in motion, or alignment of nuclei (Gache *et al.,* 2017). The binding of nuclei by different motor proteins mediates controlled positioning.

1.5.2.1 Kinesin

There are 15 families of kinesins which can be further grouped into 3 types, depending on the location of the motor domain. The most common family of kinesin is the N-kinesins, which contain an N-terminal motor domain and move towards microtubule plus-ends. Only a few kinesins fall into the other two types: M-kinesins contain its motor domain at the middle of the protein and depolymerises microtubules, whereas C-kinesins contain a C-terminal motor domain and mediate minus-ended transport (Hirokawa *et al.,* 2009).

The plus-end mediated N-kinesin kinesin-1 is the major kinesin responsible for organelle transport. There are three kinesin-1 isoforms, KIF5B, which is expressed ubiquitously, and KIF5A and KIF5C, which are only expressed in tissue of the nervous system (Kanai *et al.,* 2000). Kinesin-1 is a tetramer of two homodimers, consisting of two heavy chains and two light chains. Each heavy chain contains an N-terminal globular head motor domain to walk on microtubules, a neck linker region, a coiled-coil stalk tail for dimerization, and finally a C-terminal tail domain. Each tail domain binds one of four kinesin light chains 1-4 (KLC1-4), which in turn enables recognition and binding of cargoes (Hirokawa and Noda, 2008; Karcher *et al.,* 2002). The sequential binding and hydrolysis of ATP at the head motor domain

mediates movement along a microtubule (Hirokawa *et al.,* 2009). Other kinesins differ in the length of the coiled-coil region, number of heavy chain subunits and the inclusion of light chains, which mediate direct or indirect binding to cargo.

Kinesin-1 is mostly cytoplasmic. In a centrosome-containing cell, a large proportion of microtubule minus ends are bound to the centrosome, and kinesin-1 transports cargo away from the centrosome towards the cell periphery. Kinesin-1 also transports materials for functions such as cell survival and morphogenesis (Hirokawa and Noda, 2008). However, during myogenesis and upon nesprin-1α2 expression, it becomes localised at the NE (Wilson and Holzbaur, 2015; Espigat-Georger *et al.,* 2016). The loss of kinesin-1 in myotubes leads to impaired nuclear translocation and rotation, and ultimately nuclear clustering at the centre of the cell (Wilson and Holzbaur, 2015, 2012). Its recruitment to the NE is mediated by interaction between KLC1/2 and a LEWD motif conserved between nesprin-1, 2 and 4 (Wilson and Holzbaur, 2015; Zhou *et al.,* 2018). Eleven other kinesin family motor proteins (Fig. 1.8) such as KIF1C and KIF9 from the kinesin-3 and kinesin-9 family, respectively are also involved in nuclear positioning, albeit at a lower level, measured by their impact on the speed, time in motion, and the alignment of myonuclei during myogenesis (Gache *et al.,* 2017).

1.5.2.2 Dynein

Dynein motor proteins move towards the minus-end of microtubules. There are two classes of dynein. The first, cytoplasmic dynein, is responsible for intracellular transport. Cytoplasmic dynein-1 is the only dynein found in all microtubule-containing cells (Vale, 2003). Axonemal dyneins are the second class of dyneins and are responsible for motility in ciliary and flagellar beating (Olenick and Holzbaur, 2019).

Dynein is a 1.6 MDa multiple subunit complex containing a motor and tail domain. By itself, dynein-1 is in an inhibited state and has low affinity for microtubules. It is the binding to activator proteins which activates dynein to move along microtubules (McKenney *et al.,* 2014; Trokter *et al.,* 2012; Schlager *et al.,* 2014).

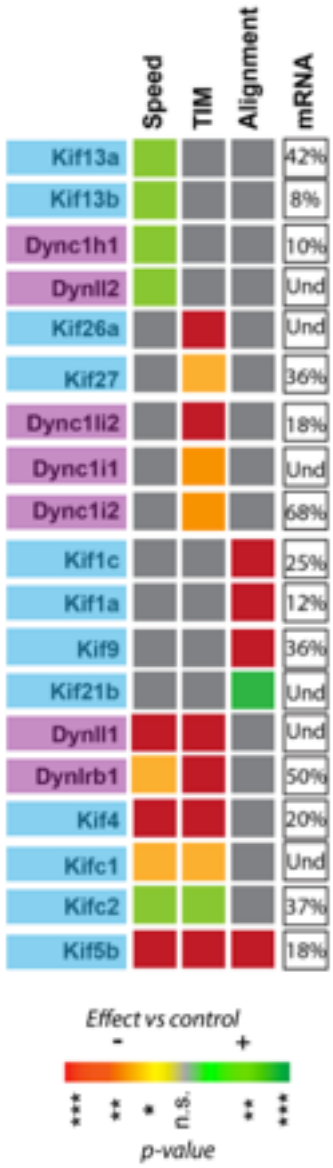


Figure 1.8 A range of kinesin and dynein motor proteins are involved in myonuclear movement by mediating nuclear speed, time in motion, or nuclear alignment

Results taken from the work of Gache *et al.* (2017), who performed a siRNA screen for motor proteins required for nuclear movement. Kinesin (blue) or dynein (purple) members were found to mediate either, or a combination of nuclear speed, time in motion (TIM), or the nuclei alignment, in 3 day old myotubes. The last column indicates the percentage of mRNA remaining after silencing.

Dynactin, a 23-subunit complex was the first dynein activator identified and is essential for its activity (Gill *et al.,* 1991). The binding of other activator co-factors such as Hook3 (Schroeder and Vale, 2016), BICD2 (McKenney *et al.,* 2014) and the centrosomal protein ninein (Redwine *et al.,* 2017) are function-dependent, and stabilise the dynein/dynactin complex (Schroeder and Vale, 2016) or facilitate cargo recognition (Redwine *et al.,* 2017).

As a minus-end directed motor protein, cytoplasmic dynein is particularly important in transporting proteins such as pericentrin and PCM1 towards the centrosome for centrosomal assembly (Denu *et al.,* 2019; Young *et al.,* 2000). Cytoplasmic dynein also mediates forward homeostatic nuclear positioning in an interphase cell by interacting with SR52-53 and the AD of nesprin-2 (Zhu *et al.,* 2017).

Dynein localises to the NE in cells entering mitosis to facilitate NE breakdown (Salina *et al.,* 2002). NE localisation of dynein also occurs in meiosis, by binding to KASH5 at the ONM, to drive chromosome movement and pairing of homologous chromosomes (Horn *et al.,* 2013b; Morimoto *et al.,* 2012). Dynein also localises to the NE in myoblasts committed to differentiation, where it is required for centration of nuclei in a nascent myotube. Disruption of dynein results in impaired nuclear translocation, rotation and nuclear clustering, as with kinesin-1 depletion (Gache *et al.,* 2017). However, whereas kinesin-1 depleted myotubes exhibited central clustering, dynein-disrupted cells showed multiple clusters of nuclei throughout the cell length (Wilson and Holzbaur, 2012). Different cytoplasmic dynein subunits or associated proteins (Fig. 1.8) modulate the speed, time in motion, and the alignment of myonuclei during myogenesis (Gache *et al.,* 2017).

1.6 The centrosome

The human centrosome consists of hundreds of proteins (Paz and Lüders, 2018). It is composed of a mother-daughter pair of perpendicular centrioles, which are surrounded by the pericentriolar material (PCM) (Mennella *et al.,* 2014), a mass of

proteins extending from the centrioles by 1 μm (Fig. 1.9). The centrosome is localised adjacent to the nucleus and is the main microtubule-organising centre (MTOC). Microtubules nucleate in radial arrays with the plus ends extending towards the cell periphery.

Centriolar satellites are dynamic granules 70-100 nm diameter which surround the centrosomes (Balczon *et al.,* 1994). Composed of over 100 proteins, centriolar satellites play roles in the maintenance and trafficking of proteins to the centrosome (Hori and Toda, 2017).

1.6.1 Pericentriolar material assembly

In an interphase cell, PCM proteins are ordered around the centrioles (Fig. 1.9). Pericentrin and centrosomal protein 152 (Cep152) bind to the mother centriole via their C-termini, whilst their N-termini extend outwards, marking the edge of the centrosome, an area termed the proximal layer (Lawo *et al.,* 2012). Other proteins are arranged into rings varying in distance from the mother centriole, showing there is order to how proteins are recruited to the PCM (Sonnen *et al.,* 2012). Cyclin-dependent kinase 5 regulatory subunit associated protein 2 (Cdk5Rap2) and Cep192 are at the surface of this layer, and bind γ-tubulin ring complexes (γ-TuRCs), a microtubule scaffold which stabilise the minus end of microtubules as it polymerises at the plus end (Lawo *et al.,* 2012; Choi *et al.,* 2010).

During mitosis, microtubule nucleation capacity increases and the PCM layer increases to accommodate this, in a process known as centrosome maturation. This is promoted by polo-like kinase 1 (PLK1) phosphorylation of proteins such as pericentrin, Cdk5Rap2 and Cep192 (Lee and Rhee, 2011; Haren *et al.,* 2009). The new, outer expansive layer of PCM proteins is less ordered and contains centrosomal proteins in concentrations higher than in the proximal layer. Such concentrations of proteins in turn recruit α/β-tubulin heterodimers at high enough concentrations for microtubule nucleation without γ-TuRCs (Fry *et al.,* 2017). After mitosis, inactivation of PLK1 leads to dephosphorylation of PCM proteins, and the PCM outer layer disassembles, leaving the proximal layer of proteins (Colicino *et*

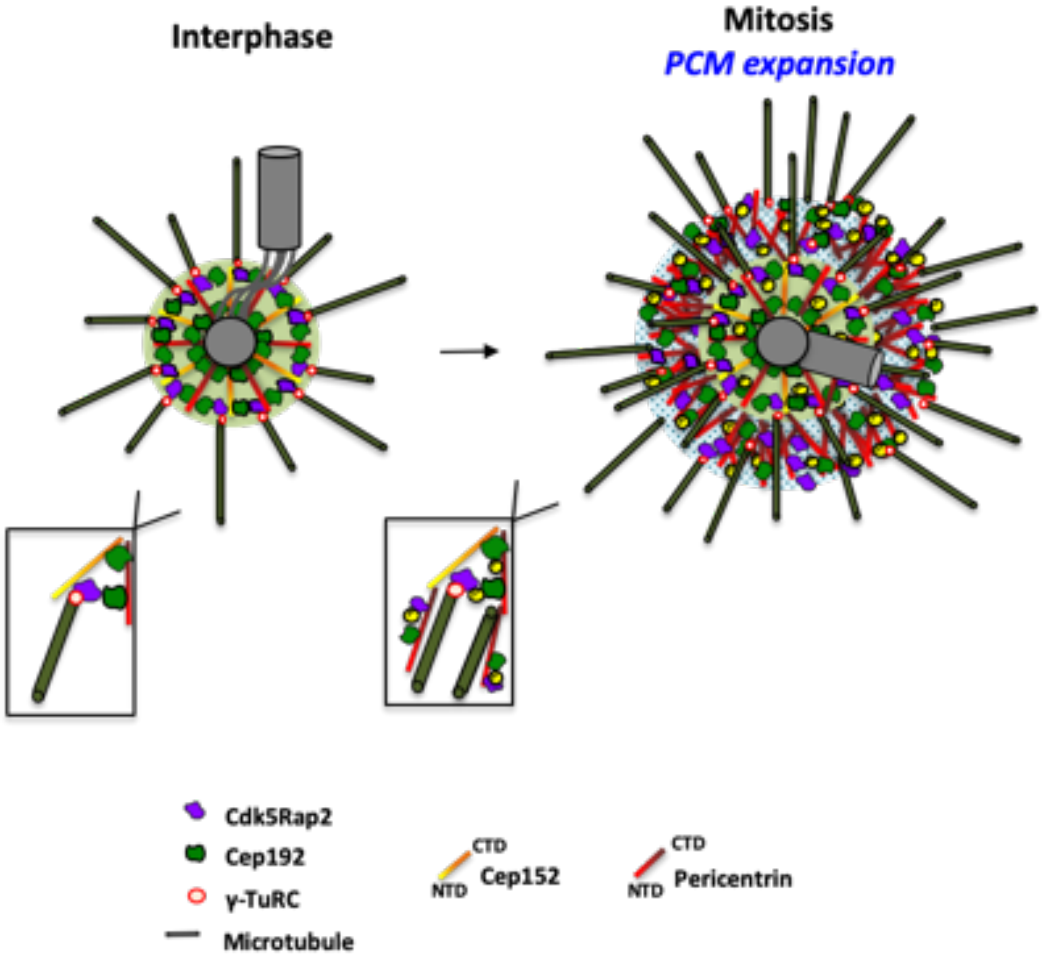


Figure 1.9 The centrosome is composed of a pair of centrioles and the PCM, a mass of proteins organised into layers

The centrosome is composed of a pair of perpendicular centrioles (grey barrels) surrounded by a mass of proteins collectively called the PCM. PCM proteins assemble around the mother centriole in an ordered manner to create rings of proteins differing in distance from the centriole. Pericentrin and Cep152 bind to the centriole via their CTD and orientate outwards to define the size of the proximal PCM layer (green circle). Proteins such as CDK5RAP2 reside at the PCM surface and binds γ-TuRCs for microtubule nucleation. Centriolar satellites (not shown) surround the centrosome and travel on microtubules to transport centrosomal proteins for centrosome assembly. During mitosis, phosphorylation of various PCM components results in the accumulation of PCM proteins into the expansive layer (blue dotted circle) to concentrate tubulin proteins for increased microtubule nucleation. From Fry *et al.,* 2017.

al., 2018; Fry *et al.,* 2017). PLK1 inactivation also promotes centrosome depletion during *D. melanogaster* oogenesis (Pimenta-Marques *et al.,* 2016).

1.6.2 Centrosome proteins

Centrosomal proteins can be responsible for microtubule nucleation, anchorage, stability or organisation. Many have a high molecular weight and contain coiled-coil regions. Due to this, it is common for a centrosomal protein to act as a scaffold to mediate recruitment of further centrosomal proteins to form the ordered PCM structure. Such relationships are not simple. For example, pericentrin is required for the localisation of CDK5RAP2, which in turn is required for γ-tubulin localisation, but pericentrin is not required for γ-tubulin localisation (Graser *et al.,* 2007; Fong *et al.,* 2008; Zimmerman *et al.,* 2004). This suggest there are redundant mechanisms or antagonistic effects between proteins. The following discussion describes a number of major centrosomal proteins and their functions at the centrosome (summarised in Fig. 1.10).

1.6.2.1 γ-tubulin

A member of the tubulin superfamily, γ-tubulin is a core component of the γ-tubulin ring complex (γ-TuRC), a template for microtubule nucleation (Gunawardane *et al.,* 2000). Other core components of the γ-TuRC are γ-tubulin complex proteins (GCPs) 2-6 (Guillet *et al.,* 2011; Gregoretti *et al.,* 2006). The γ-tubulin small complex (γ-TuSC) is first formed, consisting of a heterotetramer of two γ-tubulin molecules bound to one GCP2 and one GCP3. Multiple γ-TuSCs assemble together, by the lateral association of γ-tubulins with alternative GCP proteins GCP4, 5 and 6, to form a ring-shaped γ-TuRC (Tovey and Conduit, 2018). In humans, γ-TuRCs are assembled in the cytoplasm before transport to MTOCs where they become activated to nucleate microtubules (Choi *et al.,* 2010). Upon mitosis, γ-TuRCs rapidly accumulate at the centrosomes to amplify microtubule formation (Khodjakov and Rieder, 1999). Each γ-tubulin of the complex binds to the α-tubulin of α/β-tubulin heterodimers for protofilament formation (Kollman *et*

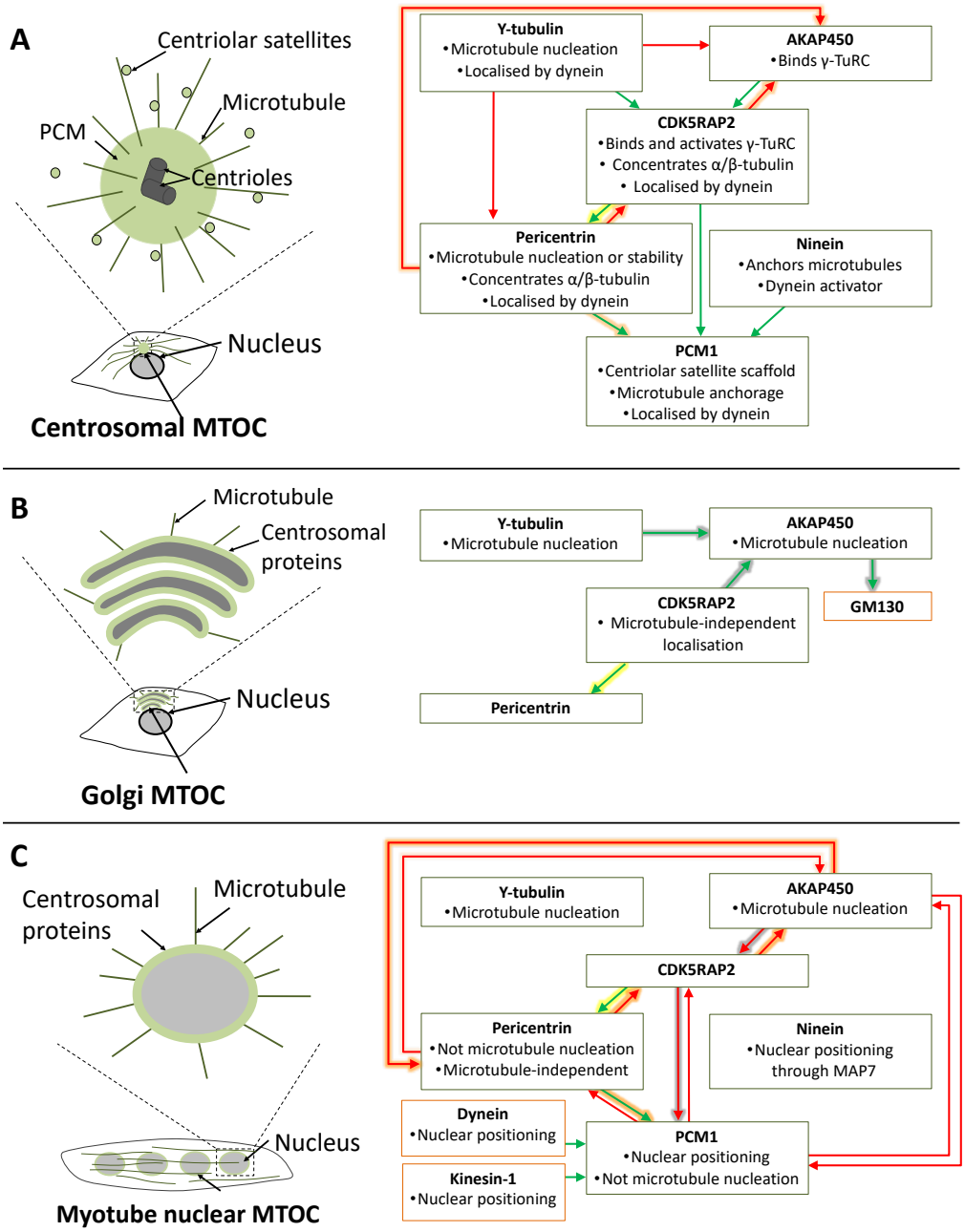


Figure 1.10 The MTOCs of the centrosome, Golgi and myotube NE and roles of their associated centrosomal proteins

Different MTOCs with associated centrosomal proteins and their functions, at the centrosome (A), Golgi (B) and myotubes (C). The centrosomal MTOC consists of a pair of centrioles, surrounding PCM and centriolar satellites. The Golgi and myotube MTOCs involve the localisation of centrosomal proteins to the Golgi membrane and NE, respectively. Centrosomal proteins present at each MTOC are in green boxes with known functions listed. Orange boxes indicate MTOC specific proteins. A protein from which a green arrow originates requires the protein where the arrow ends for localisation, whereas it does not in the case for red arrows. Yellow glowing arrows represents conserved dependencies between all three MTOCs, an orange glow represents conservation between the centrosomal and myotube nuclear MTOC and a grey glow represents a relationship not conserved at the centrosome.

*al., 2*010). The binding also stabilises and enhances the lateral formation of microtubule protofilaments, which is prone to depolymerisation at the initial stage (Tovey and Conduit, 2018).

The microtubule nucleation function of γ-tubulin is not restricted to the γ-TuRC, as single monomers of γ-tubulin are able to independently nucleate microtubules *in vitro* (Leguy *et al.,* 2000). Microtubule nucleation can be reconstituted *in vitro* by proteins that crowd α/β-tubulin heterodimers for spontaneous nucleation (Roostalu *et al.,* 2015; Woodruff *et al.,* 2017). Furthermore, *in vivo* studies showed that microtubule nucleation still occurs in the absence of γ-tubulin, though the number and structure of microtubules, as well as the rate of nucleation, is altered (Job *et al.,* 2003; Strome *et al.,* 2001).

1.6.2.2 Pericentrin

Pericentrin is a large PCM protein consisting of mostly coil-coil domains. Pericentrin B is the largest isoform at 380 kDa, and contains the pericentrin/AKAP450 centrosomal targeting (PACT) localisation domain (Gillingham and Munro, 2000) and a calmodulin binding domain at the C-terminus. Further isoforms have been detected in mice. Pericentrin S is 250 kDa, lacks the N-terminal region of pericentrin B, and is the main isoform expressed in skeletal and heart muscles. Pericentrin A is 220 kDa and contains the N-terminal region of pericentrin B, but lacks the PACT domain and calmodulin binding region (Miyoshi *et al.,* 2006). Multiple human pericentrin variants have been detected at the protein level (Mühlhans and Gießl, 2012).

Pericentrin interacts with GCP2 and GCP3 to anchor γ-TuRCs for microtubule nucleation (Takahashi *et al.,* 2002). Pericentrin and γ-tubulin colocalise on microtubules and are transported towards the centrosome by dynein (Young *et al.,* 2000). Microtubule nucleation is often observed from structures containing both centrosomal proteins. However, pericentrin depletion does not affect γ-tubulin localisation or microtubule nucleation in interphase cells, whereas in mitotic cells, pericentrin depletion leads to the loss of γ-tubulin from the spindle poles and

disrupted microtubule nucleation (Zimmerman *et al.,* 2004). Pericentrin inhibition also leads to reduced microtubule nucleation in isolated centrosomes (Takahashi *et al.,* 2002). Hence, the role of pericentrin in microtubule nucleation differs between environments, such as at different phases of the cell cycle.

Pericentrin also acts as a scaffold to recruit other proteins to the centrosome, making its role hard to differentiate from the proteins it acts as a scaffold for. It recruits PCM proteins such as CDK5RAP2 and Cep68, and centriole proteins SAS-6 and Cep57 via interaction with its PACT domain (Wang *et al.,* 2016; Graser *et al.,* 2007; Ito *et al.,* 2019; Watanabe *et al.,* 2019). Pericentrin depletion does not appear to affect microtubule anchorage or organisation in interphase SAOS cells (Zimmerman *et al.,* 2004). However, the effect of pericentrin depletion was perhaps more pronounced in interphase U2OS cells, where the microtubule network appeared less dense, suggesting defects in microtubule stability, which in turn are possibly due to mislocalisation of pericentrin alone or other centrosomal proteins (Dammermann and Merdes, 2002).

Dynein light intermediate chain 1 interacts with pericentrin (Tynan *et al.,* 2000). However, there has been no functional studies to show whether pericentrin is a dynein adaptor.

1.6.2.3 CDK5RAP2

CDK5 regulatory subunit associated protein 2 (CDK5RAP2) is a 215 kDa PCM protein consisting of mostly coil-coil domains. It contains an N-terminal CM1 motif for the binding of γ-TuRCs. Localisation to the centrosome is mediated by binding to pericentrin via a 50 residue C-terminal CM2-like motif. (Wang *et al.,* 2010).

CDK5RAP2 is transported to the centrosome by dynein (Jia *et al.,* 2013), where it is required for microtubule nucleation at the centrosome. CDK5RAP2 depleted cells contain less γ-tubulin at the centrosome in both interphase and mitotic cells (Fong *et al.,* 2008). Although γ-TuRC formation and localisation to the centrosome is

unaffected, microtubule nucleation is impaired, therefore CDK5RAP2 is thought to be the activator of γ-TuRCs (Choi *et al.,* 2010).

Pericentrin depletion leads to the loss of centrosomal CDK5RAP2 (Graser *et al.,* 2007), whereas CDK5RAP2 depletion does not affect pericentrin localisation (Fong *et al.,* 2008).

1.6.2.4 AKAP450

A-kinase anchor protein 450 (AKAP450A) is a 450 kDa PCM protein predicted to consist mostly of coiled-coil domains, and contains a C-terminal PACT domain, which targets the protein to the centrosome (Gillingham and Munro, 2000).

Both the N-terminus and C-terminus of AKAP450 interact with both GCP2 and GCP3 to anchor γ-TuRCs for microtubule nucleation, though the interaction through the C-terminus is weaker (Takahashi *et al.,* 2002). In isolated centrosomes, AKAP450 inhibition led to reduced microtubule nucleation (Takahashi *et al.,* 2002). The C-terminal third of AKAP450 localises to multiple foci throughout the cell and recruits γ-tubulin, pericentrin, CDK5RAP2, and the centrosomal proteins Cep68 and Cep170 (Kolobova *et al.,* 2017). The foci are functional MTOCs, but microtubules nucleated for 20 mins after nocodazole washout are shorter than those nucleated from the centrosome, suggesting that AKAP450 binds proteins sufficient for microtubule nucleation, but not stabilisation or maintenance.

Indeed, AKAP450, GCP2, GCP3 and pericentrin can be co-immunoprecipitated together (Takahashi *et al.,* 2002), as can AKAP450, pericentrin and CDK5RAP2 (Wang *et al.,* 2010), showing the proteins are part of a large multi-protein complex. However, AKAP450 is not required for pericentrin, γ-tubulin or CDK5RAP2 localisation at the centrosome, but itself requires CDK5RAP2 for localisation (Keryer *et al.,* 2003; Bouguenina *et al.,* 2017).

1.6.2.5 Ninein

Ninein is a 250 kDa protein consisting of non-coiled C- and N-termini and a coil-coiled central region (Bouckson-Castaing *et al.,* 1996). It localises to the mother centriole via its C-terminal domain in a microtubule-independent manner (Goldspink *et al.,* 2017; Delgehyr *et al.,* 2005).

The N-terminus of ninein interacts with γ-tubulin to recruit γ-TuRCs to the centrosome for microtubule nucleation, and anchors microtubules to the centrosome via its central region (Delgehyr *et al.,* 2005).

Ninein interacts with pericentrin, and becomes mislocalised from pericentrin depleted spindle poles, whereas pericentrin does not require ninein for localisation (Chen *et al.,* 2014). Ninein also does not require CDK5RAP2 for its localisation at the spindle poles (Barr *et al.,* 2010). It is not known whether the dependencies are conserved at the interphase centrosome.

Ninein interacts with dynein intermediate chains (Redwine *et al.,* 2017).
As purified ninein was able to activate and co-migrate with purified dynein/dynactin, ninein has classified as a dynein activator (Redwine *et al.,* 2017). Its role as a dynein activator may be for the recognition and assembly of proteins at the centrosome.

1.6.2.6 PCM1

Pericentriolar material 1 (PCM1) is 230 kDa and a major centriolar satellite protein with multiple coiled-coil domains. No major functional domains have been determined though it has been determined that the N-terminal half of the protein is required for centriolar satellite formation (Wang *et al.,* 2016).

As part of the centriolar satellites, PCM1 is mostly localised within dynamic granules concentrated around the centrosome but is also sparsely scattered

throughout the cytoplasm (Kubo *et al.,* 1999). PCM1 acts as a scaffold to hold centriolar satellites together around the centrosome, as PCM1 depletion causes the satellites to disperse throughout the cytoplasm. PCM1 is trafficked along microtubules towards the centrosome by dynein, as treatment of cells with nocodazole to depolymerise microtubules (Kubo *et al.,* 1999; Stowe *et al.,* 2012; Denu *et al.,* 2019), or with ciliobrevin D to inhibit dynein (Denu *et al.,* 2019) causes the dispersal of the granules within the cytoplasm.

Cos-7 cells depleted of PCM1, followed by nocodazole washout, showed normal centrosomal microtubule nucleation, but loss of anchorage after five minutes (Dammermann and Merdes, 2002). This suggests PCM1 is required for the anchorage but not nucleation of microtubules. In U2OS cells, displacement of PCM1 is followed by the mislocalisation of centrosomal pericentrin and ninein (Dammermann and Merdes, 2002). Indeed, PCM1 interacts with pericentrin (Miyoshi *et al.,* 2006). It is also likely to be involved in the centrosome recruitment of CDK5RAP2, as recovery of Venus-CDK5RAP2 to the centrosome after fluorescent photobleaching is slower in PCM1-depleted cells compared to control cells (Ge *et al.,* 2010). Despite an important role of PCM1 in maintaining the pericentrosomal localisation of centriolar satellites for microtubule organisation, centriolar satellites become dispersed throughout the cytoplasm late in the G2 phase of the cell cycle (Balczon *et al.,* 1994), indicating PCM1 nor centriolar satellites are required for the maintenance of proteins at the mitotic spindle.

1.7 Non-centrosomal MTOCs

The centrosome is not the sole MTOC in a cell, as microtubules also nucleate from the Golgi and centrosome protein-containing seeds in the cytoplasm (Efimov *et al.,* 2007). There are also many differentiated cells, such as muscle cells where the centrosome is eliminated (Werner *et al.,* 2017). This may be because a major role of the centrosome is in formation of the mitotic spindle, which is not required in post-mitotic differentiated cells. Therefore, PCM and centriolar satellite proteins become disassembled from the centrosome and relocalise to a new MTOC site, accompanied with reorganisation of the microtubule cytoskeleton. The remaining

centrioles may migrate to the plasma membrane and form the basal body of primary cilia, and later be eliminated from the cell (Werner *et al.,* 2017; Connolly *et al.,* 1986).

1.7.1 Golgi complex

The Golgi complex is located adjacent to the centrosome, and is formed of Golgi stacks linked together into a ribbon structure with a continuous membrane (Tassin *et al.,* 1985b). In addition to roles in post-translational modifications and protein trafficking, it also acts as a MTOC in additional to, and independently of the centrosomes (Efimov *et al.,* 2007). The Golgi ribbon is maintained by microtubules in a dynein-dependent manner, as it undergoes dramatic fragmentation and dispersal upon nocodazole treatment (Rivero *et al.,* 2009). The Golgi was identified as an alternative MTOC when microtubule nucleation was observed close to Golgi fragments in cells after nocodazole washout (Tassin *et al.,* 1985b; Chabin-Brion *et al.,* 2001).

Centrosome-derived microtubules have their minus ends at the centrosome, resulting in restricted distribution of cargos by motor proteins. By having alternative MTOCs, microtubules connect more cellular locations allowing better distribution of molecules in the cell (Zhu and Kaverina, 2013). Other than transport, Golgi-nucleated microtubules are involved in polarised cell migration (Hurtado *et al.,* 2011).

Figure 1.10 summarises the functions of MTOC proteins at the Golgi. As at the centrosomes, microtubule nucleation at the Golgi requires γ-tubulin (Efimov *et al.,* 2007) and the dynein-dynactin complex (Rivero *et al.,* 2009). CDK5RAP2 and AKAP450 bind to the cis-Golgi (Rivero *et al.,* 2009). Localisation of CDK5RAP to the Golgi is via its centrosome localising 50 residue C-terminal CM2-like motif, the same region which mediates centrosome localisation (Wang *et al.,* 2010). AKAP450 anchorage at the Golgi MTOC requires an N-terminal region which interacts with Golgi matrix protein 130 (GM130), an integral membrane protein of the Golgi (Rivero *et al.,* 2009; Wu *et al.,* 2016). This is in contrast to at the centrosome,

where the C-terminal PACT domain is responsible for localisation (Gillingham and Munro, 2000). The requirement of pericentrin for the MTOC localisation of CDK5RAP2 is conserved from the centrosome to the Golgi, whereas AKAP450 is required for CDK5RAP2 localisation only at the Golgi (Wang *et al.,* 2010). Anchorage of CDK5RAP2 at the centrosome is microtubule-dependent, whereas anchorage persists in the absence of microtubules at the Golgi (Wang *et al.,* 2010).

1.7.2 Epithelial cells

The differentiation and polarisation of epithelial cells generally involves the formation of an alternative MTOC at the apical membrane, as observed in human, mouse and *C. elegans* intestinal epithelial cells (Toya *et al.,* 2016; Yang and Feldman, 2015). During *C. elegans* intestinal epithelial cell differentiation, MTOC proteins are transferred from the centrosome to the apical membrane, yet the centrosome can be reactivated upon mitosis (Yang and Feldman, 2015). Ninein and γ-tubulin localise to the apical membrane in mouse gut organoid cells, where they promote microtubule anchorage and nucleation, with microtubule minus ends attached to the apical membrane (Goldspink *et al.,* 2017; Yang and Feldman, 2015). Unlike at the centrosome, ninein localisation at the apical membrane requires microtubules. Differentiated cells are capable of undergoing mitosis, where the centrosome must become the MTOC once again. This is achieved by the phosphorylation of the Cep192 worm orthologue SPD-2 by CDK (Yang and Feldman, 2015). It is unclear what other centrosomal components are present at the apical membrane MTOC.

1.7.3 Muscle cells

During myotube differentiation, the centrosome is disassembled and the MTOC is irreversibly transferred to the NE to form the nuclear MTOC (nMTOC), an insoluble matrix which persists in mature myofibres (Oddoux *et al.,* 2013; Srsen *et al.,* 2009). Immunoelectron microscopy of PCM1 in mouse C2C12 myotubes, using gold particles, revealed PCM1 to surround the ONM in an electron dense material 30-40

nm in thickness (Srsen *et al.,* 2009). Relocalisation of centrosomal proteins appears to initiate from the NE closest to the centrosome and occurs before the fusion of committed myoblasts (Srsen *et al.,* 2009). Some centrosomal protein seeds remain in the cytoplasm (Ralston, 1993), whereas centrioles become eliminated after myoblast fusion (Connolly *et al.,* 1986).

Proteins found at the centrosome which stain brightly at the nMTOC include pericentrin, ninein, PCM1, AKAP450 and CDK5RAP2 (Srsen *et al.,* 2009; Bugnard *et al.,* 2005). Weak staining of γ-tubulin is also observed (Srsen *et al.,* 2009; Bugnard *et al.,* 2005). Other centrosomal proteins are likely to be recruited to the NE. Microtubules nucleate from the NE (Srsen *et al.,* 2009; Bugnard *et al.,* 2005; Tassin *et al.,* 1985a) and from pericentrin (Bugnard *et al.,* 2005), PCM1 or AKAP450 (Gimpel *et al.,* 2017) seeds in the cytoplasm and become arranged into longitudinal arrays along the length of the myotube (Tassin *et al.,* 1985a). Dynein and kinesins also become recruited to the NE (Wilson and Holzbaur, 2012), and are required for nuclear centration and spreading in early myogenesis (Cadot *et al.,* 2015).

As discussed in section 1.4.5, nMTOC formation coincides with the expression of nesprin-1α2 at the NE. Nesprin-1α2 acts as the receptor for centrosomal proteins at the NE. The absence of nesprin-1 isoforms in myotubes prevents the localisation of PCM1, pericentrin, AKAP450 and CDK5RAP2 at the NE and the loss of microtubule nucleation from the NE, leading to nuclear mispositioning (Gimpel *et al.,* 2017; Espigat-Georger *et al.,* 2016). Expression of biotin-ligase fused nesprin-1α2 in differentiating C2C12 cells resulted in the biotinylation of PCM1, pericentrin and AKAP450, indicating close proximity and interaction of the proteins (Gimpel *et al.,* 2017). In addition, nesprin-1α2 and PCM1 have been shown to co-immunoprecipitate in C2C12 myotubes (Espigat-Georger *et al.,* 2016). PCM1 staining at the NE is distinct from that of NPC proteins, supporting that the LINC complex is the only NE component that mediates centrosomal protein recruitment (Srsen *et al.,* 2009). At least seven centrosome/MTOC components have been observed to be recruited to the NE (Srsen *et al.,* 2009; Bugnard *et al.,* 2005; Oddoux *et al.,* 2013). Yet, more nMTOC components are likely to be identified in the future.

Figure 1.10 summarises the functions of a number of MTOC proteins at the myotube nMTOC, which will be discussed next.

1.7.3.1 Gamma-tubulin

An essential component for microtubule nucleation at the centrosome and Golgi, the role of γ-tubulin appears to be mirrored at the nMTOC. Microinjection of γ-tubulin antibodies inhibits microtubule nucleation in both myoblasts and myotubes, whereas injection of pericentrin antibodies did not (Bugnard *et al.,* 2005). Despite this, the protein level of γ-tubulin decreases by 30% upon myogenesis, and can only be stained weakly at the NE in myotubes permeabilised with Triton X-100 and fixed by methanol (Bugnard *et al.,* 2005). It is not known whether γ-TuRCs are formed at the myonuclear nMTOC.

1.7.3.2 Pericentrin

The movement of pericentrin to the NE does not require microtubules (Zaal *et al.,* 2011). However, similar to at the centrosome, pericentrin requires PCM1, but not CDK5RAP2 nor AKAP450 for its recruitment at the nMTOC (Gimpel *et al.,* 2017; Dammermann and Merdes, 2002). Nuclear clustering and the loss of pericentrin at the NE has been observed in myotubes from an Emery-Dreifuss muscular dystrophy patient with *SUN1* mutations (Meinke *et al.,* 2014). Reduced staining of pericentrin has also been observed in an EDMD2 patient with a mutation in the *LMNA* gene (Mattioli *et al.,* 2018). Associated in the same protein complex, mutant effects of SUN1 and lamin may be transmitted through the NE, to impair the ability of nesprin-1α2 to recruit pericentrin and possibly other nMTOC or motor proteins. This is likely as the depletion of pericentrin alone in myotubes does not affect microtubule nucleation or myonuclear positioning (Gimpel *et al.,* 2017).

1.7.3.3 CDK5RAP2

It is not known whether CDK5RAP2 mediates microtubule nucleation at the nMTOC, as it does at the centrosome (Jia *et al., 2*013). Its recruitment to the NE requires pericentrin, as at the centrosome, but not PCM1 or AKAP450 (Gimpel *et al.,* 2017).

1.7.3.4 AKAP450

AKAP450 depletion in myotubes led to the loss of microtubule nucleation from the NE and clustering of myonuclei (Gimpel *et al.,* 2017). Its mechanism of anchorage to the NE is changed from the centrosome as it no longer requires CDK5RAP2 (Gimpel *et al.,* 2017). Its localisation at the myotube NE is considered to be with other centrosomal proteins to form the nMTOC (Gimpel *et al., 2*017; Holt *et al., 2*019). However, there is the possibility it also relocalises with other Golgi elements (section 1.7.3.7).

1.7.3.5 Ninein

Anchorage of ninein to the myotube NE is independent of microtubules (Bugnard *et al.*, 2005). Ninein may mediate nuclear positioning through interaction with MAP7. The *D. melanogaster* ninein orthologue Bsg25D interacts with the MAP7 orthologue Ens. Mutated Ens leads to mispositioning of myonuclei (Metzger *et al.,* 2012; Rosen *et al.,* 2019), and this is amplified when Bsg25D is also mutated (Rosen *et al.,* 2019). The loss of Bsg25D alone does not lead to mispositioning suggesting that other proteins are able to compensate for its role. Overexpression of Bsg25D lead to the displacement of Ens from microtubules and nuclear mispositioning in both myotubes and myofibres. In such myofibres, microtubules are not seen extending from the nucleus, but from concentrated Bsg25D puncta in the cytoplasm. As a result, myofiber stiffness was decreased (Rosen *et al.,* 2019). Other centrosomal proteins were not stained therefore it is not known if the results observed could be due to the mislocalisation of other nMTOC components.

1.7.3.6 PCM1

PCM1 resumes its role as a protein scaffold at the nMTOC. Knockdown of PCM1 in myotubes led to the loss of pericentrin at the NE in C2C12 myotubes (Espigat-Georger *et al.,* 2016; Gimpel *et al.,* 2017). The kinesin-1 component KLC1/2 was also mislocalised after PCM1 depletion, showing PCM1 is required in addition to nesprin-1α2 for motor protein recruitment. PCM1 depletion did not affect microtubule nucleation, but led to myonuclear mispositioning (Gimpel *et al.,* 2017; Espigat-Georger *et al.,* 2016). Pericentrin, CDK5RAP2 or AKAP450 are not required for PCM1 localisation (Gimpel *et al.,* 2017).

1.7.3.7 Golgi proteins

Upon differentiation and before fusion of myoblasts, the Golgi becomes fragmented (Lu *et al.,* 2001) and Golgi proteins GM130, α-mannosidase II, clathrin heavy chain and β-COP relocalise around the NE as a non-continuous, fragmented belt (Ralston, 1993; Zaal *et al.,* 2011). Microtubule depolymerisation does not affect the perinuclear distribution of Golgi proteins (Tassin *et al.,* 1985b; Zaal *et al.,* 2011).

The formation of the nMTOC precedes this and may be a scaffold for Golgi proteins to bind to the NE (Zaal *et al.,* 2011). This is possible as nesprin-1 null myotubes also lack the NE recruitment of GM130 (Gimpel *et al.,* 2017). Though AKAP450 and pericentrin are both found at the Golgi, their localisation to the NE is coincident with other centrosomal proteins around the whole NE and they do not completely localise with GM130. However, cytoplasmic fragments of GM130 colocalise with AKAP450 and not pericentrin (Gimpel *et al.,* 2017).

In mature myofibres microtubules are again reorganised, now into an orthogonal grid. GM130 is no longer at the NE but observed as seeds distributed around the cytoplasm (Oddoux *et al.,* 2013). These Golgi elements colocalised with γ-tubulin and pericentrin seeds, but not AKAP450 as at the cis-Golgi, and were sites of microtubule nucleation upon nocodazole washout (Oddoux *et al.,* 2013).

Microtubules nucleating from the sites eventually interconnected into arrangements similar to pre-treated cells (Oddoux *et al.,* 2013). Hence, microtubules originating from the Golgi MTOC are important upon muscle maturation when the longitudinal arrangement of microtubules changes to a grid like lattice.

1.7.4 Modifications of centrosomal proteins at different MTOCs

The presence of many centrosomal proteins is conserved at different MTOCs, though their roles, the use of the cytoskeleton for anchorage, and interdependencies on other proteins for localisation differ. Differences may be solely due to changes in the availability of interacting partners at different MTOCs, or changes in gene expression. For example, ninein binds to the centrosome to anchor microtubules, and during neuronal differentiation, alternative splicing occurs such that the centrosome localisation domain becomes absent and ninein localisation becomes cytoplasmic (Jagsi *et al.,* 2017). Alternatively, changes may be mediated through post-translational modifications. For example, CDK phosphorylates PCM1, which in turn recruits PLK1 to the centrosome, to phosphorylate substrates for primary cilia disassembly and mitosis (Wang *et al.,* 2013). Changes to centrosomal proteins which allow the change in localisation and function from the centrosome to the NE in myotubes remain elusive.

1.8 Aims and objectives

Early studies have shown the importance of microtubules in nuclear positioning during early myogenesis. Accompanying this, centrosomal proteins involved in microtubule growth and regulation are relocalised to the NE by nesprin-1. The hypothesis of this project is that, during the formation of multinucleated myotubes, the LINC complex component nesprin-1α2 becomes expressed and recruits PCM1, pericentrin, AKAP450 and other centrosomal components from the centrosome to form the nMTOC (Fig. 1.11). This is required for microtubules to be nucleated from the NE, which are used by microtubule motor proteins kinesins and cytoplasmic

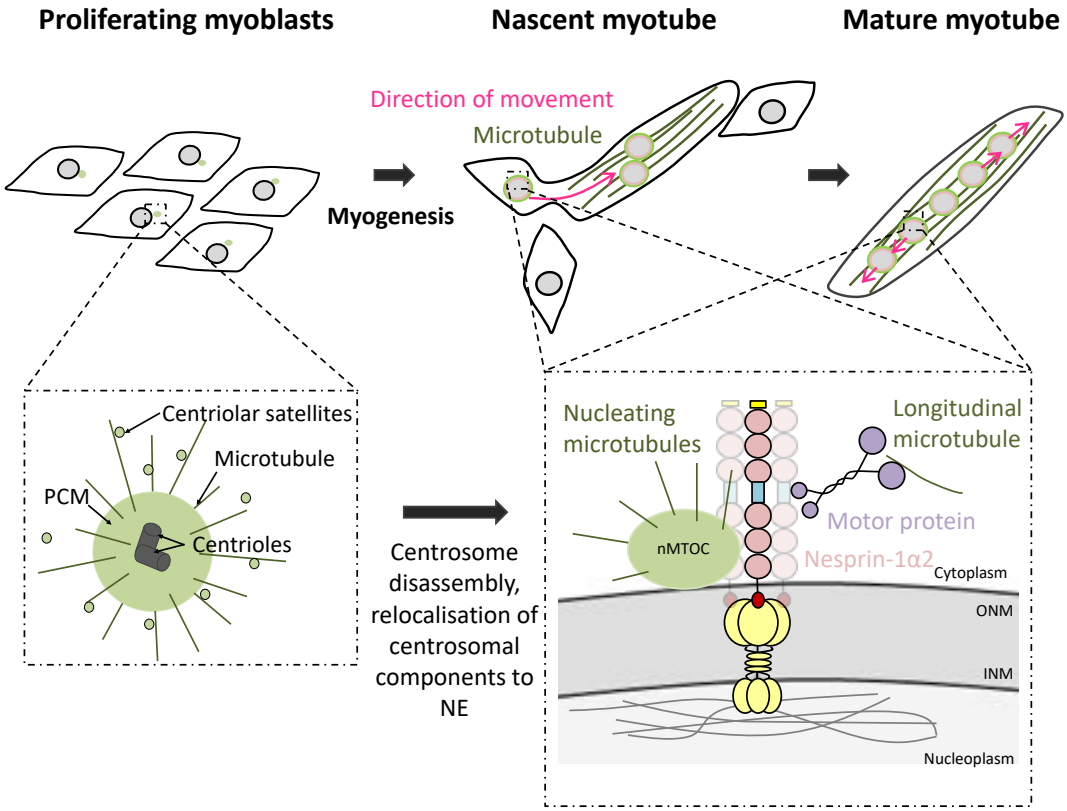


Figure 1.11 Hypothesised model of myonuclei positioning during myogenesis

In myoblasts, MTOC proteins are observed at centrosome where microtubules nucleate. Upon differentiation, nesprin-1α2 is expressed and localises to the NE. Centrosomes disassemble and centrosomal proteins are recruited by nesprin-1α2 to relocalise around the NE. Microtubules emanate from the newly formed nMTOC and become arranged in longitudinal arrays parallel to the length of the cell. The microtubules are used as tracks by motor proteins for the centration and spreading of nuclei in a developing myotube. When protein interactions between nesprin-1α2, centrosomal and motor proteins are disrupted, the organisation and maintenance of microtubules becomes disturbed, resulting in myonuclei clustering and muscle dysfunction.

dynein to position myonuclei in early myogenesis. This study aimed to understand how correct nuclear positioning is achieved, by identifying how nesprin-1α2 recruits centrosomal proteins, and how centrosomal proteins themselves relocalise to the NE. There are many interesting centrosomal protein candidates, however this study focused on PCM1, pericentrin and AKAP450, which were identified as potential nesprin-1α2 interacting partners by Gimpel *et al.* (2017). This project aimed to lead to a better molecular understanding of muscle diseases, such as which regions of nesprin-1 are required for nMTOC formation, and which MTOC proteins are responsible for the generation of microtubules required for myonuclear positioning. In the long-term, it may help discover protein targets for therapeutic drug design.

There were three aims in this project, each to be achieved with a set of objectives:

1) Characterising the dependence of centrosomal components on nesprin-1 for their localisation to the NE
    - Express GFP-nesprin-1α2 in nesprin-1 null myotubes to investigate if it is sufficient to rescue centrosomal protein recruitment to the NE
    - Express GFP-nesprin-1α2 truncation mutants in nesprin-1 null myotubes to investigate which domains are required to rescue PCM1 and AKAP450 to the NE
    - Express GFP-nesprin-1α2 point mutants associated with muscle disease, or which are required for interaction with kinesin-1, in nesprin-1 null myotubes to investigate if they impact PCM1 and AKAP450 recruitment to the NE
2) Characterising the mode by which centrosomal components are recruited to the NE
    - Express PCM1 and pericentrin truncations in myotubes to investigate their regions responsible for NE localisation
    - Deplete PCM1 in myotubes by RNAi and investigate the effects on pericentrin and AKAP450 recruitment to the NE
    - Treat myoblasts and myotubes with nocodazole to depolymerise microtubules and investigate the recruitment of PCM1 and pericentrin to the NE

3) Investigating PCM1 phosphorylation in nMTOC assembly

- Express GFP-nesprin-1α2 in non-myotube cells under different conditions in attempt to form the nMTOC in a non-myogenic environment
- Perform mass spectrometry on PCM1 to determine myotube-specific phosphorylations, to be investigated through expression of PCM1 phosphomimetics and phosphonull mutants in myoblast and myotubes

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Reagents

All chemicals were of analytical grade purity or higher. Table 2.1 lists routinely used reagents with their according supplier. Suppliers of other reagents are indicated in the text.

Table 2.1 General reagents used

Reagent	Supplier
BCA protein assay reagent	Biorad
BSA EDTA Ethidium bromide Glycerol Isopropanol Tween-20	Fisher Scientific (Loughborough, UK)
ProtoFLOWgel (30% w/v acrylamide)	Flowgen Bioscience (Nottingham, UK)
PMSF	Fluka
Nitrocellulose membrane	GE Healthcare Life Sciences Amersham
DMEM with Glutamax DMEM/F12 Foetal bovine serum Gentamycin Heat-inactivated horse serum Insulin Lipofectamine 2000 Lipofectamine 3000 Lipofectamine RNAimax Medium 199 Opti-Mem with Glutamax Penicillin/streptomycin RNAase A Trypsin-EDTA (0.5%)	Invitrogen/Gibco (Paisley, UK)
LB broth (capsules) SDS	Melford (Suffolk, UK)
Pierce™ ECL Western Blotting Substrate SuperSignal™ West Femto Maximum Sensitivity Substrate	Pierce (Rockford, USA)
X-ray film	Scientific Lab Supplies (Yorkshire, UK)
Beta-glycerophosphate TEMED	Sigma

2.1.2 DNA plasmids and primer sequences

Mammalian constructs used for transfections are listed in Table 2.2, with their origin indicated if not self-generated. Primers used in PCR reactions to sequence constructs or for cloning are listed in Table 2.3. All primers were ordered from Eurofins Genomics.

Table 2.2 Constructs for mammalian transfection

Plasmid	Antibiotic resistance	Generated by
Mammalian cell expression		
EGFP-N1α2	Kan	Qiuping Zhang (King's College London, UK)
EGFP-PCM1	Kan	Songhai Shi lab (Sloan Kettering Institute, USA)
pEGFP-C1	Kan	ClonTech (Saint-Germain-en-Laye, France)
pLEIC138-meGFP-N1a2 (SR1-SR6)	Amp	Protex (University of Leicester, UK)
eGFP-N1a2 (SR2-SR6)	Kan	
pLEIC138-meGFP-N1a2 (SR3-SR6)	Amp	Protex
pLEIC138-meGFP-N1a2 (SR4-SR6)	Amp	Protex
EGFP-N1α2(N-SR4 + KASH)	Kan	
EGFP-N1α2(N-AD + KASH)	Kan	
EGFP-N1α2(N-SR3 + KASH)	Kan	
EGFP-N1α2(N-SR2 + KASH)	Kan	
EGFP-N1α2(N + (SR2-AD)+KASH)	Kan	
EGFP-N1α2(N + (SR3-AD)+KASH)	Kan	
EGFP-N1α2(N + AD+KASH)	Kan	
EGFP-N1α2(SR1-AD + KASH)	Kan	
EGFP-N1α2(SR2-AD + KASH)	Kan	
EGFP-N1α2(LEWD/AA)	Kan	Shackleton lab member
pLEIC138-meGFP-N1α2(R429Q)	Amp	Protex
EGFP-N1α2(Y363H)	Kan	Qiuping Zhang
EGFP-N1α2(Y446C)	Kan	Qiuping Zhang
pLEIC-21 PCM1(1-1083)	Kan	Protex
pLEIC-21 PCM1(1064-2024)	Kan	Protex
pLEIC138-eGFP-PCM1 (1-331)	Amp	Protex
pLEIC138-eGFP-PCM1 (302-573)	Amp	Protex
pLEIC138-eGFP-PCM1 (544-1089)	Amp	Protex
pLEIC138-meGFP-PCM1 (1-217)	Amp	Protex
pLEIC138-meGFP-PCM1 (218-331)	Amp	Protex
EGFP-PCM1(1-1089)+KASH	Kan	

pLEICS20-Myc-PCNT(1-582)	Amp	Protex
pLEICS20-Myc-PCNT(401-800)	Amp	Protex
pLEICS20-Myc-PCNT(801-1189)	Amp	Protex
pLEICS20-Myc-PCNT(1201-1769)	Amp	Protex
pLEICS20-Myc-PCNT(1770-2627)	Amp	Protex
pLEICS20-Myc-PCNT(2628-2838)	Amp	Protex
pLEICS20-Myc-PCNT(2839-3336)	Amp	Protex
pLEICS20-Myc-PCNT(3139-3336)	Amp	Protex
pLEIC138-eGFP-PCM1 (1-331 S93A)	Amp	Protex
pLEIC138-eGFP-PCM1 (1-331 S93D)	Amp	

Table 2.3 Sequencing and cloning primers

Primer	Sequence (5'→3')
Vector sequencing primers	
pLEICS-19-Seq-F	CTGTACGGAAGTGTTACTT
pLEICS-19-Seq-R	CATCACAAATTTCACAAATA
pLEICS-21-Seq-F	CACATGGTCCTGCTGGAGTT
pLEICS-21-Seq-R	GCTGATTATGATCAGTTAT
pLEICS-12-Seq-R	AAGGCACAGTCGAGGCTGA
EGFP C F	CATGGTCCTGCTGGAGTTCGTG
EGFP C R	GTTCAGGGGGAGGTGTG
Internal sequencing primers	
CS5 PCNT 2972F	GAGAGCTGGAGGCGATGAG
C6 PCNT 3180F	TCCTTCCAAAGCAGAACGG
C19' PCNT 221F	CCATTACTGACCTGGAGAG
C20' PCNT 1988F	TGCTTTGGAGCCGGTTGT
C21 N1α2 215F	GATCAACAAGCAGTACCG
C22 N1α2 432F	AGCTAGTGTGGACTCCATC
C23 N1α2 618F	CTCCAGAAGTGGCAGCAGT
C24 N1α2 827F	AGGTCAGTCGTCATATC
C25 PCM1-F1	TGTCTGAAGAAGATGGGAGG
C26 PCM1-F2	AGCAGGAAACCATCAGCTTC
C27 PCM1-F3	TCATAGTAATGCACAGTGTG
C28 PCM1-F4	CCTGATCCAGTAGATCCAAC
C32 PCM1-1452F	GATAGCATCAAACTCAGAAC
C30 PCM1-F6	ATATTACAGGATTCACTGGC
C31 PCM1-R1	CTAGTGCACACTGGGTAGAC

Cloning primers (constructs generated by PROTEX)	
C7 PCNT 1F	GTATTTTCAGGGCGCCATGGAAGTTGAGCAAGAGCAG
C8 PCNT 582R	GACGGAGCTCGAATTTCATCACTCGAGTTCATCAACGTGA
C9 PCNT 666F	GTATTTTCAGGGCGCCCAGGCTGAACGGGCCCTTA
C10 PCNT 800R	GACGGAGCTCGAATTTCATCACTGAAGCTGCCTCATTTCAGC
C11 PCNT 800F	GTATTTTCAGGGCGCCGACCAACAGGCAGCCCAGA
C12 PCNT 1200R	GACGGAGCTCGAATTTCATCACAGGGCCAGGCCTGCGC
C13 PCNT 1200F	GTATTTTCAGGGCGCCTCGACAGCTCCGGCGCT
C14 PCNT 1769R	GACGGAGCTCGAATTTCATCAGCCAGCCTGACTGTCGCTGACTT
C15 PCNT 1769F	GTATTTTCAGGGCGCCAGTCTGCAGAGCGAGCTG
C16 PCNT 2627R	GACGGAGCTCGAATTTCATCAGAGGGACCGGGACAGCTGTT
C17 PCNT 2627F	GTATTTTCAGGGCGCCTGCGAGGTGCAGCAGGAGGTCCT
C18 PCNT 2838R	GACGGAGCTCGAATTTCATCACTCCTTCTCTCTCCTGAG
PCM1-LEICS21F	TCCGGACTCAGATCTATGGCCACAGGAGGAGGTCC
PCM1-1089-R	GTCGACTGCAGAATTTCAATGCTGATTTTGCTGGCGCA
PCM1-1063-F	TCCGGACTCAGATCTACTCAGCTAACATGGCAACAG
PCM1-LEICS21R	GTCGACTGCAGAATTTCATATACTCTGGGCTCCCAC
C35 PCM1-331R	GTCGACTGCAGAATTTCAGCCAGATAAGCTACCTGCAGT
C36 PCM1-302F	TCCGGACTCAGATCTGCACTTCTAGCTCTGCAACA
C37 PCM1-573R	GTCGACTGCAGAATTTCACTATTATTAGAAACACACTGT
C38 PCM1-544F	TCCGGACTCAGATCTCCTGTTACTAACATTCGA
C60 PCM1 218F 138	TCCGGACTCAGATCTAAAGCTAGTTCCATGCGGGA
C61 217R PCM1 138	GTCGACTGCAGAATTTCAAGTAATATAATCGCGAATTTGAACA
C46 N1SR69recE-F	TCCGGACTCAGATCTCTGGTAGCCGTGCAGCAGCTTGA
N1SR71recE-F	TCCGGACTCAGATCTATTGGCCAGCGTGAGGAGTTTG
N1SR72 recE-F	TCCGGACTCAGATCTAGTGCCCTAGAGTCACAGATC
N1recE-R	GTCGACTGCAGAATTTCAGAGTGGAGGAGGGCCAT
C85 PCM1 S93A_F	AGATACATGAGTCAGATGGCTGTCCCAGAGCAGGCAGAA
C86 PCM1 S93A_R	TTCTGCCTGCTCTGGGACAGCCATCTGACTCATGTATCT
Cloning primers (self-generated constructs)	
C51 EcoRI_N1a2F	GGACGAATTCTATGGTGGTGGCGGAGGACCTGA
C51R N1a2R_SalI	CGCTGTCGACTCAGAGTGGAGGAGGGCCAT
C48 N1a2-SR1R+KASH	GGACCGACCTGGCCCCGTCTCTTCGATTTTCAGCCTCC
C49 N1a2-KASHF+SR1	GAAAATCGAAGAGACGGGTGGCTCCGATTCCTCCCTT
C52 N1a2-263R+KASH	GGACCGACCTGGCCCAAAATGCTTGAGTCTGCGCAA
C53 N1a2-902F+SR2	AGACTCAAGCATTTTGGTGGCTCCGATTCCTCCCTT
C54 N1a2-505R+KASH	GGACCGACCTGGCCCGTGGTCCCCGGAGGTATTTTTG
C55 N1a2-902F+SR3AD	ACCTCCGGGGACCACGGTGGCTCCGATTCCTCCCTT
C56 N1a2-618R+KASH	GGACCGACCTGGCCCGTTCTGCTTCATCCTCAACTC

C57 N1a2-902F+SR4	AGGATGAAGCAGAACGGTGGCTCCGATTCCTCCCTT
C62 SR3+KASH R	GGACCGACCTGGCCCTGGGAGAGGCAGGCGGATCAG
C63 KASH+SR3 F	GATCCGCCTGCCTCTCCCACGCGGCTTCCTGTTCAGAGTC
C65 (1-31)+SR2 R	CTGCCACAATCGCCACCTTGTGACATCGCAGTTACAATC
C66 SR2+(1-31) F	CTGCGATGTCACAAGGTGGCGATTGTGGCAGAAATTTC
C74 EcoRI_PCM1_1_F	GGACGAATTCTATGGCCACAGGAGGAGGTCCCTTTG
C75 PCM1(NTD)_R+KASH	GGAATCGGAGCCACCATGCTGATTTTGCTGGCGCATAAG TTC
C76 KASH_F+PCM1(NTD)	CAGCAAAATCAGCATGGTGGCTCCGATTCCTCCCTTTCTG
C80 SR3_F+(1-31)	TGCGATGTCACAAGGATTGGCCAGCGTGAGGAGTTTGAG AC
C81 1-31_R +SR3	CTCCTCACGCTGGCCAATCCTTGTGACATCGCAGTTACAA TC
C82 AD_F+(1-31)	TGCGATGTCACAAGGGACGATGAGCACGACCTCTCAGAC
C83 1-31_R +AD	GTCGTGCTCATCGTCCCTTGTGACATCGCAGTTACAATC
C88 EcoRI_SR2F	GGACGAATTCTTGGCGATTGTGGCAGAAATTTCTG
C83 PCM1 S93D_F	AGATACATGAGTCAGATGGATGTCCCAGAGCAGGCAGAA
C84 PCM1 S93D_R	TTCTGCCTGCTCTGGGACATCCATCTGACTCATGTATCT

2.1.3 siRNA oligos

siRNA oligo sequences are listed in Table 2.4. The nesprin-1 oligo was from Thermofisher, whereas PCM1 oligos were from Integrated DNA Technologies.

Table 2.4 siRNA oligo sequences

siRNA	Sequence 5→3'
mNesprin1 92	CAGAGUUGGCCAAGCCCAUAGUCUA
mm.Ri.Pcm1.13.1 (siPCM1(1))	AGUCAGAUUCUGCAACAUGAUCUTG
mm.Ri.Pcm1.13.2 (siPCM1(2))	AAUAGUAUCCCGUAAAGCUUCAAACAU

2.1.4 Cell lines

Cell lines used are listed in Table 2.5.

Table 2.5 Cell lines

Cell line	Supplier
C2C12	ATCC (from Qiuping Zhang)
C25 (WT)	Vincent Mouly (Institute of Myology, Paris)
KM260 (*SYNE1* C.23545 G<T)	Vincent Mouly

U2OS	ATCC
293T	ATCC

2.1.5 Antibodies

Primary and secondary antibodies used for western blots and immunofluorescence staining is listed in Table 2.6 and 2.7. Dilutions used for each application and suppliers are indicated.

Table 2.6 Primary antibodies used for western blots and immunofluorescence staining

Primary antibody	Host	Dilution for WB	Dilution for IF	Supplier	Catalogue number
a-tubulin	Mouse	1/1000	1/200	Sigma	T9026
AKAP450	Rabbit	-	1/100	Sigma	HPA026109
CDK5RAP2	Rabbit	-	1/200	Bethyl	A300-554A
GFP	Mouse	1/1000	1/200	Sigma	G6539
GFP	Rabbit	1/1000	1/500	Abcam	ab6556
Nesprin-1	Rabbit	-	1/400	Didier Hodzic lab	-
Nesprin-1	Mouse	-	1/100	Glen Morris lab	MANNES1A
Nesprin-1	Mouse	1/200	-	Glen Morris lab	MANNES1E
Myc	Goat	-	1/200	Bethyl	A190-104A
Myosin heavy chain	Mouse	-	1/2000	Millipore	05-716
PCM1	Rabbit	1/1250	1/400	Bethyl	A301-149A-T
Pericentrin	Mouse	-	1/400	BD Transduction Laboratories	611814
Pericentrin	Rabbit	1/500	1/750	Biolegend	923701

Table 2.7 Secondary antibodies used for western blot and immunofluorescence staining

Secondary antibody	Dilution for WB	Dilution for IF	Supplier
Anti-mouse-HRP	1/5000	-	Sigma
Anti-rabbit-HRP	1/3000	-	Sigma
Anti-goat-HRP	1/5000	-	Invitrogen
Goat anti-rabbit Alexa Fluor® 488	-	1/500	Invitrogen
Goat anti-mouse Alexa Fluor® 488	-	1/500	Invitrogen
Donkey anti-rabbit Alexa Fluor® 594	-	1/500	Invitrogen
Donkey anti-mouse Alexa Fluor® 594	-	1/500	Invitrogen
Donkey anti-goat Alexa Fluor® 594	-	1/500	Invitrogen

2.1.6 Antibiotics for bacterial growth

A list of antibiotics and the according final working concentration is listed in **Table 2.8**.

Table 2.8 Antibiotic concentrations for bacteria growth in LB media and agar

Antibiotic	Concentration for agar plates (µg/ml)	Concentration for LB media (µg/ml)
Ampicillin	30	100
Kanamycin	50	50

2.2 Cell culture

2.2.1 Cell maintenance

C2C12, U2OS and 293T cells were cultured in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin and passaged at 70-80% confluency. For differentiation of C2C12 myoblasts, cells were washed with phosphate buffer saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.4 mM KH_2PO_4, pH 7.4) before addition of differentiation medium consisting of

DMEM/F12 medium supplemented with 2% horse serum and 1% penicillin/streptomycin. Differentiation was for 5 days with a medium change every 48 hours.

C25 (human WT) and *SYNE1* C.23545 G<T human myoblasts were cultured in KMEM medium consisting of 4 vol DMEM, 1 vol Medium 199 supplemented with 20% FBS, 5 ng/ml hEGF (Life Tech), 0.5 ng/ml bFGF (Life Tech), 0.2 µg/ml dexamethasone (Sigma), 5 µg/ml insulin (Sigma) and 25 µg/ml fetuin (Life Tech). Cells were passaged at 80% confluency. For differentiation, cells were washed with PBS before addition of differentiation media consisting of DMEM/F12 supplemented with gentamycin (50 µg/ml) and insulin (10 µg/ml) for 5 days until harvest.

All cells were maintained at 37°C/5% CO_2 in a humidified incubator on 10 cm plates. For passaging, cells were washed with PBS before incubation with 1 ml 0.05% trypsin-EDTA at 37°C/5% for the detachment of cells from the plate surface. After trypsin inactivation by addition of growth medium, the cell pellet was retrieved from spinning the cell suspension at 244 x g for 5 min and resuspended in fresh cell medium to be added to new plates at the appropriate density.

2.2.2 Cell storage and recovery

Cells were frozen down in liquid nitrogen in FBS + 10% DMSO. For recovery, cells were thawed in a 37°C water bath, spun down with complete medium and the cell pellet was resuspended in 10 ml complete medium and re-plated on a 10 cm plate.

2.2.3 Cell counting

The cell density of the resuspended pellet was calculated when seeding myoblasts onto glass coverslips. 10 µl of the cell suspension was pipetted onto a

haemocytomer, and cells were counted at the four corner squares. The following equation was used to work out the cell density:
Cell density (cells/ml) = average number of cells per square x 10,000

The appropriate number of cells was seeded with volumes made up accordingly with complete medium. For each well in a 24-well plate, 2.5 x 10^4 C2C12 cells, 6.5 x 10^4 C25 or KM260, and 2.0 x 10^4 U2OS cells were seeded.

2.2.4 Collagen coating of coverslips

Collagen coated coverslips were used for culturing of myotubes for immunofluorescence microscopy. Glass coverslips measuring 13 mm in diameter were incubated for 1 h at room temperature in rat-tail collagen (Corning) diluted to 50 µg/ml in 0.02 N acetic acid, washed with PBS then air-dried.

2.2.5 Transient transfection of plasmids

C2C12, C25 and *SYNE1* C.23545 G<T cells were transfected using lipofectamine 3000 (Invitrogen). For transfection of myoblasts, cells were seeded, transfected the next day, and harvested after another day. For transfection of C2C12 myotubes, myoblasts were differentiated the day after transfection, for 5 days. For transfection of human C25 and *SYNE1* C.23545 G<T myotubes, myoblasts were seeded, then differentiated the next day. On the fifth day of differentiation, myotubes were transfected and harvested the next day. For transfection of cells in a 24-well plate, 1.5 µl lipofectamine 3000 was diluted in 25 µl Opti-MEM. 1 µl p3000 and 0.5 µg plasmid was diluted in another 25 µl Opti-MEM. Both mixtures were then mixed well together and incubated for 20 mins to form liposome-DNA complexes before addition to cells.

U2OS cells were transfected using lipofectamine 2000. For transfection in a 24-well plate, 0.8 µl lipofectamine was diluted in 25 µl Opti-MEM, and 0.2 µg DNA was

diluted in another 25 µl Opti-MEM. The solutions were mixed together, incubated for 20 minutes before addition to cells. Cells were fixed after 24 h.

293T cells were transfected using polyethylenimine (PEI; Sigma). For transfection in a 6-well surface, 2 µg DNA was diluted in 100 µl PBS. After vortexing, 5 µl PEI was added to the mixture, followed by another vortex. After 20 mins incubation, the mixture was added to the cells. Cells were harvested after 24 h.

Volumes of reagents used for transfections in 6-well plates were multiplied by 5, whereas transfections in 10 cm plates were multiplied by 20.

2.2.6 RNAi knockdown of proteins in myotubes

For depletion of proteins in C2C12 myotubes, cells were seeded on glass coverslips, and depending on the experiment, either transfected the next day, then differentiated after another day; or differentiated the next day and transfected after a further 48 h. For each transfection in a 24-well plate, 0.4 µl 20 µM siRNA and 2 µl RNAiMax Lipofectamine (Invitrogen) were first diluted separately in 30 µl opti-mem, then combined and incubated for 5 min before addition drop-by-drop to cells. Differentiation medium was replaced every 48 h. After incubation for the appropriate number of days, cells were fixed and permeabilised -20˚C methanol.

2.2.7 Nocodazole treatment of cells

For short-term depolymerisation of microtubules in myotubes, nocodazole (Sigma) was added to cells at a final concentration of 5 µg/µl for between 30 min and 120 min. Cells were then fixed in -20˚C methanol.

To culture and differentiate myoblasts in the absence of microtubules, the growth medium of myoblasts was changed, 4 h after seeding to differentiation medium with 0.5 µg/µl nocodazole. Cells were differentiated in this medium for 48 h before fixation in -20˚C methanol.

2.2.8 Centrinone treatment of cells

U2OS cells were seeded on glass coverslips the day before treatment. The next day, centrinone (MedChemExoress) was added to pre-warmed cell medium at 100 nM and filter-sterilised before adding to the U2OS cells. After 48 h of drug treatment, cells were fixed in -20˚C methanol.

2.3 Protein analysis

2.3.1 Generation of cell lysates for western blot

For the generation of cell lysates for western blotting, cells grown on a 6-well plate were trypsinised and centrifuged at 244 x g for 5 mins to retrieve the cell pellet. After a PBS wash, the cell pellet was lysed in 30 µl RIPA buffer (50 mM tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 1% triton x-100, 0.1% SDS, supplemented with 1x protease inhibitor cocktail (Roche), 1 mM PMSF, 5 mM sodium fluoride and 50 mM beta-glycerophosphate) and incubated on ice for 30 min before centrifugation at 13,000 rpm in a microcentrifuge for 10 min at 4˚C to isolate the clarified cell lysate.

2.3.2 BCA assay

The Bio-Rad BCA assay was used to measure the protein concentration of clarified cell lysates according to the manufacturer's protocol. Briefly, diluted protein samples and BSA solutions of known concentrations were incubated for 15 min with the assay reagents before measuring at an absorbance of 650 nM. A standard curve was produced from the BSA samples and used to determine the protein concentration of unknown samples.

2.3.3 Immunoprecipitation by GFP-Trap beads

For immunoprecipitation, cells were cultured and harvested from 10 cm plates by scraping in cold PBS. After centrifugation at 244 x g for 5 mins, the pellet was resuspended in lysis buffer (20 mM Tris pH 7.4, 100 mM NaCl, 1% NP-40 10% glycerol, 5% EDTA, supplemented with 1x protease inhibitor cocktail (Roche), 1 mM PSMF, 5 mM sodium fluoride and 50 mM beta-glycerophosphate) and incubated on ice for 30 min, followed by sonication 3x 15 secs at amplicon 2 with 30 sec pauses in between. Solubilised proteins were retrieved by centrifugation at 13,000 rpm in a microcentrifuge, for 10 min at 4˚C. Lysates were incubated overnight with 10 µl GFP-Trap (ChromoTek) beads on a rotating wheel. Beads were washed three times with lysis buffer at 500 x g for 5 min before resuspension in 60 µl sample buffer and boiled at 95˚C for 5 min. Storage was at -20˚C.

2.3.4 SDS-PAGE

Handcast gels were made at the appropriate percentage (Table 2.9) and run at 170 V for 60-90 min submerged in SDS buffer (25 mM tris, 192 mM glycine, 0.1% SDS), using the Mini-PROTEAN® 3 Cell system. 3 µl of protein marker (Thermofisher pre-stained plus) was used alongside protein samples. To stain proteins directly, the gel was incubated in Instant Blue (Expedeon). For western blotting, gels were prepared for the transfer of proteins onto nitrocellulose membranes.

Table 2.9 Components for resolving and stacking gel

	Resolving gel			**Stacking gel**
Gel concentration	6%	7.5%	10%	3%
Protogel	1.2 ml	1.5 ml	2.0 ml	325 µl
Lower buffer pH 8.8	1.5 ml	1.5 ml	1.5 ml	
Upper buffer pH 6.8				625 µl
dH_2O	3.3 ml	3.0 ml	2.5 ml	1.5 ml
10% APS	75 µl	75 µl	75 µl	75 µl
TEMED	5µl	5µl	5µl	5µl

2.3.5 Chemiluminescent Western blot

Semi-dry transfer was done when probing proteins less than 220 kDa. The gel and nitrocellulose membrane with a pore size of 0.45 µm (Amersham) were soaked in blotting buffer (25 mM Tris, 192 mM glycine, 10% v/v methanol) before blot sandwich assembly on the Amersham ECL Semi-Dry blotter, according to the manual. Transfer was run for 1 h at 70 mA per gel using the Amersham TE 70 semi-dry blotting transfer unit.

Wet transfer was used for probing proteins greater than 220 kDa. The gel and membrane were soaked in transfer buffer (50 mM Tris, 384 mM glycine, 0.05% SDS, 10% methanol) for 15 min before sandwich assembly. Transfer was run at 25 V, 14 h at 4˚C.

After transfer, Ponceau S staining was used to check for the presence of protein on the membrane. The membrane was then blocked in 5% milk/TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6) for 1 h before incubation with primary antibody (dilutions listed in Table 2.6) in blocking solution. The membrane was washed 3x 5 min in TBST before incubation with a HRP conjugated secondary antibody in blocking solution (dilutions listed in Table 2.7). After washing 4x 10 min, the membrane was incubated in ECL reagent for 1-5 min according to the manufacturer's manual and visualised using X-ray films and an automated film processor.

2.3.6 Mass spectrometry

15x 10 cm plates of C2C12 myoblasts were grown and transfected using 30 µl lipofectamine 3000, 20 µl p3000 and 10 µg DNA per plate as described in section 2.2.4. For analysis of myoblast proteins, cells were harvested the next day by scraping on ice in PBS. For myotube proteins, cells were differentiated the next day and cells were harvested after 3 days of differentiation.

Cell pellets were collected and lysed in 5 ml RIPA buffer and prepared as on 2.3.1, but using 60 µl GFP-Trap beads and using 5 ml RIPA buffer per wash, 5 times.

Proteins were run on 10% handcast gels and stained overnight with Instant Blue (Expedeon). In one experiment, gel sections were cut out and digested by the PNACL service at the University of Leicester, ready to be used for mass spectrometry. In another experiment, gel excision and protein digest was performed in the lab using an In-Gel Tryptic Digestion Kit (Thermo Scientific) according to the protocol. Briefly, gel pieces excised with a clean scalpel were destained in acetonitrile and ammonium bicarbonate until clear. TCEP and iodoacetamide were used as the reduction and alkylating reagent. Gel pieces were digested with trypsin in digestion buffer overnight. The product was submitted for LC-mass spectrometry by the Proteomics Research Technology Platform, at the University of Warwick. Results were analysed using the Scaffold4 software.

2.3.7 Immunofluorescence staining and imaging

Sterile glass coverslips were used for immunofluorescence microscopy. For their preparation, coverslips were rinsed 3x in dH_2O and incubated in 1 M HCl for 30 mins. After rinsing 3x in dH_2O, coverslips were incubated in 100% ethanol for 30 min before being air-dried and baked for 3h, 250˚C. Cells were seeded on cooled coverslips.

All cells grown on glass coverslips were fixed by -20˚C methanol for at least 20 mins at -20˚C. Cells were then rinsed in PBS 3x 5 mins, and then blocked for 30 min in 1% BSA/PBS. Primary antibody solutions were made in 3% BSA/PBS and incubated on coverslips for 1 h, followed by 3x 5 min PBS washes. Secondary antibody (Table 2.7) solutions were made at 1/500 dilutions in 3% BSA/PBS, with 0.5 µg/ml DAPI. Following 3x 5 min PBS washes, coverslips were mounted on microscope slides on mounting medium (80% glycerol, 3% n-propyl gallate) and sealed with nail varnish.

Images were taken using the VisiTech Infinity3 confocal laser microscope using the acquisition software Voxcell. Images were processed using FIJI.

2.4 Molecular techniques

2.4.1 Cloning

Recombination-based cloning in pLEIC vectors was performed by the University of Leicester cloning service PROTEX (see Table 2.2), by providing a DNA template alongside a pair of primers (see Table 2.3), containing sequence complementary both to the vector of interest and the insert.

Constructs self-generated in the lab (see Table 2.2) was by overlap PCR and ligation, where four primers were used to generate each construct (see Table 2.3). For PCR amplification of the desired insert, a 5' outer primer was designed with an ECoRI restriction site followed by the starting sequence of the insert. The 3' outer primer was designed with the 3' sequence of the insert flanked by a SalI restriction digest site. These were paired with overlapping internal primers to firstly amplify the desired sequences to be fused in a 10 µl PCR reaction. Then, only the outer primers were used for the second overlapping PCR reaction to generate the whole insert in a 25 µl reaction. For the DNA template in the second PCR, 0.5 µl of each PCR product were diluted in 600 µl, and 1 µl of this dilution was used. PCR reactions were performed using KOD polymerase (Millipore) according to the manufacturer's protocol.

The PCR product containing the insert was purified using a gel extraction kit (Qiagen) according to the manufacturer's protocol. Then, co-digestion reactions with ECoRI and SalI (Fermentas) for the purified PCR product and pEGFP-C1 vector were set up separately and performed at 37°C overnight. After column PCR purification (Qiagen), the digested vector and insert were ligated using DNA ligase (Fermentas) in a total volume of 10 µl (Fermentas) using a vector:insert molar ratio of 1:3 for 30 min at 22°C. 1 µl of the ligation reaction was used for

transformation into 50 µl DH5α cells, as detailed in 2.4.5. All new constructs were sequenced (section 2.4.4) using primers (see table 2.1.2) covering the whole insert.

For site directed mutagenesis, 10 ng template plasmid was used in a 10 µl PCR reaction, using KOD polymerase with overlapping primers containing the required mutation. 0.2 µl DpnI was added to the resulting reaction and incubated 2 h at 37°C to digest the template DNA. Transformation was subsequently performed as in section 2.4.3.

2.4.2 DNA agarose gels

Agarose gels were made by dissolving agarose powder in 1x TBE (0.1 M tris, 0.1 M boric acid, 2 mM EDTA) followed by the addition of ethidium bromide at 1/15,000. Once set, gels were run at 80 V in 1x TBE buffer for at least 20 min or until adequate gel separation.

2.4.3 Bacterial transformation

Competent *E. Coli* DH5α cells were thawed on ice and 50 µl was incubated with 1 µl ligation reaction for cloning, or plasmid for plasmid amplification for 30 min followed by heat shocking for 42°C, 45 sec and recovery on ice for 2 min. 400 µl LB or SOC medium was added the cells and incubated shaking at 210 rpm, 37°C for 1 h before plating on LB + Kan (50 µg/ml) or LB + Amp (50 µg/ml) agar. Plates were incubated overnight at 37°C.

2.4.4 Sequencing

For sequencing reactions to be run by PNACL at the University of Leicester, the following PCR reaction was set up for each sample:

BigDye terminator v3.1	0.5µl
5x buffer	1.75 µl
5 µM primer	0.75 µl
Plasmid	200 ng
dH_2O	to 10 µl

and performed at the following conditions for 28 cycles:

96°C 10 sec
50°C 5 sec
60°C 4 min

The PCR product was cleaned using gel cartridges (EdgeBio), which were firstly prepared for use by spinning at 3000 rpm for 3 min in a benchtop microcentrifuge. The PCR reaction mix was applied to the gel and centrifuged at again at 3000 rpm for 3 min to remove unincorporated dye terminators. The resulting sequencing sample was submitted to PNACL to be read using a 3730 automated sequencer.

Alternatively, plasmids and primers were sent to Source Biosciences (Nottingham), who performed the entire process.

DNA sequences were aligned to the expected sequence using the Ape1 software.

2.4.5 Midi and maxi prep

Maxi-preps were done kit-free. A 5 ml starter culture was grown for 8 hours and the whole volume was used to inoculate an overnight 250 ml culture at 210 rpm, 37˚C. In the morning, cells were spun down at 3500 rpm for 20 min in an ultracentrifuge. All subsequent spins were performed under the same conditions unless otherwise stated. The pellet was resuspended in 25 ml resuspension buffer (Tris pH8, 5 mM EDTA), then lysed with 8 ml lysis buffer (1% SDS, 0.2 M NaOH), and incubated at room temperature for 5 min. Next, 8 ml neutralisation buffer (3M potassium acetate, pH 5.5) was added and mixed by inversion to precipitate proteins. After centrifugation at the condition previously stated, the supernatant was passed through a piece of miracloth to remove precipitates. Then, 0.7 vol isopropanol was added for DNA precipitation and the resulting pellet after centrifugation was washed with 70% v/v EtOH before resuspension in 2 ml tris-EDTA (TE; 10 mM tris pH 8, 1 mM EDTA). RNA was precipitated by addition of 2.5 ml 5M LiCl. After centrifugation the resulting supernatant was subjected to isopropanol precipitation as before. After another 70% EtOH wash the pellet was

resuspended in 500 µl TE, and incubated with 50 µg RNase (Invitrogen) at 37˚C for 20 min with shaking to remove contaminating RNA. An equal volume of 13% PEG 8000/1.6M NaCl was added and incubated on ice for 30 min to precipitate DNA, which was spun down at 13,000 rpm in a benchtop microcentrifuge at 4˚C. The DNA was resuspended in 800 µl TE and phenol/chloroform extracted, and centrifuged at 14,000 rpm for 10 min. The upper phase was split into 2 tubes. For each, sodium acetate DNA precipitation was performed by addition of 0.1 vol 3M sodium acetate and 2.5 vol 100% EtOH. The DNA was recovered by spinning at 14,000 rpm, 5 min at 4˚C and washed with 1 ml 70 % EtOH. The pellets were each resuspended in 100 µl TE, combined and the DNA concentration was measured using a spectrophotometer.

For midi-preps, an overnight colony was added to 50 ml LB and the appropriate antibiotic to prepare an overnight culture. DNA purification was performed using the NucleoBond® Xtra Midi (Macherey-Nagel) or ZymoPURE™ Plasmid Midiprep Kit according to the manufacturer's manual, or kit free as with the maxi-prep protocol with reagent volumes scaled down accordingly to 20% of the original volumes.

For mini-preps, a colony was picked and grown in 3 ml LB and the appropriate antibiotic overnight. DNA was extracted using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) or GenElute Plasmid DNA Miniprep Kit (Sigma-Aldrich), according to the manufacturer's protocol.

2.5 Statistical analysis

Graphpad Prism 8 was used for data analysis. The one-way analysis of variance (ANOVA) was used to determine a statistical difference between means of independent experiments between 3 or more groups. The Turkey's post hoc test was used to determine statistical differences between each group. A statistical significance was defined by p-values < 0.05.

Chapter 3

Characterising the dependence of centrosomal components on nesprin-1 for their localisation to the NE

3.1 Introduction

Nesprins form the cytoplasmic facing component of the LINC complex and can bind different cytoskeletal filaments for different cellular processes. Myotubes express two nesprin-1 isoforms: the full length nesprin-1G and nesprin-1α2, by an internal promoter in the *SYNE1* gene (Zhang *et al.,* 2001). Nesprin-1G contains two N-terminal actin-binding CH domains, 74 SRs with an adaptive domain (AD) separating SR71 and SR72, and the NE-localising KASH domain at the C-terminus (Fig. 1.6). Nesprin-1α2 is a muscle specific isoform, which becomes expressed in myoblasts upon commitment to differentiation into multinucleated myotubes (Duong *et al.,* 2014; Holt *et al.,* 2016). It lacks the CH domains and first 68 SRs of the full-length protein, and instead contains an isoform specific 31 amino acid sequence followed by the C-terminal 6 SRs, AD and KASH domain of nesprin-1G (Fig. 1.6). The requirement of nesprin-1 isoforms in muscle development is illustrated by nuclear mispositioning in cultured nesprin-1 null myotubes (Espigat-Georger *et al.,* 2016) and defects in nuclear positioning and anchorage in muscle fibres from nesprin-1 knockout mice (Zhang *et al.,* 2009a).

Upon myogenesis, centrosomes disassemble and centrosomal proteins such as PCM1, pericentrin and AKAP450, along with microtubule-motor proteins kinesins and dynein relocalise to the surface of the NE. The transfer of centrosomal proteins, which contain MTOC properties, to the NE forms the nuclear MTOC (nMTOC). Microtubules nucleated from myonuclei are used by motor proteins to move a new nucleus to the centre of the nascent myotube, and later for myonuclei to spread along the myotube length. In nesprin-1 null myotubes, PCM1, pericentrin and AKAP450 and motor proteins fail to be recruited to the NE, and myonuclei become clustered (Gimpel *et al.,* 2017; Espigat-Georger *et al.,* 2016). This shows that nesprin-1 isoforms are required for the localisation of MTOC and motor proteins to the NE. In contrast, nesprin-2 depletion in myotubes does not affect pericentrin recruitment to the NE (Chang *et al.,* 2015a).

Nesprin-1α2 is thought to be the isoform responsible for recruiting and binding nMTOC associated proteins. Firstly, its expression and localisation to the NE upon

myogenesis coincides with the NE localisation of centrosomal proteins mentioned, along with CDK5RAP2, γ-tubulin and others (Srsen *et al.,* 2009; Gimpel *et al.,* 2017; Espigat-Georger *et al.,* 2016), and microtubule motor proteins (Espigat-Georger *et al.,* 2016) . Secondly, nesprin-1α2, but not nesprin-1G knockout mice show muscular dysfunction, mislocalisation of the kinesin-1 component kinesin heavy chain (KHC) and nuclear mispositioning in muscle fibres (Stroud *et al.,* 2017). Additionally, exogenous expression of mouse nesprin-1α is sufficient to rescue pericentrin localisation at the NE in myotubes depleted of nesprin-1 isoforms (Gimpel *et al.,* 2017).

The details of how nesprin-1, or nesprin-1α2 recruits centrosomal proteins to the myonuclear envelope is yet to be explored. However, depletion of PCM1 in myotubes leads to reduced localisation of pericentrin at the NE, indicating that nesprin-1α2 recruits PCM1, which acts a scaffold itself to facilitate recruitment of further MTOC proteins (Gimpel *et al.,* 2017; Espigat-Georger *et al.,* 2016).

How microtubule motor proteins kinesin and dynein are recruited to the NE is better defined. Kinesin-1 is recruited to the NE via interaction of its light chain components, KLC1/2 with a conserved KLC-binding region, the LEWD motif, which is present in the adaptive domain (AD) of nesprin-1, 2 and 4. Indeed, bacteria expressed GST-nesprin-1α interacts with KLC1/2 from myotube extracts, but this is abolished upon mutation of the nesprin-1α LEWD motif to LEAA (Zhou *et al.,* 2017). Nesprin-2 fragments containing the AD also interact with KLC2, but not upon LEWD/LEAA mutation (Wilson and Holzbaur, 2015). Furthermore, PCM1 depletion in myotubes leads to a reduction of KLC1/2, in addition to pericentrin, at the NE (Espigat-Georger *et al.,* 2016; Gimpel *et al.,* 2017), suggesting a relationship between PCM1 and kinesin-1 in addition to their dependency on nesprin-1. Dynein also interacts with nesprin-2 fragments containing the AD, though the exact residues involved have not yet been elucidated (Zhu *et al.,* 2017).

The aim of this chapter was to characterise how nesprin-1, specifically nesprin-1α2 acts as a centrosome/MTOC protein receptor. Firstly, nesprin-1 and centrosomal protein antibodies were tested on myoblast and myotube cell lines for

use in microscopy and western blot examine localisation and expression. Then, rescue experiments were used to confirm that nesprin-1α2 is sufficient for localisation of PCM1 and AKAP450 to the NE in myotubes. Finally, expression of various nesprin-1α2 truncations and mutant constructs in nesprin-1 null myotubes were explored, to investigate the nesprin-1α2 domains and residues required for the recruitment of PCM1 and AKAP450 to the NE.

3.2 Results

3.2.1 Identifying nesprin-1α2 as the centrosomal protein receptor

The aim here was to show that nesprin-1 isoforms are required for the recruitment of centrosomal proteins to the NE in myotubes, and that nesprin-1α2 alone is sufficient for this.

3.2.1.1 Nesprin-1 and centrosomal protein expression and localisation in C2C12 myoblasts and myotubes

To show the change in localisation of nesprin-1 and centrosomal proteins during myogenesis, nesprin-1 and centrosomal protein antibodies were used to stain C2C12 myoblasts, an immortalised mouse cell line, and again upon differentiation for 5 days into myotubes. The nesprin-1 antibody was generated to target a region shared by nesprin-1G and nesprin-1α2, but only recognises nesprin-1α2 in immunofluorescent microscopy (Gimpel *et al.,* 2017). In myoblasts nesprin-1 staining was at the centrosomes (Fig. 3.1), thought to be a cross reaction as previously seen in HeLa cells (Randles *et al.,* 2010). PCM1 and pericentrin were at the centriolar satellites and centrosomes, respectively, whereas the AKAP450 antibody did not stain C2C12 myoblasts. Upon differentiation into myotubes, NE staining of nesprin-1 at the NE became apparent (Fig. 3.2), coinciding with the localisation of PCM1 and pericentrin proteins to the NE. AKAP450 and CDK5RAP2 were also localised at the NE in myotubes. AKAP450 puncta staining was also observed in the cells, which may indicate its association with Golgi elements in myotubes.

To confirm that a change in nesprin-1 isoform expression accompanies the change in localisation, whole cell myoblast and myotube extracts were prepared with RIPA buffer and detected by western blot (Fig. 3.3). Nesprin-1G is >1000 kDa and was difficult to detect in western blots, with a degradation product of 300-400 kDa being more prominent. Consistent with this, a strong band corresponding to the

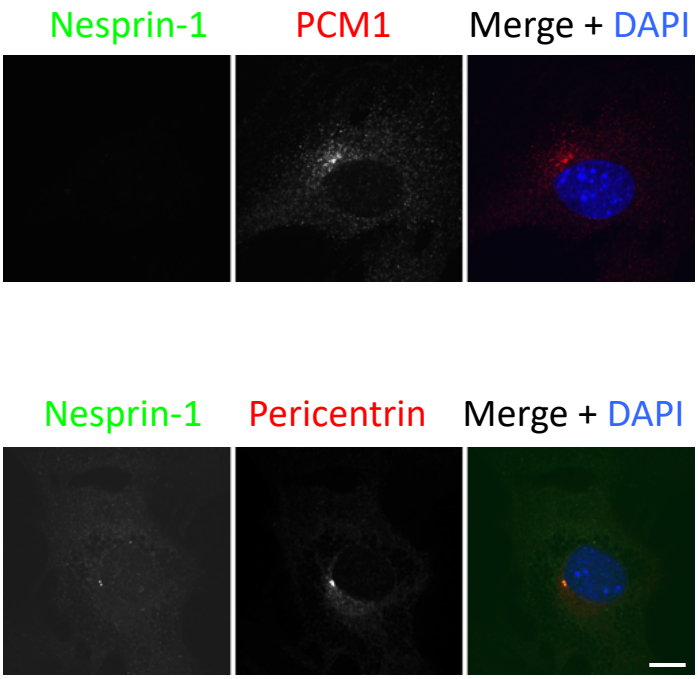


Figure 3.1 PCM1 and pericentrin are localised at the centriolar satellites and centrosomes, respectively, in C2C12 myoblasts

C2C12 myoblasts were fixed in -20°C methanol and co-stained with antibodies against nesprin-1 (green) and either PCM1 or pericentrin (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

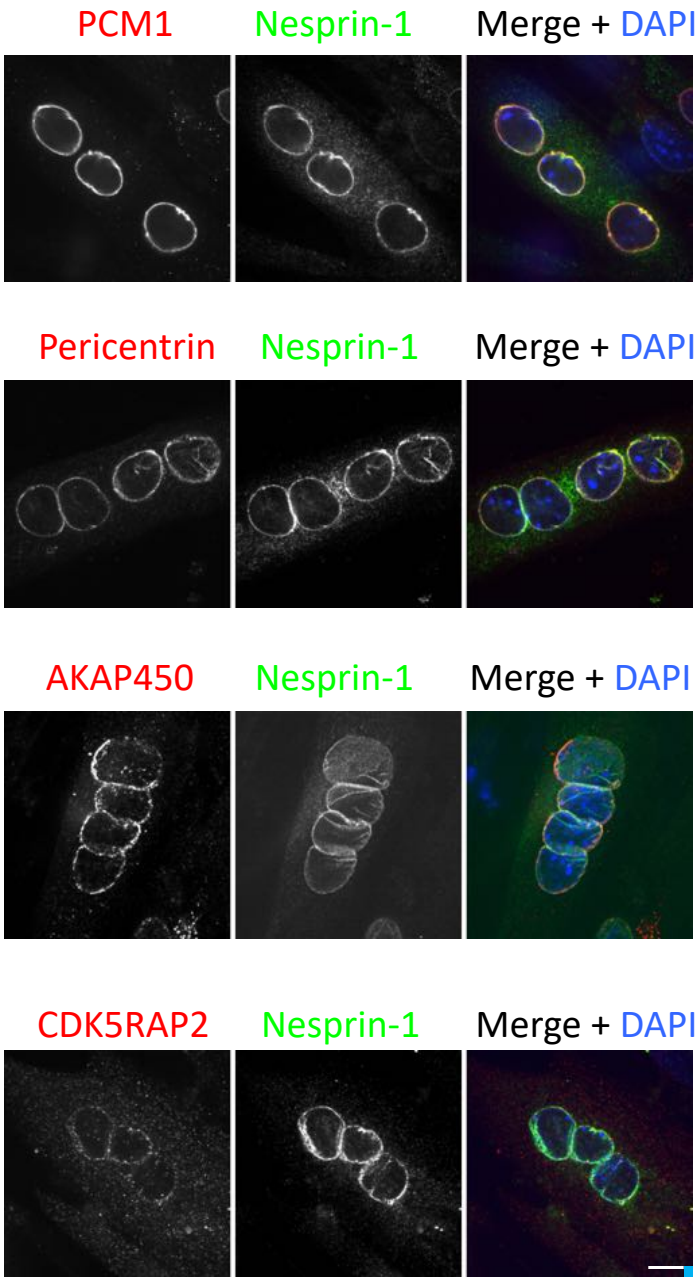


Figure 3.2 PCM1, pericentrin, AKAP450, CDK5RAP2, and nesprin-1 are localised around the NE in myotubes

C2C12 myotubes differentiated for 5 days were fixed in -20°C methanol and co-stained with antibodies against nesprin-1 (green) and either PCM1, pericentrin, AKAP450 or CDK5RAP2 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

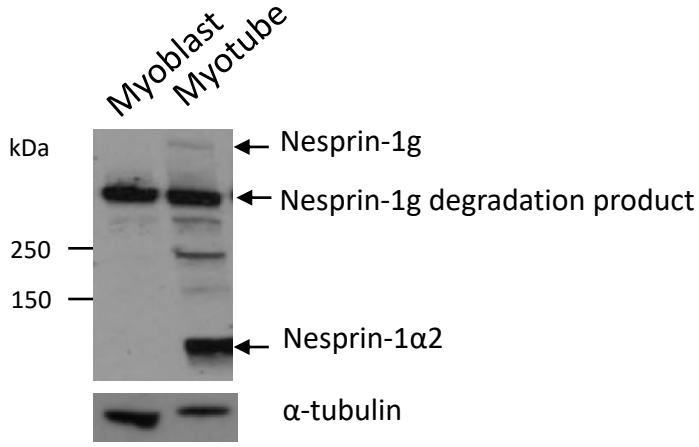


Figure 3.3 Nesprin-1G is expressed in myoblasts and myotubes, whereas nesprin-1α2 is only expressed in myotubes

C2C12 myoblasts and 5 day old myotubes were lysed with RIPA buffer, and 40 μg of protein extracts were immunoblotted with nesprin-1 and α-tubulin antibodies.

degradation product was observed in both myoblast and myotubes, whereas nesprin-1G was only faintly detected in myotubes. A smaller 112 kDa band was detected only in myotubes, consistent in size with the muscle specific nesprin-1α2 isoform. This is also in agreement with the detection of nesprin-1α2 mRNA during myogenesis (Duong *et al.,* 2014).

3.2.1.2 Nesprin-1 isoforms are required for the recruitment of centrosomal proteins to the NE during myogenesis

To confirm the requirement of nesprin-1 for centrosomal protein localisation at the NE, nesprin-1 isoforms were depleted in C2C12 myotubes by RNA interference with siRNA oligos previously confirmed in the lab. Immunofluorescence microscopy showed that knockdown of nesprin-1 from the NE led to mislocalisation of PCM1, pericentrin and AKAP450 from the NE (Fig. 3.4). Nesprin-1 knockdown was performed 48 hours after differentiation yet many cells already express nesprin-1α2 and PCM proteins at the NE after 24 h. This shows that PCM proteins already at the NE must become mislocalised after nesprin-1 depletion, showing that nesprin-1 is required for continued anchorage of proteins at the NE. The centrosomal proteins became mislocalised throughout the cytoplasm, possibly bound to Golgi fragments, which has previously been show to also become mislocalised in nesprin-1 null myotubes (Gimpel *et al.,* 2017).

The *SYNE1* (23545 G>T) human myoblast cell line carries a homozygous mutation resulting in an early stop codon. This leads to mRNA instability of both nesprin-1 isoforms, and as a result the cells are effectively nesprin-1 null (Holt *et al.,* 2016). To verify the requirement of nesprin-1 for PCM protein recruitment to the NE, wildtype (WT) and *SYNE1* (23545 G>T) myoblasts and myotubes were stained with antibodies against nesprin-1, PCM1, AKAP450, CDK5RAP2, in addition to myosin heavy chain (MHC) as a myotube marker (Fig. 3.5). Pericentrin was not explored as pericentrin antibodies used in this study do not target pericentrin in human myotubes.

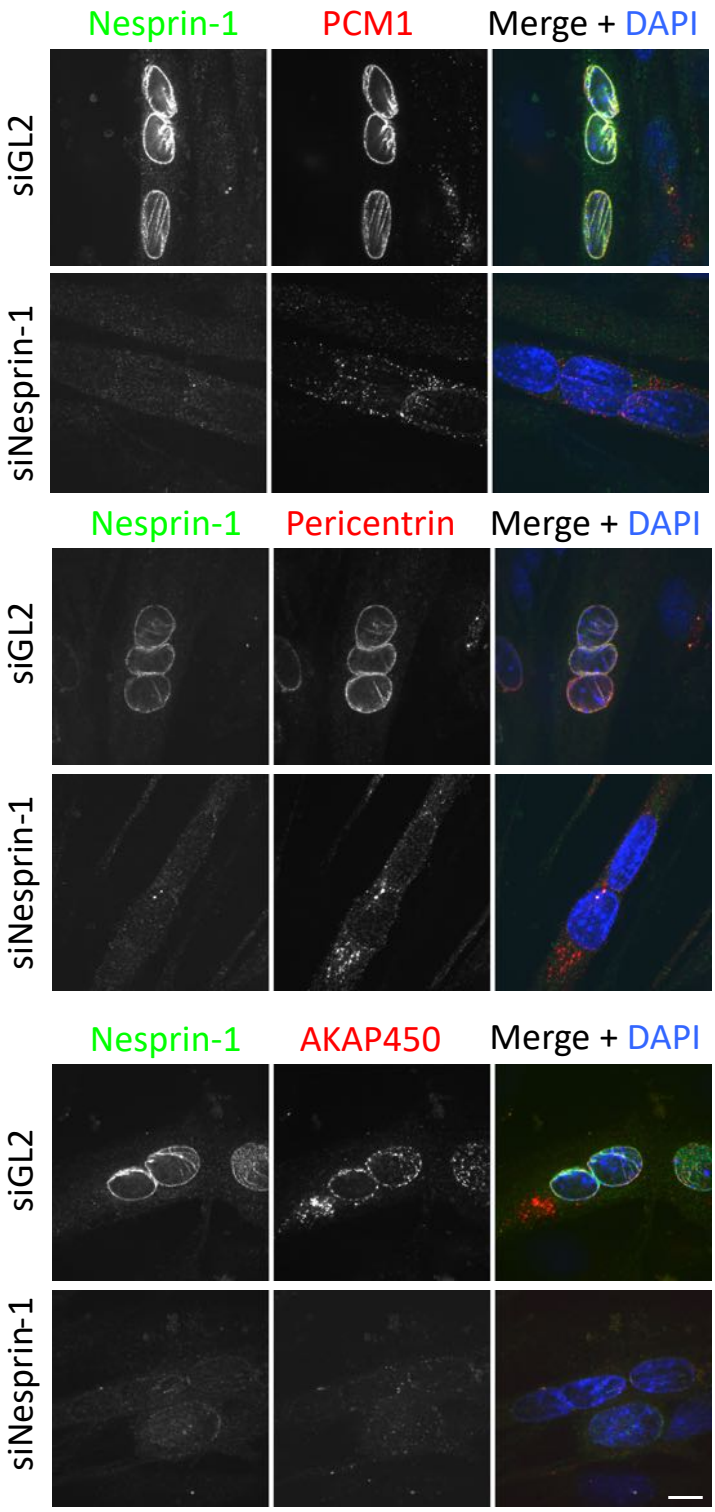


Figure 3.4 Nesprin-1 is required for the localisation of PCM1, pericentrin and AKAP450 to the NE

C2C12 myoblasts were differentiated for 2 days, then were transfected with siGL2 or siNespin-1 oligos and differentiated for 4 further days. Cells were fixed in -20°C methanol and co-stained with antibodies against nesprin-1 (green) and PCM1, pericentrin or AKAP450 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

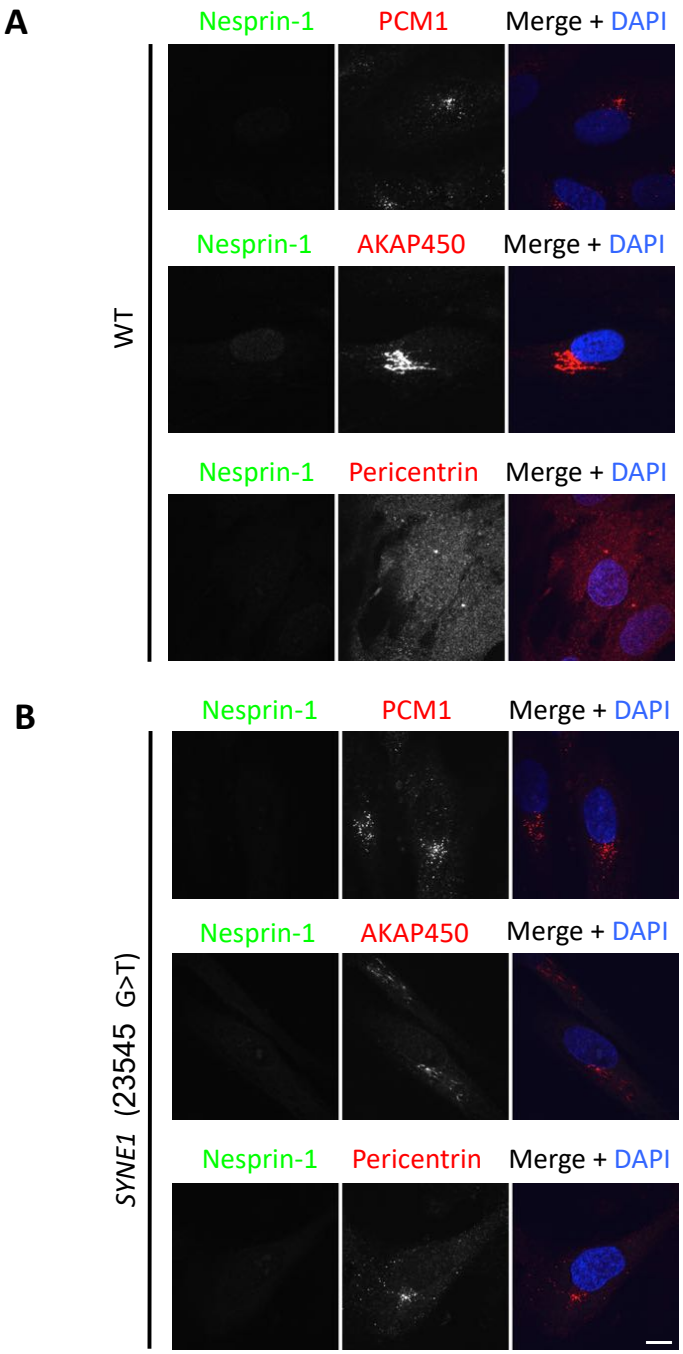


Figure 3.5 PCM1, AKAP450 and pericentrin protein localisation is unaffected in SYNE1 *(23545 G>T)* myoblasts

Wildtype (A) and *SYNE1* (23545 G>T) (B) myoblasts were fixed in -20°C methanol and co-stained with antibodies against nesprin-1 (green) and either PCM1, AKAP450 or pericentrin (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

Nesprin-1 and centrosomal proteins were previously shown to be mislocalised in *SYNE1* (23545 G>T) myotubes (Gimpel *et al.,* 2017). In myoblasts, nesprin-1 staining by the MANNES1A antibody (epitope shown in Fig. 1.6) was not observed in the wildtype or *SYNE1* (23545 G>T) cells (Fig. 3.5). Nesprin-1 staining at the human myoblast NE using the same antibody was previously reported (Holt *et al.,* 2016). The difference in results may be due to differences in fixation. Localisation of PCM1, pericentrin and AKAP450 is the same in myoblasts in both cell lines, showing that nesprin-1 is not required for localisation of centrosome proteins to centrosomes and centriolar satellites in myoblasts. In contrast, whilst nesprin-1 and all PCM proteins tested were at the NE of WT myonuclei, they were absent from the NE of *SYNE1* (23545 G>T) myonuclei (Fig. 3.6), showing that nesprin-1 is responsible for PCM protein localisation to the NE. Again, AKAP450 puncta staining in the cells may indicate its association with Golgi elements, and mislocalised PCM1 and CDK5RAP2 in *SYNE1* (23545 G>T) myonuclei may now also be associated with cytoplasmic Golgi fragments (Gimpel *et al.,* 2017).

As expected, nesprin-1 isoforms were detectable by western blot in the WT myoblast and myotubes, but not *SYNE1* (23545 G>T) cells (Fig. 3.7). PCM1 and pericentrin protein expression levels were unaffected in *SYNE1* (23545 G>T) myotubes, indicating that whilst absence of nesprin-1 impairs their recruitment to the NE, this does not regulate the stability of centrosomal proteins. The AKAP450 and CDK5RAP2 antibodies did not work for western blotting.

3.2.1.3 GFP-nesprin-1α2 expression in nesprin-1 null myotubes is sufficient for the recruitment of PCM1 and AKAP450 to the NE

As both nesprin-1α2 expression and nMTOC formation occur only in myotubes, this suggests that nesprin-1α2 is responsible for centrosomal protein recruitment to the NE. In nesprin-1 depleted myotubes, mycBirA*-nesprin-1α expression was shown to be sufficient to rescue pericentrin to the NE (Gimpel *et al.,* 2017). To test whether the short nesprin-1α2 isoform is sufficient for recruiting further MTOC proteins to the NE, GFP-nesprin-1α2 was expressed in *SYNE1* (23545 G>T)

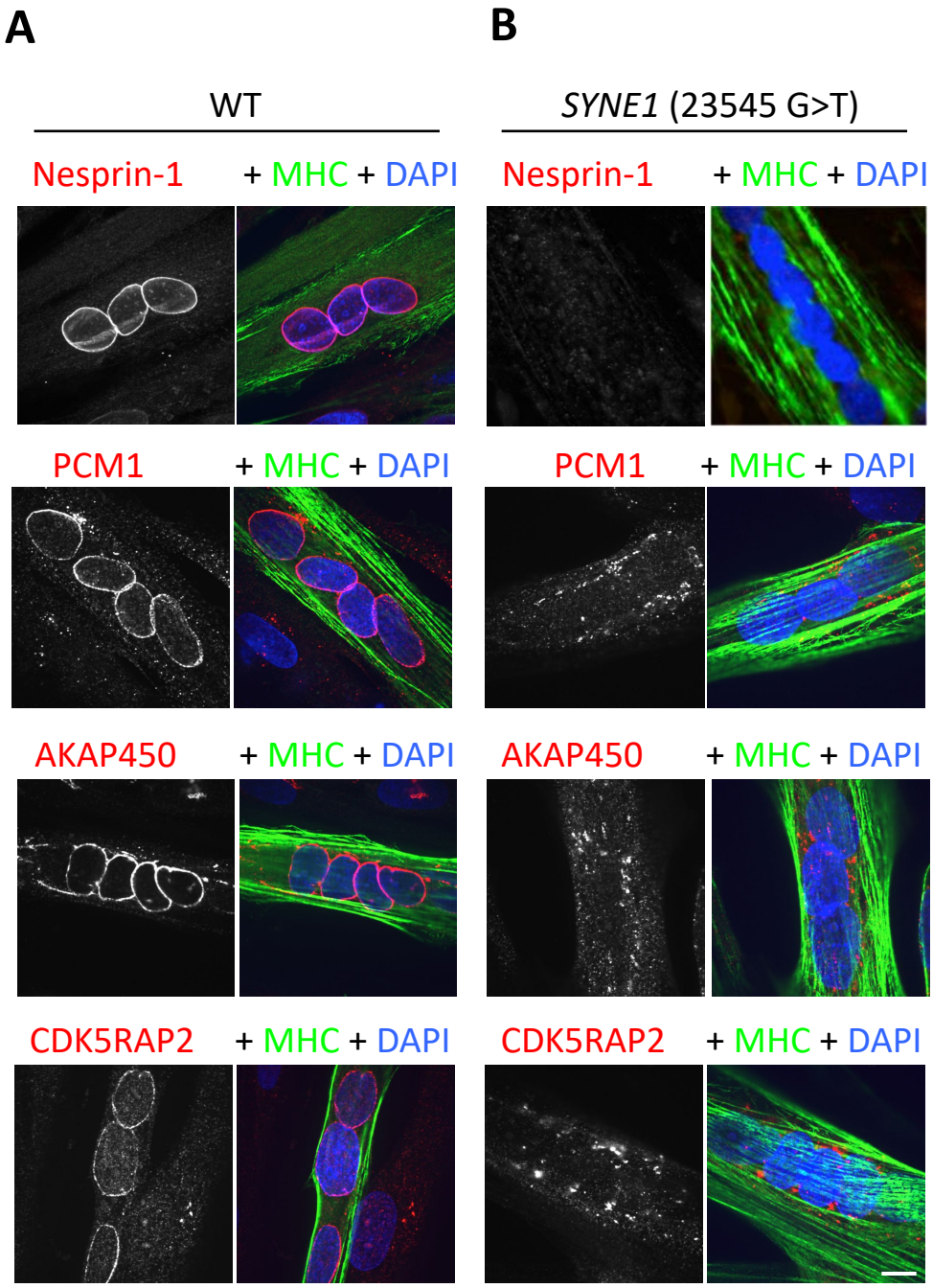


Figure 3.6 PCM1, AKAP450 and CDK5RAP2 are absent from the NE in *SYNE1* (23545 G>T) myotubes

Wildtype (A) and *SYNE1* (23545 G>T) (B) myoblasts were differentiated for 5 days. Myotubes were fixed in -20°C methanol and co-stained with antibodies against myosin heavy chain (MHC; green) and either nesprin-1, PCM1, AKAP450, or CDK5RAP2 (red). DNA was stained with DAPI (blue). Scale bar = 10 µm.

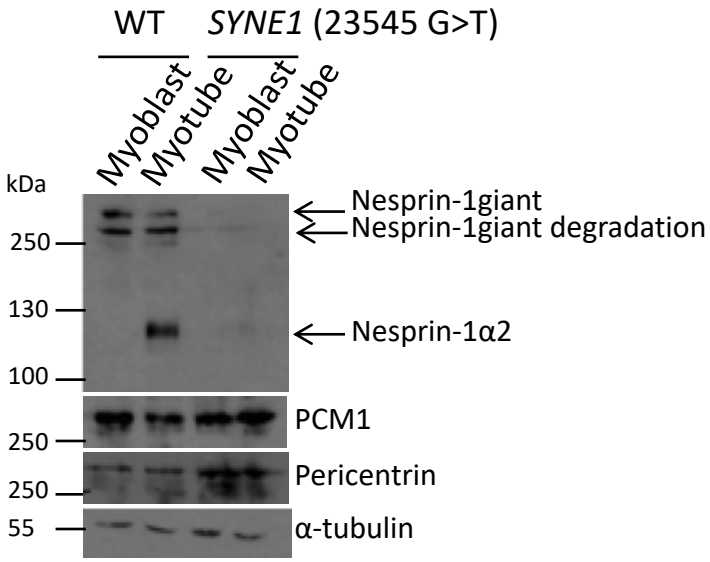


Figure 3.7 PCM1 and pericentrin protein levels are unaffected in *SYNE1* (23545 G>T) myotubes

Wildtype and *SYNE1* (23545 G>T) myoblasts and 5 day old myotubes were lysed with RIPA buffer and 40 μg extracts were immunoblotted with nesprin-1, PCM1, pericentrin or α-tubulin antibodies.

myotubes. Myotubes expressing GFP-nesprin-1α2, but not GFP, showed rescue of PCM1 and AKAP450 localisation to the NE (Fig. 3.8). Although this result does not invalidate nesprin-1G to also be a MTOC protein receptor, nesprin-1α2 alone is able to recruit and anchor multiple MTOC components at the NE in myotubes.

3.2.2 Identifying the domains of nesprin-1α2 required for PCM1 and AKAP450 recruitment to the NE

Having identified nesprin-1α2 to be sufficient to recruit PCM1 and AKAP450 to the NE of myotubes, the next aim was to identify the nesprin-1α2 domains involved. The domains of nesprin-1α2 are clearly identified: it contains an N-terminus 31 residue isoform-specific sequence, followed by 3 SRs around 100 residues each, the adaptive domain, 3 further SRs, and the C-terminal transmembrane KASH domain (Rajgor and Shanahan, 2013). Nesprin-1α2 truncation mutants were generated by the removal of known domains, and expressed in *SYNE1* (23545 G>T) myotubes to investigate their capabilities to recruit PCM1 and AKAP450 to the NE. The recruitment of PCM1 and AKAP450 to the NE was quantitively analysed, to show whether the removal of individual domains result in reduced or complete abrogation of recruitment.

3.2.2.1 Developing an indirect method for measuring centrosomal protein fluorescence intensity at the NE

Investigating the recruitment of PCM1 and AKAP450 to the NE required measuring their fluorescence staining intensity at the NE. As NE fluorescence staining intensity is variable around a single NE, it was desirable to measure the staining intensity around the whole NE and obtain an average value. For this, a method was required to select the NE of a cell, which was independent of PCM1 or AKAP450 recruitment to the NE.

DAPI staining was utilised to identify the position of the NE, and mark the area in which the fluorescence staining intensity of PCM1 or AKAP450 should be

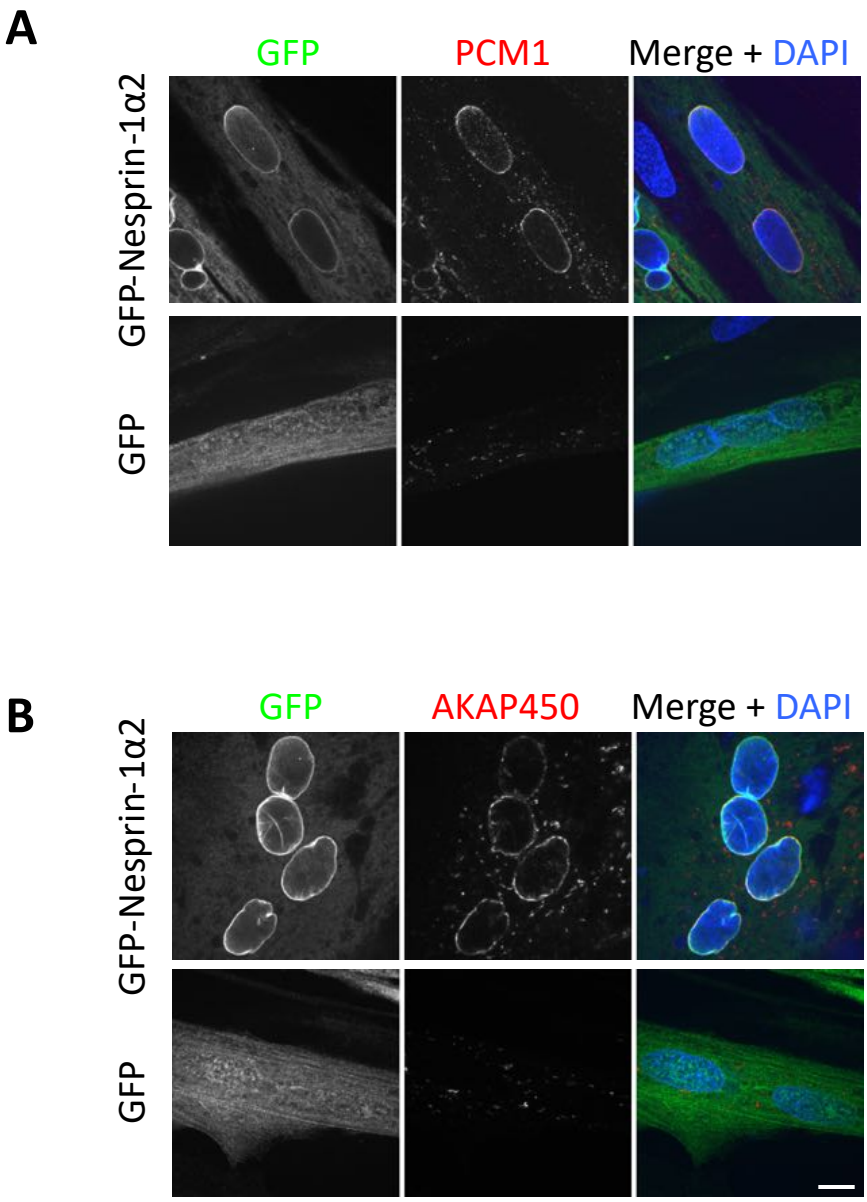


Figure 3.8 GFP-Nesprin-1α2 recruits PCM1 and AKAP450 to the NE in *SYNE1* (23545 G>T) myotubes

5 day old *SYNE1* (23545 G>T) myotubes were transfected with GFP-nesprin-1α2 or GFP. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and (A) PCM1 or (B) AKAP450 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

measured (Fig. 3.9). Using the DAPI image, nuclei in a single slice image were detected by selecting all pixels with an intensity above a cut-off value (Fig. 3.9A). This thresholding detects all nuclei staining which may belong from multiple cells, requiring manual selection of nuclei from a single myotube. The outline of the selected nuclei marks the NE (Fig. 3.9B). A limitation of this method is that the NE of a nucleus which is touching another nucleus is not selected. The outline of the selected nuclei was modified to be 8 pixels in thickness, which was empirically determined to be the best thickness to represent NE staining (Fig. 3.9C). This is the region of interest (ROI), which marks the NE (Fig. 3.9E) and can be applied on another channel (Fig. 3.9D) to measure the fluorescence staining intensity at the NE. Due to staining and variation of NE thickness even within one nucleus, the ROI will not completely align with the actual NE. In this method, one myotube measurement is defined from the number of nuclei fitting in the 95x64 μm field of vision. This may or may not contain all the myonuclei of a single cell.

It should be noted that the recruitment of PCM1 and AKAP450 to the NE by GFP-nesprin-1α2 in *SYNE1* (23545 G>T) myotubes varied between cells in the same culture. This is illustrated by Fig. 3.10. GFP-nesprin-1α2 expression in *SYNE1* (23545 G>T) myotubes did not recruit the proteins to the NE in every cell (Fig. 3.10A' and 3.10A''', 3.10B' and 3.10 B'''). Non-transfected WT myotubes all displayed PCM1 at the NE (not shown). Interestingly, a population of WT myotubes expressing GFP-nesprin-1α2 exhibited the loss of PCM1 or AKAP450 at the NE (not shown). This showed that at times, overexpressed nesprin-1α2 exhibited a dominant-negative effect on centrosomal protein recruitment. The proportion of GFP-nesprin-1α2 expressing myotubes which showed PCM1 staining at the NE was comparable between the WT and *SYNE1* (23545 G>T) cell lines (Fig. 3.10C; images not shown). Perhaps between its dominant-negative effect, and role to recruit PCM1 and AKAP450, some GPF-nesprin-1α2 expressing myotubes show partial recruitment of the proteins (Fig. 3.10A'' and Fig. 3.10B''). One explanation of this is that when GFP-nesprin-1α2 is also observed in the cytoplasm, it is in the endoplasmic reticulum (ER), since it is continuous with the NE. GFP-nesprin-1α2 in the ER and NE may compete for centrosomal proteins, leading to reduced or the abolishment of centrosomal proteins at the NE. However, this does not offer a full

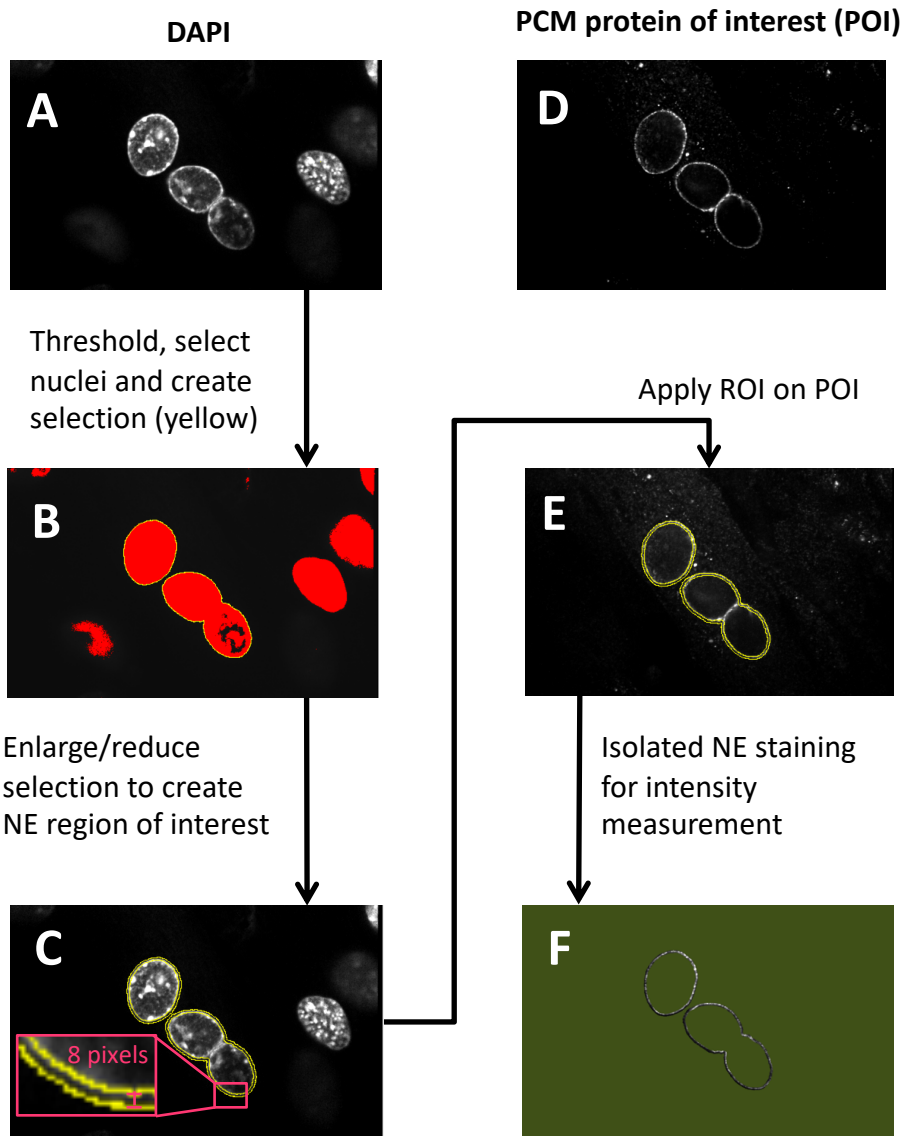


Figure 3.9 Utilising DAPI staining to isolate the area of the NE, in order to measure the fluorescence staining intensity of proteins at the NE

The DAPI channel (A) is thresholded (B, red) to identify the edge of nuclei which is considered to be the NE. Outlines of nuclei to be measured are selected (B, yellow) and used to create a ROI such that the thickness of the NE to be measured is 8 pixels (C, yellow). This ROI is applied on the channel with the POI (D, E) to locate the NE, and staining intensity of the region is measured (F).

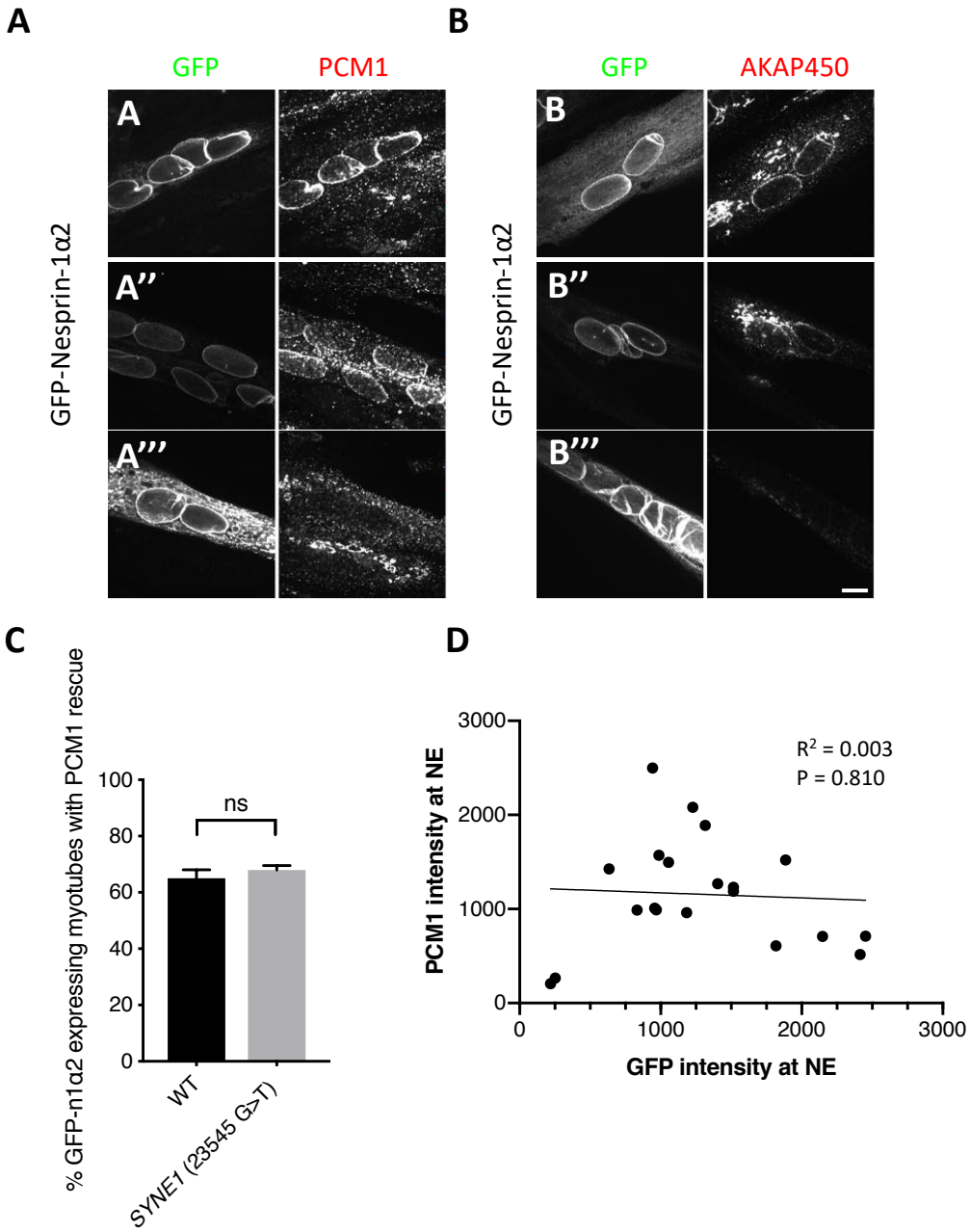


Figure 3.10 GFP-nesprin-1α2 mediates varied recruitment of PCM1 and AKAP450 to the NE in *SYNE1* (23545 G>T) myotubes

SYNE1 (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (A) or AKAP450 (B) (red). Scale bar = 10 μm. Different rows indicate variable centrosomal protein recruitment within one experiment. (C) Histogram showing average percentage of GFP-nesprin-1α2 expressing WT and *SYNE1* (23545 G>T) myotubes with PCM1 recruitment at the NE. 20 myotubes were analysed per experiment. Data shows means and SEM, n=3. *P=<0.05 using a two-tailed unpaired t-test. (D) Pearson's correlation showing relationship between GFP-nesprin-1α2 expression level at the NE against level of PCM1 recruitment, both measured by fluorescence intensity staining measurements. 20 myotubes were analysed.

explanation as there are cells where GFP-nesprin-1α2 is only at the NE, and yet do not recruit PCM1 to the NE. This difference in centrosomal protein recruitment was anticipated to lead to variation in the measurements.

As the expression level of GFP-nesprin-1α2 varied between *SYNE1* (23545 G>T) myotubes, it was considered that the expression level may impact on centrosomal protein recruitment. To test this, the fluorescence staining intensity of GFP and PCM1 at the NE was measured in 20 *SYNE1* (23545 G>T) myotubes expressing GFP-nesprin-1α2 (Fig. 3.10D). The Pearson's correlation coefficient between the values was 0.003, indicating there is no relationship between GFP-nesprin-1α2 expression level at the NE and PCM1 recruitment. Therefore, any GFP-nesprin-1α2 expressing myotubes, regarding of expression level were used for analysis.

3.2.2.2 Nesprin-1α2 residues 1-31 contribute towards the recruitment of PCM1 to the NE, whereas results for AKAP450 are inconclusive

To identify the nesprin-1α2 region responsible for recruiting PCM1 and AKAP450 to the NE, a set of GFP-tagged nesprin-1α2 C-terminal mutants was generated. The N-terminal isoform-specific 31 residues was removed in the first mutant, and further mutants were generated by sequentially removing SR1, SR2, SR3+AD, SR4 and SR5, and their expression was confirmed by western blot (Fig. 3.11). C2C12 myoblasts were chosen to generate lysates for western blots due to the short period required for culturing and comparable transfection rate compared to myotubes. The constructs were then transfected into *SYNE1* (23545 G>T) myotubes to explore the rescue of PCM1 (Fig. 3.12) and AKAP450 (Fig. 3.13) recruitment to the NE.

As expected, for PCM1, NE recruitment was observed in GFP-nesprin-1α2 expressing cells, but not in GFP expressing cells. Recruitment of PCM1 to the NE was also observed in cells expressing GFP-nesprin-1α2(SR1-SR6), but not in GFP-nesprin-1α2(SR2-SR6) or further truncations. To measure the recruitment of

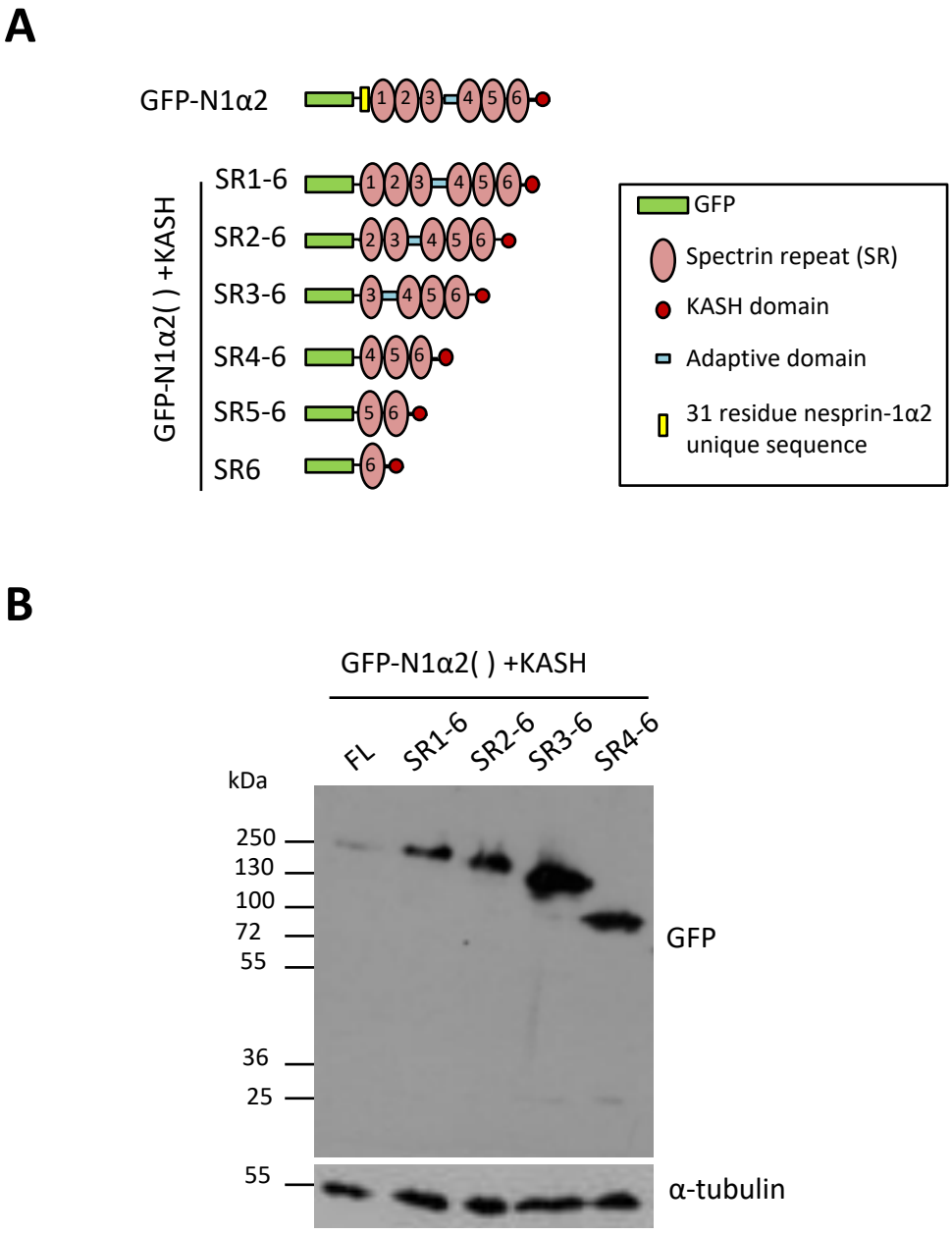


Figure 3.11 Schematic representation and western blot of GFP-nesprin-1α2 N-terminal truncations

(A) Schematic representation of GFP-nesprin-1α2 truncation mutants generated by consecutive deletion of domains from the N-terminus. (B) C2C12 myoblasts were transfected with the constructs for 24 h. Cells were lysed with RIPA buffer and 50 μg of whole cell extracts were analysed by immunoblotting with GFP antibody.

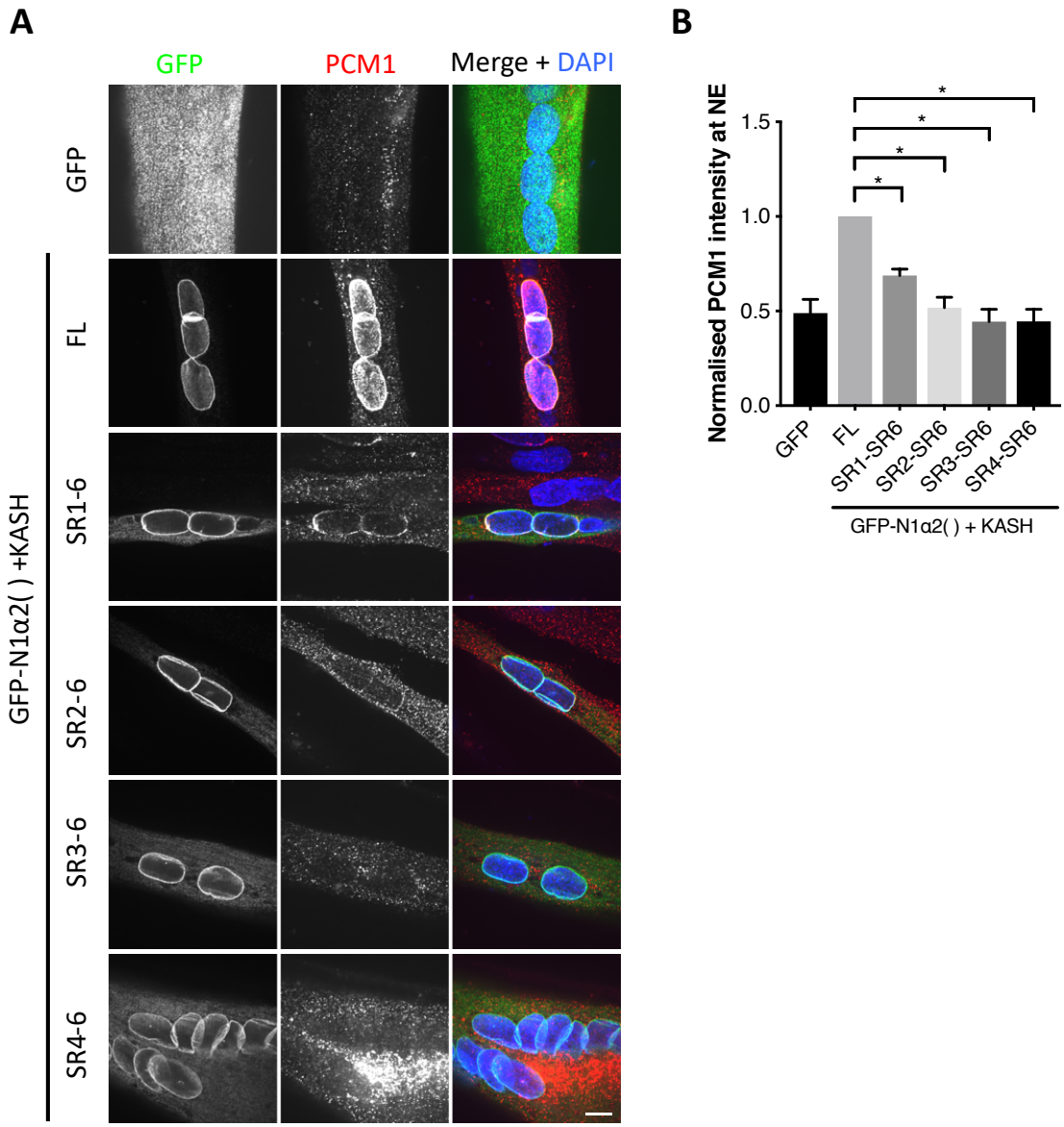


Figure 3.12 Nesprin-1α2 residues 1-31 and SR1 are required for the recruitment of PCM1 to the NE in *SYNE1* (23545 G>T) myotubes

(A) *SYNE1* (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2 truncations as indicated. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) Histogram showing PCM1 fluorescence intensity at the NE of nuclei expressing each GFP-nesprin-1α2 construct, and GFP alone. Average intensities were normalised to full-length (FL) GFP-nesprin-1α2, set at 1.0. Nuclei from 20 myotubes were analysed per experiment. Data shows means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test. All significant differences are shown.

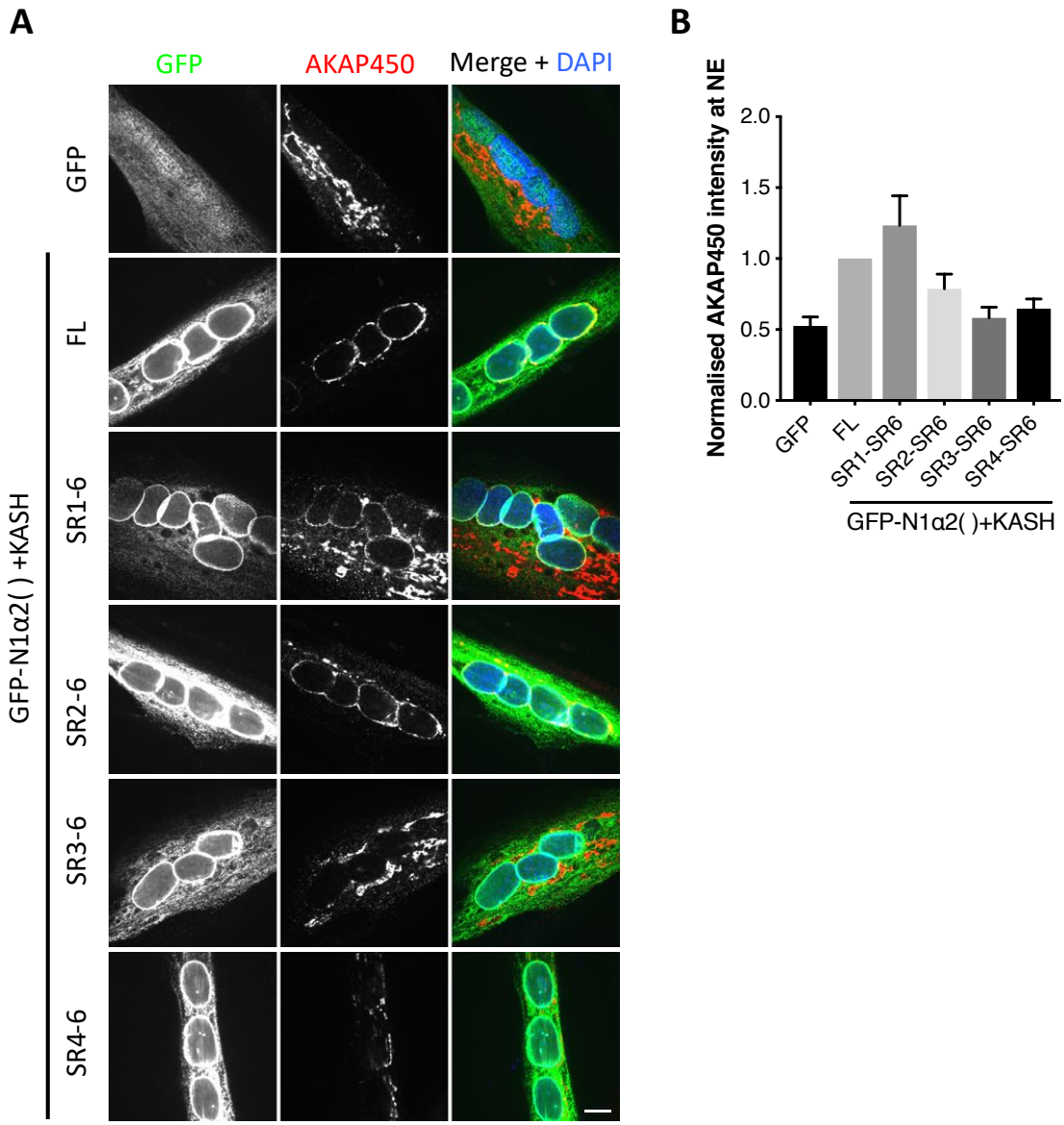


Figure 3.13 Nesprin-1α2 residues 1-31 may not be required for the rescue of AKAP450 to the NE in nesprin-1 null myotubes

(A) *SYNE1* (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2 truncations as indicated. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and AKAP450 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) Histogram showing AKAP450 fluorescence intensity at the NE of nuclei expressing each GFP-nesprin-1α2 construct, and GFP alone. Average intensities were normalised to full-length (FL) GFP-nesprin-1α2, set at 1.0. Nuclei from 20 myotubes were analysed per experiment. Data show means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test. All significant differences are shown.

PCM1 to the NE, the average fluorescence staining intensity of PCM1 was measured at the NE in myotubes expressing each mutant, and normalised to the measurement of GFP-nesprin-1α2 expressing myotubes, which was set to 1. There was no statistical significance between GFP-nesprin-1α2 and GFP in the recruitment of PCM1 to the NE, despite there being a clear difference by observation. However, compared to the full-length protein PCM1 recruitment was significantly reduced with all truncations, indicating the N-terminal 31 residues of nesprin-1α2 is required for PCM1 to fully recruit to the NE. Although the recruitment of PCM1 was higher in GFP-nesprin-1α2(SR1-SR6) compared to other truncations, there were no significant differences between any truncations.

Again, for AKAP450, NE recruitment was observed in GFP-nesprin-1α2, but not GFP expressing myotubes. Recruitment was also observed in cells expressing GFP-nesprin-1α2(SR1-SR6) and GFP-nesprin-1α2(SR2-SR6), though recruitment was in fewer cells and visibly weaker in the latter cells, whereas recruitment was abolished in further truncations. This was partially reflected by quantification, where compared to the full-length protein, recruitment was not reduced until GFP-nesprin-1α2(SR2-SR6), and further truncations were further reduced at similar levels to GFP. There were no significant differences between any of the truncation mutants in their ability to recruit AKAP450 to NE, resulting in inconclusive data. Furthermore, compared to the full-length protein, GFP-nesprin-1α2(SR1-SR6) recruited PCM1 at higher levels, suggesting that the N-terminal isoform specific sequence is not required for the recruitment of AKAP450, unlike PCM1. However, this requires further investigation as there was a large variation in the fluorescence intensity measurements for GFP-nesprin-1α2(SR1-SR6) expressing cells. The role of N-terminal nesprin-1α2 regions in AKAP450 recruitment was further explored in section 3.2.10.

3.2.2.3 The nesprin-1α2 region N-AD recruits PCM1 and AKAP450 to the NE

To again narrow the region of nesprin-1α2 required for the recruitment of PCM1 and AKAP450, further GFP-tagged nesprin-1α2 mutants were generated, by deletion of non-KASH domains from the C-terminal end. The C-terminal KASH domain was retained in the mutants for NE localisation. All mutants included the N-terminal isoform-specific 31 residues, referred to as "N". Mutants generated were GFP-nesprin-1α2(N-SR4), GFP-nesprin-1α2(N-AD), GFP-nesprin-1α2(N-SR3) and GFP-nesprin-1α2(N-SR2), and their expression was confirmed by western blot (Fig. 3.14). Constructs were transfected into *SYNE1* (23545 G>T) myotubes as before to investigate the rescue of PCM1 (Fig. 3.15) and AKAP450 localisation to the NE (Fig. 3.16).

For PCM1, NE recruitment was observed in GFP-nesprin-1α2, but not GFP expressing cells. Recruitment was also observed in cells expressing GFP-nesprin-1α2(N-SR4) and GFP-nesprin-1α2(N-AD), but not in cells expressing GFP-nesprin-1α2(N-SR3) nor GFP-nesprin-1α2(N-SR2). Quantitative analysis showed a significant statistical difference between the fluorescence intensity staining of PCM1 at the NE of cells expressing GFP-nesprin-1α2 and GFP. Compared to GFP-nesprin-1α2, GFP-nesprin-1α2(N-AD) was able to recruit PCM1 to NE at the same level, whereas it was significantly reduced in GFP-nesprin-1α2(N-SR3) and GFP-nesprin-1α2(N-SR2) expressing cells, where PCM1 recruitment was similar to GFP expressing cells. Furthermore, compared to the full-length protein, there was more PCM1 at the NE in GFP-nesprin-1α2(N-SR4) expressing cells, suggesting a structural hindrance in the full-length protein which is overcome after removal of SR5-SR6, allowing easier recruitment of PCM1. However, the difference was not significant.

By observation, the mutants behaved similarly for the recruitment of AKAP450 to the NE. Again, there was a significant difference between the fluorescence intensity staining of AKAP450 at the NE of cells expressing GFP-nesprin-1α2 and GFP. Again,

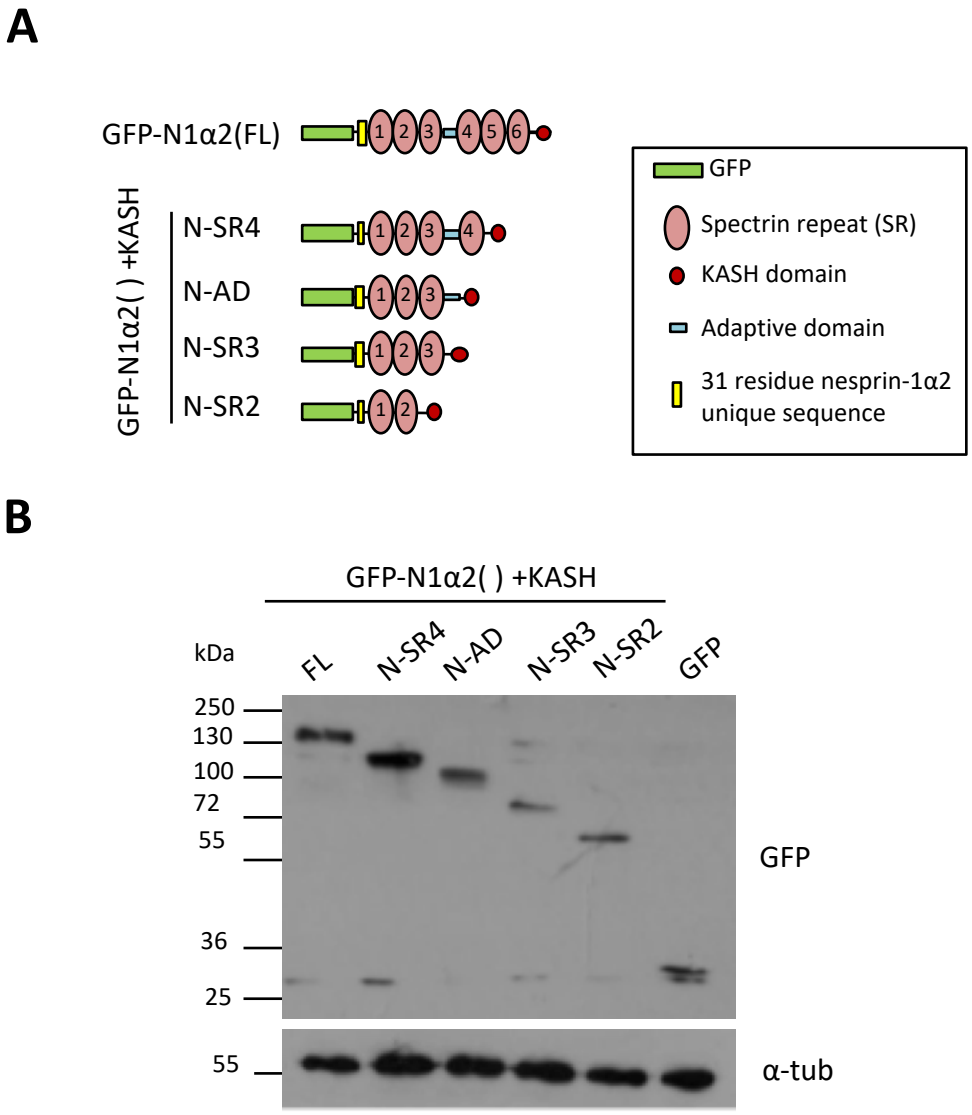


Figure 3.14 Schematic representation and western blot of nesprin-1α2 C-terminal truncations

(A) Schematic representation of GFP-nesprin-1α2 truncation mutants generated by selected deletion of domains from the C-terminal end. (B) C2C12 myoblasts were transfected with the constructs in for 24 h. Cells were lysed with RIPA buffer and 50 µg of whole cell extracts were analysed by immunoblotting with GFP antibody.

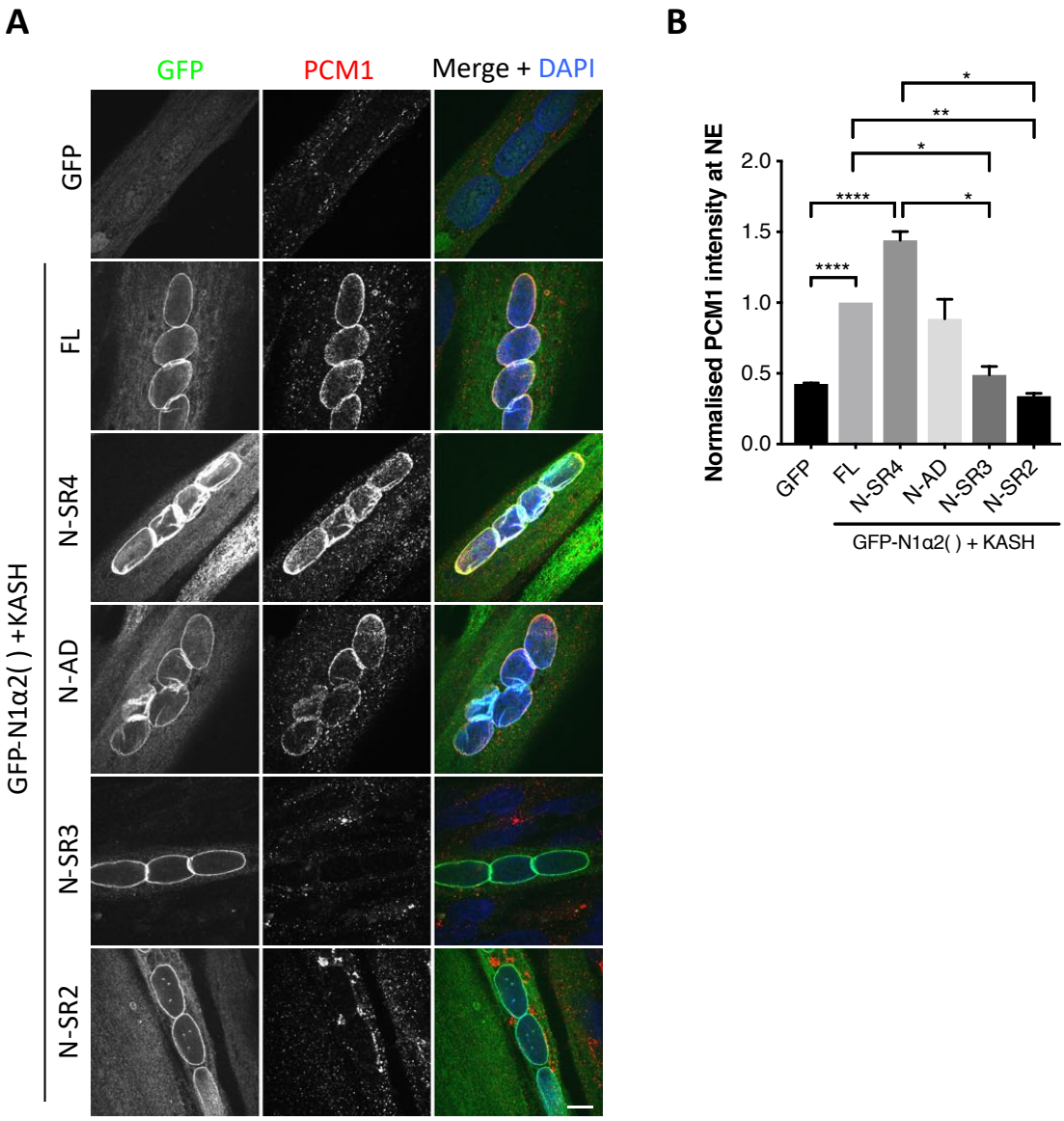


Figure 3.15 Nesprin-1α2(N-AD) is sufficient for the rescue of PCM1 to the NE in *SYNE1* (23545 G>T) myotubes

(A) *SYNE1* (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2 truncations as indicated. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) Histogram showing PCM1 fluorescence intensity at the NE of nuclei expressing each GFP-nesprin-1α2 construct, and GFP alone. Average intensities were normalised to full-length (FL) GFP-nesprin-1α2, set at 1.0. Nuclei from 20 myotubes were analysed per experiment. Data show means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test. All significant differences are shown.

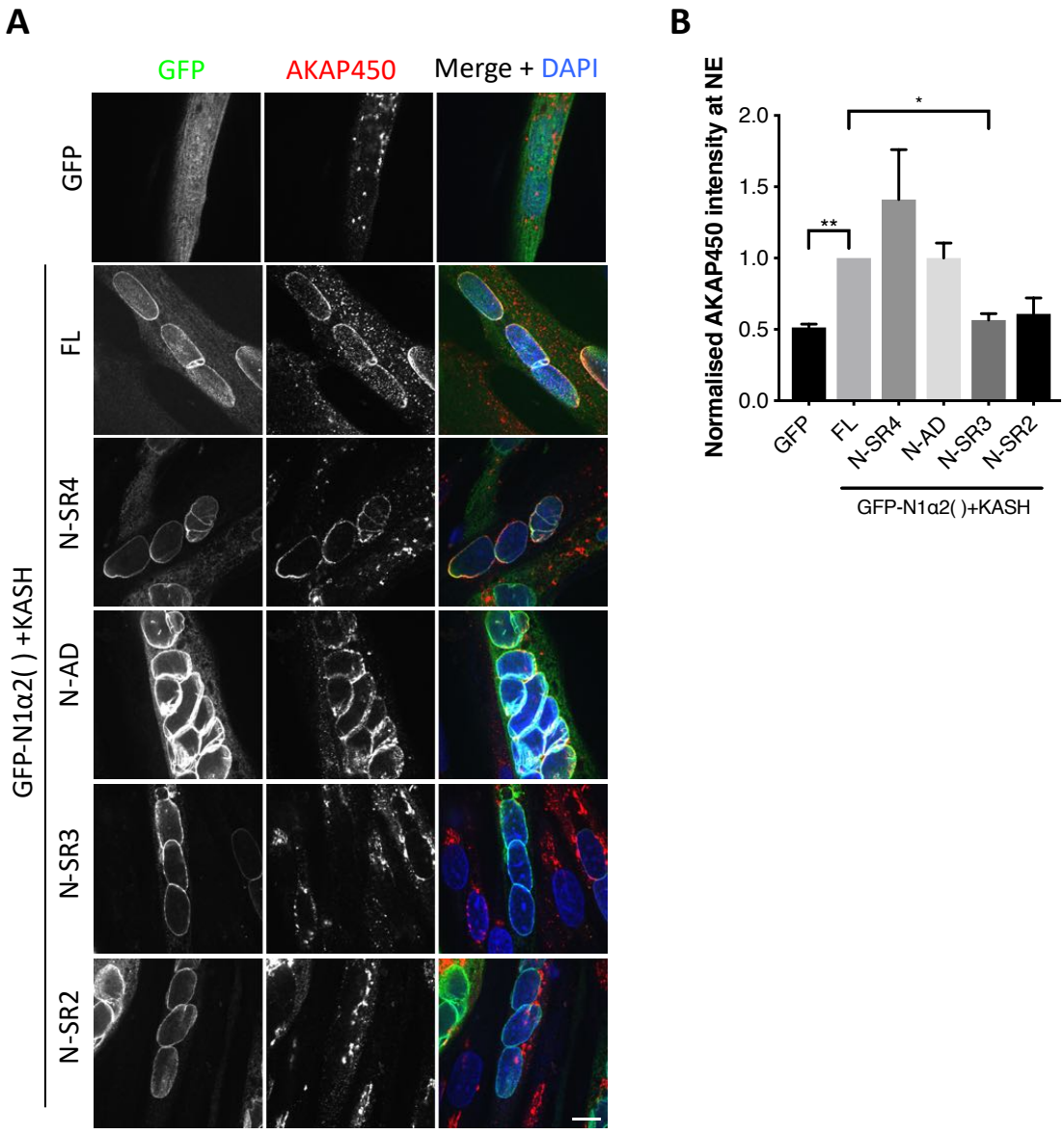


Figure 3.16 Nesprin-1α2(N-AD) is sufficient for the rescue of AKAP450 to the NE in *SYNE1* (23545 G>T) myotubes

(A) SYNE1 (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2 truncations as indicated. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and AKAP450 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) Histogram showing AKAP450 fluorescence intensity at the NE of nuclei expressing each GFP-nesprin-1α2 construct, and GFP alone. Average intensities were normalised to full-length (FL) GFP-nesprin-1α2, set at 1.0. Nuclei from 20 myotubes were analysed per experiment. Data show means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test. All significant differences are shown.

GFP-nesprin-1α2(N-AD) was the shortest mutant capable of recruiting AKAP450 at levels comparable to the full-length protein. Despite there being no significant difference in results between GFP-nesprin-1α2 and GFP-nesprin-1α2(N-SR2) expressing cells, a significant difference was observed between GFP-nesprin-1α2 and GFP-(N-SR3). Also, as with PCM1, compared to the full-length protein, there was more AKAP450 at the NE in GFP-nesprin-1α2(N-SR4) expressing cells, but the difference was not significant.

This set of mutants showed that the nesprin-1α2 region N-AD is sufficient to fully recruit PCM1 and AKAP450 to the NE in myotubes, and that further removal of the AD completely abrogates their recruitment.

3.2.2.4 The nesprin-1α2 fusion mutant 1-31+SR2-AD has reduced ability to recruit PCM1 and AKAP450 to the NE

To test whether the three SRs in nesprin-1α2(N-AD) are essential for the recruitment of PCM1 and AKAP450, they were sequentially deleted from the N-terminal end to generate GFP-nesprin-1α2(N + (SR2-AD)), GFP-nesprin-1α2(N + (SR3-AD)) and GFP-nesprin-1α2(N + AD)) and their expression was confirmed by western blot (Fig. 3.17). Constructs were transfected into *SYNE1* (23545 G>T) myotubes as before to investigate the rescue of PCM1 (Fig. 3.18) and AKAP450 (Fig. 3.19) localisation to the NE. The full-length GFP-nesprin-1α2 was used to represent GFP-nesprin-1α2(N-AD), as they were previously shown to recruit PCM1 and AKAP450 to the NE at comparable levels.

For PCM1, there was NE recruitment observed in GFP-nesprin-1α2, but not GFP expressing cells. Recruitment was also observed in cells expressing GFP-nesprin-1α2(N + (SR2-AD)), though it was apparent that it was in fewer cells and recruitment was weaker compared to the full-length protein. There was no PCM1 recruitment to the NE in cells expressing GFP-nesprin-1α2(N + (SR3-AD)), nor GFP-nesprin-1α2(N + AD)). Quantitative analysis showed a significant statistical difference between the fluorescence intensity staining of PCM1 at the NE of cells

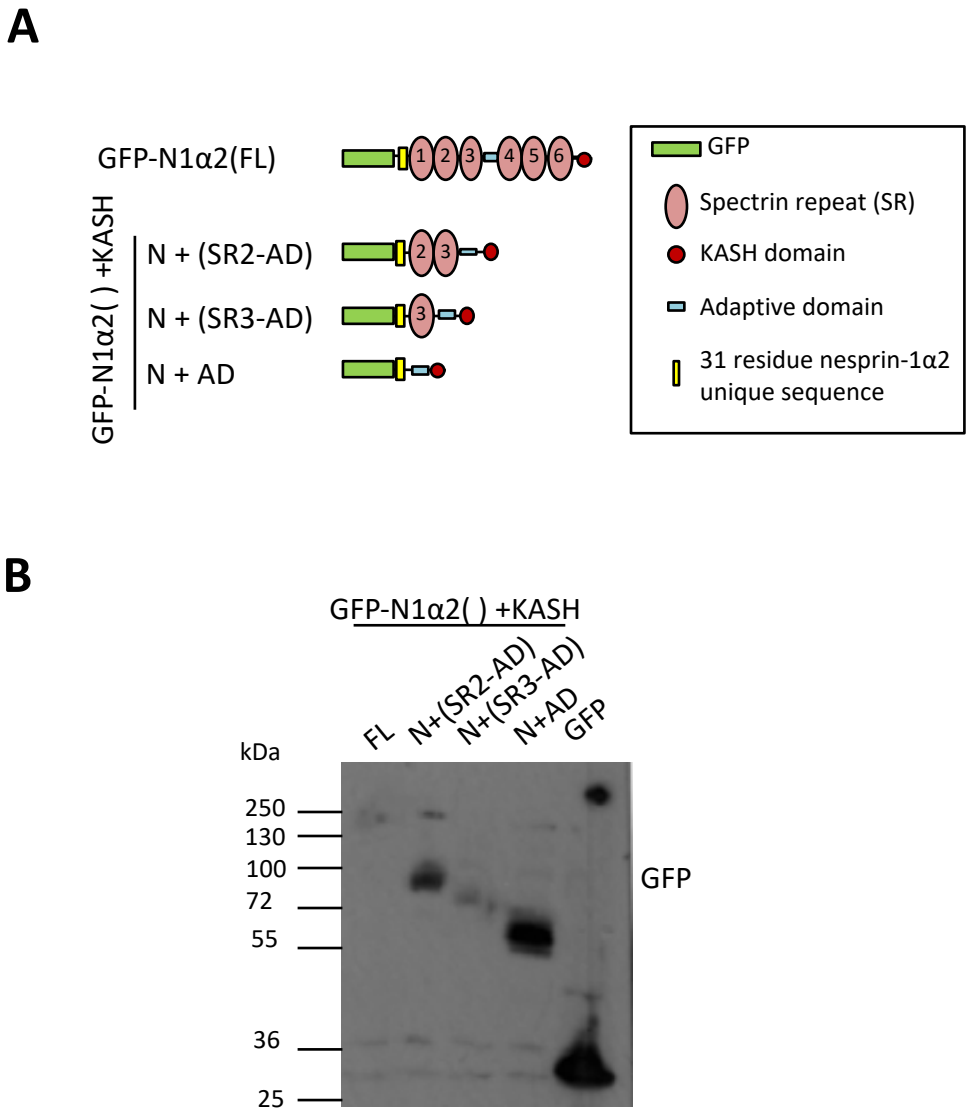


Figure 3.17 Schematic representation and western blot of nesprin-1α2(N-AD) truncation constructs

(A) Schematic representation of GFP-nesprin-1α2 truncation mutants generated by selected deletion of domains from the C-terminal end. (B) C2C12 myoblasts were transfected with the constructs in for 24 h. Cells were lysed with RIPA buffer and 50 μg of whole cell extracts were analysed by immunoblotting with GFP antibody.

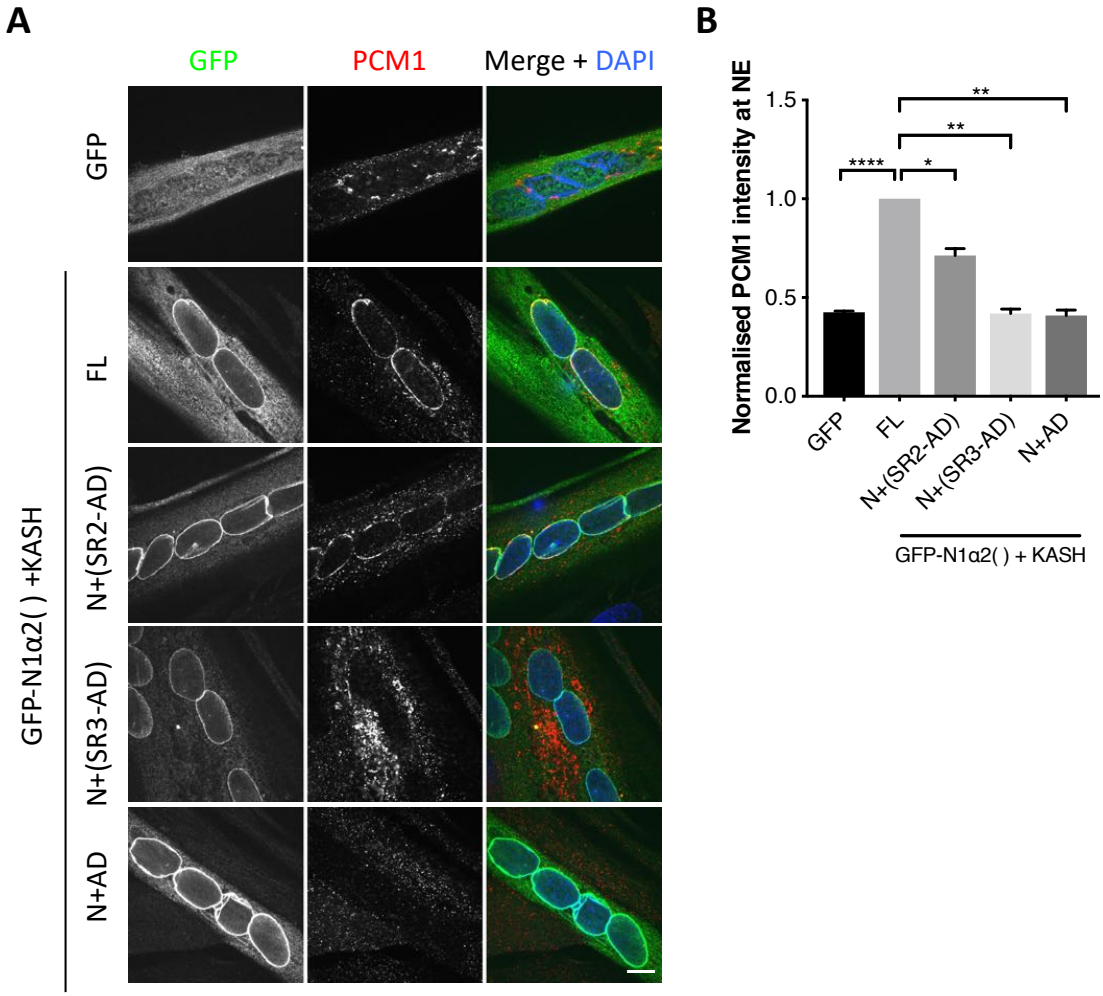


Figure 3.18 Nesprin-1α2(N+SR2-AD) partially rescues PCM1 recruitment to the NE in *SYNE1* (23545 G>T) myotubes

(A) *SYNE1* (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2 truncations as indicated. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) Histogram showing PCM1 fluorescence intensity at the NE of nuclei expressing each GFP-nesprin-1α2 construct, and GFP alone. Average intensities were normalised to full-length (FL) GFP-nesprin-1α2, set at 1.0. Nuclei from 20 myotubes were analysed per experiment. Data show means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test. All significant differences are shown.

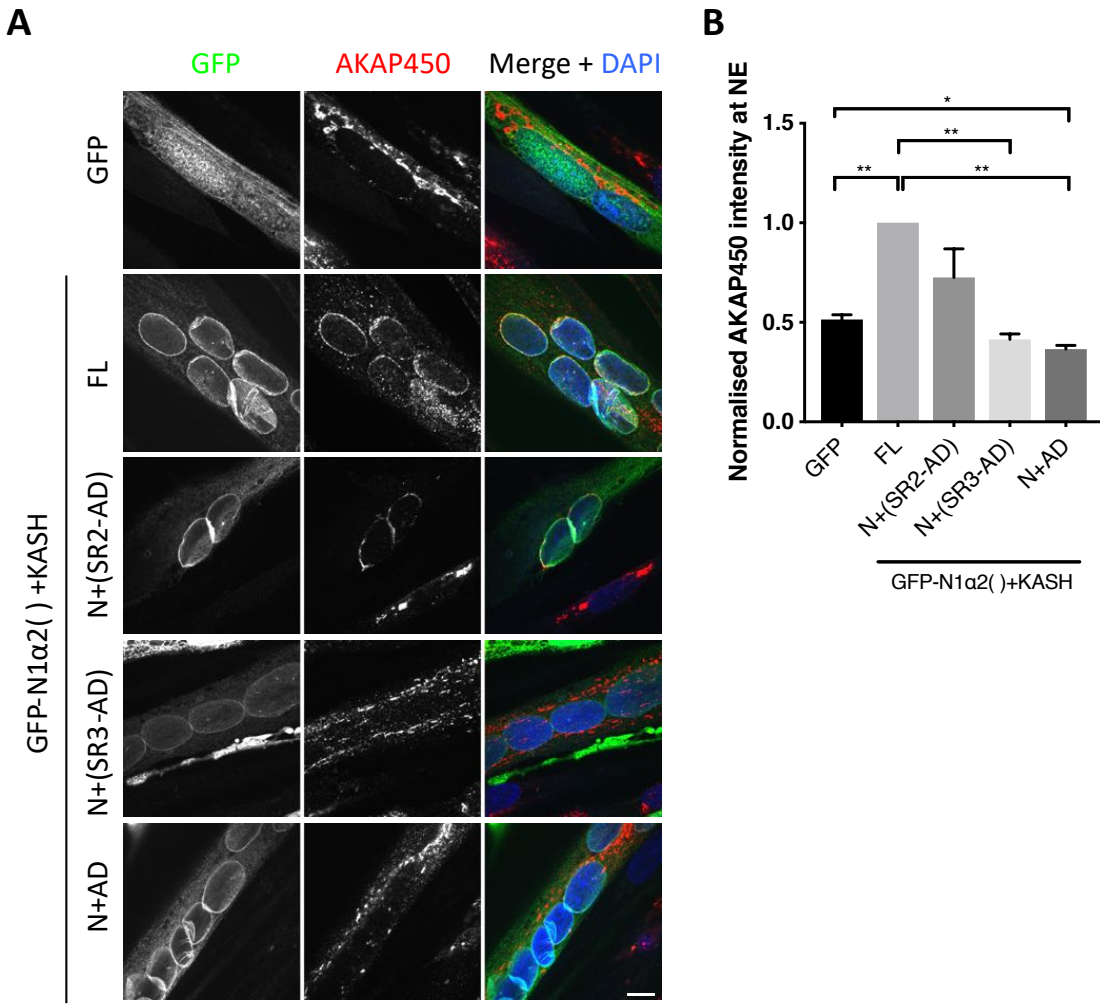


Figure 3.19 Nesprin-1α2(N+SR2-AD) may partially rescue AKAP450 recruitment to the NE in *SYNE1* (23545 G>T) myotubes

(A) *SYNE1* (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2 truncations as indicated. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and AKAP450 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) Histogram showing AKAP450 fluorescence intensity at the NE of nuclei expressing each GFP-nesprin-1α2 construct, and GFP alone. Average intensities were normalised to full-length (FL) GFP-nesprin-1α2, set at 1.0. Nuclei from 20 myotubes were analysed per experiment. Data show means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test. All significant differences are shown.

expressing GFP-nesprin-1α2 and GFP. There were also significant statistical differences between the full-length protein and all truncations, showing all truncation mutants were less able to recruit PCM1. However, despite GFP-nesprin-1α2(N + (SR2-AD)) recruiting more PCM1 compared to other truncations and GFP, the difference was not significant. This does not support that GFP-nesprin-1α2(N + (SR2-AD)) is able to recruit PCM1 at a greater level than further truncations, despite it being observed.

By observation, this set of mutants behaved similarly for the recruitment of AKAP450 to the NE. Again the trend was reflected in quantitative analysis, which showed a significant statistical difference between the recruitment of AKAP450 to the NE of cells expressing GFP-nesprin-1α2 and GFP. Recruitment of AKAP450 was significantly higher in GFP-nesprin-1α2 expressing cells, compared to those expressing GFP-nesprin-1α2(N + (SR3-AD)), and GFP-nesprin-1α2(N + AD)), showing that the mutants lose the capability to recruit to AKAP450. Although GFP-nesprin-1α2(N + (SR2-AD)) recruited AKAP450 less than the full-length protein, and more than shorter mutants, none of the differences were significant. This does not support that GFP-nesprin-1α2(N + (SR2-AD)) is able to recruit AKAP450, despite it being observed.

From this set of mutants, it was observed that GFP-nesprin-1α2(N + (SR2-AD)) partially recruits PCM1 and AKAP450 to the NE, whereas removal of SRs completely abrogates all recruitment. However, the observations were not supported by significant differences.

3.2.2.5 Nesprin-1α2 residues 1-31 facilitate PCM1 and AKAP450 recruitment to the NE in nesprin-1α2 C-terminal truncations

So far, this chapter showed that nesprin-1α2(N-AD) contains domains sufficient for recruiting PCM1 and AKAP450 to the NE, at levels comparable to the full-length protein. It then showed that nesprin-1α2(N + (SR2-AD)) was able to partially recruit the proteins, though this was only observed in cells, and not supported by

significant differences between the relevant groups. The isoform-specific residues 1-31 was found to be required for the maximum recruitment of PCM1 to the NE, however its role in AKAP450 was inconclusive due to a large variation in the data.

To re-evaluate a role for the nesprin-1α2 residues 1-31 in PCM1 and AKAP450 recruitment to the NE, the residues were removed from GFP-nesprin-1α2(N-AD) and GFP-nesprin-1α2(N + (SR2-AD)), to generate GFP-nesprin-1α2(SR1-AD) and GFP-nesprin-1α2(SR2-AD), respectively (Fig. 3.20). Expression was confirmed by western blot (Fig. 3.20), and constructs were transfected into *SYNE1* (23545 G>T) myotubes as before to investigate the rescue of PCM1 (Fig. 3.21) and AKAP450 (Fig. 3.22) localisation to the NE.

For PCM1, PCM1 recruitment was observed in cells expressing both GFP-nesprin-1α2(N-AD), and GFP-nesprin-1α2(SR1-AD), but recruitment was much weaker in the latter cells. No recruitment was observed in GFP expressing cells. This was reflected by quantitative analysis, though, again not fully supported by significant differences. GFP-nesprin-1α2(N-AD) recruited PCM1 at levels significantly higher than GFP, but neither GFP proteins were significantly different from GFP-nesprin-1α2(SR1-AD). PCM1 recruitment was observed in cells expressing GFP-nesprin-1α2(N + (SR2-AD)), but not GFP-nesprin-1α2(SR2-AD). Quantification showed that GFP-nesprin-1α2(N + (SR2-AD)) recruited PCM1 at significantly higher levels than GFP-nesprin-1α2(SR2-AD), showing the latter mutant had decreased capacity to recruit PCM1. However, neither GFP-proteins were significantly different to GFP only.

By observation, this set of mutants behaved similarly for the recruitment of AKAP450 to the NE, and the pattern was reflected by quantitative analysis and significant differences. GFP-nesprin-1α2(N-AD) recruited PCM1 to the NE significantly higher than GFP-nesprin-1α2(SR1-AD), and GFP only. GFP-nesprin-1α2(SR1-AD), in turn also recruited PCM1 to the NE significantly higher than GFP only. This supports that residues 1-31 in GFP-nesprin-1α2(N-AD) plays a partial role in the recruitment of AKAP450 to the NE. GFP-nesprin-1α2(N + (SR2-AD)) was shown to recruit AKAP450 to the NE at levels significantly higher than both

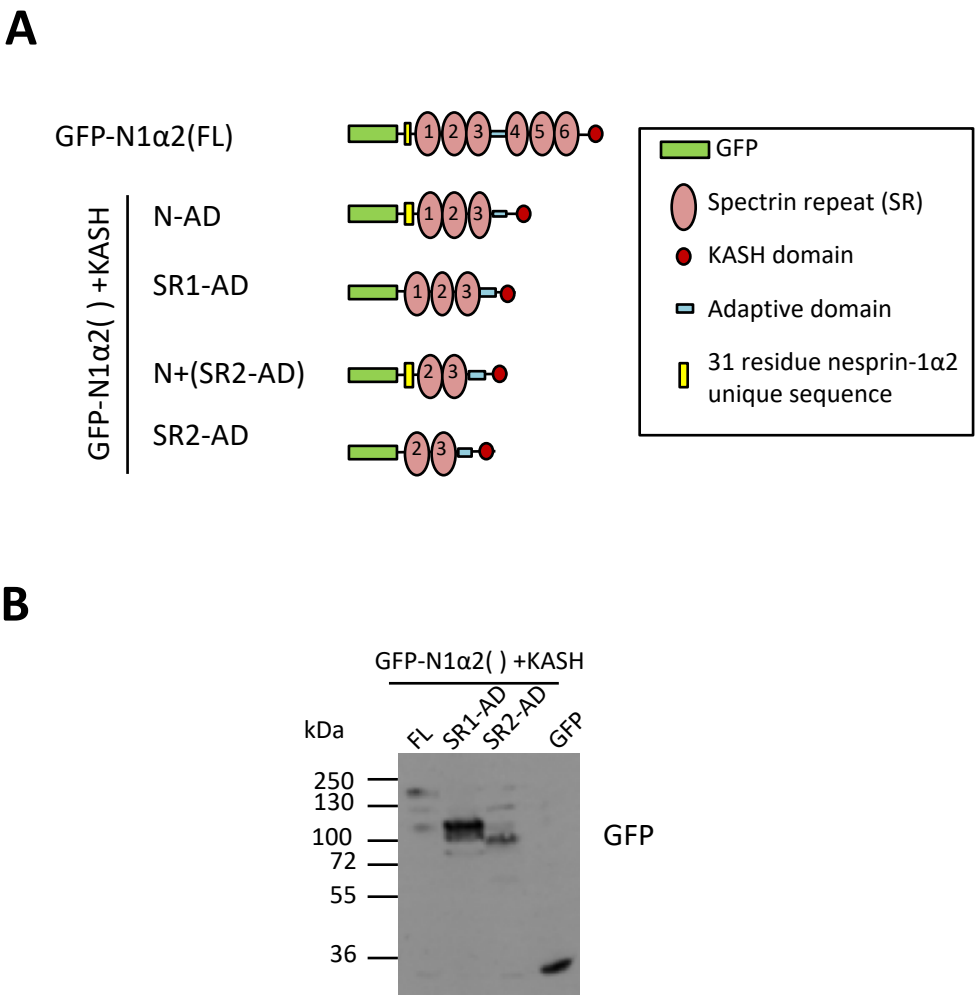


Figure 3.20 Schematic representation and western blot of GFP-nesprin-1α2(N-AD) and GFP-nesprin-1α2(N+(SR2-AD))

(A) Schematic representation of GFP-nesprin-1α2 fusion proteins generated by selected deletion of domains from the C-terminal end. (B) C2C12 myoblasts were transfected with the constructs in for 24 h. Cells were lysed with RIPA buffer and 50 µg of whole cell extracts were analysed by immunoblotting with GFP antibody.

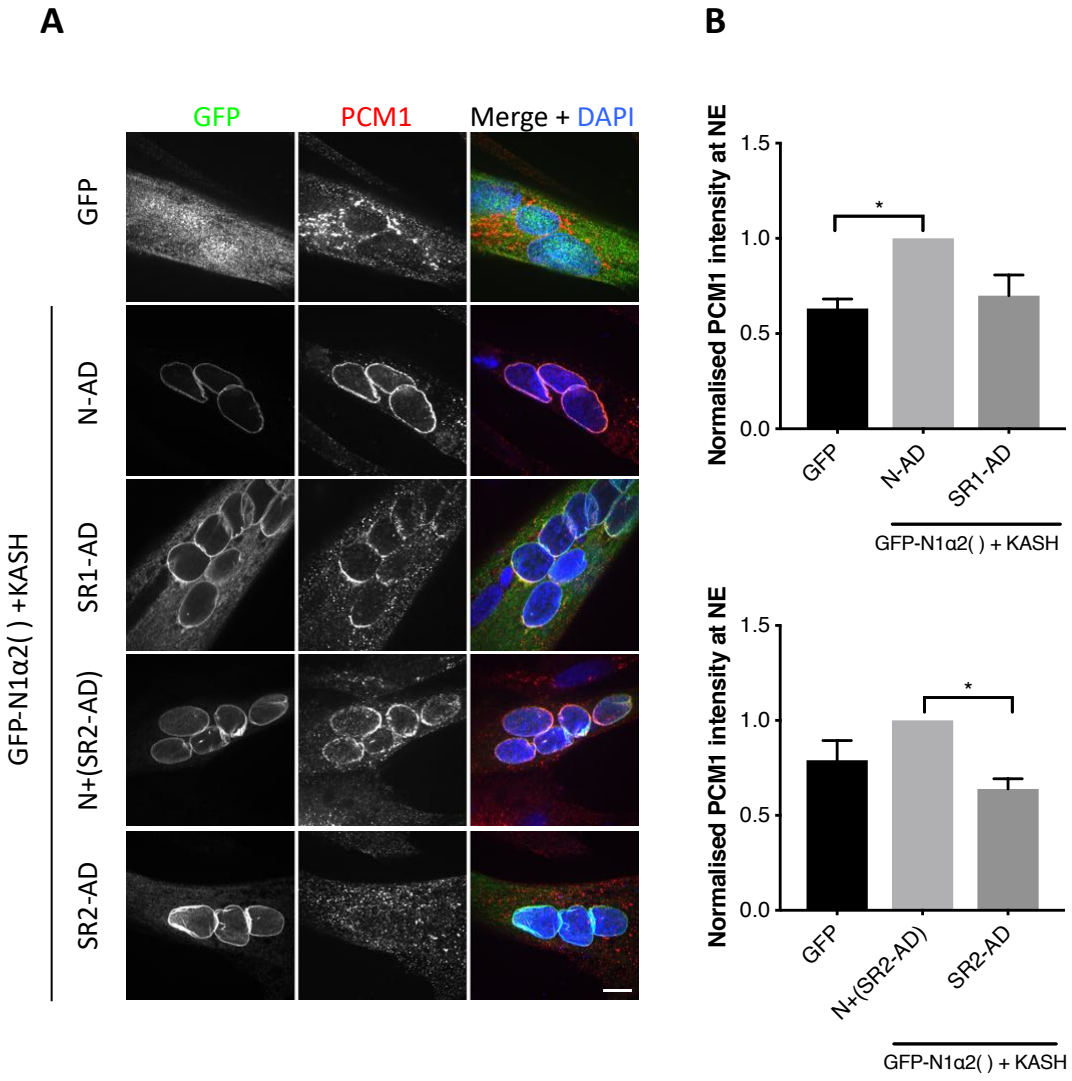


Figure 3.21 Nesprin-1α2 residues 1-31 in nesprin-1α2(N-AD) and nesprin-1α2(N+(SR2-AD)) truncation mutants facilitate the recruitment of PCM1 to the NE in *SYNE1* (23545 G>T) myotubes

(A) *SYNE1* (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2 truncations as indicated. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) Histogram showing PCM1 fluorescence intensity at the NE of nuclei expressing each GFP-nesprin-1α2 construct, and GFP alone. Average intensities were normalised to full-length (FL) GFP-nesprin-1α2, set at 1.0. Nuclei from 20 myotubes were analysed per experiment. Data show means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test. All significant differences are shown.

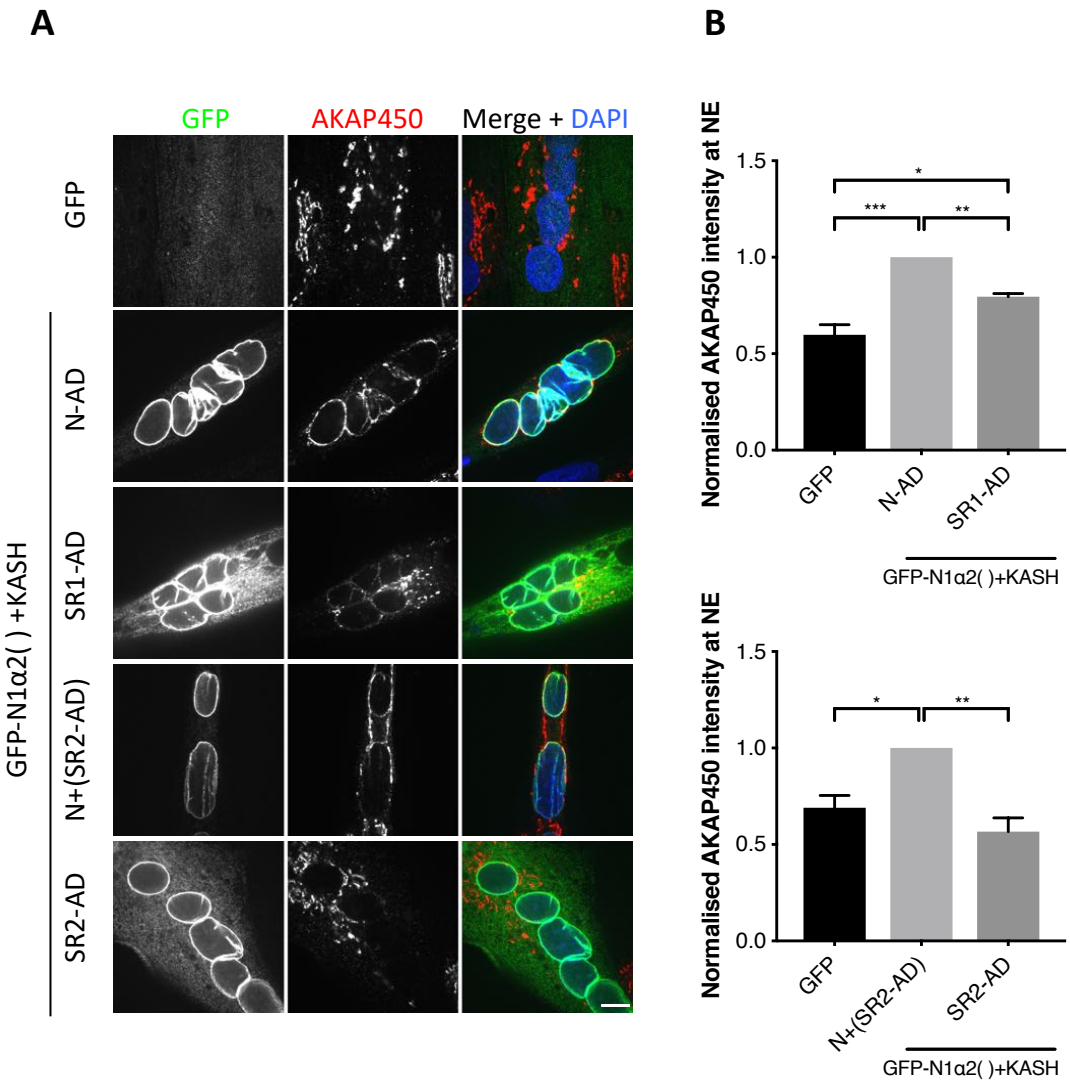


Figure 3.22 Nesprin-1α2 residues 1-31 in nesprin-1α2(N-AD) and nesprin-1α2(N+(SR2-AD)) truncation mutants facilitate the recruitment of AKAP450 to the NE in *SYNE1* (23545 G>T) myotubes

(A) *SYNE1* (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2 truncations as indicated. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and AKAP450 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) Histogram showing AKAP450 fluorescence intensity at the NE of nuclei expressing each GFP-nesprin-1α2 construct, and GFP alone. Average intensities were normalised to full-length (FL) GFP-nesprin-1α2, set at 1.0. Nuclei from 20 myotubes were analysed per experiment. Data show means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test. All significant differences are shown.

GFP-nesprin-1α2(SR2-AD) and GFP. There was no significant difference between GFP-nesprin-1α2(SR2-AD) and GFP, supporting that GFP-nesprin-1α2(SR2-AD) does not recruit AKAP450 to the NE. This shows that residues 1-31 in GFP-nesprin-1α2(N + (SR2-AD)) is essential for any recruitment of AKAP450 to the NE.

From these set of truncations, the role of the nesprin-1α2 residues 1-31 in facilitating the recruitment of PCM1 to the NE was supported by observation and trend, but not by significant difference. Whereas, for the first time, the same residues were shown to play a role in recruiting AKAP450 to the NE.

3.2.3 Investigating whether point mutations located in SR3 or the AD of nesprin-1α2 impact recruitment of PCM1 and AKAP450 to the NE

The expression of nesprin-1α2 mutants in *SYNE1* (23545 G>T) myotubes showed that GFP-nesprin-1α2(N-AD) is sufficient for recruiting PCM1 and AKAP450 to the NE, and that removal of the AD abolishes all recruitment. Residues within SR3 and the AD have been identified to be required for kinesin-1 binding, or are mutated in muscle disease (Wilson and Holzbaur, 2015; Zhou *et al.,* 2017). Therefore, the next aim was to explore whether these nesprin-1α2 point mutations impact upon PCM1 and AKAP450 recruitment to the NE in myotubes.

3.2.3.1 NE recruitment of PCM1, but not AKAP450 requires the kinesin-1 binding LEWD motif

The microtubule motor protein kinesin-1 is required for the spreading and alignment of nuclei along a myotube during early myogenesis (Espigat-Georger *et al., 2*016; Wilson and Holzbaur, 2015; Cadot *et al., 2*015). Its light chain components KLC1/2 are localised around the NE in myotubes, and interact with nesprin-1α2 (Wilson and Holzbaur, 2015; Zhou *et al.,* 2017). A LEWD motif conserved in the AD between nesprin-1, nesprin-2 and nesprin-4, is required for this interaction, as co-immunoprecipitation experiments using nesprin-1 or

nesprin-2 LEAA mutants show lost or reduced interaction with KLC1/2 (Zhou *et al.,* 2017; Wilson and Holzbaur, 2015). Furthermore, data from the Shackleton lab shows that unlike WT GFP-nesprin-1α2, GFP-nesprin-1α2(LEAA) does not rescue any KLC1/2 recruitment to the NE in *SYNE1* (23545 G>T) myotubes (A. Haworth, unpublished data). To investigate whether the LEWD motif is required for the recruitment of PCM1 and AKAP450 to the NE, wildtype GFP-nesprin-1α2 and GFP-nesprin-1α2(LEAA) mutant expression were confirmed by western blot (Fig. 3.23) and expressed in *SYNE1* (23545 G>T) myotubes as before to investigate the rescue of PCM1 (Fig. 3.24) and AKAP450 localisation to the NE (Fig. 3.25).

Recruitment of PCM1 to the NE was observed in cells expressing GFP-nesprin-1α2 or GFP-nesprin-1α2(LEAA), though recruitment appeared to be weaker in the latter cells. PCM1 was not observed at the NE in GFP expressing cells. This was reflected by quantitative analysis, where WT GFP-nesprin-1α2 recruits PCM1 at a level significantly higher than GFP-nesprin-1α2(LEAA) and GFP, and the recruitment by GFP-nesprin-1α2(LEAA) is in turn significantly higher than by GFP only.

Recruitment of AKAP450 to the NE was observed in cells expressing GFP-nesprin-1α2 or GFP-nesprin-1α2(LEAA), but not in GFP expressing cells. Quantitative analysis showed that the recruitment of AKAP450 was lower in WT GFP-nesprin-1α2 compared to GFP-nesprin-1α2(LEAA), however this was not clear by observation and the difference was not significant. Whereas, both proteins recruited AKAP450 to the NE at significantly higher levels than in GFP-expressing cells.

This shows that the LEWD motif, which is critical for any localisation of KLC1/2 to the myotube NE, is also required for PCM1, but not AKAP450 to be fully recruited to the NE.

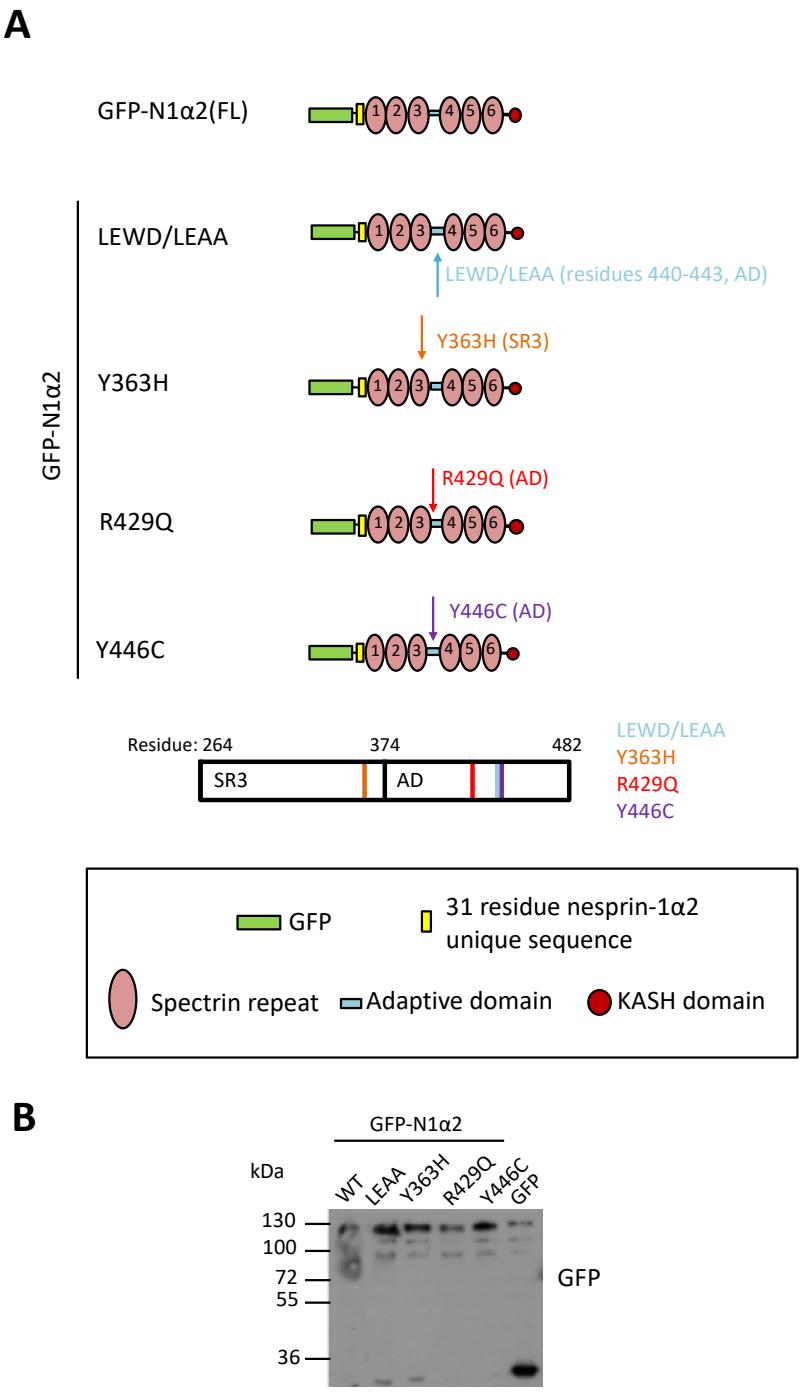


Figure 3.23 Schematic representation and western blot of GFP-nesprin-1α2 point mutants

(A) Schematic representation of GFP-nesprin-1α2 point mutants, with their position in the nesprin-1α2 domain affected indicated. (B) C2C12 myoblasts were transfected with the constructs in for 24 h. Cells were lysed with RIPA buffer and 50 µg of whole cell extracts were analysed by immunoblotting with GFP antibody.

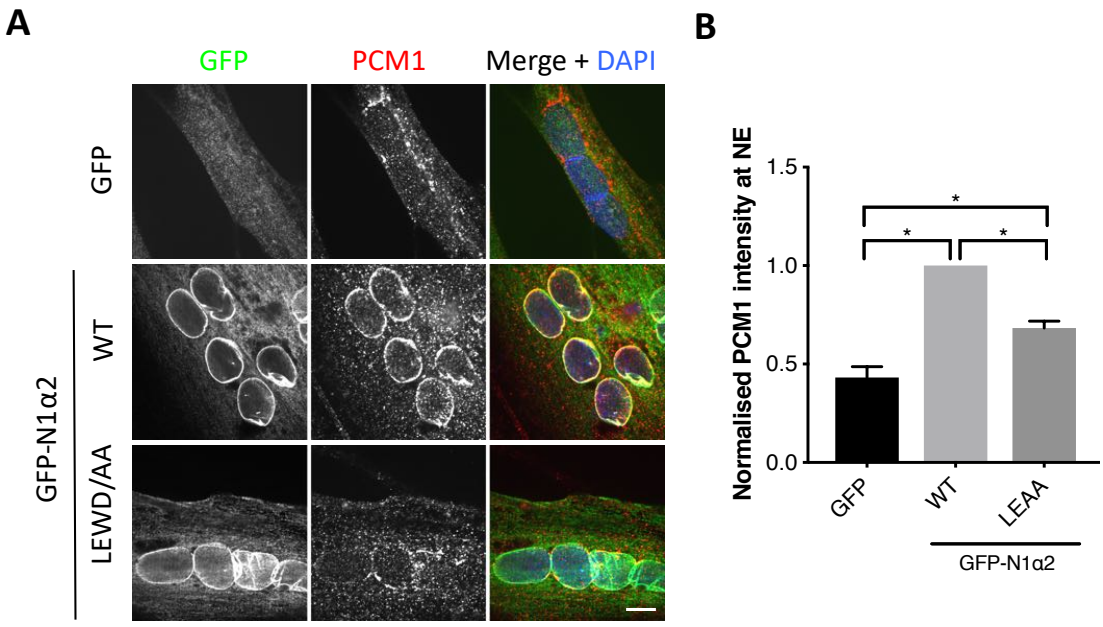


Figure 3.24 The GFP-nesprin-1α2(LEWD/AA) mutation, needed for KLC1/2 binding, results in reduced PCM1 recruitment at the NE in *SYNE1* (23545 G>T) myotubes

(A) *SYNE1* (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2 truncations as indicated. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) Histogram showing PCM1 fluorescence intensity at the NE of nuclei expressing each GFP-nesprin-1α2 construct, and GFP alone. Average intensities were normalised to full-length (FL) GFP-nesprin-1α2, set at 1.0. Nuclei from 20 myotubes were analysed per experiment. Data show means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test. All significant differences are shown.

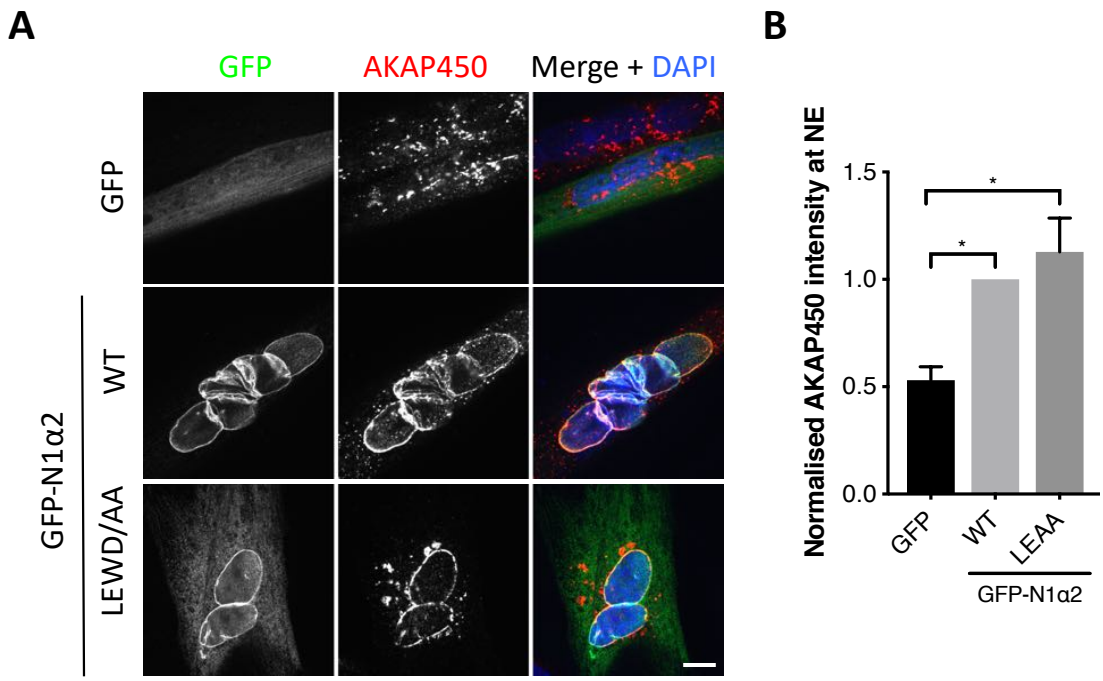


Figure 3.25 The GFP-nesprin-1α2(LEWD/AA) mutation, needed for KLC1/2 binding, does not affect AKAP450 recruitment to the NE in *SYNE1* (23545 G>T) myotubes

(A) *SYNE1* (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2 truncations as indicated. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and AKAP450 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) Histogram showing AKAP450 fluorescence intensity at the NE of nuclei expressing each GFP-nesprin-1α2 construct, and GFP alone. Average intensities were normalised to full-length (FL) GFP-nesprin-1α2, set at 1.0. Nuclei from 20 myotubes were analysed per experiment. Data show means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test. All significant differences are shown.

3.2.3.2 NE recruitment of PCM1 and AKAP450 are not affected in R429Q/Y363H/Y446C nesprin-1α2 disease mutations

Several *SYNE1* mutations have been associated with muscle disorders, many of which are within the region coding for nesprin-1α2 (Zhou *et al.,* 2017, 2018), supporting an essential role of the isoform in myogenesis. A mutation corresponding to nesprin-1α2 R429Q from a dilated cardiomyopathy patient has been shown to interact weaker with KLC1/2 compared to the wildtype protein (Zhou *et al.* 2017). This point mutation is also in the AD and is 13 residues upstream of the LEWD motif (Fig. 3.23). Zhou *et al.* (2017) identified two further uncharacterised *SYNE1* point mutations, corresponding to nesprin-1α2 Y363H, which is in SR3, and Y446C, which is in the AD, from an EDMD and a DCM patient, respectively (Fig. 3.23).

To investigate whether the point mutations impact upon the localisation of PCM1 and AKAP450 to the myotube NE, nesprin-1α2 constructs containing the point mutations were obtained from Qiuping Zhang and re-cloned to contain an N-GFP tag. WT GFP-nesprin-1α2, GFP-nesprin-1α2(Y363H), GFP-nesprin-1α2(R429Q), GFP-nesprin-1α2(Y446C) and GFP expression were confirmed by western blot and expressed in *SYNE1* (23545 G>T) myotubes as before, to investigate the rescue of PCM1 (Fig. 3.26) and AKAP450 localisation to the NE (Fig. 3.27).

Recruitment of PCM1 to the NE was observed in all GFP-nesprin-1α2, but not GFP expressing cells. Quantitative analysis showed that compared to the WT protein, GFP-nesprin-1α2(Y363H) and GFP-nesprin-1α2(R429Q) recruited slightly less PCM1 to the NE, though the differences were not significant. GFP-nesprin-1α2(Y446C) also recruited PCM1 at comparable levels to the WT protein. However, the WT protein and GFP-nesprin-1α2(Y363H) were the only proteins which showed a significant increase in PCM1 recruitment compared to GFP. Despite nesprin-1α2 R429Q and Y446C being observed to recruit PCM1 at similar levels to the wildtype protein, this could not be quantitively concluded.

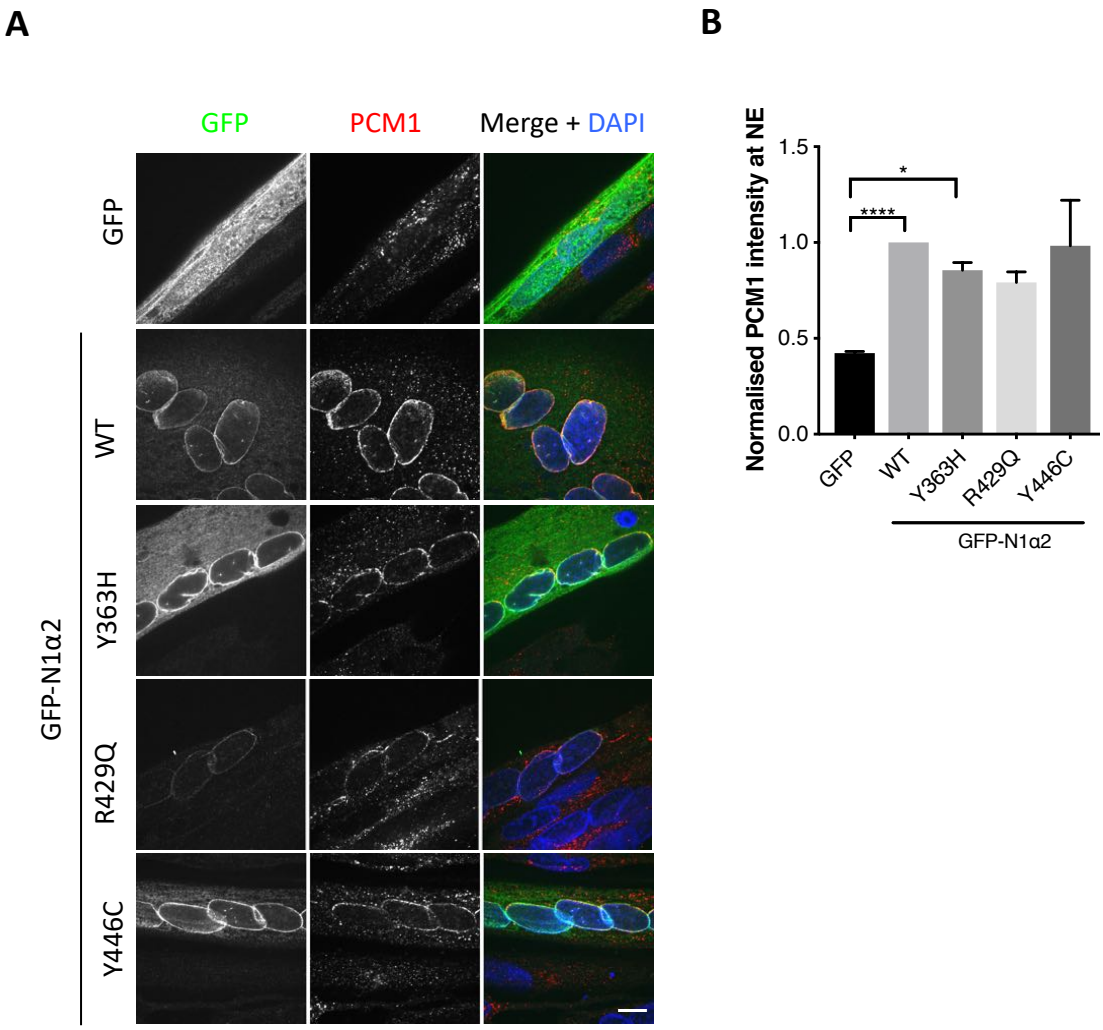


Figure 3.26 PCM1 recruitment to the NE is unaffected by nesprin-1α2 mutants which reduce rescue of KLC1/2 in *SYNE1* (23545 G>T) myotubes

(A) *SYNE1* (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2 truncations as indicated. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) Histogram showing PCM1 fluorescence intensity at the NE of nuclei expressing each GFP-nesprin-1α2 construct, and GFP alone. Average intensities were normalised to full-length (FL) GFP-nesprin-1α2, set at 1.0. Nuclei from 20 myotubes were analysed per experiment. Data show means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test. All significant differences are shown.

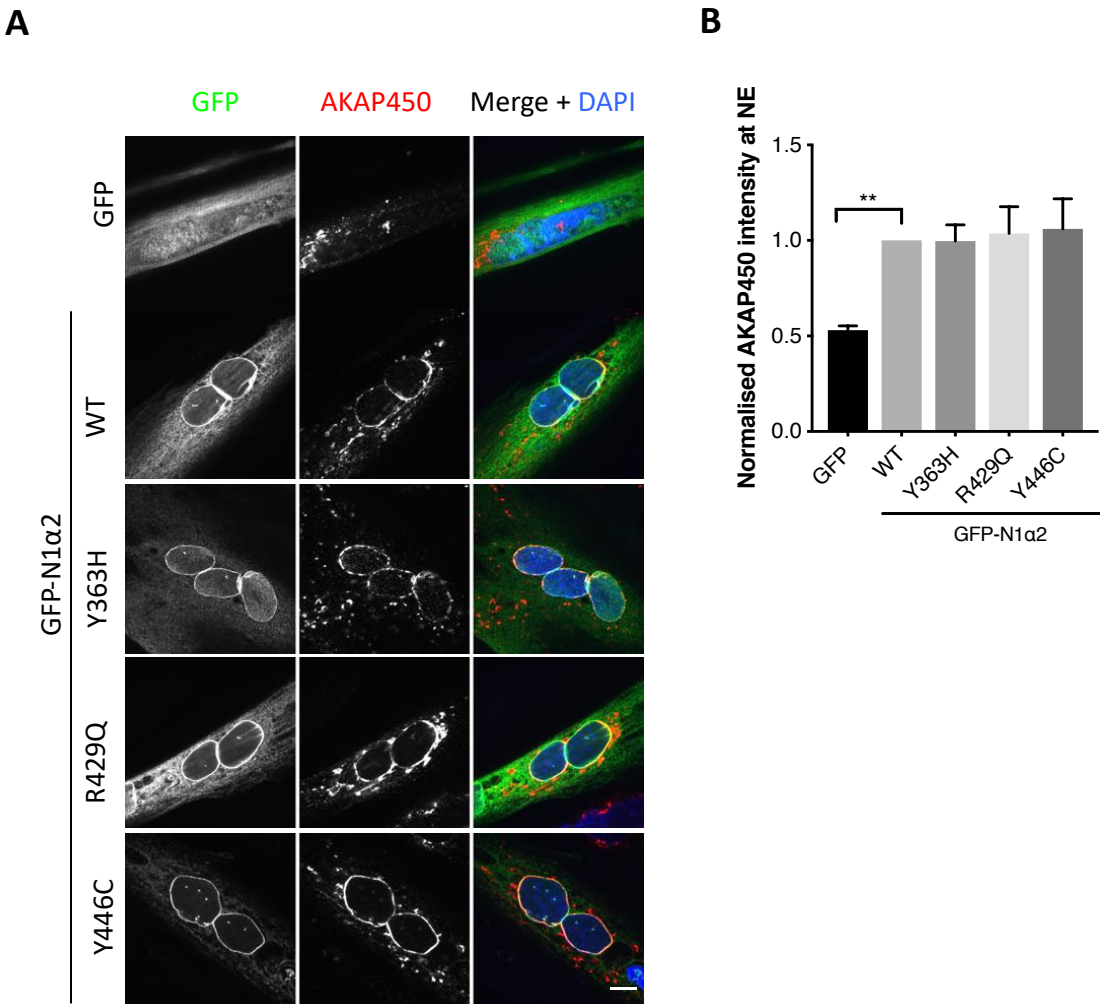


Figure 3.27 AKAP450 recruitment to the NE is unaffected by nesprin-1α2 mutants which reduce rescue of KLC1/2 in *SYNE1* (23545 G>T) myotubes

(A) *SYNE1* (23545 G>T) myotubes differentiated for 5 days were transfected with GFP-nesprin-1α2 truncations as indicated. After 24 h, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and AKAP450 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) Histogram showing AKAP450 fluorescence intensity at the NE of nuclei expressing each GFP-nesprin-1α2 construct, and GFP alone. Average intensities were normalised to full-length (FL) GFP-nesprin-1α2, set at 1.0. Nuclei from 20 myotubes were analysed per experiment. Data show means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test. All significant differences are shown.

By observation, all GFP-nesprin-1α2 proteins recruited AKAP450 to the NE similarly. Quantitative analysis showed a significant difference in the recruitment of AKAP450 between GFP-nesprin-1α2 and GFP, however there were no significant differences between the mutants with wildtype protein or GFP. Despite all GFP-nesprin-1α2 proteins appearing to recruit AKAP450 to the NE similarly, this could not be quantitively concluded.

Work using this set of mutants showed that PCM1 and AKAP450 localisation to the NE is not affected by R429Q/Y363H/Y446C nesprin-1α2 point mutants. However, this was not supported quantitively by significant differences.

3.3 Discussion

This chapter demonstrated that the muscle specific nesprin-1α2 is sufficient for the recruitment of nMTOC components PCM1 and AKAP450 to the NE. Then, a series of GFP-nesprin-1α2 mutants was used to identify the domains and residues required for the recruitment of PCM1 and AKAP450. Fig. 3.28 compares the recruitment between GFP-nesprin-1α2 and different mutants in a heatmap. For simplicity, the fluorescence intensity values from the FL and GFP construct are given as 100 and 0 respectively. Other values were normalised between the values, and any values greater than 100 or less than 0 were set to 100 or 0, respectively.

Although the domains required for the recruitment of PCM1 and AKAP450 to the NE were identified in this study, only future studies can indicate if recruitment of the proteins are by direct or indirect interaction with nesprin-1α2.

3.3.1 Measuring the recruitment of PCM1 and AKAP450 to the NE by fluorescence staining intensity measurements

Much of work in this chapter relied on analysing the level of PCM1 or AKAP450 recruitment to the NE in *SYNE1* (23545 G>T) myotubes expressing GFP-nesprin-1α2 proteins. This was achieved by measuring fluorescence intensity staining of the centrosomal proteins at the NE. However, consistent measurements were limited by GFP-nesprin-1α2 proteins which would recruit centrosomal proteins to the NE of all, some or no myonuclei, in myotubes within the same culture. One reason why centrosomal proteins may not be recruited to the NE is when the overexpressed protein is at both the NE and in the ER. In such cases, ER localised GFP-nesprin-1α2 proteins may compete for the recruitment of centrosomal proteins. In addition, in cells with no centrosomal protein recruitment at the NE, the centrosomal protein may be dispersed within the area of, but not associated with, the NE. Such localisation would be regarded as being at the NE during image analysis, and increase the overall fluorescence intensity staining measurement.

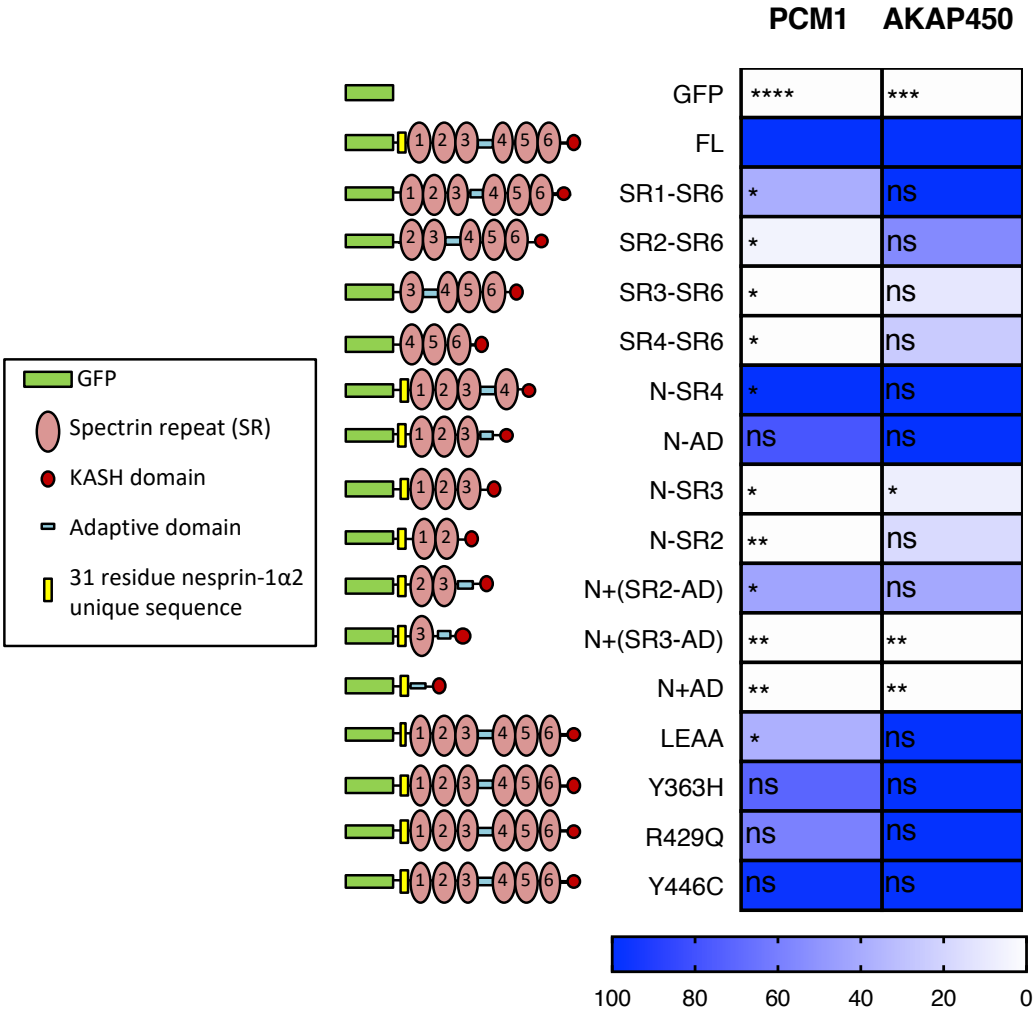


Figure 3.28 3.29 PCM1 and AKAP450 recruitment to the NE by nesprin-1α2 mutants

Heatmap showing the mean fluorescence intensity staining values of PCM1 and AKAP450 for each nesprin-1α2 mutant, normalised between 0 (GFP rescue) and 100 (full-length (FL) rescue). Values below 0 or above 100 are set to 0 and 100 respectively. Significant differences to the FL construct are indicated.

This was likely to have contributed to data variation, resulting in obvious observed differences between different GFP-nesprin-1α2 proteins not being supported by a statistical significance, despite an increase or decrease in protein recruitment also being reflected after quantification. Further repeats of the experiments would likely have led to a statistical significant difference between such results.

3.3.2 Nesprin-1α2 as the PCM1 and AKAP450 receptor

Two nesprin-1 isoforms are expressed during myogenesis: the ubiquitously expressed nesprin-1G, and the muscle-specific nesprin-1α2. This chapter confirmed that nesprin-1α2 is sufficient to recruit centrosomal proteins to the myotube NE to form the nMTOC, as reported by Gimpel *et al. (*2017). In agreement, nesprin-1α2 interacts with the centrosomal protein PCM1 in myotubes (Espigat-Georger *et al.,* 2016).

Aside from its N-terminal 31 residues, nesprin-1α2 is identical to the C-terminal end of nesprin-1G. It is therefore interesting why centrosomal components might only be recruited by nesprin-1α2. An explanation, as suggested by the current results, is that the nesprin-1α2 isoform-specific sequence is what provides its unique properties. This region may help protein folding to provide accessible binding sites, or be the recipient of post-translational modifications to activate the protein as a MTOC component receptor. Another possibility is that when nesprin-1G is bound at the NE, the N-terminal 68 SRs block the binding sites of the last 6 SRs. As an example of how the long rod domain could disrupt C-terminal SRs near the NE, exogenously expressed nesprin-3 at the NE interacts with the N-terminal actin binding domain of nesprin-1G, creating an arch where nesprin-1G is tethered to the NE at both ends of the protein (Lu *et al.,* 2012). The role of nesprin-1G in recruiting centrosomal proteins to the NE has not been explored in *in vitro*, most likely due the inevitable difficulty of exogenously expressing a >1000 kDa protein in mammalian cells, and no method to only knockdown nesprin-1α2 in myotubes, due to sequence similarity to the C-terminal of nesprin-1G. However, this could be investigated in the nesprin-1G and nesprin-1α2 knockout mouse model,

successfully generated by Stroud *et al. (*2017), by staining for the presence of centrosomal proteins at the nuclei of mouse myofibres.

Although many *SYNE-1* mutations in muscular dystrophy patients are in the region coding for nesprin-1α2, there are some which only affect nesprin-1G (Sandra *et al.,* 2019; Zhou *et al.,* 2017, 2018). There is possibly another role for nesprin-1G in myogenesis, which is not involved in nuclear positioning.

3.3.3 Structural stabilisation of nesprin-1α2 by residues 1-31 and SR1

There are no reported roles of the muscle-specific nesprin-1α2 N-terminal 31 residues, or of SRs in nMTOC formation. It is interesting that compared to the full-length protein, GFP-nesprin-1α2(SR1-SR6) recruits less PCM1, whereas there is no recruitment observed with GFP-nesprin-1α2(SR2-SR6) and further truncations, yet GFP-nesprin-1α2(N+(SR2-AD)) is able to weakly recruit PCM1. This suggests that the nesprin-1α2 residues 1-31 and SR1 play independent, yet additive roles in contributing towards PCM1 recruitment, perhaps by structural stabilisation of sites within nesprin-1α2 to facilitate protein folding. The role of nesprin-1α2 residues in AKAP450 recruitment was less defined. Analysis revealed GFP-nesprin-1α2(SR1-SR6) to recruit higher levels of AKAP450, compared to the full-length protein, however it was not significantly higher and individual fluorescence staining values for GFP-nesprin-1α2(SR1-SR6) were highly variable. Extra repeats of the experiments would be highly desirable to confirm the recruitment of AKAP450 by GFP-nesprin-1α2(SR1-SR6). However, the use of C-terminal nesprin-1α2 truncations revealed nesprin-1α2 residues 1-31 and SR1 to have additive roles in the recruitment of AKAP450 to the NE, as with PCM1.

If nesprin-1α2 residues 1-31 and SR1 do stabilise nesprin-1α2 for the recruitment of PCM1 and AKAP450, a question is whether the function of this N-terminal protein cap can be substituted by a non-specific protein sequence. In this case, the N-terminal GFP-tag may have played a stabilisation role, and led to higher

recruitment of proteins. In this case there could be instances where an observed rescue would not be seen without GFP. Further evaluation of this cannot be by switching the GFP-tag to the C-terminus of nesprin-1α2, as it contains the NE localising KASH domain, which interacts with SUN1/2 for NE anchorage. Instead, utilisation of a smaller N-terminal tag, such as a FLAG-tag, or even the use of untagged proteins is a possibility. The nesprin-1 MANNES1A antibody targets either SR6 or the KASH domain, as it can detect GFP-nesprin-1α2(SR6 + KASH) in *SYNE1* (23545 G>T) myoblasts (not shown). The same experiment may be done with a GPP-KASH construct to determine the actual antibody binding site. If it is the KASH domain, untagged truncations, as generated in this chapter can be expressed and stained by MANNES1A. This approach may still be used if the antibody epitope is at SR6, as SR6 does not impact upon protein rescue. In this case, SR6 would have to be included in every truncation generated for detection in cells.

If a non-specific N-terminal cap on nesprin-1α2(SR2-AD) is able to recruit PCM1 and AKAP450, this suggests nesprin-1G may also be capable of recruiting centrosomal components to the NE. Though unable to exogenously express full-length nesprin-1G, expressing N-terminal truncations of the protein, comprising greater than six SRs would show if a protein cap too high in molecular weight can prevent protein recruitment.

3.3.4 The AD of nesprin-1α2 co-operates with SRs to recruit PCM1 and AKAP450 to the NE

SRs have traditionally been considered as molecular spacers to separate domains within a protein (Djinovic-Carugo *et al.,* 2002). More recently they have been considered to contain protein binding sites, for example, nesprin-2 SR48-49 directly interacts with BIN1 (D'Alessandro *et al.,* 2015). The AD is an unstructured region and stabilises the SRs of nesprin-1α2, preventing unfolding (Zhong *et al.,* 2010). The AD also interacts with dynein (Zhu *et al.,* 2017), and contains the LEWD

motif required for a nesprin to interact with KLC1/2 (Wilson and Holzbaur, 2015; Zhou *et al.,* 2017).

The minimum truncation capable of recruiting PCM1 and AKAP450 to the same extent as the FL protein is GFP-nesprin-1α2(N-AD). Further removal of the AD abolishes all recruitment, yet GFP-nesprin-1α2(N + AD) is not sufficient to recruit PCM1 nor AKAP450 to the NE, indicating that co-operation of the AD with SRs is required. It is possible that the AD stabilises the SRs, enabling the latter to interact with proteins for PCM1 and AKAP450 recruitment. Indeed, nesprin-1α2 SR1-SR3 are evolutionary more conserved compared to SR4-SR6, and so are more likely to contain residues for binding with other proteins (Autore *et al.,* 2013). Alternatively, the AD may directly recruit PCM1 and AKAP450 to the NE, and require SRs solely as a molecular spacer. Indeed, the AD interacts with kinesin-1 and dynein (Zhu *et al.,* 2017; Zhou *et al.,* 2018; Wilson and Holzbaur, 2015), and therefore may interact with other proteins. To test this, SR1, SR2 and SR3 in GFP-nesprin-1α2(N-AD) could be replaced with SR4, SR5, and SR6. As nesprin-1α2 and PCM1 have been found to interact by co-immunoprecipitation (Espigat-Georger *et al.,* 2016), co-immunoprecipitations of PCM1 with nesprin-1α2 truncations would complement this data.

3.3.5 Nesprin-1α2 recruits PCM1 and AKAP450 through similar but not identical domains

Similar observations of PCM1 and AKAP450 recruitment to the NE in *SYNE1* (23545 G>T) myotubes by nesprin-1α2 mutants suggest there may be interdependency between the proteins for localisation. However, Gimpel *et al.* (2017) showed that PCM1 or AKAP450 depletion in myotubes does not affect the NE localisation of each other. It would be desirable to obtain antibodies allowing for the triple staining of GFP, PCM1 and AKAP450, to investigate if proteins are recruited in the same cells. The relationship between the PCM1 and AKAP450 as part of the nMTOC is further explored in chapter 4.

It is clear that nesprin-1 isoforms are required for the localisation of PCM1 and AKAP450 to the NE. However, nesprin-1 null myotubes display mislocalisation of the Golgi protein GM130 from the NE (Gimpel *et al., 2*017), and AKAP450 is known to bind to GM130 in centrosome-containing cells (Rivero *et al.,* 2009; Wu *et al.,* 2016). It is therefore possible that AKAP450 is recruited to a myotube nucleus in two distinct layers: by binding to the surface of the NE with other centrosomal proteins, and by binding on the surface of the NE-localised Golgi. It would be interesting to investigate whether GM130 requires the same nesprin-1α2 truncation mutants as AKAP450 for NE localisation.

3.3.6 A potential relationship between the recruitment of PCM1 and kinesin-1 to the NE in myotubes

Muscle cells are the only mammalian cells in which centrosomal proteins localise at the NE, suggesting recruitment is mediated by a muscle-specific system. On the other hand, NE localisation of kinesin-1 is not limited to muscle cells and can be observed in cells expressing nesprin-4 (Roux *et al.,* 2009), or nesprin-2 (Schneider *et al.,* 2011), to function in cell polarisation. Kinesin-1 and dynein also localise to the NE through association with the nuclear pore complex in non-muscle cells during mitotic entry (Splinter *et al.,* 2010). Hence, in non-myotubes, the NE localisation of kinesin-1 is not accompanied by PCM1. However, PCM1 depletion in myotubes leads to the reduction of NE-localised KLC1 (Espigat-Georger *et al.,* 2016), indicating a myotube-specific relationship between the proteins at the NE.

The LEWD motif conserved in the AD of nesprin-1, nesprin-2 and nesprin-4, is essential for its interaction with KLC1/2 (Wilson and Holzbaur, 2015; Zhou *et al.,* 2018). Furthermore, unlike the wildtype protein, GFP-nesprin-1α2(LEAA) cannot recruit KLC1/2 to the NE of *SYNE1* (23545 G>T) myotubes (A. Haworth, unpublished data). Using the same mutant protein, this study showed that the LEAA mutant results in only partial recruitment of PCM1 to the myotube NE, though AKAP450 recruitment is unaffected. As PCM1 depletion in myotubes leads to reduced KLC1/2 localisation (Espigat-Georger *et al.,* 2016), and it is possible the

proteins are dependent on each other for their localisation at the NE. However, it may be that PCM1 itself requires the LEWD motif to be fully localised to the NE.

The mutant nesprin-1α2(R429Q) was found to have significantly reduced interaction with KLC1/2 (Zhou *et al.,* 2018), but it is not known whether GFP-nesprin-1α2(R429Q) impacts KLC1/2 recruitment to the myotube NE. However, neither this mutation, nor the uncharacterised muscle disease associated mutations Y363H and Y446C was found to impact PCM1 and AKAP450 recruitment to the NE. In the future, the potential interdependency of PCM1 and KLC1/2 for their localisation, can be investigated by the depletion of KLC1/2 in myotubes, followed by analysis of PCM1 localisation to the NE.

3.3.7 Consequences of reduced centrosomal proteins at the NE

Heavy reduction of pericentrin localisation at the NE has been observed in myotubes from muscular dystrophy patients with mutations in *SUN1* (Meinke *et al.,* 2014) and *LMNA* (Mattioli *et al.,* 2018), genes coding for proteins associated with the LINC complex which interact with nesprins (Mislow *et al.,* 2002; Stewart-Hutchinson *et al.,* 2008; Sosa *et al.,* 2012). The myotubes containing a *SUN1* mutation also displayed microtubule nucleation defects. It was not certain whether the defect was directly due to the mislocalisation of pericentrin or through other downstream effects of the SUN1 mutation. However, AKAP450 depleted myotubes lose the capability to nucleate microtubules (Gimpel *et al.,* 2017), indicating there are indeed consequences of impaired nMTOC formation. As this study, amongst other published data, suggested that centrosomal proteins are interdependent on each other for their localisation to the NE (Espigat-Georger *et al.,* 2016; Gimpel *et al.,* 2017), reducing or abrogating the recruitment of one centrosomal protein, may lead to the reduction of further proteins, with individual effects adding up to lead to a dysfunctional nMTOC.

3.3.8 Summary

Having confirmed nesprin-1α2 as a NE bound centrosomal protein receptor, the role of nesprin-1α2 domains in the recruitment of PCM1 and AKAP450 to the myotube NE was explored. The N-terminal sequence of nesprin-1α2 was found to facilitate recruitment of PCM1 and AKAP450 to the NE, whereas the AD was found to co-operate with SRs and be essential for their recruitment. PCM1, similar to KLC1/2, requires the LEWD motif for recruitment. There may be some interdependency between the localisation of PCM1 and AKAP450 to the NE, a relationship previously unreported. In addition to the new understandings, a set of nesprin-1α2 truncation mutants was generated, that can be used to investigate the nesprin-1α2 domains responsible for the recruitment of other proteins to the NE.

Chapter 4
Characterising the mode by which centrosomal proteins are recruited to the NE

4.1 Introduction

The microtubule organising centre (MTOC) is the site from which microtubules nucleate. The centrosome is the main MTOC in proliferating cells, and consists of a central pair of centrioles surrounded by a mass of proteins termed the pericentriolar material (PCM). The roles of PCM proteins at the centrosome are connected. For example, pericentrin and AKAP450 bind the microtubule nucleation template complex γ-TuRCs (Delaval and Doxsey, 2010; Kolobova *et al.,* 2017), whereas CDK5RAP2 is required for the latter to nucleate microtubules (Choi *et al.,* 2010). The centrosomes are surrounded by electron-dense granules termed the centriolar satellites. Pericentriolar material 1 (PCM1) is a major centriolar satellite component, and is involved in microtubule and dynein-mediated shuttling of PCM proteins to the centrosome (Young *et al.,* 2000; Dammermann and Merdes, 2002), and the anchorage of microtubules to the centrosome (Dammermann and Merdes, 2002). Hundreds of PCM and centriolar satellite proteins have identified through mass spectrometry techniques (Paz and Lüders, 2018).

Upon myogenesis, the site of the major MTOC is transferred to the NE, as the centrosome of a committed myoblast disassembles, and centrosomal proteins, including the ones discussed relocalise at the outer nuclear membrane (Gimpel *et al.,* 2017). Data from chapter 3 showed that nesprin-1α2 acts as the receptor to recruit centrosomal proteins to the NE. However, is unclear how the proteins, once released from the centrosome are able to relocalise to the NE to form part of the multi-protein nMTOC. There are three factors to consider: the protein would require a localisation domain, it may require the presence of another centrosomal protein at the NE, and it would require a method of transportation, by either by diffusion or by utilising the cytoskeleton.

Protein localisation requires a localisation domain or motif, to interact with a protein at its destination. Pericentrin contains a C-terminal PACT domain, which is shown to be sufficient for centrosome localisation (Martinez-Campos *et al.,* 2004; Gillingham and Munro, 2000). The same domain is sufficient for NE localisation in

the presence of nesprin-1α2 in myoblasts (Gimpel *et al.,* 2017). Indeed, tandem affinity purification indicated that nesprin-1 and pericentrin are interacting partners (Falk *et al.,* 2018), and biotin identification has been used to show that pericentrin, alongside PCM1, AKAP450 and AKAP6, are close in proximity to nesprin-1α2 in myotubes (Gimpel *et al.,* 2017). PCM1 and nesprin-1α2 have been shown to interact in myotubes by co-immunoprecipitation (Espigat-Georger *et al.,* 2016), however the localisation domain of PCM1 has not been identified. AKAP450 also contains a C-terminal PACT domain, which is required for centrosomal localisation (Gillingham and Munro, 2000). However, it is not known if it is sufficient for NE localisation in myotubes. Further studies defining the interacting domains of MTOC proteins and nesprin-1α2 would present a better picture of the nMTOC at the nesprin-1α2 interface.

Proteins in a multi-protein complex may be interdependent on each other for their recruitment. This may be because binding of a ligand protein to a receptor results in the conformational change of either protein, which allows further proteins to bind (Laskowski *et al.,* 2009). Alternatively, a ligand may act as a scaffold to physically localise other proteins of the complex. This appears to be the case in centrosomal MTOC formation, where PCM1 is a scaffold for other centrosomal proteins such as pericentrin, ninein and centrin (Dammermann and Merdes, 2002), and pericentrin is a scaffold for proteins such as CDK5RAP2 (Buchman *et al.,* 2010). This role of PCM1 and pericentrin appear to be conserved at the nMTOC, as PCM1 and pericentrin depletion leads to the reduction of pericentrin and CDK5RAP2, respectively, at the NE (Gimpel *et al.,* 2017; Espigat-Georger *et al.,* 2016).

The physical transfer of a protein to a location may be through diffusion, or by utilisation of the cytoskeleton. In centrosome-containing cells, PCM1 and pericentrin are transported along microtubules by dynein towards the centrosome (Young *et al.,* 2000; Dammermann and Merdes, 2002). It is not known whether PCM1 requires microtubules for its transfer to the NE in a differentiating myoblast. The anchorage of proteins at a location may also require the cytoskeleton. Centriolar satellites are dynamic and have no physical structure to bind, and are

instead held in position by microtubules (Kubo *et al.,* 1999; Stowe *et al.,* 2012). It is possible that microtubules also anchor PCM1 at the NE. On the other hand, studies for pericentrin show that microtubules are not needed for its anchorage at the centrosome or NE (Musa *et al.,* 2003; Gimpel *et al.,* 2017; Zaal *et al.,* 2011).

This chapter aimed to identify how centrosomal MTOC proteins localise to the myotube NE as discussed. Regions of PCM1 and pericentrin responsible for localisation to the centrosomal and nMTOC were identified. Next, the interdependency of pericentrin and AKAP450 on PCM1 for their localisation at the NE was quantitatively explored. Finally, the role of microtubules in centrosomal protein localisation and anchorage at the NE was investigated.

4.2 Results

4.2.1 Identifying the PCM1 and pericentrin regions responsible for NE localisation

The first aim was to identify the region of PCM1 and pericentrin responsible for centrosome and NE localisation. It was particularly interesting to determine whether localisation at the two MTOCs utilise the same localisation region.

4.2.1.1 PCM1(1-331) is the minimum fragment required for NE localisation in myotubes

To investigate the region of PCM1 responsible for centriolar satellite and NE localisation, PCM1 truncation mutants were generated for use in localisation studies. As expected, endogenous PCM1 was localised at the centriolar satellites in centrosome-containing myoblasts, but relocated to the NE in myotubes (Fig. 4.1). Full-length GFP-PCM1, gifted from Songhai Shi was verified by western blot (Fig. 4.2). 293T cells were used to generate lysates for western blotting due to the low transfection efficiency of GFP-PCM1 in C2C12 myoblasts, likely due to its high molecular weight. Next, GFP-PCM1 was expressed in myoblasts and myotubes to verify that it behaves like endogenous PCM1 (Fig 4.3). Cells were co-stained with GFP and PCM1 antibodies. The PCM1 antibody used targets residues 1925-1975, which is present in both the endogenous and exogenous protein. In C2C12 myoblasts, GFP and PCM1 staining showed that GFP-PCM1 localised throughout the cytoplasm instead of the centriolar satellites, possibly due to the overexpressed protein flooding the cell. However there was a region of concentrated fluorescence staining adjacent to the NE by both antibodies, which was likely to be the centriolar satellites. In myotubes, the protein was localised around the NE as expected.

In order to map the PCM1 region that binds to the myotube NE, a series of GFP-PCM1 truncation mutants was generated. Human PCM1 is 2024 residues and

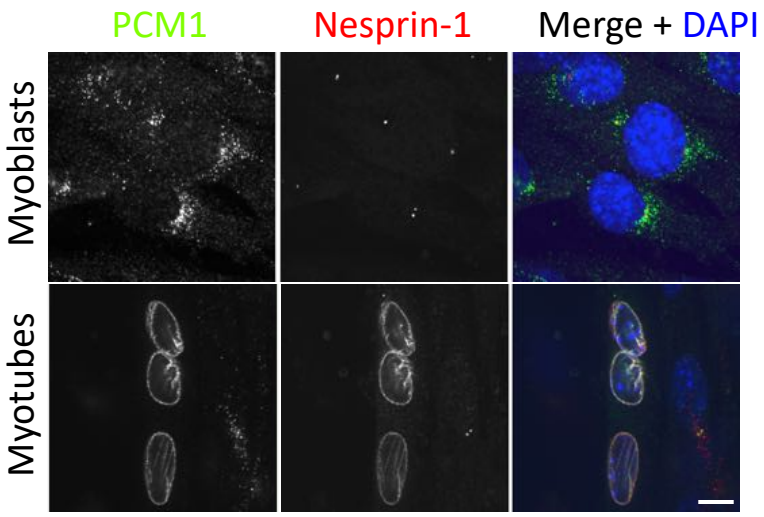


Figure 4.1 PCM1 localises to the centriolar satellites in myoblasts and to the NE in myotubes

C2C12 myoblasts and 5 day old myotubes were fixed in -20°C methanol and co-stained with antibodies against PCM1 (green) and nesprin-1 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

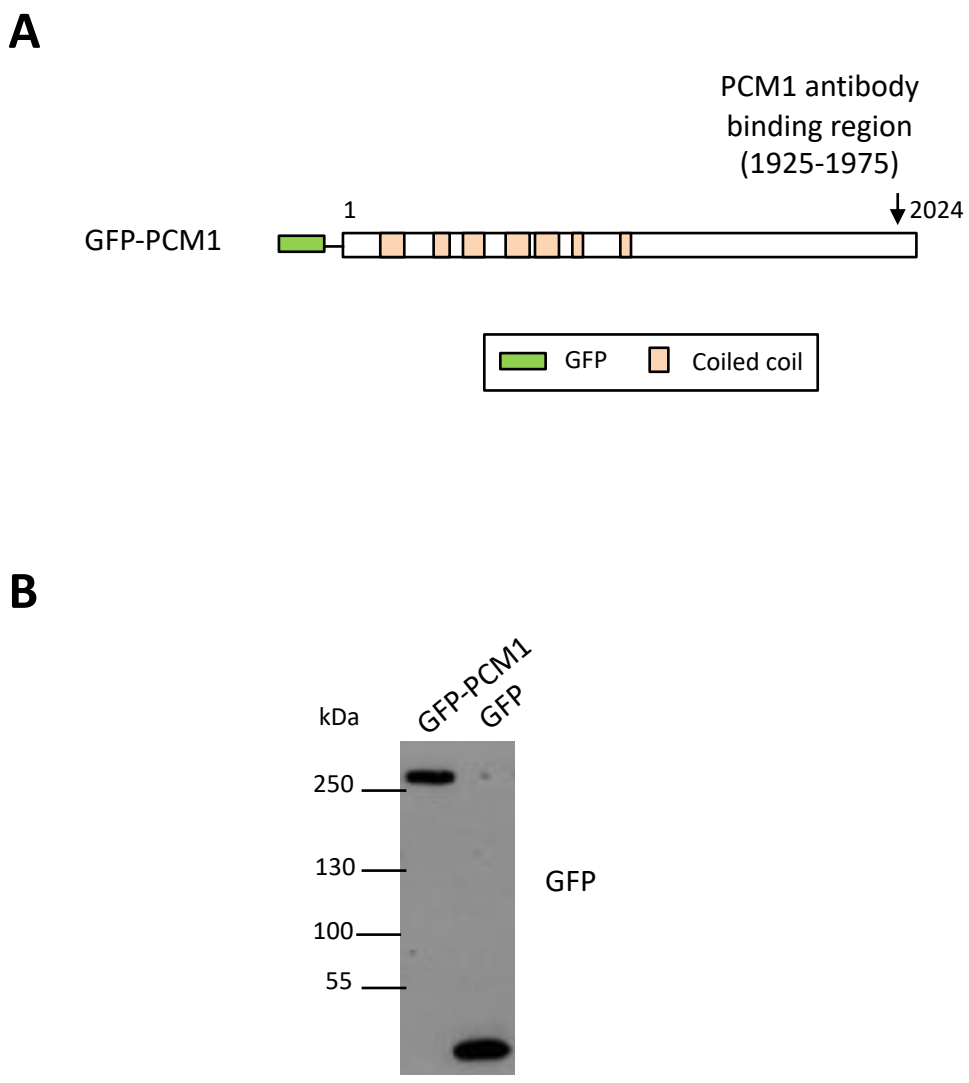


Figure 4.2 Schematic representation and western blot of GFP-PCM1

(A) Schematic representation of GFP-PCM1. (B) 293T cells were transfected with GFP or GFP-PCM1 for 24 h. Cells were lysed with RIPA buffer and 50 µg of whole cell extracts were analysed by immunoblotting with GFP antibody.

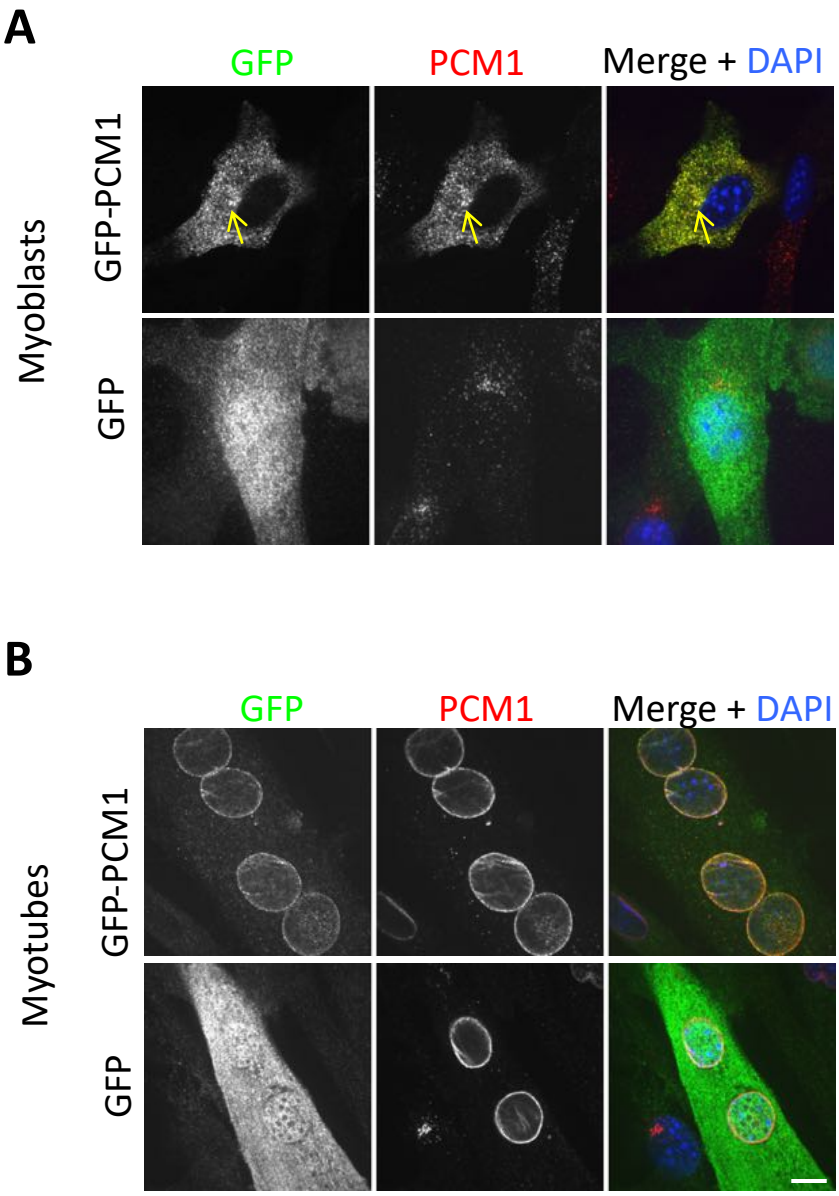


Figure 4.3 GFP-PCM1 localises to the cytoplasm in myoblasts and to the NE in myotubes

C2C12 myoblasts were transfected with GFP-PCM1 or GFP and fixed with -20°C methanol the next day for the study of myoblasts (A), or differentiated for 5 days for the study of myotubes (B). Cells were co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Yellow arrows indicate concentrated staining of proteins adjacent to the NE, reminiscent of centriolar satellites. Scale bar = 10 μm.

contains seven coiled-coil regions which are mostly in the N-terminal half of the protein (Fig. 4.2). These were used as the start or end point of PCM1 truncations to improve the likelihood of correct protein folding. PCM1 mutants were expressed in myoblasts and myotubes and their localisation were examined by immunofluorescence microscopy.

Firstly, the 2024 residue PCM1 was split into two halves, GFP-PCM1(1-1089) and GFP-PCM1(1062-2024). Protein expression was confirmed by western blot (Fig. 4.4) and localisation was investigated in myoblasts and myotubes (Fig. 4.5). In C2C12 myoblasts, GFP-PCM1(1-1089) localised to the centriolar satellites and GFP-PCM1(1062-2024) localised in the nucleus. In myotubes, GFP-PCM1(1-1089) colocalised to the NE with endogenous PCM1, whereas GFP-PCM1(1062-2024) remained inside the nucleus. Although PCM1 antibody targets residues within GFP-PCM1(1062-2024), it did not stain the overexpressed protein well in the nucleus. This may be due to the PCM1 antibody-epitope being inaccessible in the nucleus. It is not clear why only the truncated, but not full-length GFP-PCM1 clearly associates with centriolar satellites, however the current data shows that the N-terminal half of PCM1 is responsible for both centriolar satellite and NE localisation. The nuclear localisation of GFP-PCM1(1062-2024) may be due to the presence of a putative bipartite nuclear localisation signal sequence (NLS) within residues 1723-1741 (Fig. 4.4), identified using the NLS predictor cNLS Mapper. PCM1 has not been otherwise reported to be inside the nucleus.

To further narrow the domain responsible for NE localisation, GFP-PCM1(1-1089) was truncated into three fragments: GFP-PCM1(1-331), GFP-PCM1(302-573) and GFP-PCM1(544-1089). Correct expression was confirmed by western blot (Fig. 4.6), and localisation was investigated in myoblast and myotubes by immunofluorescence microscopy (Fig. 4.7). The PCM1 antibody epitope was not present in any of these truncations, therefore it only stained for endogenous PCM1. In myoblasts, GFP-PCM1(1-331) and GFP-PCM1(302-573) were localised throughout the cytoplasm whereas GFP-PCM1(544-1089) co-localised with endogenous PCM1 at the centriolar satellites. In myotubes, GFP-PCM1(302-573) was again in the cytoplasm. Both GFP-PCM1(1-331) and GFP-PCM1(544-1089)

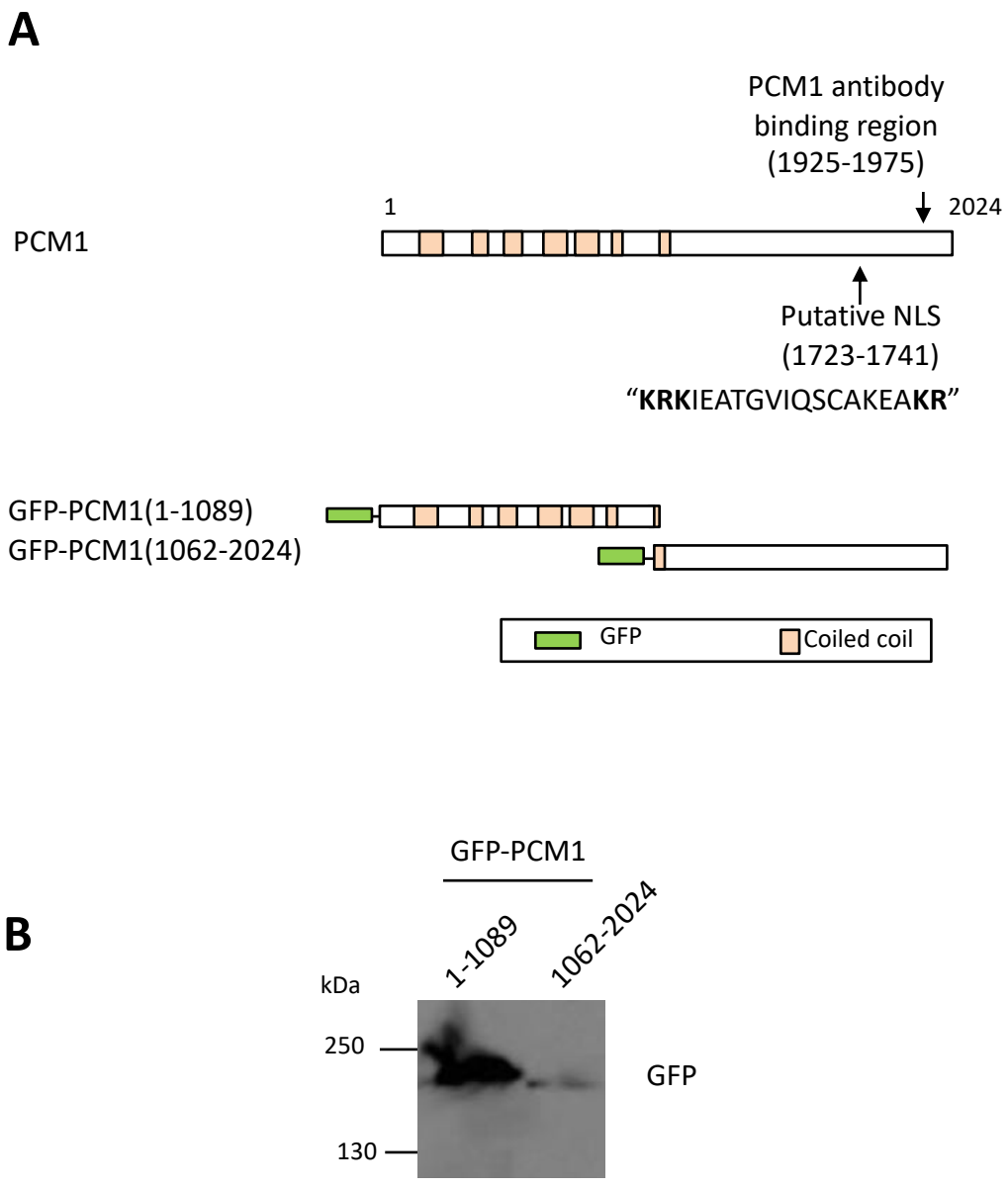


Figure 4.4 . Schematic representation and western blot of PCM1 N-terminal and C-terminal constructs

(A) Schematic showing GFP-PCM1 truncation constructs. The position of the PCM1 antibody binding site and the putative bipartite nuclear localisation signal (NLS) is indicated. The NLS sequence is shown with basic residues in bold. (B) C2C12 cells were transfected with the constructs for 24 h. Cells were lysed with RIPA buffer and 50 µg of whole cell extracts were analysed by immunoblotting with GFP antibody.

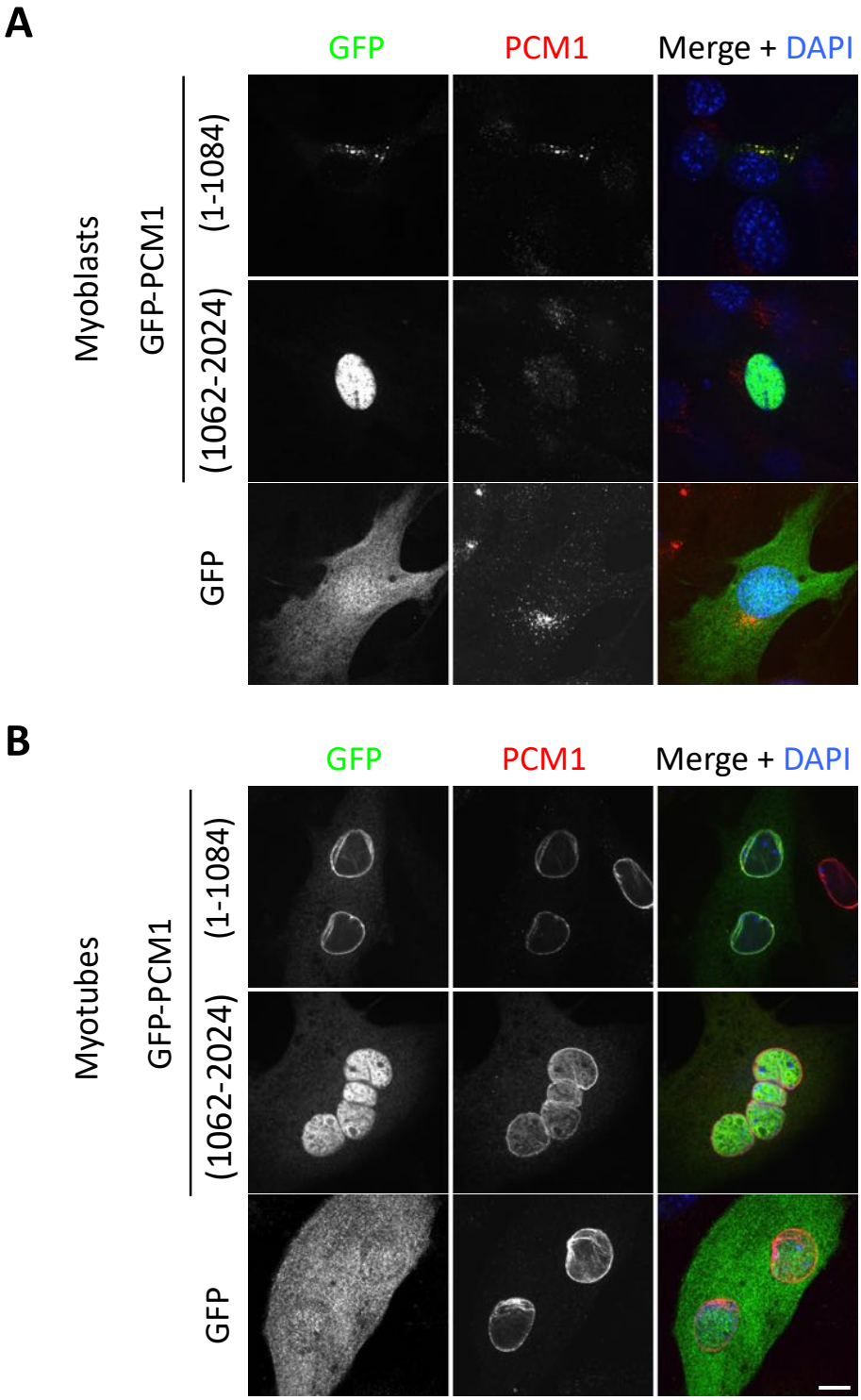


Figure 4.5 PCM1(1-1089) localises to the MTOC whereas PCM1(1062-2024) is imported into the nucleus in both myoblasts and myotubes

GFP-PCM1(1-1089), GFP-PCM1(1062-2024) and GFP were transfected into C2C12 myoblasts and fixed the next day for the study of myoblasts (A), or differentiated for 5 days for the study of myotubes (B). Cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

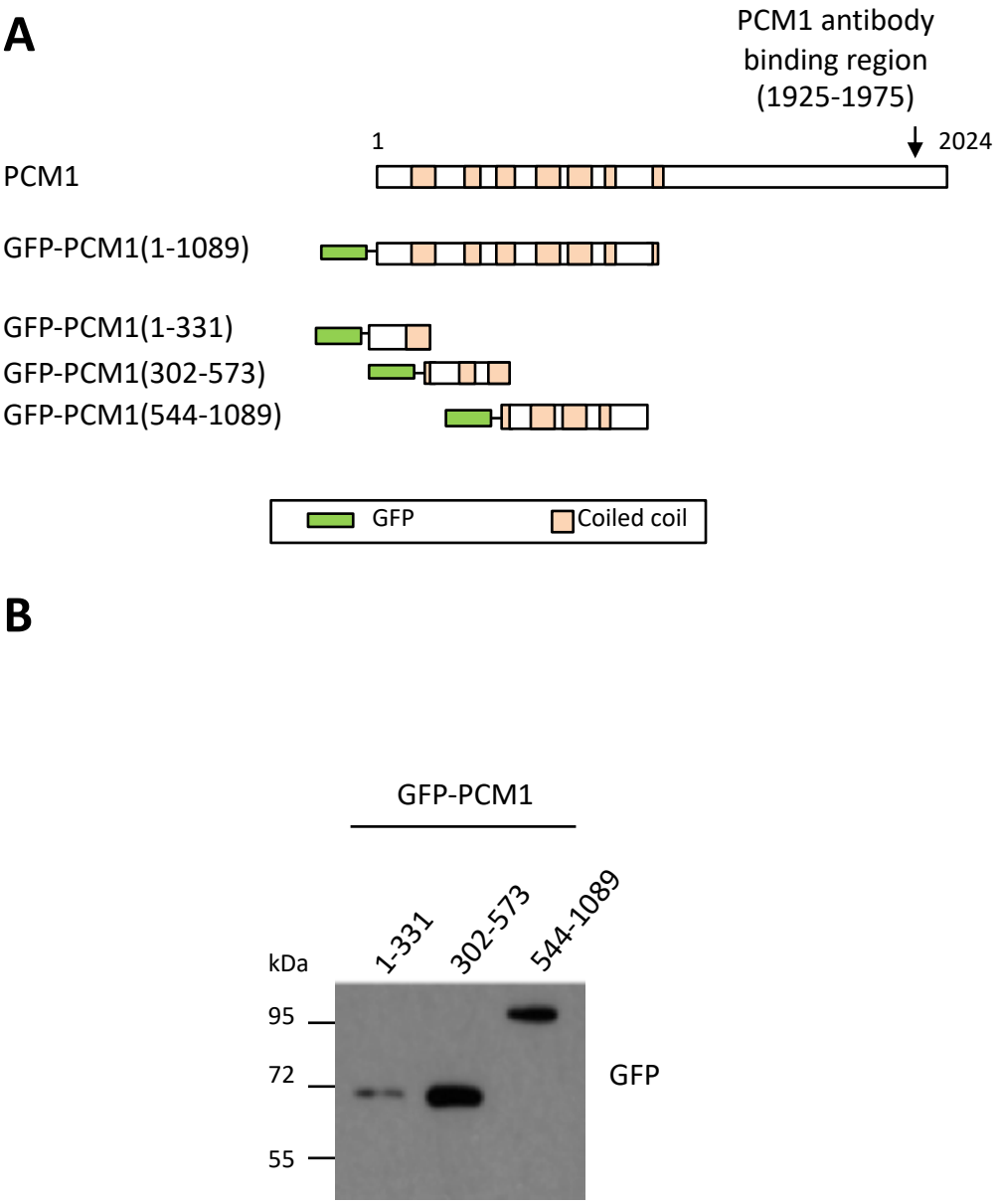


Figure 4.6 Schematic representation and western blot of PCM1(1-1089) truncations

(A) Schematic representation showing GFP-PCM1(1-1089) truncations. (B) C2C12 cells were transfected with the constructs for 24 h. Cells were lysed with RIPA buffer and 50 µg of whole cell extracts were analysed by immunoblotting with GFP antibody.

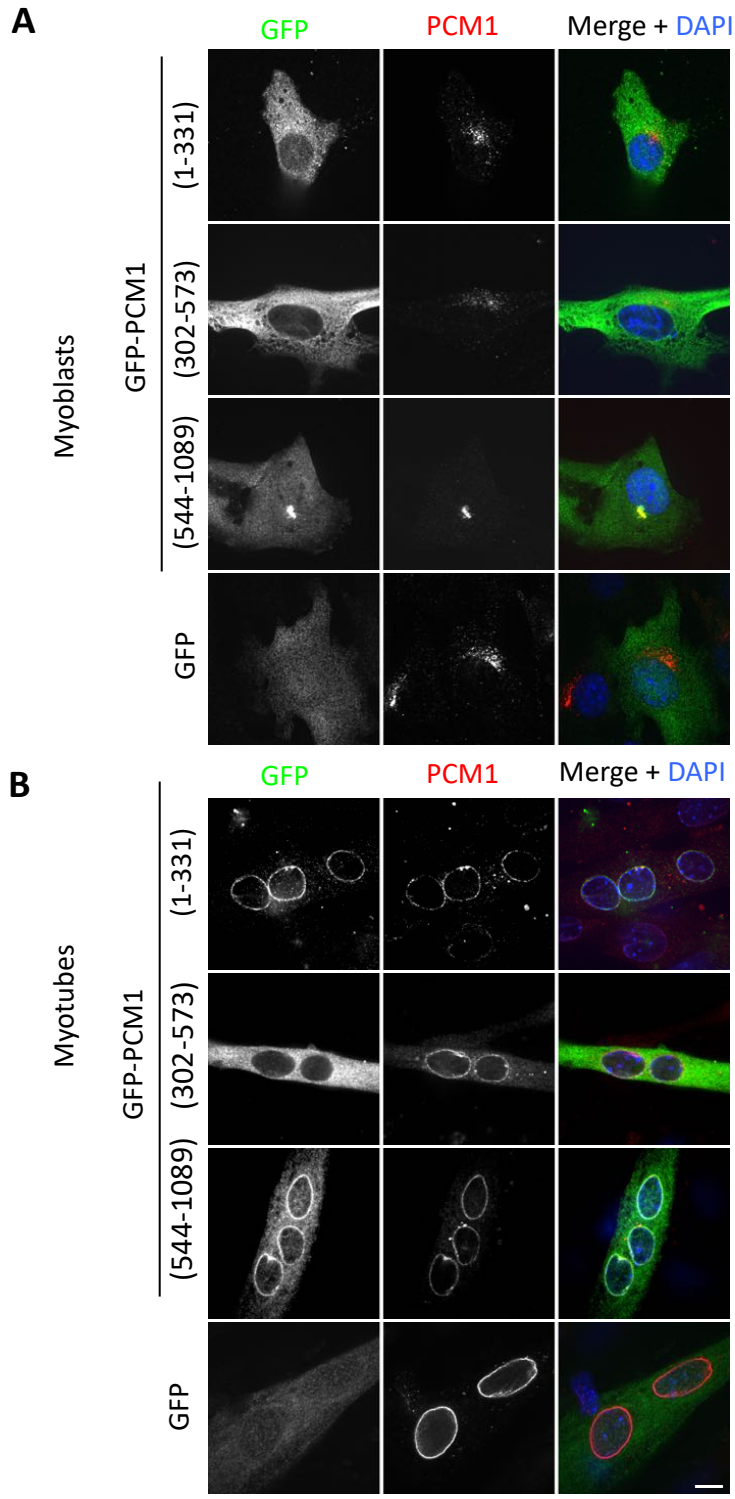


Figure 4.7 GFP-PCM1(544-1089) localises to the centriolar satellites, whereas both GFP-PCM1(1-331) and GFP-PCM1(544-1089) localise to the NE in myotubes

GFP-PCM1 truncations were transfected into C2C12 myoblasts and fixed the next day for the study of myoblasts (A), or differentiated for 5 days for the study of myotubes (B). Cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

showed NE localisation, however, GFP-PCM1(544-1089) localisation at the NE appeared less efficient as cytoplasmic staining was also observed in many cells.

Since PCM1 is rich in coiled-coil regions, there is the likelihood for the colocalisation of PCM1 constructs with the endogenous protein to be due to self-association with endogenous PCM1. To investigate this, endogenous PCM1 was depleted in C2C12 myoblasts using a siRNA oligo that targets the C-terminal half of PCM1 (verification of the oligo is in section 4.2.2.1). Then, relevant GFP-PCM1 constructs were transfected the next day to test their localisation in the absence of endogenous PCM1. Cells were fixed the next day to study myoblasts, or differentiated for 3 days for the study of myotubes (Fig. 4.8). In PCM1-depleted myoblasts, GFP-PCM1(544-1089) was localised in foci similar to centriolar satellites adjacent to the nucleus. This indicates PCM1(544-1089) localises to centriolar satellites adjacent to the nucleus independent of endogenous PCM1. GFP-PCM1(1-331) was not transfected into PCM1-depleted myoblasts, due to its cytoplasmic localisation in the cells. In PCM1-depleted myotubes, GFP-PCM1(544-1089) was no longer at the NE and was present only in the cytoplasm, whereas GFP-PCM1(1-331) remained localised at the NE. This indicates only PCM1(1-331) binds to the NE independently, whereas the observation of PCM1(544-1089) at the NE was due to association with endogenous PCM1. Interestingly, it shows that different residues of PCM1 mediate localisation at the centriolar satellites (544-1089) in myoblasts, and at the NE in myotubes (1-331).

Next, PCM1(1-331) was truncated into two sections and protein expression confirmed by western blot (Fig. 4.9). This was to examine whether binding to the NE could be further narrowed down (Fig. 4.10). The N-terminal portion, PCM1(1-217) is predicted to be unstructured whereas the C-terminal half PCM1(218-331) is a coiled-coil. Both proteins localised to the cytoplasm in myoblasts. However, localisation varied between myotubes. In some cells, both overexpressed proteins were weakly localised around the NE, and colocalised with endogenous PCM1 (Fig. 4.10B). In other myotubes, the proteins were mainly cytoplasmic and drove endogenous PCM1 away from the NE (Fig. 4.10C). To determine whether binding of the PCM1(1-331) truncations to the NE was due to binding to endogenous

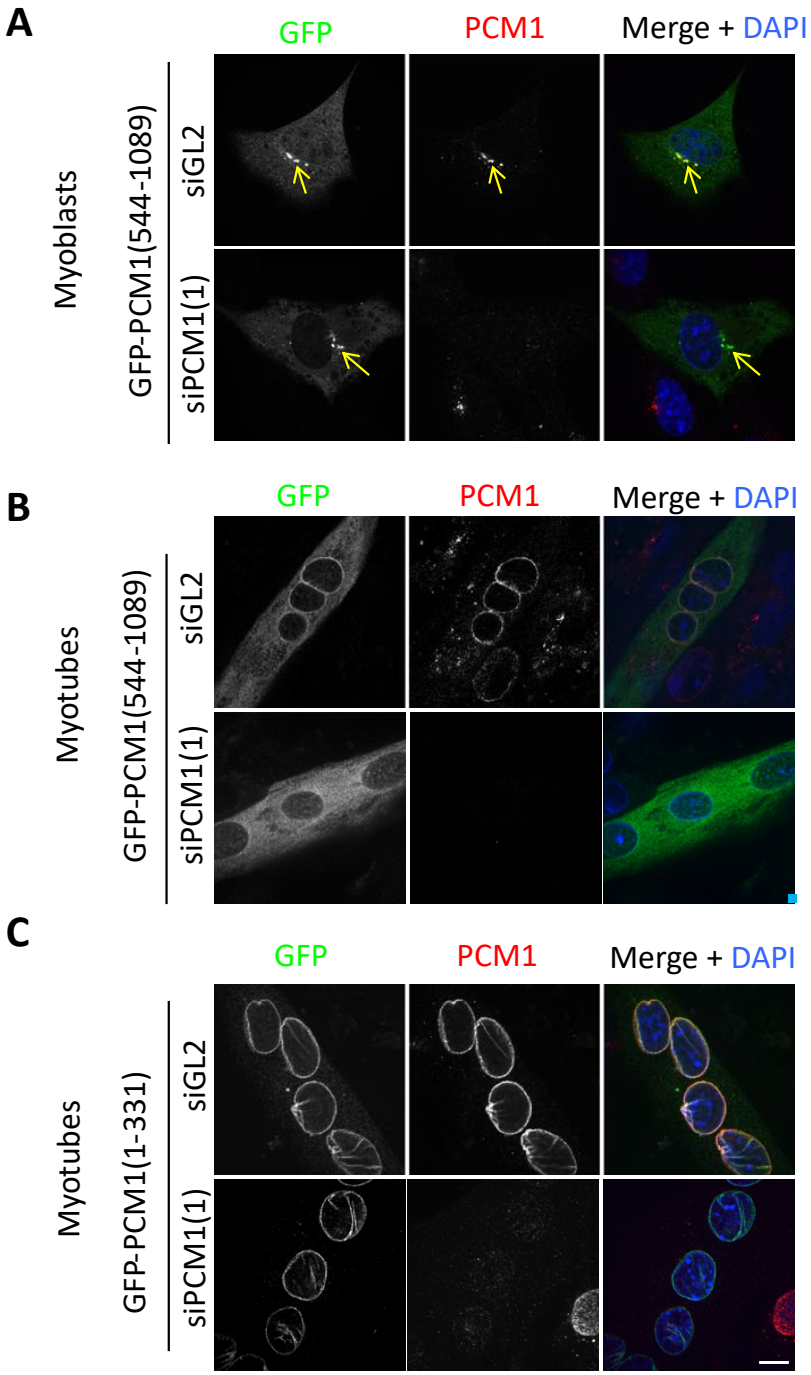


Figure 4.8 In absence of endogenous PCM1, GFP-PCM1(544-1089) localises to the centriolar satellites, whereas only GFP-PCM1(1-331) localises to the myotube NE

(A) C2C12 myoblasts were transfected with siGL2 or siPCM1(1) and then transfected with GFP-PCM1(544-1089) the next day. Cells were fixed after a further 24 h. (B+C) C2C12 cells differentiated for 2 days were transfected with siGL2 or siPCM1(1) oligos and then transfected with GFP-PCM1(544-1089) or GFP-PCM1(1-331) the next day. Cells were fixed after 3 further days of differentiation. Cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. Yellow arrows indicate centriolar satellite staining.

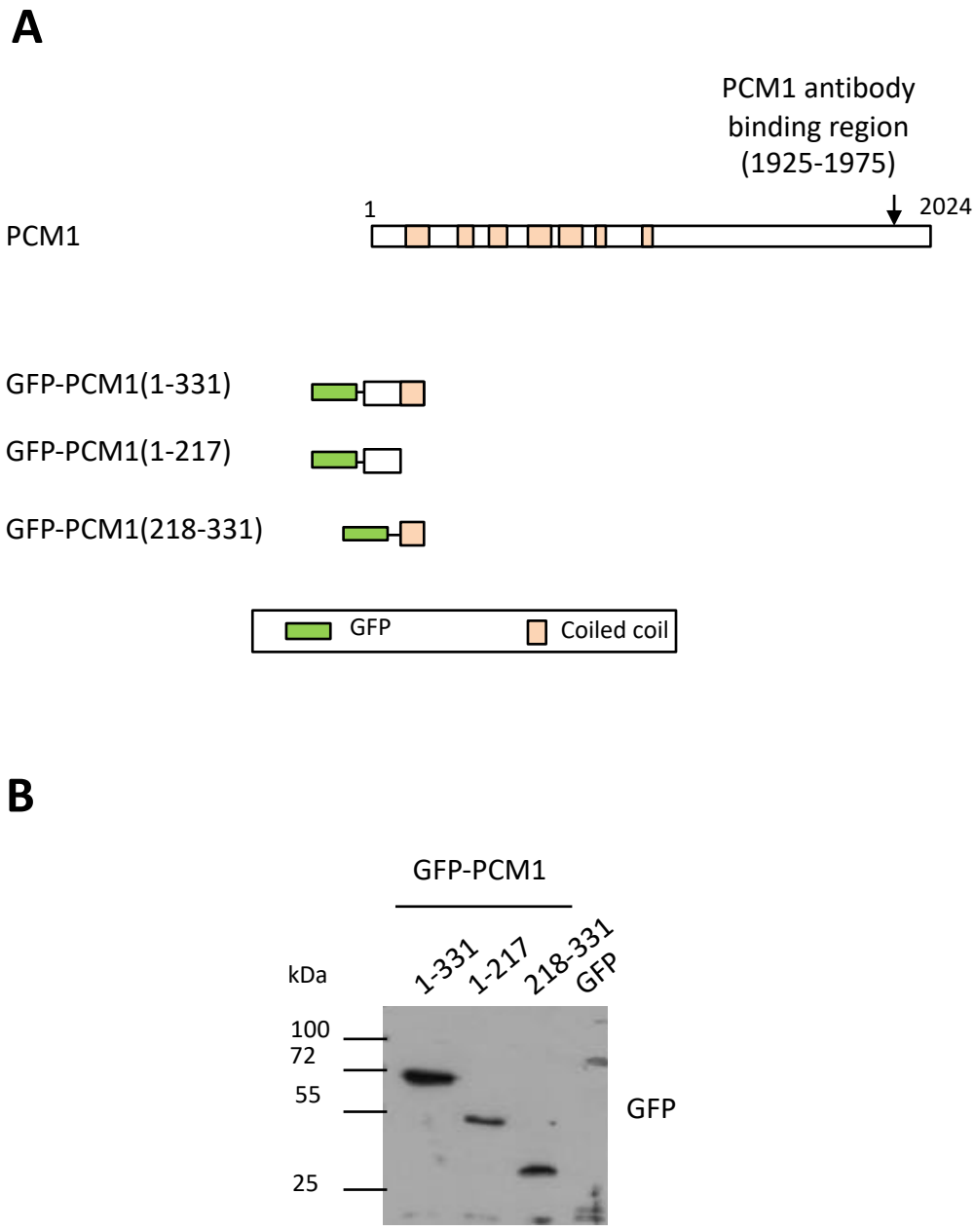


Figure 4.9 Schematic representation and western blot of PCM1(1-331) truncations

(A) Schematic representation of GFP-PCM1(1-331) and further and truncations. (B) C2C12 cells were transfected with the constructs for 24 h. Cells were lysed with RIPA buffer and 50 µg of whole cell extracts were analysed by immunoblotting with GFP antibody.

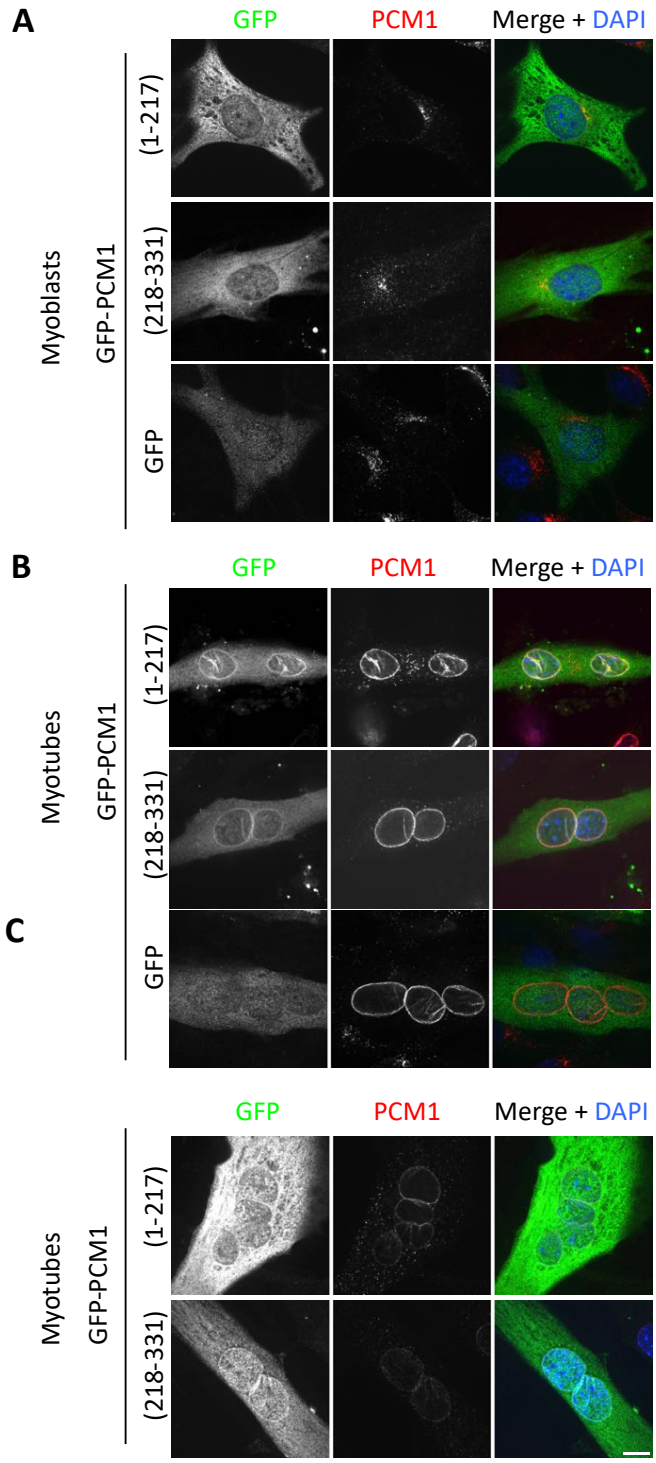


Figure 4.10 GFP-PCM(1-217) and GFP-PCM1(218-331) show weak localisation to the NE in myotubes

GFP-PCM(1-217) and GFP-PCM1(218-331) were transfected into C2C12 myoblasts and fixed the next day for the study of myoblasts (A), or differentiated for 5 days for the study of myotubes (B+C). Cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 µm.

PCM1, the constructs were expressed in PCM1 depleted myotubes. Both mutants remained bound to the NE after PCM1 depletion (Fig. 4.11). This suggests that both the unstructured and coiled-coil region are sufficient for weak NE binding but both are required together for strong association as seen with GFP-PCM1(1-331). Quantifying the immunofluorescent staining of the constructs at the NE would verify this.

4.2.1.2 PCM1(1-331) interacts with nesprin-1α2 in myotubes

The mapping studies presented in this chapter demonstrated that the minimal PCM1 fragment to independently and fully localise to the NE in C2C12 myotubes is PCM1(1-331). To test whether this localisation is by interaction with nesprin-1α2, the three N-terminal PCM1 fragments, GFP-PCM1(1-331), GFP-PCM1(302-573) and GFP-PCM1(544-1089) were expressed in C2C12 myotubes, and lysates were generated for use in GFP-trap co-immunoprecipitation. Cell lysates were incubated with GFP-trap beads to purify GFP-tagged proteins and interaction partners. Of the three PCM1 truncations, only GFP-PCM1(1-331) was able to pull-down nesprin-1α2 (Fig. 4.12). There was some interaction between nesprin-1α2 and GFP only, however it was statistically significantly less than with GFP-PCM1(1-331). Thus, GFP-PCM1(1-331) is the region responsible for localising PCM1 to the NE, and this is through interaction with nesprin-1α2.

4.2.1.3 The PACT domain is the sole NE localising domain of pericentrin

The next aim was to investigate the region of pericentrin responsible for its localisation at the centrosome and at the myotube NE. Other than the N- and C-terminal 200 residues which are thought to be unstructured, pericentrin is predicted to be composed of coiled-coil regions (Gillingham and Munro, 2000). The ~77 residue PACT domain at the non-coiled-coil C-terminus of pericentrin is required for its localisation to the centrosome (Gimpel *et al.,* 2017; Gillingham and Munro, 2000), and is sufficient by itself for localisation to the NE in upon

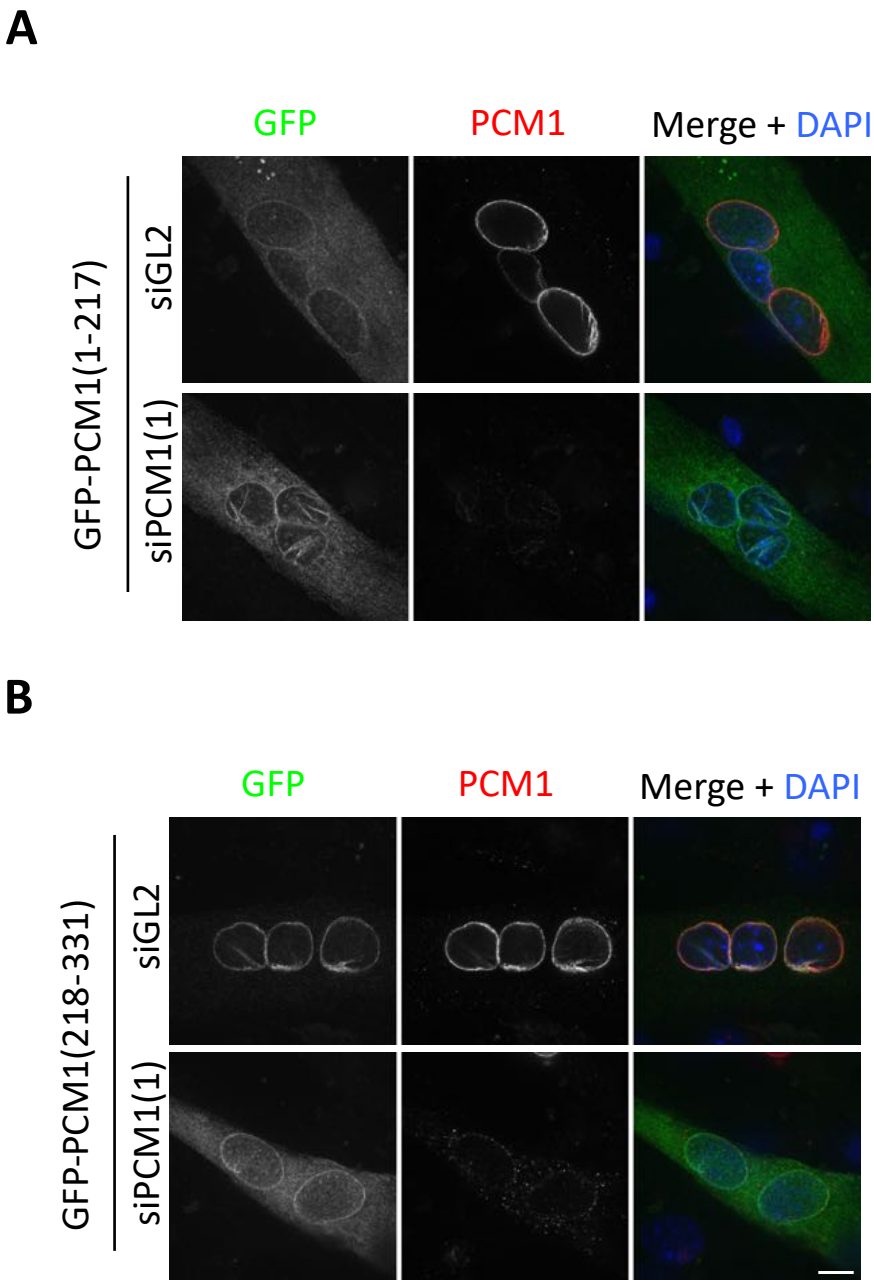


Figure 4.11 GFP-PCM1(1-217) and GFP-PCM1(218-331) are able to localise to the NE independently of endogenous PCM1

C2C12 cells differentiated for 2 days were first transfected with siGL2 or siPCM1(1), then transfected with GFP-PCM1(1-217) (A) or GFP-PCM1(218-331) (B) the day after. Cells were fixed after 3 further days of differentiation. Cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 µm.

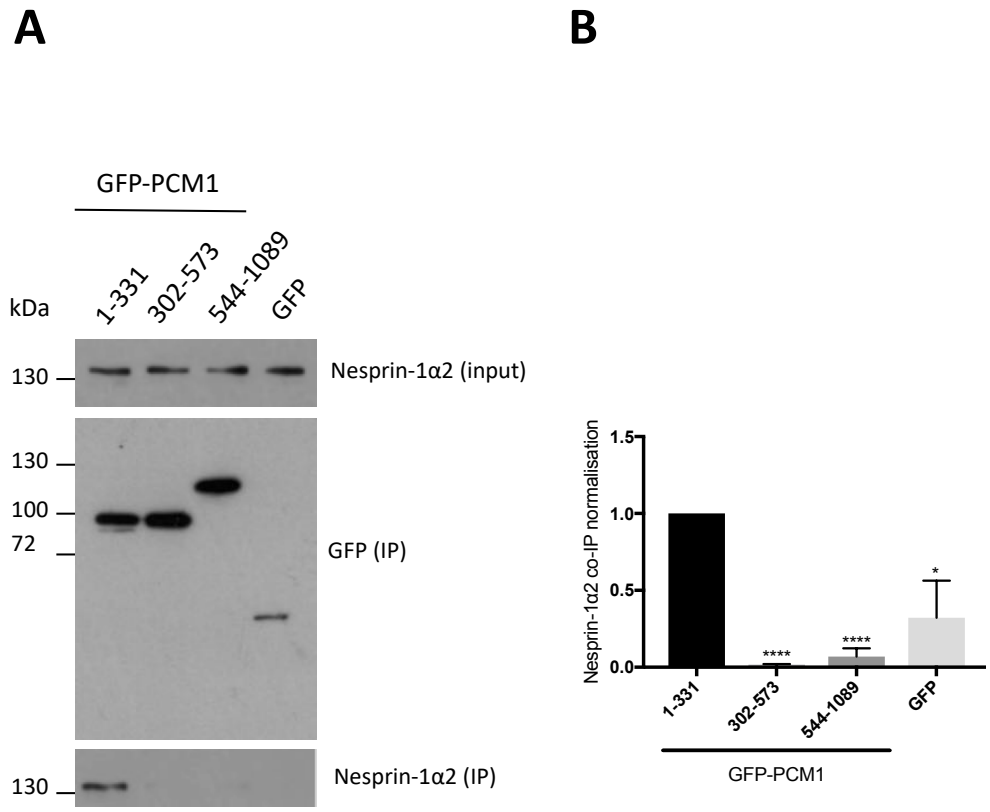


Figure 4.12 GFP-PCM1(1-331) interacts with nesprin-1α2 in C2C12 myotubes

(A) C2C12 myotubes transfected with GFP-PCM1(1-331), GFP-PCM1(302-573), GFP-PCM1(544-1089), or GFP were lysed with IP buffer and incubated with GFP-trap beads to isolate GFP-fused proteins and interacting partners. Eluates were immunoblotted with nesprin-1 and GFP-antibodies. (B) Histogram showing normalised levels of nesprin-1α2 immunoprecipitated with each GFP protein. Data shows means and SEM, n=3. Significant differences are shown to PCM1(1-331). *P=<0.05 using Tukey's multiple comparisons test.

expression of nesprin-1α2 in C2C12 myoblasts (Gimpel *et al.,* 2017). To confirm the role of the PACT domain and to identify any other NE localisation domains, eight fragments of the 3330 residue pericentrin were generated with an N-myc tag (Fig. 4.13), and their localisation in myoblasts and myotubes was investigated (Fig 4.14.). The first six fragments collectively covered residues 1-2838, a region with no characterised domains. Fragment 7, Myc-pericentrin(2839-3330) comprised the remaining C-terminus of pericentrin, which included the PACT domain (3139-3216). Fragment 8, Myc-pericentrin(3139-3330) contained residues from the PACT domain to the C-terminus of pericentrin. Western blot of the first six truncations showed bands at the expected molecular weight (Fig. 4.13B). Bands were not observed for the two PACT domain containing fragments, which was likely due to poor transfection efficiency and, potentially, their low molecular weight. The PACT domain-containing fragments were therefore assumed to be correct based on sequencing alone.

All constructs were expressed in myoblasts and myotubes and localisation was investigated by co-staining with pericentrin and myc antibodies for immunofluorescence microscopy (Fig. 4.14). Full-length FLAG-pericentrin-myc, a gift from Kunsoo Rhee, localised to the centrosomes in myoblasts and the NE in myotubes. Fragments 7 + 8, containing the PACT domain, localised similarly to full-length pericentrin as expected, strongly suggesting correct identity of the constructs. All other truncations localised to the cytoplasm, indicating the absence of an alternative centrosome or NE localisation domain. The epitope used to generate the pericentrin antibody is unknown, however pericentrin staining remained at the MTOC with expression of all truncations apart from myc-pericentrin(1201-1769), where pericentrin staining also corresponded with the overexpressed protein in the cytoplasm in both myoblasts and myotubes. It is therefore likely that the pericentrin antibody epitope is contained within this truncation, and the colocalisation was not due to a dominant effect of the truncation attracting endogenous pericentrin to the cytosol. This data showed that unlike PCM1, the pericentrin localisation domain (PACT domain) for the centrosome and NE is shared, and that none of the fragments associate with the endogenous protein.

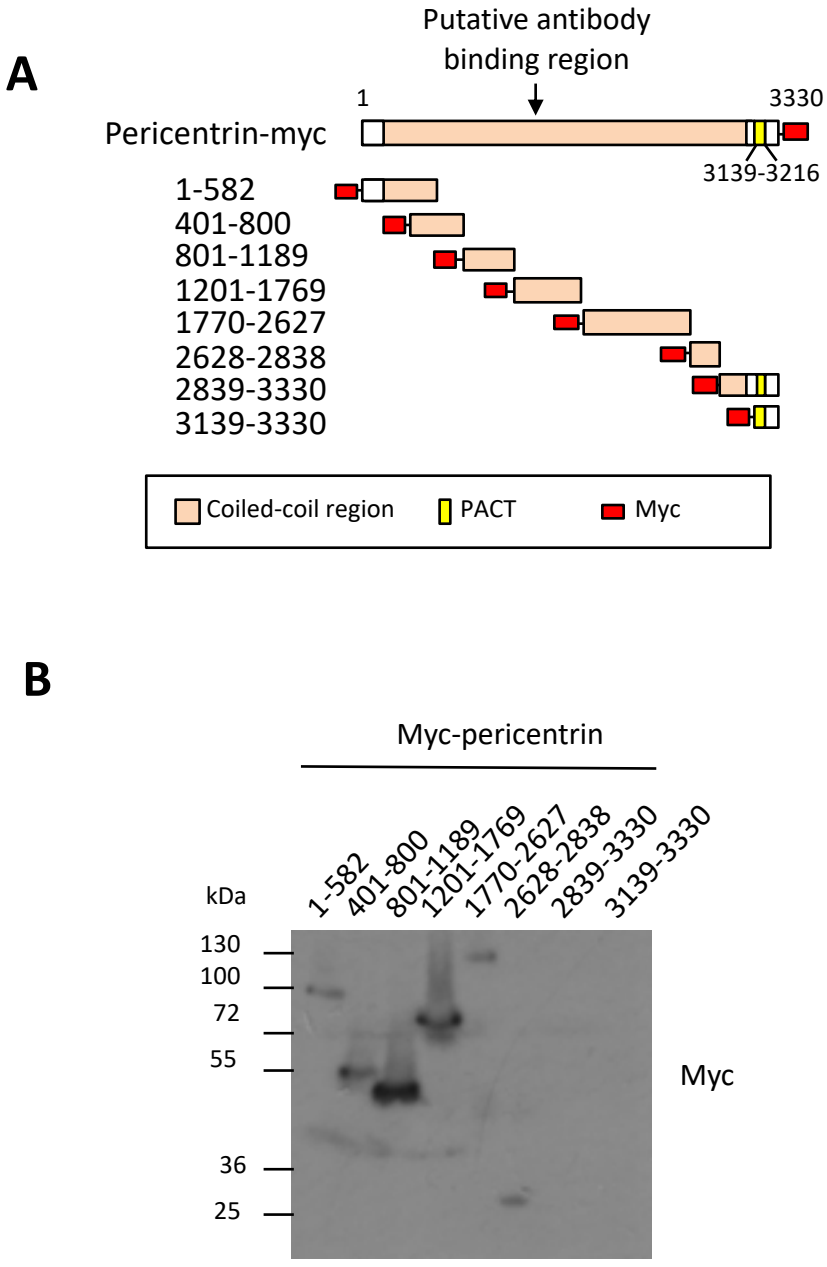


Figure 4.13 Schematic representation and western blot of myc-pericentrin fragments

(A) Schematic representation of FLAG-pericentrin-myc and truncation mutants. (B) C2C12 cells were transfected with the truncation constructs for 24 h. Cells were lysed with RIPA buffer and 50 µg of whole cell extracts were analysed by immunoblotting with myc antibody.

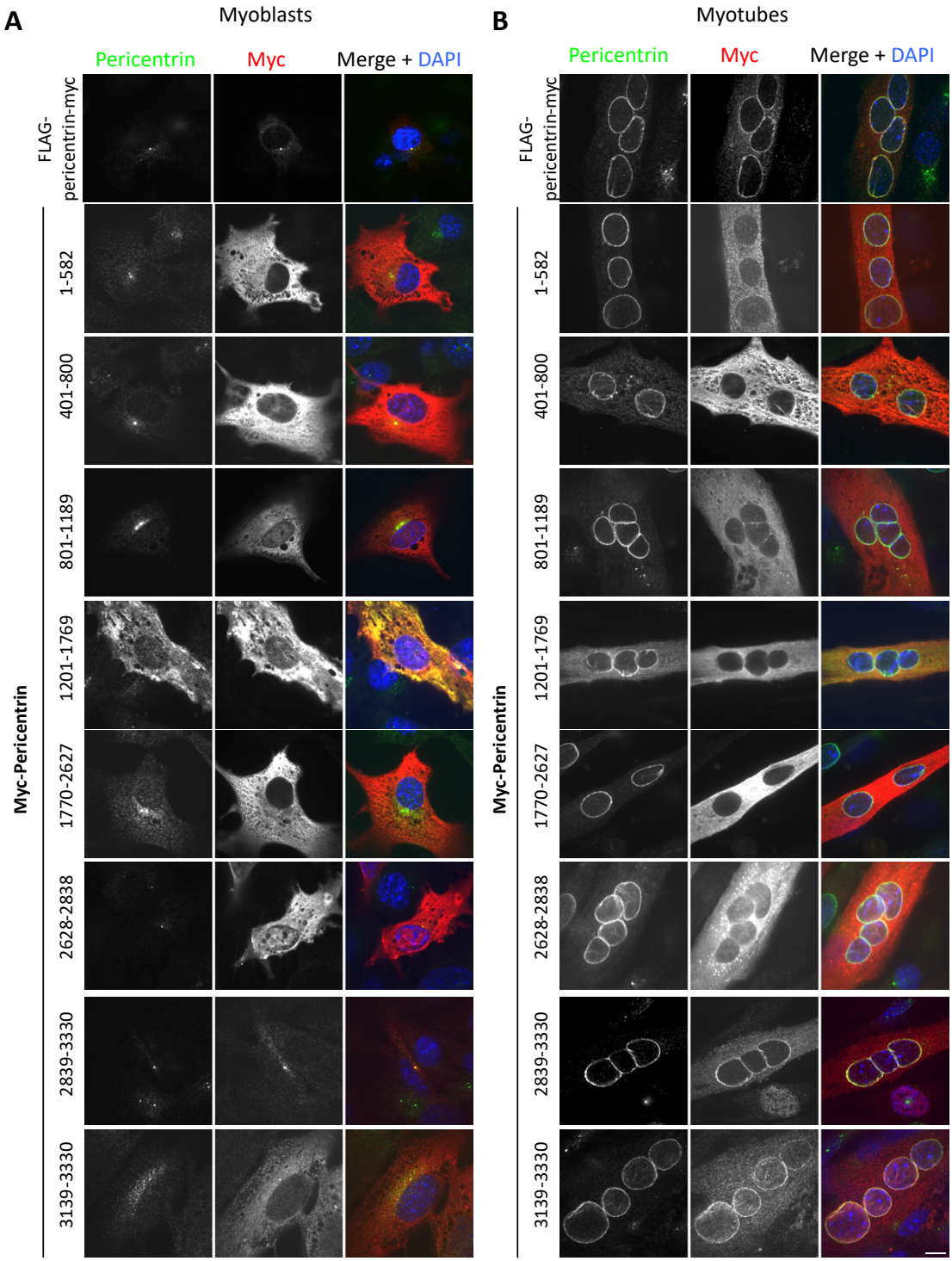


Figure 4.14 Only PACT domain-containing pericentrin fragments localise to the MTOC in myoblasts and myotubes

Myc-pericentrin truncations were transfected into C2C12 myoblasts and fixed the next day for the study of myoblasts (A), or differentiated for 5 further days for the study of myotubes (B). Cells were fixed in -20°C methanol and co-stained with antibodies against pericentrin (green) and myc (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

Interaction studies between PACT-containing pericentrin fragments and nesprin-1α2 were not carried out due to low expression levels of the mutants.

4.2.2 Investigating the interdependency of PCM components in their recruitment to the NE

Centrosomal proteins at the centrosome are interdependent for their localisation. As a centriolar satellite protein, PCM1 is a scaffold for proteins such as pericentrin and ninein at the centrosome (Dammermann and Merdes, 2002). C2C12 myotubes depleted of PCM1 also show reduced localisation or complete mislocalisation of pericentrin at the NE (Gimpel *et al.,* 2017; Espigat-Georger *et al.,* 2016), whereas AKAP450 is unaffected (Gimpel *et al.,* 2017). This suggests PCM1 may also be a protein scaffold at the NE. However no quantification was performed in the published studies, making firm conclusions difficult to draw. The objective here was to apply a quantitative method for a more rigorous analysis of this observation.

4.2.2.1 Using nesprin-1 staining as a direct marker for the NE localisation of centrosomal proteins

PCM1 was depleted from C2C12 myotubes with two different mouse-targeting siRNA oligos. Both oligos were shown to deplete PCM1 in C2C12 myoblasts and myotubes, by immunofluorescence and western blot (Fig. 4.15), allowing the role of PCM1 in the NE localisation of pericentrin and AKAP450 to be explored. At this time, all antibodies for PCM1, pericentrin and AKAP450 were raised in rabbit, so co-staining of MTOC proteins could not be performed. Instead, for each oligo, three sets of coverslips were prepared and stained for PCM1, pericentrin or AKAP450 along with nesprin-1, localisation of which is unaffected by PCM1 depletion (Fig. 4.15D). By conducting the experiments and preparing the coverslips in parallel, the efficiency of PCM1 depletion could be examined by the coverslip co-stained with PCM1 and nesprin-1.

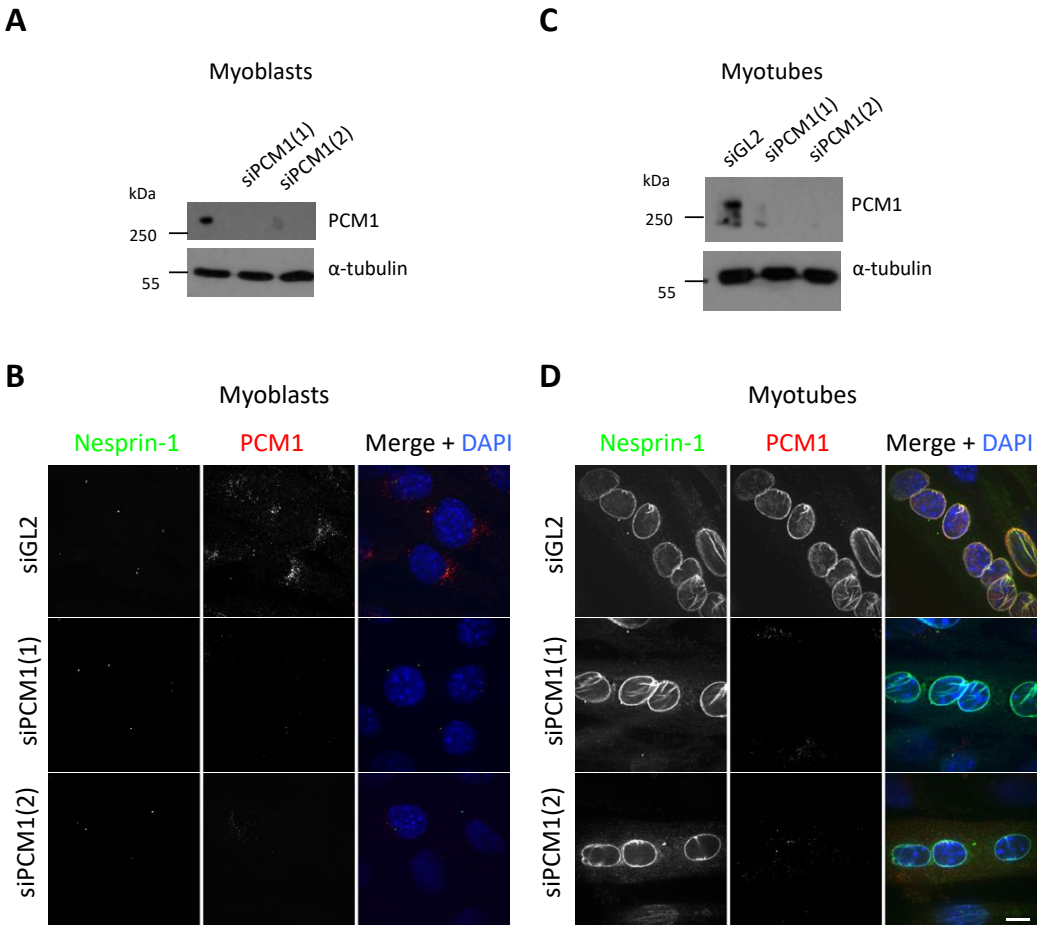


Figure 4.15 RNAi mediated depletion of PCM1 in C2C12 myoblasts and myotubes

C2C12 myoblasts or 2 day old myotubes were transfected with siGL2, siPCM1(1) or siPCM1(2) for 24 h. Myoblasts were fixed the next day, and myotubes were fixed after 4 further days. 40 µg RIPA protein extracts were immunoblotted by PCM1 or α-tubulin antibody in myoblasts (A) and myotubes (C). Cells were fixed in -20°C methanol and co-stained with antibodies against nesprin-1 (green) and PCM1 (red) in myoblasts (B) and myotubes (D). DNA was stained with DAPI (blue). Scale bar = 10 µm.

Nesprin-1 was used as a NE marker to quantify the binding of pericentrin and AKAP450 at the NE after PCM1 depletion. DAPI staining was not used to find the NE as in section 3.2.2.1, because nesprin-1 is a direct and more accurate NE marker that colocalises with PCM proteins, even with variations in the thickness of NE staining. Line scans, where a line is drawn across the diameter of a nucleus to plot the intensity value for each pixel along the line, were used to confirm that nesprin-1 can be used as a marker for MTOC proteins. NE localisation of proteins can be identified by two peaks in the graph, representing the brightest staining at the two points at the NE. In C2C12 myotubes co-stained with nesprin-1 and either PCM1, pericentrin or AKAP450, line scans showed peaks for nesprin-1 and all MTOC proteins at the same pixel (Fig. 4.16A). It should be noted that there is the potential of line scans picking up NE proteins at nuclear folds, as shown in the image co-stained with pericentrin and nesprin-1, to give more than two peaks. In this case, the outer peaks should be used. Since line scans only take intensity reading from two points of the NE yet staining around the NE is not always equal, measuring fluorescence intensity around the whole NE is preferable for measuring intensity. To do this, Fiji was used to measure the fluorescence intensity of a MTOC component in an image. Firstly, thresholding was applied to the nesprin-1 image to identify the stained area, which corresponded to the NE. This area was selected as the region of interest (ROI), then applied to the protein of interest (POI) image, to measure MTOC protein staining around the NE (Fig. 4.16B). NE intensity measurements for each myotube in this study were made from two adjacent myonuclei.

4.2.2.2 PCM1 does not anchor AKAP450 to the NE in myotubes

To investigate the role of PCM1 on AKAP450 recruitment and localisation to the NE, C2C12 cells were differentiated for 48 h before transfection with siGL2 or siPCM1 oligos (siPCM1(1) and siPCM1(2)) and differentiated for another four days before fixation. Cells were stained with nesprin-1 and PCM1 or AKAP450 antibodies (Fig. 4.17). To prevent bias during imaging, myotubes were chosen by identification from only the nesprin-1 stained channel.

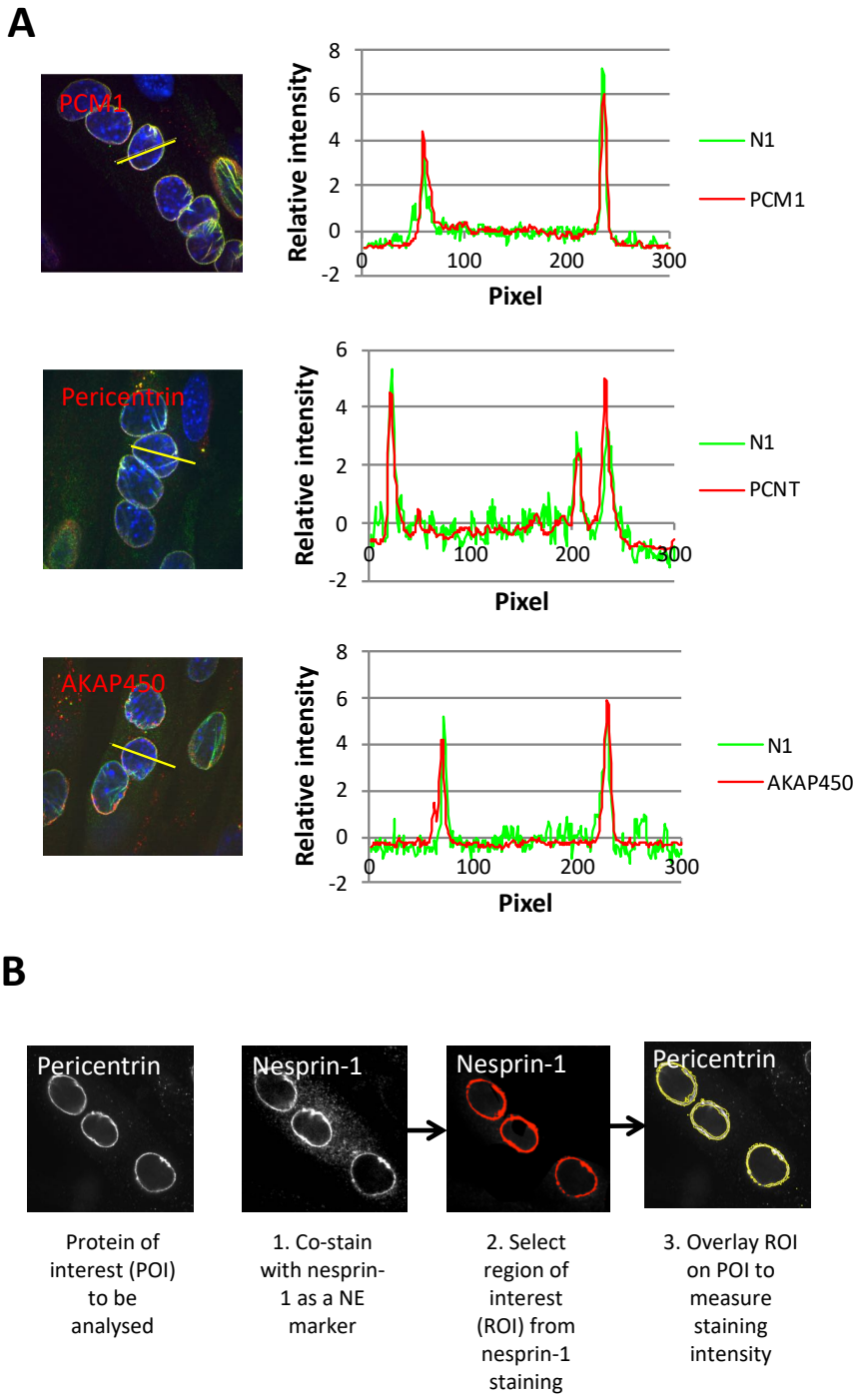


Figure 4.16 Measuring the NE staining intensity of centrosomal proteins using nesprin-1 as a NE marker

(A) C2C12 myotubes differentiated for 5 days were fixed in -20°C methanol and co-stained with antibodies against nesprin-1 (green) and either PCM1, pericentrin (PCNT) or AKAP450 (red). DNA was stained with DAPI (blue). Line scans plotted by a 300 pixel line (yellow) show co-localisation of centrosomal proteins with nesprin-1 at the NE. (B) To measure the staining intensity of a centrosomal protein at the NE, the region of interest (ROI) is first selected using the nesprin-1 staining, then the intensity of centrosomal protein staining within that ROI is measured.

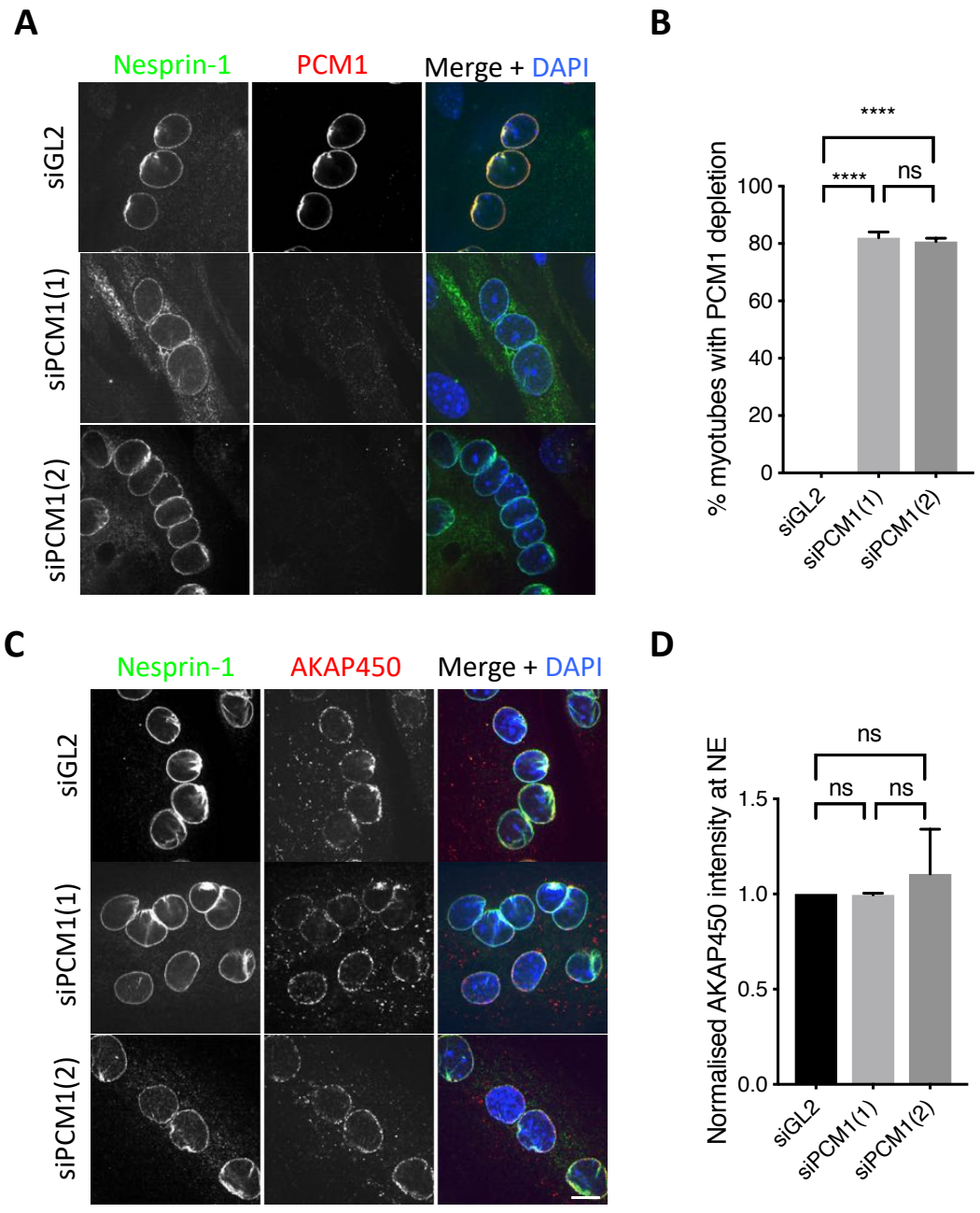


Figure 4.17 PCM1 is not required for AKAP450 localisation to the NE in myotubes.

Two day old myotubes were transfected with siGL2, siPCM1(1) or siPCM1(2) and differentiated for 4 further days. Cells were fixed in -20°C methanol and co-stained with antibodies against nesprin-1 (green) and PCM1 (A) or AKAP450 (C) (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. (B) For each oligo treated coverslip stained with PCM1,100 myotubes were observed by immunofluorescence to determine the number of PCM1-depleted cells. (D) For each oligo treated coverslip stained with AKAP450, the fluorescence staining intensity of AKAP450 at the NE was measured in 2 nuclei of 20 myotubes per experiment. Average intensities were normalised to staining of siGL2 treated cells, set at 1.0. Data shows means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test.

PCM1 stained coverslips generated in parallel showed that PCM1 had been depleted in the experiments. Both siPCM1 oligos led to depletion of PCM1 in around 80% of myotubes. For each siRNA treatment, cells stained for AKAP450 were imaged, and localisation to the NE was measured using nesprin-1 as the marker. Intensity measurements were normalised to staining in siGL2 treated cells, which was given an intensity measurement of 1.0. AKAP450 localisation to the NE was not affected by PCM1 depletion and fluorescence intensity relative to control treatment was measured at 1.00 and 1.11 with siPCM1(1) and siPCM1(2), respectively, quantitatively agreeing with the findings of Gimpel *et al.* (2017) that PCM1 does not play a role in NE recruitment of AKAP450.

4.2.2.3 PCM1 is required for maximum localisation of pericentrin to the NE in myotubes

The requirement of PCM1 for the localisation of pericentrin to the NE was examined in the same way as AKAP450 (Fig. 4.18). As transfections and staining of coverslips with PCM1, pericentrin and AKAP450 were done together, it was known that PCM1 depletion was successful in 80% of myotubes. Myotubes treated with siPCM1(1) had significantly weaker pericentrin staining than control cells at 0.64, whereas cells treated with siPCM1(2) retained pericentrin staining comparable to siGL2 treated cells at 1.03 (Fig. 4.18B+C). It was possible that off-target effects may have caused the lower, averaged pericentrin localisation in siPCM1(1) treated myotubes. Regardless, these results were inconclusive, and required further investigation. Gimpel *et al.* (2017) and Espigat-Georger *et al.* (2016) both reported weaker staining of pericentrin after PCM1 knockdown in myotubes, and the PCM1 oligos used in this study contained the same sequence as those of Gimpel *et al.* (2017). The difference in findings could be due to the timing of transfections employed in each study, where cells were transfected during the myoblast stage (Gimpel *et al.,* 2017) or 24 h after differentiation (Espigat-Georger *et al.,* 2016), whereas transfection in this study was performed 48 h after differentiation. Expression of nesprin-1α2 and MTOC protein localisation to the NE already occurs

A

Day	1	2	3	4	5	6	7	8
Old	Seed	Diff		*Trans*				Fix
New	Seed	*Trans*	Diff					Fix

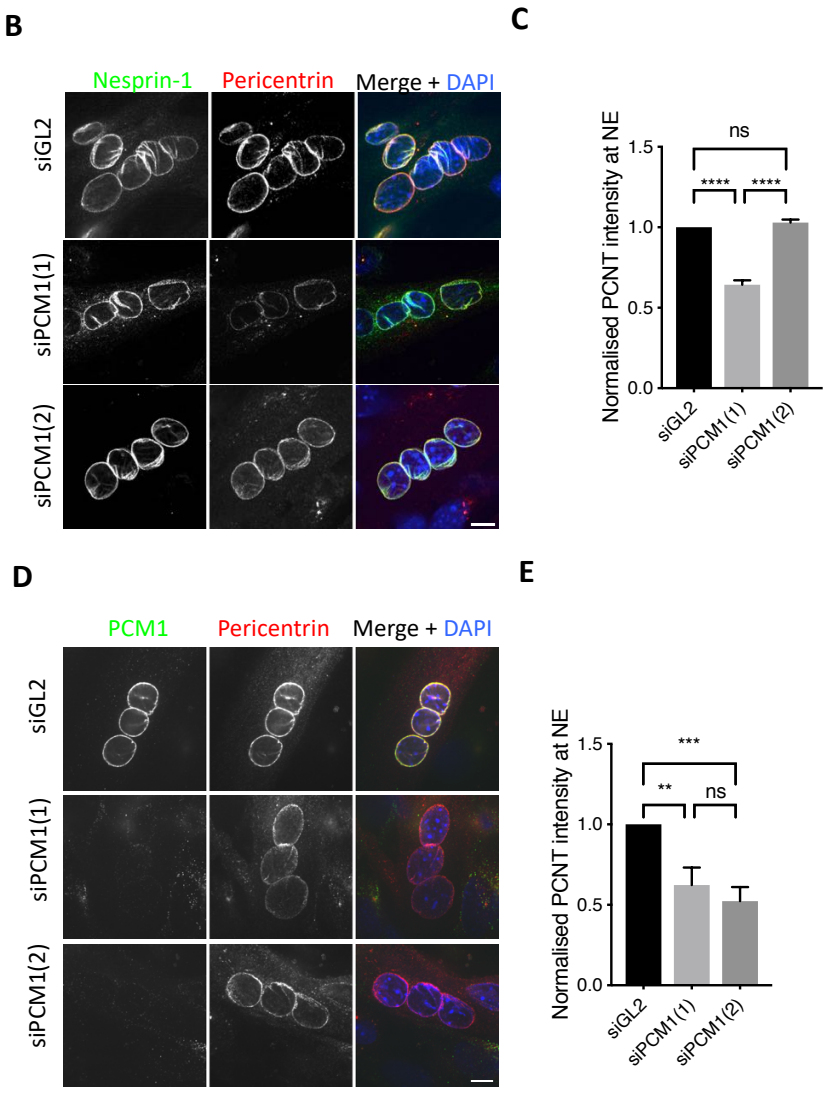


Figure 4.18 Depleting PCM1 at the myoblast stage reduces the localisation of pericentrin to the NE upon myogenesis

(A) Schematic showing 2 experiment protocols for PCM1-depleted myotubes, with different timeframes for the transfection (trans) and differentiation (diff) of C2C12 cells. In the "old" protocol, two day old myotubes were transfected with siGL2, siPCM1(1) or siPCM1(2) and differentiated for 4 further days (B). In the "new" protocol, myoblasts were transfected with siGL2, siPCM1(1) or siPCM1(2) and differentiated the next day for 5 further days (D). Cells were fixed in -20°C methanol and co-stained with antibodies against PCM1 (green) and pericentrin (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. For each oligo, the fluorescence staining intensity of pericentrin (PCNT) was measured in 2 nuclei of 20-25 myotubes per experiment. Average intensities were normalised to staining of siGL2 treated cells, set at 1.0. Data shows means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test

in a number of cells after 24 h of differentiation and increases towards 48 h (Zhou *et al.,* 2017). It may be that within the first 48 h of differentiation, PCM1 recruits pericentrin to the NE for binding with nesprin-1α2, after which pericentrin becomes independently anchored at the NE. In these cells at this timepoint, depletion of PCM1 would no longer affect the localisation of pericentrin at the NE, but still would in cells yet to commit to differentiation. To explain why only cells treated with siPCM1(1) showed reduced pericentrin staining, siPCM1(2) may contain an off-target effect towards a gene required for differentiation. In this case, PCM1 depletion at 48 h would inhibit residual myoblasts from to committing to differentiation, leaving only myotubes with pericentrin already anchored at the NE to be studied at the end of the experiment.

To investigate this, C2C12 myoblasts were transfected with siRNA and siPCM1 oligos, induced to differentiate the next day, and fixed after five days of differentiation. Comparison of the timeframes between the old and new protocol is shown in Fig. 4.18A. The new time of transfection, also used by Espigat-Georger *et al.* (2016), allowed PCM1 depletion before differentiation. At the end of the experiment, many myotubes treated with siPCM1 oligos had NE staining of PCM1, likely due to the extended period of culturing after siRNA treatment leading to PCM1 mRNA which is no longer degraded. However, at this time, a mouse pericentrin antibody was obtained to allow co-staining of PCM1 and pericentrin, ensuring that all myotubes imaged were PCM1 positive, or negative, depending on the oligo treatment. Fluorescence intensity measurements were this time obtained using the DAPI staining to delineate the NE (as in section 3.2.2.1), as it was anticipated that there would be cells which had no NE staining in any channel.

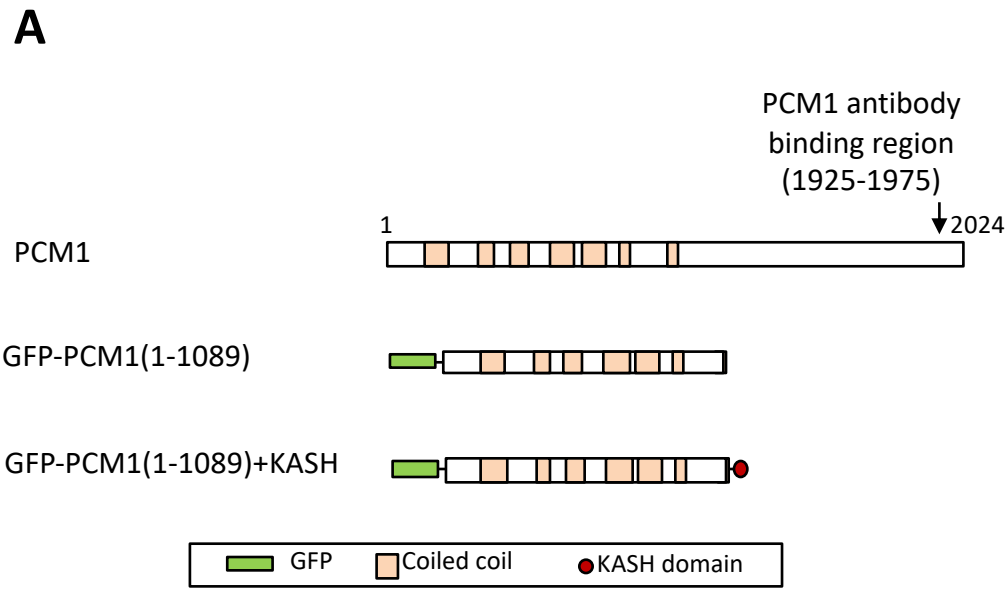
This time, both PCM1 oligo treated myotubes showed significantly reduced pericentrin staining at the NE compared to siGl2 treatment (Fig. 4.18D+E). There were noticeably fewer differentiated cells in all samples treated with siPCM1(2) (not shown), supporting the theory that the oligo may have an off-target effect on a protein required for the onset of differentiation. If PCM1 must already be depleted at the time of differentiation to negatively affect pericentrin recruitment, this suggests that PCM1 is only required for the recruitment and not anchorage of

pericentrin. There is not sufficient data to confirm this at this stage, and from the data, it can only be concluded that PCM1 is required for the full localisation of pericentrin at the myotube NE.

4.2.2.4 Forced NE recruitment of PCM1 in the absence of nesprin-1α2 leads to the recruitment of AKAP450 to the myonuclear rim

PCM1 depletion does not affect the NE localisation of AKAP450, but reduces NE localisation of pericentrin in myotubes. PCM1 may recruit pericentrin directly to bring it close to nesprin-1α2, or another protein, for anchorage at the NE. If so, NE-tethered PCM1 may be sufficient to recruit pericentrin to the NE even in the absence of nesprin-1α2, using the nesprin-1 null, human *SYNE1* (23545 G>T) myotubes. Unfortunately, there was not an available pericentrin antibody to stain human pericentrin. The relationship between PCM1 and pericentrin will be further explored in C2C12 myoblasts in chapter 5.

However, despite that PCM1 depletion does not affect localisation of AKAP450 to the NE, it may still play a role in recruiting AKAP450 to the NE. To test this, primers were designed to generate constructs to express GFP-PCM1 in two halves, with a C-terminal fusion to the NE localising KASH domain of nesprins. Only cloning of the N-terminal half of PCM1, which contains the NE localisation signal, was successful. Expression of the this construct, GFP-PCM1(1-1089)-KASH, was confirmed by western blot (Fig. 4.19), then transfected into *SYNE1* (23545 G>T) myotubes, to investigate whether it could rescue the localisation of AKAP450 to the NE. GFP-PCM1(1-1089)-KASH localised to the NE in *SYNE1* (23545 G>T) myotubes, whereas GFP-PCM1(1-1089) alone localised to the cytoplasm due to the absence of nesprin-1α2 as its NE anchor (Fig. 4.20). GFP-PCM1(1-1089)-KASH was observed to weakly recruit endogenous PCM1 to the NE in a number of myotubes, but at a lesser extent than observed with GFP-nesprin-1α2. This was probably due to association of endogenous PCM1 to the exogenous protein. Due to this, any recruitment of AKAP450 to the NE by the construct cannot be attributed solely to the N-terminal half of PCM1.



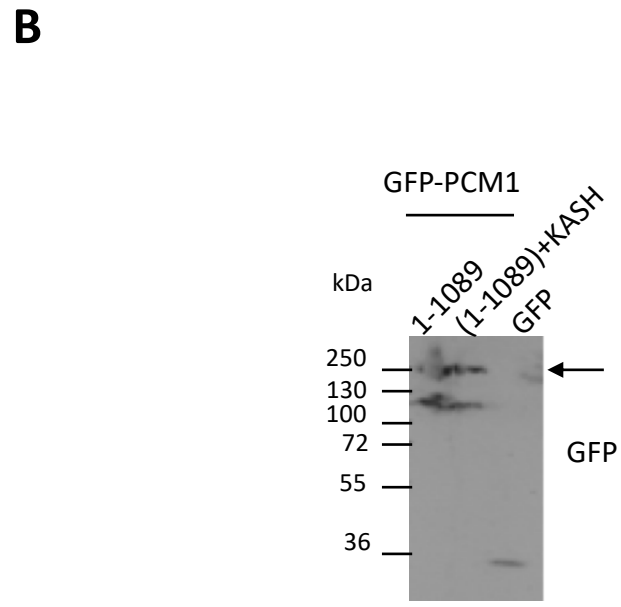


Figure 4.19 Schematic representation and western blot of GFP-PCM1(1-1089)-KASH

(A) Schematic representation of GFP-PCM1(1-1089), GFP-PCM1(1-1089)-KASH and GFP. B) C2C12 cells were transfected with the constructs for 24 h. Cells were lysed with RIPA buffer and 50 µg of whole cell extracts were analysed by immunoblotting with GFP antibody.

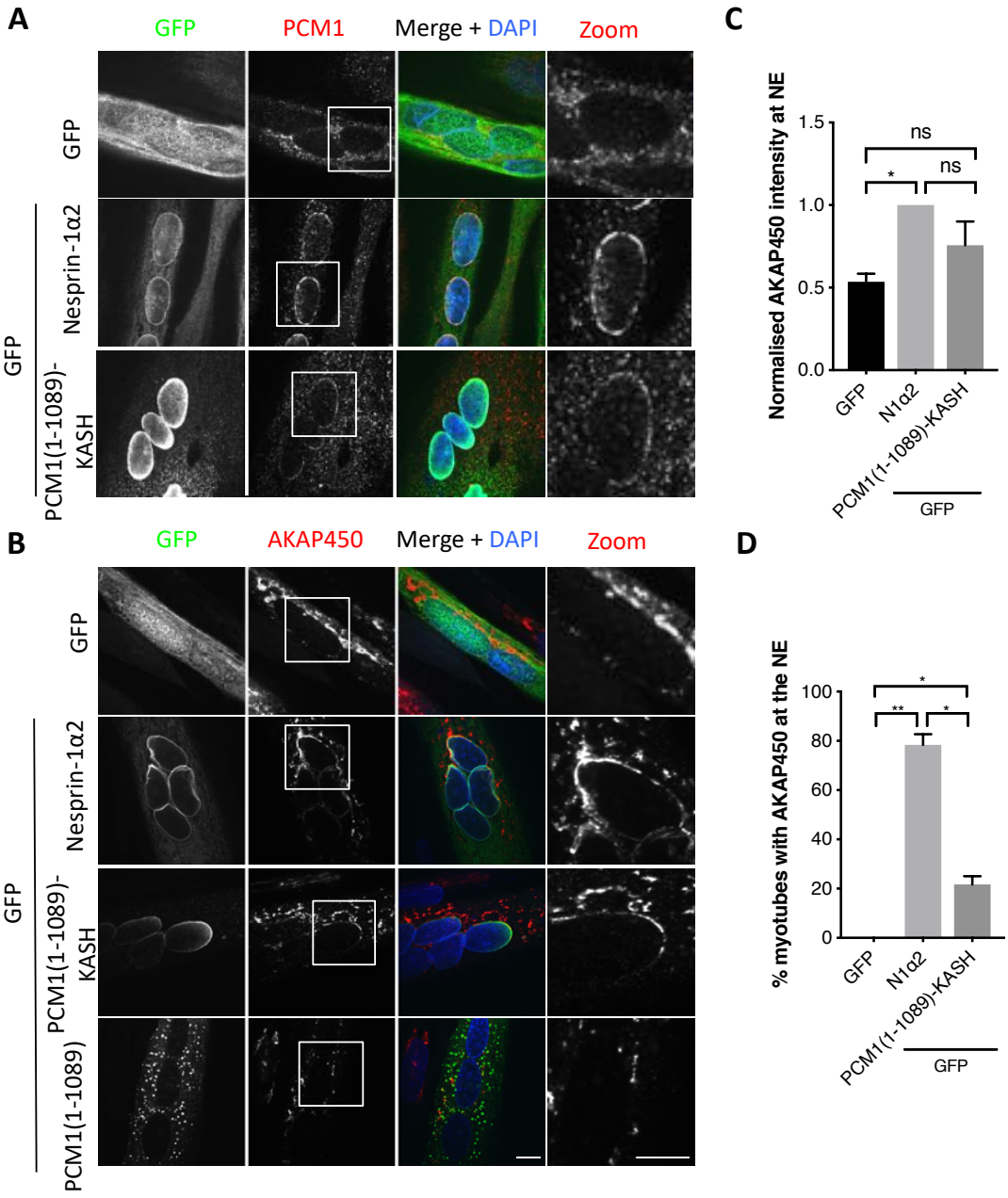


Figure 4.20 Forced recruitment of PCM1 to the NE weakly recruits AKAP450 to the nuclear rim in nesprin-1 null myotubes

SYNE1 (23545 G>T) myotubes differentiated for 5 days were transfected with GFP constructs as indicated. The next day, cells were fixed in -20°C methanol and co-stained with antibodies against GFP (green) and PCM1 (A) or AKAP450 (B) (red). DNA was stained with DAPI (blue). All scale bars = 10 μm. (C) Histogram showing normalised AKAP450 intensity at the NE of cells expressing different GFP constructs. Intensity of cells expressing GFP-nesprin-1α2 is given a value of 1.0. (D) Histogram showing the percentage of myotubes with observed AKAP450 fluorescence staining at the NE when expressing different GFP constructs. Nuclei from 20 myotubes were analysed per experiment. Data shows means and SEM, n=3. *P=<0.05 using Tukey's multiple comparisons test.

There was no AKAP450 recruitment to the NE by GFP-PCM1(1-1089) or by GFP only, whereas some recruitment was observed in myotubes expressing GFP-PCM1(1-1089)-KASH (Fig. 4.20). Recruitment was less consistent than in cells expressing GFP-nesprin-1α2, in that fewer nuclei had AKAP450 at the NE, and the staining was around the nuclear rim of just one nucleus rather than covering the whole NE of a number of myonuclei. Despite the positive recruitment being reflected in the quantified fluorescence staining of AKAP450 at the NE, there was no significant difference in the recruitment between GFP-PCM1(1-1089)-KASH and GFP (Fig. 4.20C). Instead, the number of myotubes with visible rescue at the nuclear rim was considered (Fig. 4.20D). Visible AKAP450 recruitment to the NE was observed in 21.7% of cells expressing GFP-PCM1(1-1089)-KASH. This was significantly different to both GFP and GFP-nesprin-1α2 expressing cells, where 0.0% and 78.3% cells, respectively, showed AKAP450 recruitment to the NE. This preliminary evidence suggests that PCM1 is able to bypass nesprin-1α2 to recruit AKAP450 to the myotube NE, and the weak localisation of AKAP450 suggests the recruitment may be indirect.

4.2.3 Investigating the relationship between centrosomal protein recruitment to the NE and microtubules in myotubes

So far in this chapter, the NE localising regions of PCM1 and pericentrin and a relationship between MTOC proteins for nMTOC formation have been identified. In non-muscle cells, PCM1 and pericentrin are transported along microtubules by dynein towards the centrosome (Young *et al.,* 2000; Dammermann and Merdes, 2002). Indeed, microtubules are required for the localisation of centriolar satellite granules next to the nucleus (Kubo *et al.,* 1999; Stowe *et al.,* 2012; Denu *et al.,* 2019). Microtubule depolymerisation has also been reported to cause the detachment of the centrosome from the nucleus (Salpingidou et al., 2007) and lead to reduced centrosomal pericentrin and γ-tubulin (Young et al., 2000). Therefore, the next aim was to investigate whether microtubules mediate the transport and anchorage of PCM1 and pericentrin to the myotube NE, as part of the nMTOC.

4.2.3.1 PCM1 and pericentrin anchorage at the NE does not require microtubules

To investigate whether anchorage of MTOC proteins at the NE is microtubule-dependent, microtubules were depolymerised in C2C12 myoblasts and myotubes by the addition of nocodazole for a range of times between 0 to 120 mins. Cells were co-stained with nesprin-1 as a myotube marker, and PCM1 (Fig. 4.21) or pericentrin (Fig. 4.22). In myoblasts treated for 60 min, PCM1 was still observed at granules concentrated next to the nucleus, though centriolar satellites appeared slightly dispersed in some cells. After 90 and 120 min of treatment, centriolar satellite granules were obviously dispersed throughout the cytoplasm, confirming the role of microtubules for the placement of centriolar satellites (Kubo *et al.,* 1999; Stowe *et al.,* 2012; Denu *et al.,* 2019). In myotubes, strong PCM1 staining was maintained at the NE after all nocodazole treatment times including 120 min, indicating that its anchorage at the NE as part of the nMTOC does not require microtubules.

Pericentrin localisation at the myoblast centrosome remained the same after 60 min with nocodazole or DMSO treatment. Centrosome staining in cells remained after 120 min. The level of pericentrin staining at the centrosome was not noticeably different, and was not analysed. However, the distance between the centrosome and the nucleus had visibly increased, indicating that although centrosomes remain intact, they lose anchorage next to the nucleus, as previously described (Salpingidou *et al.,* 2007). The time at which this occurs corresponds to the dispersal of centriolar satellites throughout the cell. In myotubes, strong NE localisation was maintained even at 120 min, indicating that pericentrin anchorage at the NE as part of the nMTOC does not require microtubules.

These results confirm that in myoblasts, microtubules are needed to anchor PCM1 and pericentrin, as part of the centriolar satellites and centrosomes, respectively. However, after relocalisation to the NE, both proteins become anchored to the NE independently of microtubules.

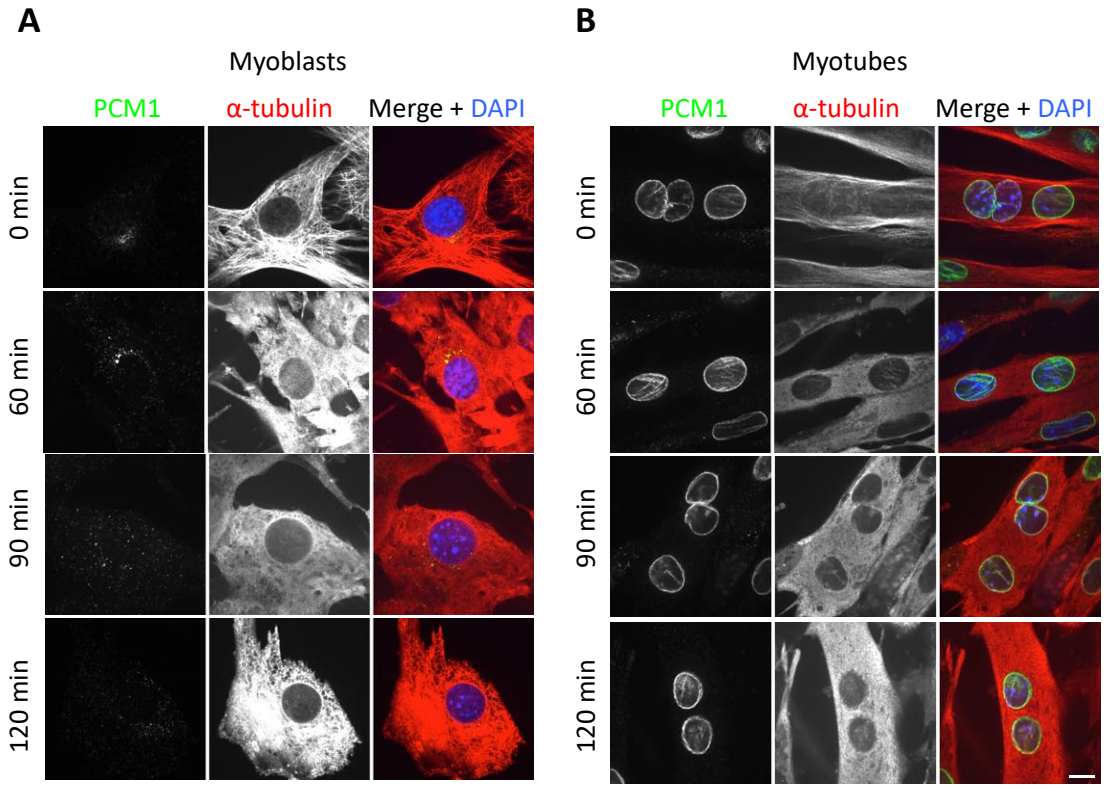


Figure 4.21 Microtubules anchor PCM1, as a component of the centriolar satellites next to the nucleus in myoblasts, but not as part of the nMTOC at the NE in myotubes

C2C12 myoblasts (A) or myotubes differentiated for 5 days (B) were treated with 5 µg/ml nocodazole for the times stated at 37°C and fixed in -20°C methanol and co-stained with antibodies against PCM1 (green) and α-tubulin (red). DNA was stained with DAPI (blue). Scale bar = 10 µm.

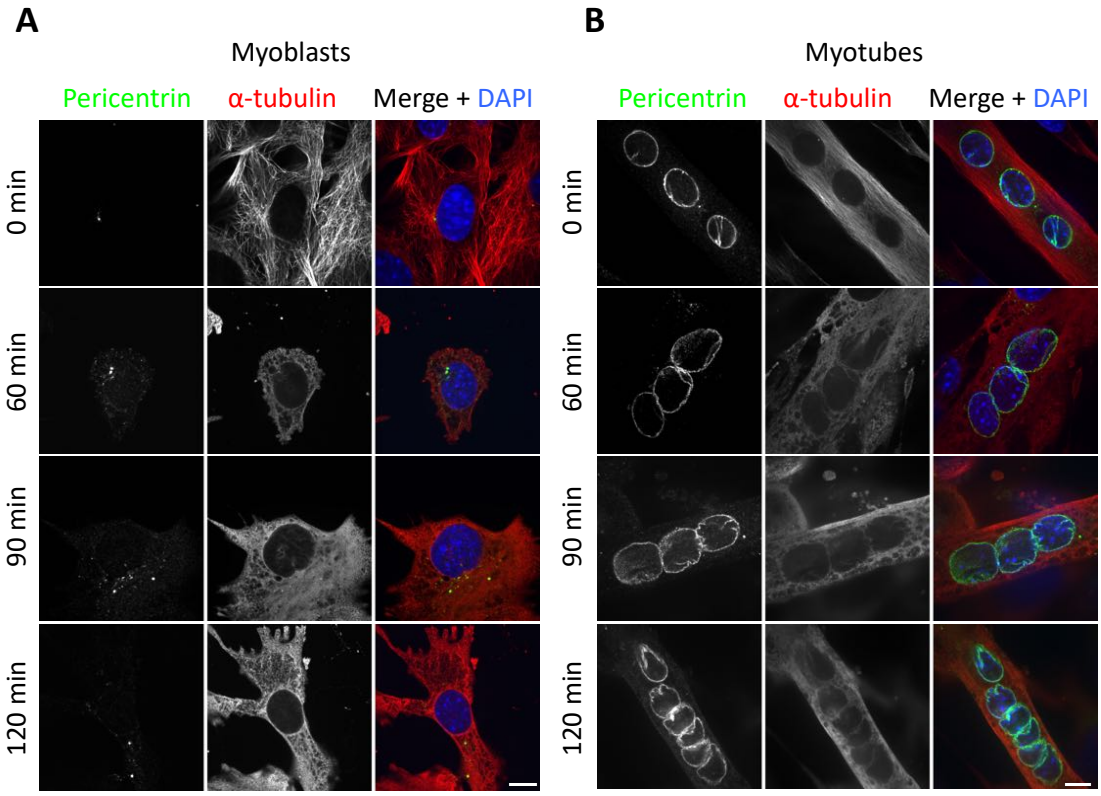


Figure 4.22 Microtubules anchor pericentrin, as a component of the centrosome next to the nucleus in myoblasts, but not as part of the nMTOC at the NE in myotubes

C2C12 myoblasts (A) or myotubes differentiated for 5 days (B) were treated with 5 μg/ml nocodazole for the times stated at 37°C and fixed in -20°C methanol and co-stained with antibodies against pericentrin (green) and α-tubulin (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

4.2.3.2 PCM1 and pericentrin relocalisation to the NE is not microtubule-dependent

The anchorage of PCM1 and pericentrin at the NE does not require microtubules, however their transport from the centrosome to the NE may do. To investigate this, myoblasts were simultaneously differentiated and incubated with a low concentration of nocodazole, to explore if nMTOC formation still occurred, as performed by Zaal *et al.* (2011). Differentiation and nocodazole treatment was for 48 h. At this stage, most cells were committed myoblasts, where PCM1 and pericentrin are already at the NE, but before fusion with other cells. Cells treated with DMSO or nocodazole were co-stained with PCM1 (Fig. 4.23) or pericentrin (Fig. 4.24), and MHC or α-tubulin in parallel experiments. Cells stained with α-tubulin showed that nocodazole treatment was effective in depolymerising major microtubule structures, though small fibres, mostly near the cell periphery remained. MHC staining at the NE identified cells undergoing myogenesis. Cells co-stained with PCM1 or pericentrin and MHC revealed that all differentiating cells treated with nocodazole were still able to recruit PCM1 and pericentrin to the NE. This shows that during myogenesis, PCM1 and pericentrin relocalise to the NE in a microtubule-independent manner.

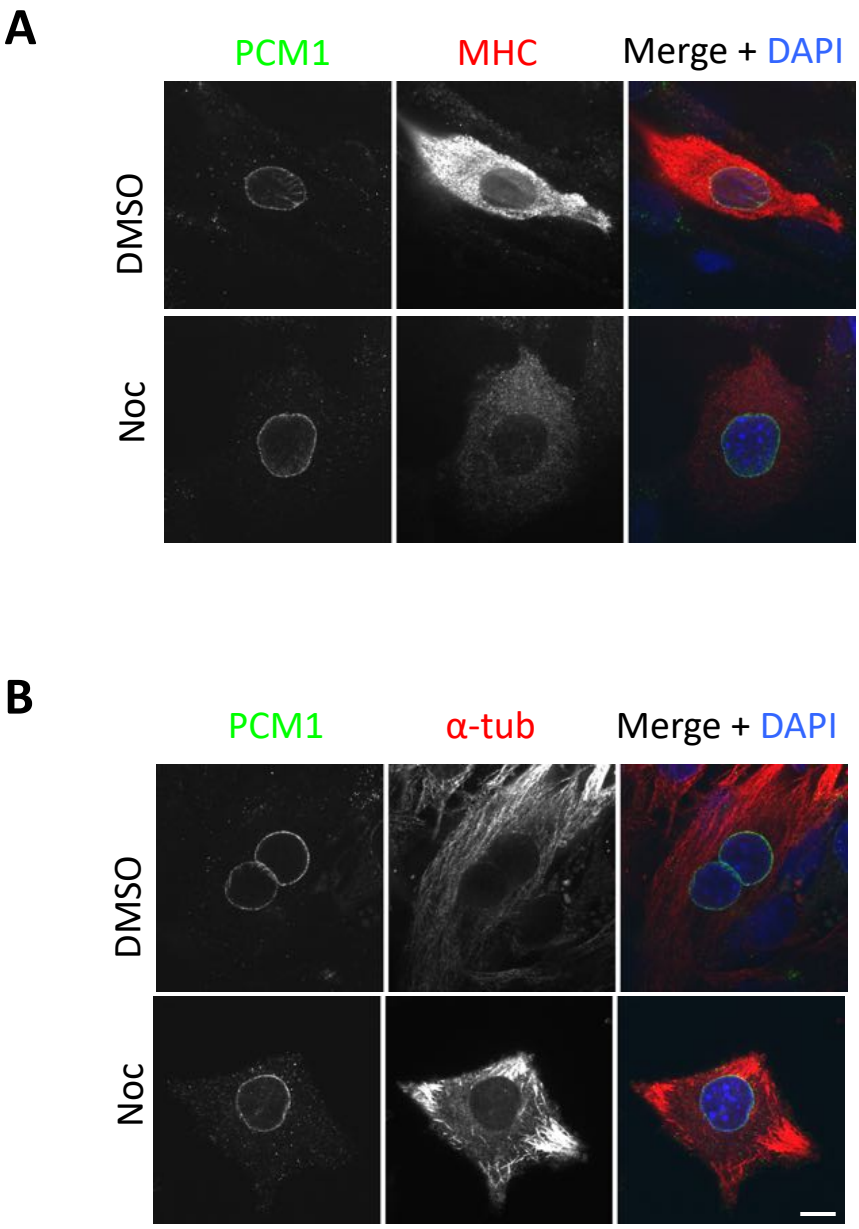


Figure 4.23 Microtubules are not required for the relocalisation of PCM1 to the NE during myogenesis

C2C12 myoblasts were seeded in growth medium. After 4 hours, the cell medium was switched to differentiation medium with 0.5 µg/ml nocodazole. After 2 days of differentiation and drug treatment, cells were fixed in -20°C methanol and co-stained with antibodies against PCM1 (green) and MHC (A) or α-tubulin (α-tub) (B) red. DNA was stained with DAPI (blue). Scale bar = 10 µm.

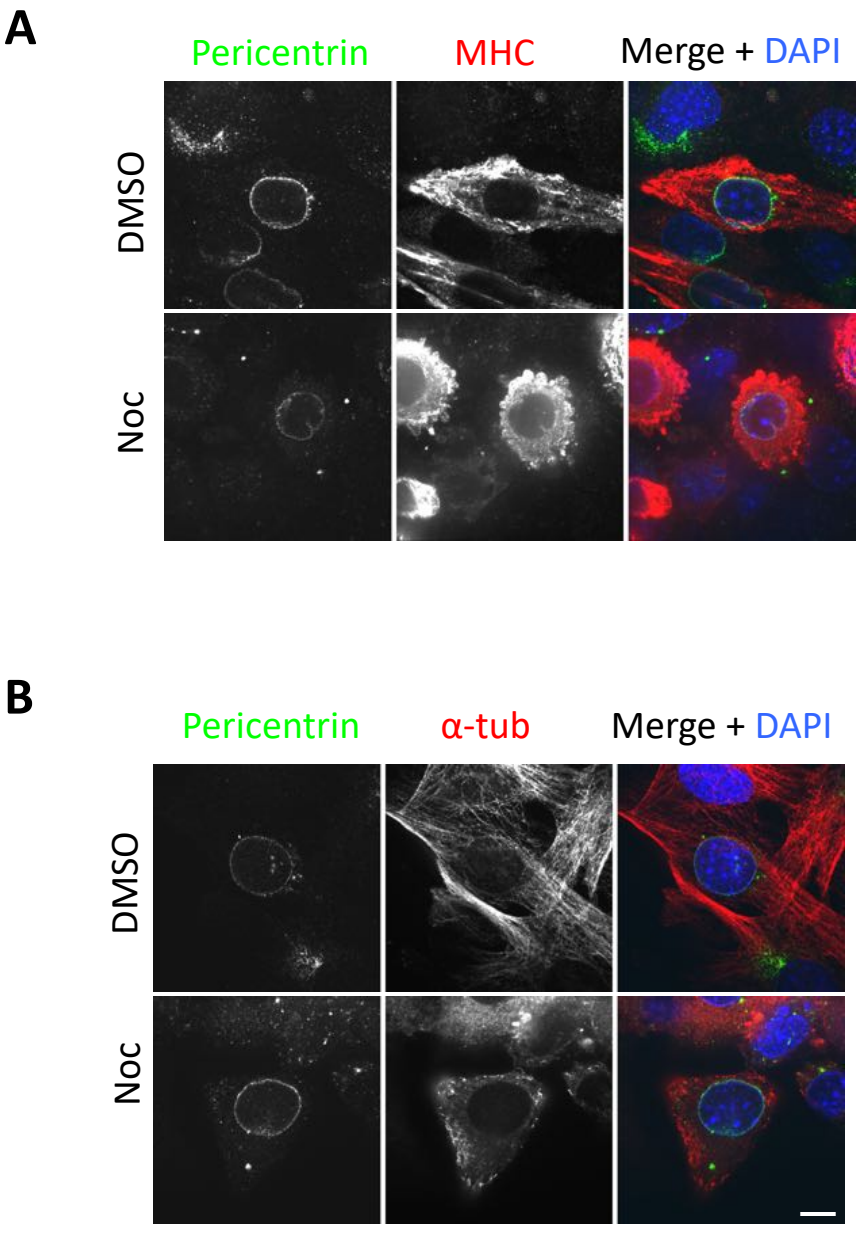


Figure 4.24 Microtubules are not required for the re-localisation of pericentrin to the NE during myogenesis

C2C12 myoblasts were seeded in growth medium. After 4 hours, the cell medium was switched to differentiation medium with 0.5 µg/ml nocodazole. After 2 days of differentiation and drug treatment, cells were fixed in -20°C methanol and co-stained with antibodies against pericentrin (green) and MHC (A) or α-tubulin (α-tub) (B) red. DNA was stained with DAPI (blue). Scale bar = 10 µm.

4.3 Discussion

This chapter identifies the NE localisation domains of PCM1 and pericentrin, the relationship between PCM1 with pericentrin and AKAP450 for nMTOC formation, and the requirement of microtubules in the assembly and maintenance of pericentrin and PCM1 at the centrosomal and nuclear MTOC.

4.3.1 The unstructured and coiled-coil regions of PCM1(1-331) work together for NE localisation and interact with nesprin-1α2

Deletion mapping studies in this chapter revealed the first 331 residues of PCM1 to be the minimal region for strong localisation to the NE. The fragment consists an N-terminal unstructured region and a C-terminal coiled-coil. Both regions yielded a protein product which was either weakly bound to the NE, where endogenous PCM1 is also retained, or a product absent from the NE which also displaced endogenous PCM1. Displacement of endogenous PCM1 from the NE when the fragments are localised in the cytoplasm may be a result of interaction between the overexpressed protein and endogenous PCM1. This suggests both regions are involved in binding to the NE, but must work together for strong NE localisation.

Out of GFP-PCM1(1-331), GFP-PCM1(302-573) and GFP-PCM1(544-1089), only PCM1(1-331) was found interact with nesprin-1α2. This is in agreement with a previous study showing that endogenous PCM1 and nesprin-1α2 interact in myotubes (Espigat-Georger *et al.,* 2016). This indicates that in myotubes, PCM1(1-331) is responsible for NE localisation by interaction with nesprin-1α2.

4.3.2 The centrosome and nuclear MTOC targeting motifs may be different or the same depending on the protein

Localisation studies of the PCM1 truncations generated in this study have strongly suggested that the centriolar satellite and NE localisation domains of PCM1 are different. It could not be confirmed whether the granular dots observed with GFP-

PCM1(544-1089) in PCM1-depleted myotubes corresponded to centriolar satellites as the cells were co-stained with GFP and PCM1, and PCM1 is depleted in the cell. However in other studies, expression of full-length PCM1 in PCM1-depleted cells also allows re-formation of similar structures assumed to be centriolar satellites (Odabasi *et al.,* 2019; Wang *et al.,* 2016). Expression of a mouse PCM1 truncation equivalent to human PCM1(1-1203) in PCM1 KO cells was able to form large dots which were also regarded as centriolar satellites, whereas a truncation equivalent to human PCM1(596-2024) was dispersed throughout the cytoplasm (Wang *et al.,* 2016). Combining this with the current results, the motif for centriolar satellite localisation may be contained within or at least overlap with PCM1 residues 544-596. Triple staining with an alternative centriolar satellite marker whose presence in the cell is unaffected by PCM1 depletion, such as Cep131 (Wang *et al.,* 2016), would ultimately confirm if the dots observed are associated with centriolar satellites.

This is different to pericentrin whose localisation to the centrosomes and NE was found to be via the C-terminal pericentrin-AKAP450 centrosomal targeting (PACT) domain. This same 77 residue domain has been shown to be recruited to the NE in myoblasts exogenously expressing nesprin-1α2 (Gimpel *et al.,* 2017). The current studies show that only the PACT domain is able to localise to the NE in myotubes, indicating it is the only pericentrin region responsible for its recruitment to the nMTOC. AKAP450 also contains a conserved PACT domain which may also be responsible for its NE localisation. This is yet to be confirmed. Furthermore, potential presence of other localisation domains in AKAP450 has not been examined.

Other proteins also vary between having the same or different domains for localisation to different MTOCs. The Golgi localisation domain of AKAP450 is contained at the N-terminal end of the protein, in contrast to the C-terminal centrosomal localising PACT domain (Wu *et al.,* 2016). In contrast, CDK5RAP2 binds to the centrosome and Golgi by the same motif (Wang *et al.,* 2010). The requirement for a different localisation motif may be for binding to different proteins at different MTOCs. For example, the Golgi localisation domain of

AKAP450 interacts with the Golgi-specific protein GM130 (Wu *et al.,* 2016). Localisation via a different localisation motif may also allow an alternative spatial arrangement of the protein, for different protein function or to evade spatial constraints. Although MTOC functions may be similar at different locations, they are unlikely to be identical. Also, at the centrosome, proteins are compact and based around the centrioles, whereas at the NE or Golgi, they are associated with expansive flat membrane surfaces. Due to this, centrosomal proteins may be under different spatial constraints at different MTOCs if localised by the same region.

4.3.3 Protein interactions and interdependency at the nMTOC

At the multi-protein complex centrosome, proteins are interdependent on each other for their recruitment. PCM1 is large protein with coiled-coil regions, allowing it to scaffold pericentrin, amongst other proteins. Indeed, PCM1 depletion leads to the reduction of pericentrin at the centrosome (Dammermann and Merdes, 2002), and PCM1 has been found to interact with pericentrin (Li *et al.,* 2000). This chapter used a quantitative method to show the requirement of pericentrin on PCM1 for its localisation is conserved at the nMTOC, as observed by other groups (Gimpel *et al.,* 2017; Espigat-Georger *et al.,* 2016). Depletion of PCM1 resulted in reduced recruitment of pericentrin to the myonuclei NE, suggesting that the nMTOC is also a multi-protein complex, where PCM1 is a major pericentrin scaffold, but there are other unknown proteins at the NE which also recruit pericentrin to the NE.

Gimpel *et al.* (2017) also showed that PCM1 depletion in myotubes does not affect AKAP450 localisation, and the current data confirms this through quantitative analysis. However, this chapter shows evidence that forced binding of PCM1(1-1089) to the myotube NE, in the absence of nesprin-1 isoforms, weakly recruits AKAP450 to the NE. This provides further evidence towards a multi-protein nMTOC, where PCM1, amongst other proteins recruit AKAP450 to the NE. The results suggest that upon PCM1 depletion, other AKAP450 scaffolding proteins, which are themselves recruited by nesprin-1α2, are sufficient to maintain full AKAP450 localisation to the NE.

Overall, evidence indicates that PCM1 recruits both pericentrin and AKAP450 to the nMTOC. Only recruitment of pericentrin, not AKAP450 is reduced upon PCM1 depletion, suggesting there are different levels of interdependencies between proteins, and that there may be an order in which they are recruited to the NE, as at the centrosome (Lawo *et al.,* 2012; Sonnen *et al.,* 2012; Fry *et al.,* 2017).

4.3.4 PCM1 and pericentrin involvement with microtubules in myotubes

This chapter also explores the involvement of microtubules in nMTOC formation and maintenance. By staining for PCM1 and pericentrin, it was found that microtubules are required for the placement of both centriolar satellites and the centrosome next to the nucleus (Kubo *et al.,* 1999; Stowe *et al.,* 2012; Salpingidou *et al.,* 2007). Instead, work from this chapter shows that anchorage of the nMTOC, by staining for PCM1 and pericentrin, no longer requires microtubules. This was in agreement with Zaal *et al. (*2011), who only studied pericentrin. Centriolar satellites are dynamic organelles without a physical anchor, and therefore may require microtubules for localisation. A centrosome is tethered to the nucleus by microtubules during interphase (Salpingidou *et al.,* 2007), and also requires microtubules for movement in the cell during mitosis (Tanenbaum and Medema, 2010). As the nMTOC is formed from early myogenesis and persists even in a mature muscle fibre (Oddoux *et al.,* 2013), centrosomal proteins may directly bind to nesprin-1α2 for persistent anchorage.

Finally, this chapter shows that PCM1 and pericentrin are able to relocalise to the NE in myotubes in the absence of microtubules. This means the movement cannot be mediated by the microtubule-motor proteins kinesin or dynein. The rearrangement of the microtubule cytoskeleton during myogenesis may be incompatible with MTOC protein movement along the fibres at the same time. Instead, simple diffusion or an actin-based transport system may be responsible. This also raises a question regarding the role of PCM1 in the localisation of pericentrin in myotubes. At the centrosomal MTOC, PCM1 transports pericentrin

from the cytoplasm to the centrosome along microtubules (Kubo *et al.,* 1999). This cannot be also the case in myotubes, therefore PCM1 may be involved in the localisation of pericentrin by a muscle-specific mechanism.

4.3.5 Summary

This set of studies identifies the minimum region of PCM1, residues 1-331, and pericentrin, the PACT domain, for their recruitment to the myotube NE, and finds that the PCM1 region interacts with nesprin-1α2. It also shows that PCM1 is involved in recruiting both pericentrin and AKAP450 to the NE through mechanisms of different importance. This suggests an ordered recruitment of MTOC components to the NE. Finally, data shows that nMTOC formation is microtubule-independent, and upon relocation to the NE, PCM1 and pericentrin no longer require microtubules for anchorage as required at the centrosomal MTOC.

Chapter 5

Regulating nMTOC assembly by phosphorylation

5.1 Introduction

It is well established that upon myogenesis, nesprin-1α2 is expressed and localised at the NE. The centrosomes of myoblasts disassemble, and centrosomal proteins relocalise to form the nuclear MTOC (nMTOC) at the NE, from where microtubules now nucleate (Srsen *et al.,* 2009; Duong *et al.,* 2014; Gimpel *et al.,* 2017; Zhou *et al.,* 2017). This all occurs before myoblast fusion (Srsen *et al.,* 2009; Fant *et al.,* 2009). The process which initiates and mediates centrosomal protein relocalisation to the NE is unclear.

The pericentrin/AKAP450 centrosomal targeting (PACT) domain contains the localisation domain of pericentrin (Gillingham and Munro, 2000). dsRed-PACT localises to the centrosomes in C2C12 myoblasts whereas, when it is co-expressed with GFP-nesprin-1α, it shows some NE localisation in addition to centrosomal localisation (Gimpel *et al.,* 2017). The localisation of full-length pericentrin and other MTOC components in GFP-nesprin-1α2 expressing non-myotube cells has not otherwise been examined. However, this does suggest that the expression and localisation of nesprin-1α2 at the NE is only in part responsible for nMTOC formation and that other events, such as post-translational modifications (PTMs) are required to release MTOC proteins from the centrosome.

The NE localisation of PCM proteins is unique to muscle cells in mammals. However, upon fusion of a differentiated muscle cell with an osteosarcoma U2OS cell, PCM1 and pericentrin are able to localise around the NE of the U2OS cell nucleus (Fant *et al.,* 2009). NE fluorescence staining of the proteins are weaker in the U2OS cell compared to the muscle cell, suggesting that even though the cells are fused and sharing the same environment, only the muscle cell nucleus could utilise the optimal conditions for nMTOC formation. The U2OS centrosome also disappeared, suggesting that a myogenic environment promotes centrosome disassembly. The trigger which initiated centrosome disassembly and transition of PCM proteins to the NE in the U2OS cell was not identified. It may have been due to myogenic factors shared from the muscle cell such as nesprin-1α2, phosphorylated proteins or muscle-specific kinases.

Indeed, it is not uncommon for the function of a protein to be mediated or maintained by protein phosphorylation. Upon mitosis, PLK1 phosphorylates a number of centrosomal proteins such as pericentrin, CDK5Rap2 and Cep192 for centrosome expansion and maturation (Fry *et al.,* 2017). PLK1 phosphorylation of pericentrin is also required for bipolar spindle formation (Kim and Rhee, 2014) and centriole separation during mitotic exit (Kim *et al.,* 2015). PLK4 phosphorylates PCM1 S372 for both cilium formation and centriolar satellite integrity (Hori *et al.,* 2016), whereas CDK1 phosphorylates PCM1 for cilium disassembly (Wang *et al.,* 2013).

Unsurprisingly, the onset of myogenic differentiation and fusion is also regulated by kinases. At least eight kinases are involved in myocyte formation, the stage where PCM proteins relocalise to the NE (Knight and Kothary, 2011). One example of a kinase which is involved in myogenesis is, again, PLK1, whose mRNA levels are upregulated in primary myoblasts upon differentiation, and in muscle satellite cells upon muscle regeneration *in vivo* (Jia *et al.,* 2019). PLK1 is required for proliferation and survival of myoblasts in both embryonic development and adult myogenesis as PLK1 depletion leads to the cells becoming arrested at M-phase and undergoing apoptosis (Jia *et al.,* 2019). Though its substrates here are likely to be proteins of the cell cycle, it may also be required to activate proteins for nMTOC formation, particularly as it is known to phosphorylate pericentrin and PCM1 in other cell types.

The aim of this chapter was to investigate the triggers required for nMTOC formation in myotubes. Firstly, experiments were done in attempt to recruit MTOC proteins to the NE of non-myotube cells. For this, nesprin-1α2 was exogenously expressed in non-myotubes to investigate whether it is sufficient to recruit PCM1 and pericentrin to the NE. Next, the expression levels and localisation of PCM1 and pericentrin were varied to investigate if its abundance or its localisation in the centrosome or cytosol affects nMTOC formation. The NE localising GFP-PCM1(1-1089)-KASH was also expressed in myoblasts to investigate if it could recruit pericentrin to the NE in the absence of nesprin-1α2 and myogenic factors. Finally,

mass spectrometry was carried out on GFP-PCM1(1-331), which contains the NE localisation domain of PCM1. This was in attempt to identify residues which are phosphorylated during myogenesis, and to study if they are involved in nMTOC formation by mediating its own recruitment to the NE, or mediating the recruitment of other proteins, due to its role as a protein scaffold.

5.2 Results

5.2.1 PCM proteins remain at the myoblast centrosome upon expression of GFP-nesprin-1α2

Nesprin-1α2 becomes expressed upon myoblast differentiation, co-incident with centrosome disassembly and centrosomal protein recruitment to the NE. The presence of exogenous nesprin-1α2 in non-myotubes may be sufficient to drive relocation of PCM1 and other PCM components from the centrosomes to the NE, simply by acting as their receptor. To examine this, GFP-N1α2 and GFP were expressed in C2C12 myoblasts. Endogenous PCM1 and pericentrin were not visible at the NE in GFP-nesprin-1α2 nor GFP expressing cells but instead remained at the centriolar satellites or centrosome, respectively (Fig. 5.1). Since PCM1 is localised in dynamic granules and there is a cytoplasmic pool of pericentrin (Liu *et al.,* 2010), there is a population of both proteins which should be free bind to the NE upon the correct conditions. Therefore, expression of nesprin-1α2 in a myoblast is not sufficient to trigger disassembly of the centrosomes or initiate nMTOC formation by recruiting proteins free in the cytoplasm.

5.2.2 Myc-PACT remains at the myoblast centrosome in the presence of GFP-nesprin-1α2

As shown in chapter 4, the C-terminal PACT domain of pericentrin is sufficient for binding to both the centrosome and myotube NE, in agreement with others (Gillingham and Munro, 2000; Gimpel *et al.,* 2017). As a smaller truncation of pericentrin, it may have the properties to bind to nesprin-1α2 at the NE in a non-myotube cell. To investigate this, Myc-PACT, containing pericentrin residues 2839-3330 was co-transfected into myoblasts with GFP-nesprin-1α2 (Fig. 5.2). Myc-PACT expressed alone colocalised with pericentrin at the centrosome. When co-transfected with the NE localising GFP-nesprin-1α2, Myc-PACT remained at the

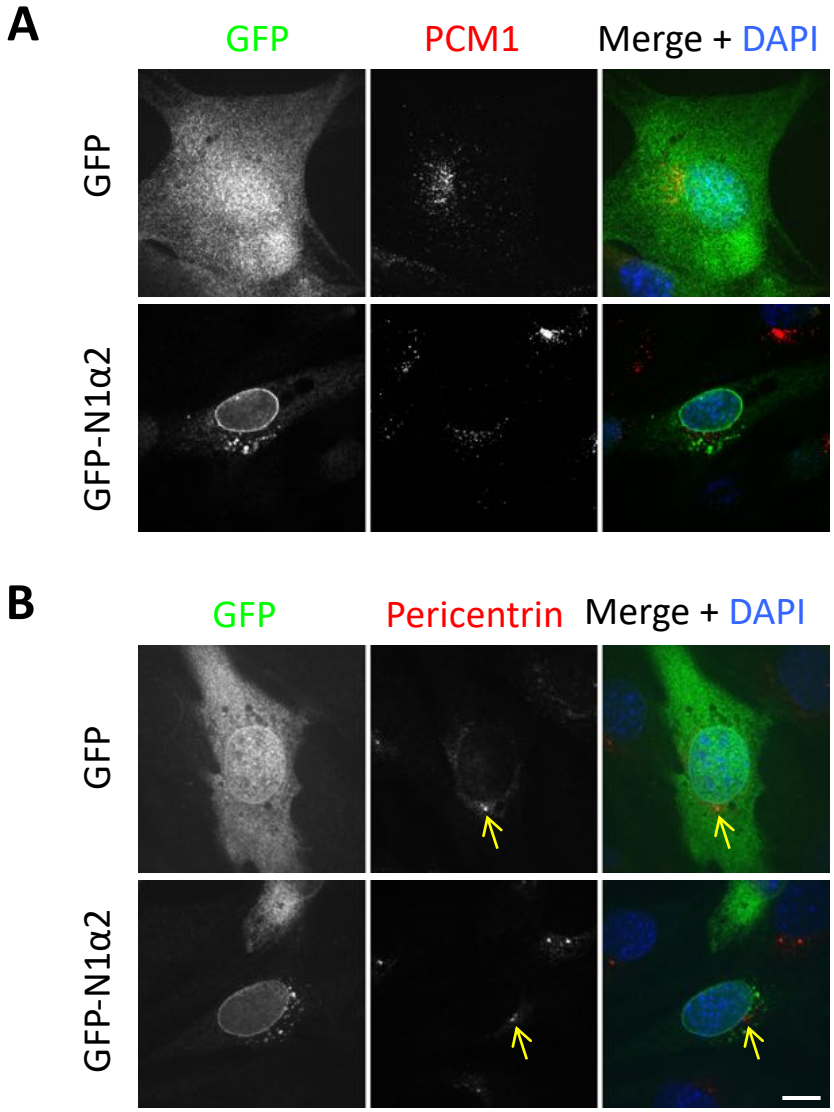


Figure 5.1 GFP-nesprin-1α2 expression in myoblasts is not sufficient to recruit PCM1 or pericentrin to the NE

C2C12 myoblasts were transfected with GFP-nesprin-1α2 or GFP and fixed with -20°C methanol the next day. Cells were co-stained with antibodies against GFP (green) and PCM1 (A) or pericentrin (B) (red). DNA was stained with DAPI (blue). Scale bar = 10 μm. Yellow arrows point towards the centrosome.

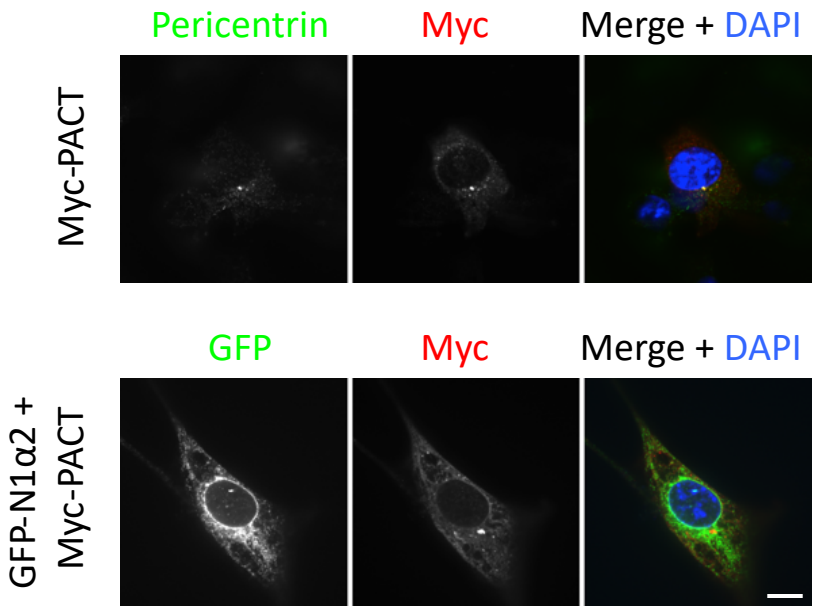


Figure 5.2 The NE localisation domain pericentrin Myc-PACT remains at the myoblast centrosome upon co-expression with GFP-nesprin-1α2

C2C12 myoblasts were transfected with Myc-PACT alone or with GFP-nesprin-1α2 and fixed with -20°C methanol the next day. Cells were co-stained with antibodies against GFP or pericentrin (green) and Myc (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

centrosome structure. This result suggests that either the PACT domain preferentially binds to its interacting partner at the centrosome rather than with nesprin-1α2, or overexpression of nesprin-1α2 is not sufficient to recruit the PACT domain to the NE in non-myotubes.

5.2.3 GFP-PCM1(1-331) remains in the myoblast cytoplasm in the presence of GFP-nesprin-1α2

In chapter 4, the centriolar satellite and NE localisation domains of PCM1 were found to be contained within residues 544-1089 and 1-331, respectively. In non-myotubes, the centriolar satellite localisation domain may be dominant over the NE localisation domain and prevent localisation with exogenously expressed nesprin-1α2. As GFP-PCM1(1-331) only contains the NE localisation motif, it would be exempt from the inhibitory effects of the centriolar satellite localising domain. Furthermore its cytoplasmic localisation provides a large amount of free protein to bind to nesprin-1α2. GFP-PCM1(1-331) was transfected with HA-nesprin-1α2 in myoblasts to investigate whether the NE localisation motif-containing domain can be driven to the NE of a non-myotube cell (Fig. 5.3). HA-nesprin-1α2 was localised at the NE with large surrounding aggregates. GFP-PCM1(1-331) localisation was in the cytoplasm in both the absence and presence of HA-nesprin-1α2 and there was no association with HA-nesprin-1α2 at the NE or its cytoplasmic aggregates.

5.2.4 PCM1 does not localise with GFP-nesprin-1α2 in U2OS cells after centrinone-mediated loss of centrosomes

Chapters 3 and 4 have both suggested an interdependency of MTOC proteins for their localisation at the NE. It is possible that multiple PCM components must co-operate and form a multi-protein complex for optimal NE localisation. One method of investigating this is by disassembling the centrosome to free all PCM proteins, in

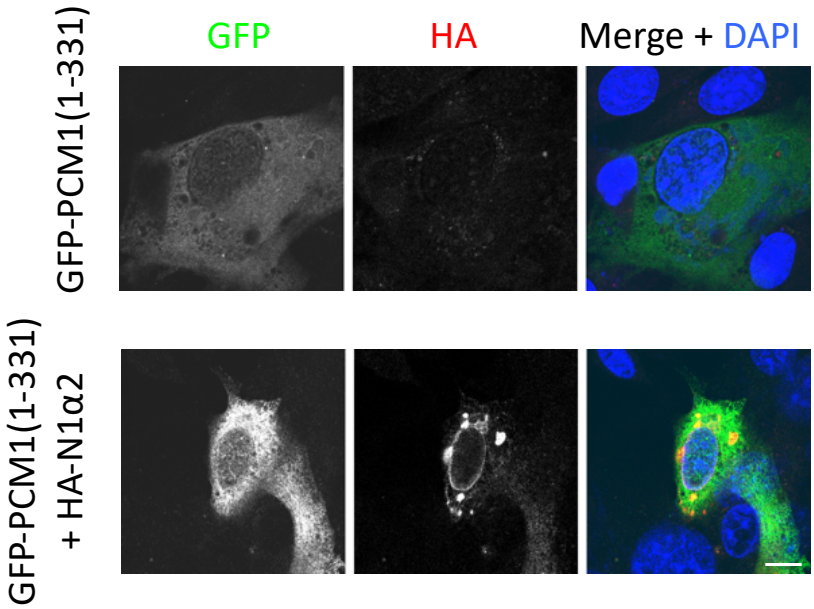


Figure 5.3 The NE localisation domain containing GFP-PCM1(1-331) fragment remains at the myoblast cytoplasm upon co-expression with HA-nesprin-1α2

C2C12 myoblasts were transfected with GFP-PCM1(1-331) alone or with HA-nesprin-1α2 and fixed with -20°C methanol the next day. Cells were co-stained with antibodies against GFP (green) and HA (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

the presence of GFP-nesprin-1α2. This would lead to greater amounts of free centrosomal protein in the cytosol, and may facilitate nMTOC formation if centrosome localisation of proteins is dominant over NE localisation. The reversible PLK4 inhibitor, centrinone, was used to achieve this. Incubation of cells with the drug inhibits centriole assembly during cell division, resulting in cells devoid of centrosomal structures. The centrosomes are fully reformed upon centrinone washout, showing centrosomal proteins remain present and active (Wong *et al.,* 2015).

U2OS cells were transfected with GFP-nesprin-1α2, followed by treatment with DMSO or centrinone to explore if there is nMTOC formation in the absence of centrosomes. U2OS cells were used due to the prolonged incubation time with centrinone, which led to spontaneous differentiation and nMTOC formation in myoblasts. PCM1 was stained at granules concentrated next to the nucleus in DMSO-treated cells but was dispersed throughout the cytoplasm in centrinone-treated cells, and remained undetectable at the NE in images in all samples (Fig. 5.4). This suggests that centrosomal localisation of MTOC proteins does not inhibit their binding to overexpressed nesprin-1α2 at the NE of non-myotubes, and that the availability of multiple MTOC components free in the cytosol is not sufficient for nMTOC formation. Together with previous results, this suggests that factors which are only supplied upon induction of myogenesis are required for centrosomal proteins to localise to the NE.

5.2.5 Pericentrin is partially recruited to the NE in myoblasts expressing GFP-PCM1(1089)-KASH

Pericentrin requires PCM1 for localisation at both the centrosome (Dammermann and Merdes, 2002) and the myotube NE as shown in chapter 4, in agreement with others (Gimpel *et al.,* 2017; Espigat-Georger *et al.,* 2016). The aim here was to investigate whether NE localising GFP-PCM1(1-1089)-KASH would be able to recruit pericentrin to the myoblast NE, by the interaction and the dependency of pericentrin on PCM1 for localisation. It is not known whether the interaction

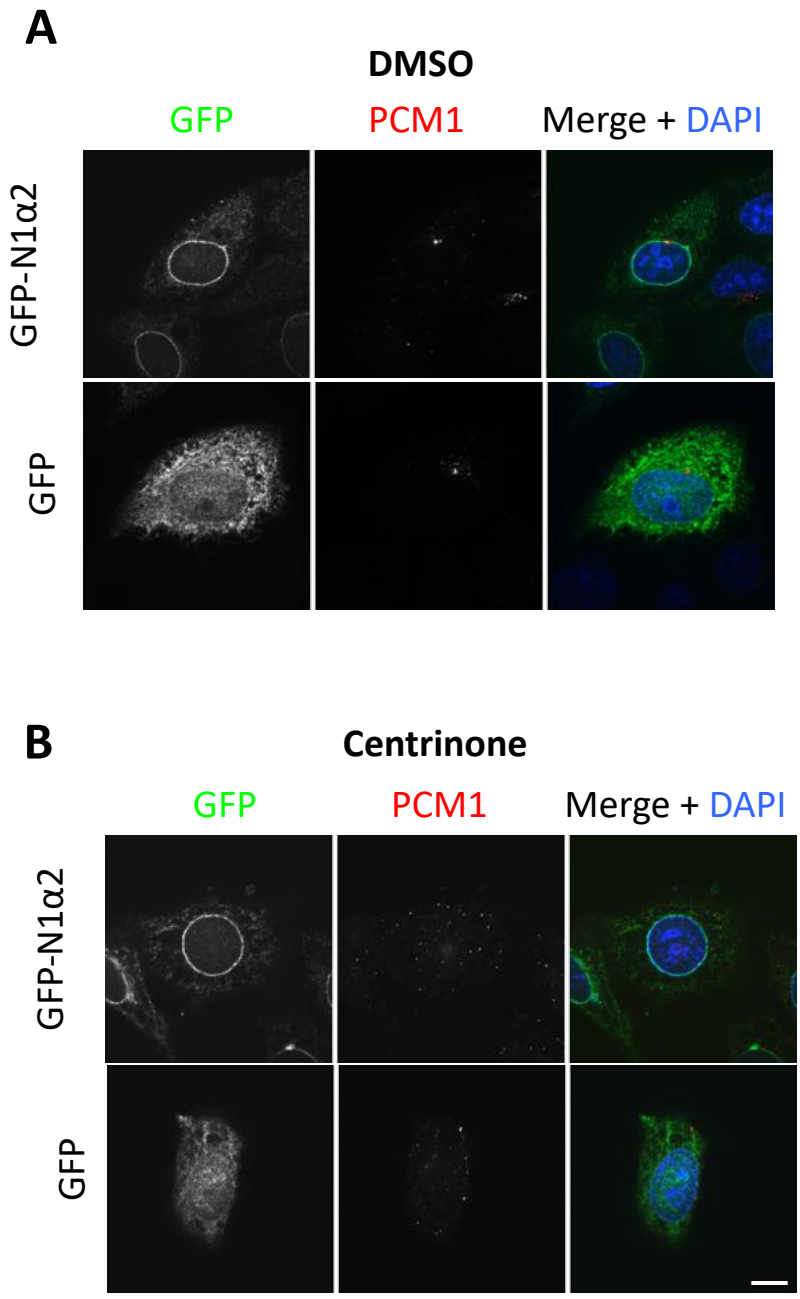


Figure 5.4 PCM1 does not localise with GFP-nesprin-1α2 in myoblasts upon centrinone-mediated centrosome depletion in U2OS cells

U2OS cells were transfected with GFP-nesprin-1α2 or GFP and treated with DMSO (A) or centrinone (B) the next day for 2 further days until fixation with -20°C methanol. Cells were co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

involves the N-terminal half of PCM1, however it was the only KASH-containing PCM1 construct available. Myoblasts were transfected with GFP-nesprin-1α2, GFP-PCM1(1-1089)-KASH or GFP, followed by analysis of pericentrin fluorescence intensity at the NE to investigate if overexpression of the proteins promoted pericentrin recruitment to the NE (Fig. 5.5).

Average fluorescence intensities were normalised to GFP, as GFP-nesprin-1α2 does not recruit pericentrin to the myoblast NE. In addition, myoblasts may spontaneously differentiate, and by normalising values to GFP, the increase in fluorescence intensity due to spontaneously differentiating cells is accounted for. Compared to GFP only expressing cells, in cells expressing GFP-PCM1(1-1089)-KASH, pericentrin appeared to be generally more dispersed in foci around the centrosome, and a greater number of cells exhibited weak pericentrin recruitment to the NE in the region adjacent to the centrosome. Measurement of pericentrin fluorescence intensity at the NE, as described in section 3.2.2.1, revealed a statistically significant 1.6-fold increase in staining in PCM1(1-1089)-KASH expressing cells compared with control cells expressing only GFP. In cells expressing GFP-nesprin-1α2, there was also some dispersal of pericentrin around the centrosome but no increased visible recruitment to the NE compared to GFP. There was a statistically non-significant 1.3-fold increase in NE staining in GFP-nesprin-1α2 expressing cells compared with control cells expressing only GFP. The increase could be due to the accumulation, but not association of pericentrin in the vicinity of the NE. Perhaps for the same reason, there was also no significant difference in fluorescence intensity between cells expressing GFP-PCM1(1-1089)-KASH and GFP-nesprin-1α2. Therefore, forced localisation of PCM1(1-1089)-KASH to the myoblast NE may be able to bypass nesprin-1α2 and unknown myogenic factors to weakly recruit pericentrin to the NE.

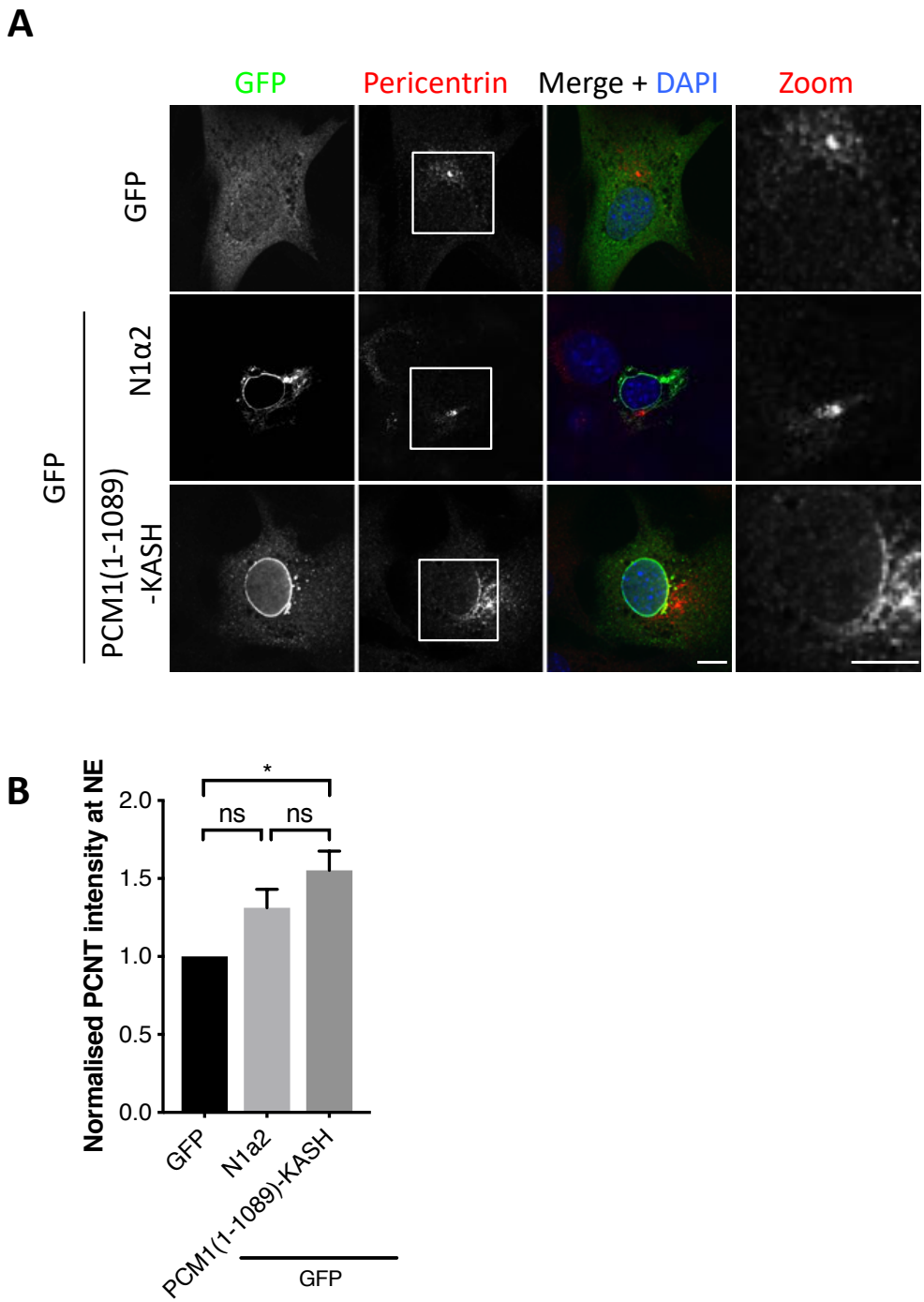


Figure 5.5 GFP-PCM1(1-1089)-KASH and GFP-nesprin-1α2 partially recruits pericentrin to the myoblast NE

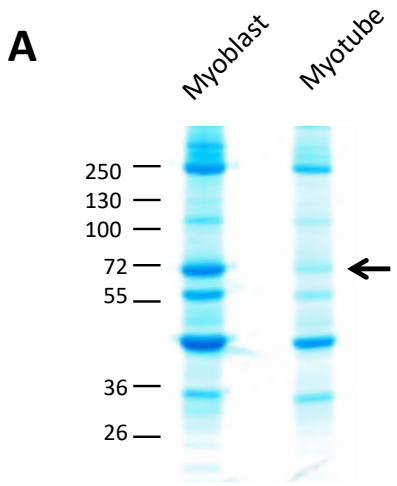
C2C12 myoblasts were transfected with GFP, GFP-nesprin-1α2 or GFP-PCM1(1-1089)-KASH and fixed with -20°C methanol the next day. (A) Cells were co-stained with antibodies against GFP (green) and pericentrin (red). DNA was stained with DAPI (blue). All scale bars = 10 μm. (B) Histogram showing normalised pericentrin (PCNT) intensity at the NE of cells expressing GFP, GFP-nesprin-1α2 or GFP-PCM1(1-1089)-KASH. Intensity of cells expressing GFP-nesprin-1α2 is given a value of 1. Nuclei from 20 myotubes were analysed per experiment. Data shows means and SEM, N=4. *P=<0.05 using Tukey's multiple comparisons test.

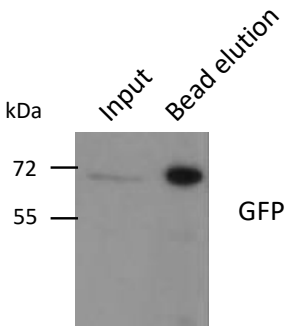
5.2.6 Identification of PCM1 S93 as a possible phosphorylation site during myogenesis

The nMTOC cannot be generated in a non-myotube cell simply by expression of the PCM protein receptor nesprin-1α2, together with an abundancy of MTOC proteins or their truncations in the cell. For example, the NE localising domain PCM1(1-331) is not recruited to the myoblast NE by overexpressing nesprin-1α2. On the other hand, artificial tethering of PCM1(1-1089) at the NE by a KASH domain appears to be able to partially recruit pericentrin to the myoblast NE. It can therefore be speculated that a myotube-specific phosphorylation is required to activate the PCM1 NE localisation domain for its recruitment to the NE, where it readily recruits other MTOC proteins. Indeed, PCM proteins undergo phosphorylation during the cell cycle to promote centrosome maturation prior to mitotic entry (Lee and Rhee, 2011). It is likely phosphorylations are also required for the formation of the nMTOC and reorganisation of the microtubule organisation during myogenesis.

A mass spectrometry approach was used to identify potential myotube-specific phosphorylations in PCM1(1-331). GFP-PCM1(1-331) (sequence in Appendix A) was expressed in C2C12 myoblasts and myotubes and cells were lysed with RIPA buffer. GFP-Trap beads were used to perform a large-scale purification of the protein. Purified proteins were separated on a SDS-PAGE gel (Fig. 5.6A), and the appropriate protein band was excised for trypsin digestion and liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Due to time limitations, mass spectrometry was only carried out twice. Western blot (Fig. 5.6B) and mass spectrometry confirmed the presence of GFP-PCM1(1-331) in all samples. Dephosphorylation of PCM1 residues for nMTOC formation was also considered a possibly, however there were no myoblast-specific phosphorylations. Myotube-specific phosphorylations may also be detected in myoblasts due to spontaneously differentiating cells. The amount of protein used





C

	Expt 1		Expt 2	
	MB	MT	MB	MT
S65	✓	✓	✓	✓
S68	✓	✓	✓	✓
S69	✓	✓	✓	✓
Y88	x	x	✓	x
S90	x	x	x	✓
S93	x	✓	✓	✓
S157	NC	✓	✓	✓
T158	NC	x	✓	✓
S159	NC	✓	✓	✓
T165	x	x	x	✓

✓ = phosphorylation
x = no phosphorylation
NC = not covered

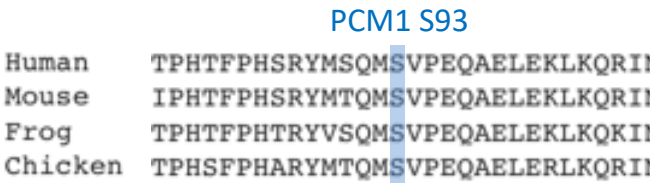


Figure 5.6 Mass spectrometry of GFP-PCM1(1-331) identifies PCM1 S93 to be phosphorylated in myotubes

(A) C2C12 myoblasts or myotubes transfected with GFP-PCM1(1-331) were lysed with RIPA buffer. Protein extracts were incubated with GFP-Trap beads for purification of the overexpressed protein. Proteins were eluted by boiling of the beads with SDS sample buffer and resolved on a SDS-page gel. The bands corresponding to GFP-PCM1(1-331) were excised for mass spectrometry (position of bands indicated by the arrow). Proteins were digested with trypsin and analysed by LC-MS/MS. (B) Western blot of mass spectrometry samples confirming identification of GFP-PCM1(1-331). (C) Table showing phosphorylated residues as identified by mass spectrometry in myoblasts (MB) and myotubes (MT) from two experimental repeats. Residues not found to be phosphorylated in any sample or were not covered are not included. (D) Conservation of PCM1 S93 between human, mouse, frog and chicken.

for mass spectrometry was not normalised so the number of instances a phosphorylated residue was detected in a sample could not be compared between myoblasts and myotubes. Possibly linked to this, there were no myotube-only phosphorylated residues that were present in both repeats. All residues found to be phosphorylated in at least one sample are listed in Figure 5.6C. A full list of serine-tyrosine-threonine residues and their phosphorylation status in each sample and experiment is listed in Appendix B. In experiment 1, 6 residues were phosphorylated. S65, S68 and S69 were phosphorylated in both myoblasts and myotubes and only S93 became phosphorylated upon differentiation. S157 and S159 were also phosphorylated in myotubes however neither residues were covered in the myoblast sample. In experiment 2, S65, S68 and S69 were again phosphorylated in both samples, as were S93, S157, S158 and S159. S90 and T165 showed myotube-specific phosphorylation only in experiment 2.
Y88 was phosphorylated in myoblasts. Of residues phosphorylated in both myotube samples, residues S65, S68 and S69 were also phosphorylated in both myoblast samples and were also reported over 100 times on Phosphosite, a database that documents phosphorylation sites. These residues were disregarded for further studies as frequently phosphorylated residues are likely involved in major cell events such as during mitosis. This left S93, S157 and 159, of which only S93 was covered in all 4 samples and has been previously reported to be phosphorylated in human skeletal muscle (Lundby *et al.,* 2012). Therefore, S93 was chosen for further investigation. The residue is conserved between human, mouse, chicken and frog (Fig. 5.6D), further suggesting a biological function. The residue is within a sequence that forms a casein kinase II (CK2) consensus phosphorylation motif pSXX[E/D] (Schwartz and Gygi, 2005).

5.2.7 PCM1(1-331) S93A/D mutated proteins show the same localisation in myoblasts and myotubes compared to the WT protein

To test whether PCM1 S93 phosphorylation is involved in nMTOC formation, the GFP-PCM1(1-331) construct was mutated to generate PCM1(1-331) S93A/S93D

phosphonull and phosphomimic mutants and their expression confirmed by western blot (Fig. 5.7). Constructs were transfected into myoblasts and myotubes and co-stained with GFP and PCM1 antibodies to investigate the effects of mutations on protein localisation. The PCM1 antibody epitope was not present in the constructs and therefore stained endogenous PCM1. In myoblasts (Fig. 5.8), WT and both mutant GFP-PCM1(1-331) constructs localised to the cytoplasm and the centriolar satellite localisation of endogenous PCM1 was unaffected. This indicates that the phosphorylation state of S93 does not affect PCM1(1-331) localisation in myoblasts. In myotubes (Fig. 5.9), both phosphonull and phosphomimetic mutants localised to the NE similarly to WT GFP-PCM1(1-331). NE localisation of endogenous PCM1 and pericentrin was unaffected in all experiments. Thus the phosphorylation status of S93 does not influence recruitment of either PCM1(1-331) itself, or pericentrin, to the myonuclei NE.

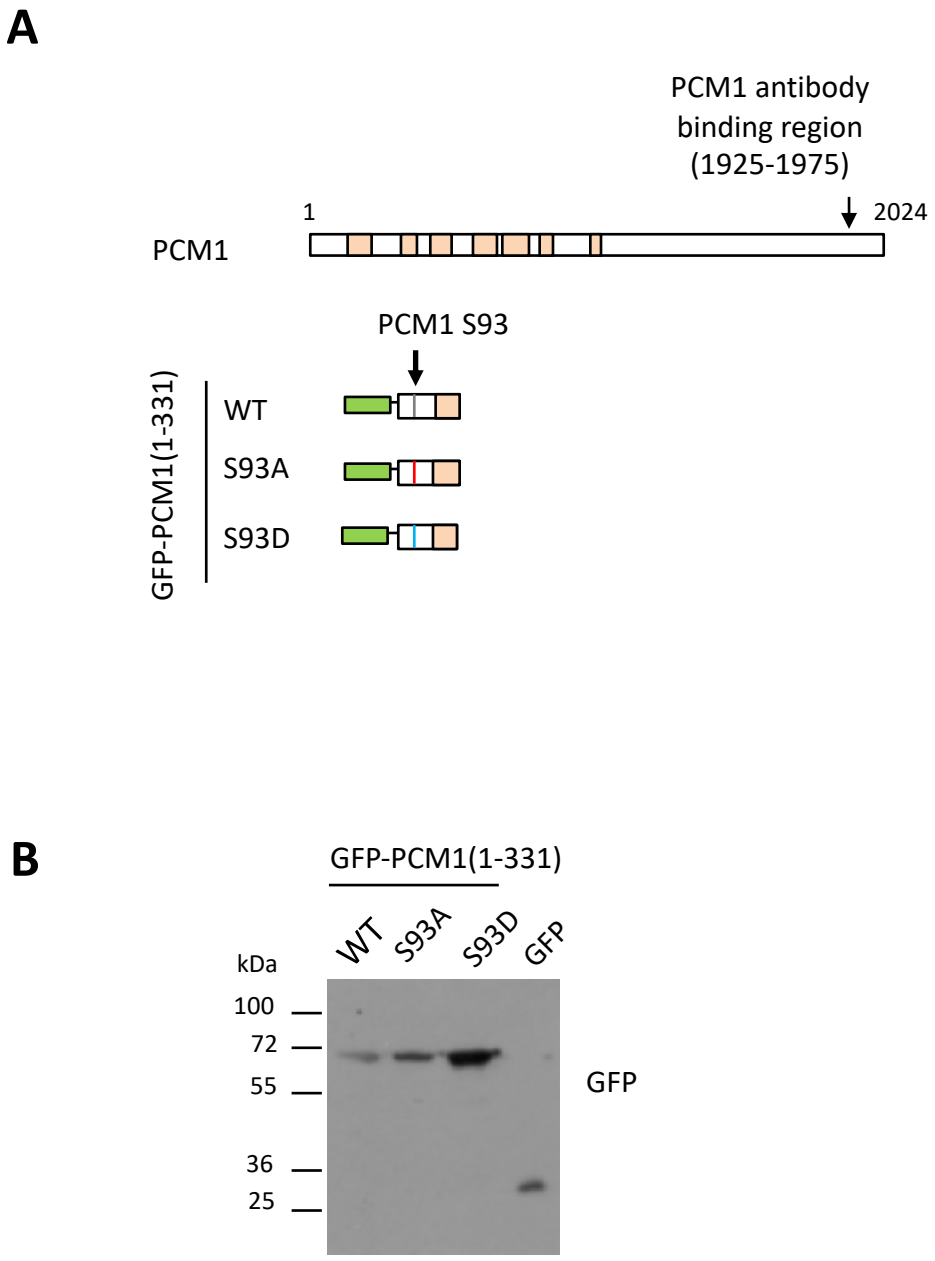


Figure 5.7 Schematic representation and western blot of GFP-PCM1(1-331)-S93A/S93D mutants

(A) Schematic representation of GFP-PCM1(1-331)-S93A/S93D mutants. (B) C2C12 cells were transfected with the constructs in for 24 h. Cells were lysed with RIPA buffer and 50 µg of whole cell extracts were analysed by immunoblotting with GFP antibody.

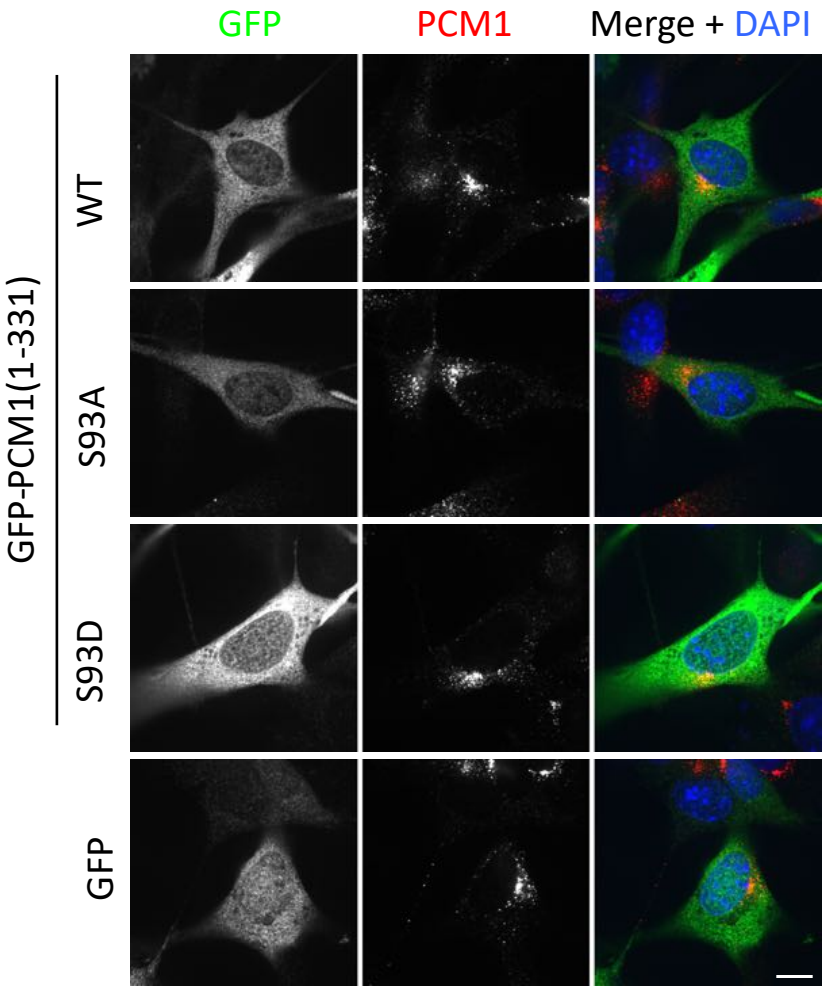


Figure 5.8 GFP-PCM1(1-331) WT and S93A/S93D mutants localise to the cytoplasm in myoblasts

C2C12 myoblasts were transfected with GFP-PCM1(1-331) WT and mutant constructs and fixed with -20°C methanol the next day. Cells were co-stained with antibodies against GFP (green) and PCM1 (red). DNA was stained with DAPI (blue). Scale bar = 10 µm.

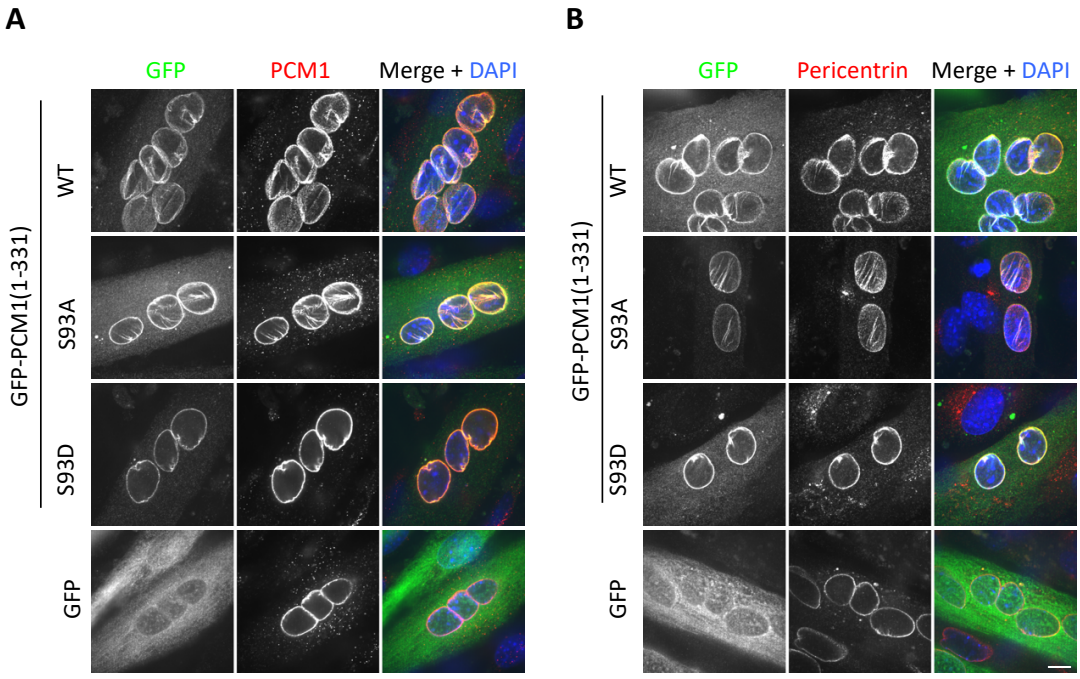


Figure 5.9 WT GFP-PCM1(1-331)-S93A/S93D mutants localise to the NE in myotubes

C2C12 myoblasts were transfected with GFP-PCM1(1-331) WT and mutant constructs and differentiated the next day for 5 days before fixation with -20°C methanol. Cells were co-stained with antibodies against GFP (green) and PCM1 (A) or pericentrin (B) (red). DNA was stained with DAPI (blue). Scale bar = 10 μm.

5.3 Discussion

This chapter shows that exogenous expression of nesprin-1α2 alone in myoblasts does not lead to the disassembly of centrosomes, or recruit PCM1 and pericentrin to the NE. Interestingly, NE-tethered PCM1(1-1089) may partially recruit pericentrin to the NE in myoblasts, indicating that an unknown myogenic factor must be provided for the relocalisation of PCM1 to the NE, where it readily acts as a protein scaffold for pericentrin and likely other proteins. The missing factor was postulated to be a myotube-specific phosphorylation. PCM1 post-translational modifications during myogenesis was explored in GFP-PCM1(1-331), which contains the NE localisation region, by mass spectrometry. S93 was identified as a phosphorylation candidate.

5.3.1 Nesprin-1α2 expression and presence of PCM proteins in the cytoplasm is not sufficient for nMTOC formation in non-myotube cells

GFP-nesprin-1α2 expressed in non-myotubes does not induce centrosome disassembly or recruit PCM1 and pericentrin to the NE. Overexpression of HA-nesprin-1α2 with Myc-PACT in C2C12 myoblasts is insufficient to drive Myc-PACT to the NE as it remained at the centrosome. This contrasts with the study performed by Gimpel *et al.* (2017), where dsRed-PACT localised to both the centrosome and NE, also in C2C12 myoblasts expressing GFP-nesprin-1α2. However, that study did not stain cells expressing the construct with a centrosome marker to show that the construct is as expected. There may also be a difference between the PACT sequence in dsRed-PACT and the Myc-PACT used in this study. It would be desirable to obtain the dsRed-PACT construct and repeat the experiment using both PACT constructs to further explore this.

The NE localising domain containing GFP-PCM1(1-331) also remained unrecruited to the NE, and remained localised in the cytosol in myoblasts even upon HA-nesprin-1α2 expression. Hence, the results in this study show that GFP-nesprin-

1α2 cannot recruit full-length or minimal localisation domains of pericentrin or PCM1 to the NE, and the availability of centrosomal proteins free in the cell is not the limiting factor for nMTOC formation. As PCM1(1-331) does not contain the centriolar satellite localising region, it should be free to localise to the NE. This is why it was hypothesised that phosphorylation of the PCM1 NE localisation domain is required for its binding to the NE.

There was still no nMTOC formation in centrinone-treated and GFP-nesprin-1α2 expressing cells, showing that simultaneous availability of multiple PCM protein components with GFP-nesprin-1α2 does not promote nMTOC formation. It also showed that an active centrosome does not sequester proteins from a potential forming nMTOC. This is the case in differentiated intestinal epithelial cells, where the active MTOC swaps between the apical membrane and centrosome. When two such cells are fused, the centrosome becomes the sole MTOC within minutes, showing it is the dominant MTOC (Yang and Feldman, 2015). It should be noted that centrinone is a PLK4 inhibitor, and although PLK4 mRNA expression decreases upon myogenesis (Jia *et al.,* 2019), PLK4 may be essential for nMTOC formation. If so, an alternative method to mediate the loss of centrosomes should be utilised, such as depletion of the PLK4-interacting centriole protein SAS-6 (Arquint and Nigg, 2016).

It may be the process of centrosome disassembly that activates nMTOC formation, however details of this process is unclear. Centrosome depletion also occurs during *D.melanogaster* oogenesis, where inactivation of PLK1 leads to disassembly of the PCM and centrioles (Pimenta-Marques *et al.,* 2016). Pimenta-Marques *et al.* (2016) showed that forced tethering of active PLK1 to the centriole prevents PCM and centriole disassembly in oocytes. PLK1 could be tethered to the centrioles of myoblasts to determine if inactivation is required for centrosome disassembly in myogenesis. On the other hand, PLK1 interacts with PCM1 to phosphorylate proteins for the disassembly of the primary cilium before mitotic entry (Seeley *et al.,* 2010). This interaction is mediated by the phosphorylation of PCM1 by CDK1 (Seeley *et al.,* 2010). PLK1 and CDK1 inhibitors may be used to determine whether

these kinases phosphorylate substrates needed for centrosome disassembly during myogenesis.

5.3.2 GFP-PCM1(1-1089)-KASH partially recruits pericentrin to the myoblast NE

All unsuccessful attempts of generating the nMTOC in non-myotubes suggested that myogenic factors are required for nMTOC formation. Instead of recruiting centrosomal proteins to the NE by exogenous expression of the myotube-specific nesprin-1α2, GFP-PCM1(1-1089)-KASH was investigated to see if it can readily recruit pericentrin to the NE if itself is forced to localise to the NE. This was the only condition where pericentrin, could be induced to localise to the NE in a non-myotube cell. Localisation was still partial, further indicating there are other requirements for optimum nMTOC formation.

The observation that pericentrin is weakly recruited to the NE of myoblasts with GFP-PCM1(1089)-KASH can be explained by the interaction between PCM1 and pericentrin at the centrosome (Delaval and Doxsey, 2010), which is maintained at the nMTOC. It may also be due to the dependence of pericentrin on PCM1 for its localisation, which is conserved from the centrosome to the myotube NE. However, the increased NE localisation of pericentrin upon GFP-PCM1(1089)-KASH may be due to the exogenous protein promoting differentiation. To have ruled out this possibility, the experiment should have utilised triple staining and a third antibody towards a myogenic marker to exclude differentiating cells from the study. Alternatively, myoblasts expressing the different constructs could have been subjected to PCR to detect mRNA levels of myogenic markers to explore the differentiation status of the cell culture as a whole.

5.3.3 Mass spectrometry analysis of GFP-PCM1(1-331) to identify phosphorylation sites in myoblasts and myotubes

From the discussed results, phosphorylation of PCM1 during myogenesis could be required for its own localisation to the NE, and/or disassembly of the centrosomes to allow other centrosomal proteins to bind to the NE. This study utilised mass spectrometry to identify phosphorylated residues in GFP-PCM(1-331). Eight serines, threonines, and tyrosine residues, S44, S45, S233, S252, Y254, T3, S320, and T325 were not covered in myotubes in any experiment and they should not be disregarded to be involved in myogenesis. The coverage of PCM1(1-331) in the mass spectrometry samples ranged from 63% to 84%. Alternative digestive enzymes could be used for different coverage of the protein. Furthermore PCM1 is 2024 residues whereas only PCM1(1-331) was utilised in mass spectrometry, due to low transfection efficiency of longer fragments. Being able to identify phosphorylated residues in the whole PCM1 sequence in the future would be ideal to identify further residues possibly involved in nMTOC formation.

Of the residues found to be phosphorylated in myotubes, only S93 was further studied as phosphorylation of this residue was previously reported in human skeletal muscle (Lundby *et al.,* 2012). Lundby *et al.* (2012) also identified phosphorylation of PCM1 S533, S537 and S497 in human skeletal muscle. These residues are not in the GFP-PCM1(1-331) construct used in this study so it is unknown whether the same residues are phosphorylated in cultured mouse myoblast and myotubes. According to the localisation domains identified in this study, S533, S537 and S497 are located between the NE and centriolar satellite localising regions.

The residues surrounding PCM1 S93 form the consensus motif which is phosphorylated by CK2. CK2 is known to phosphorylate a number of substrates and can be in various cell locations including the nucleus, cytoplasm and Golgi (Litchfield, 2003). Due to this, CK2 is involved in many functions such as cell survival (Litchfield, 2003) and the cell cycle (Hanna *et al.,* 1995). CK2 is also

involved in the differentiation of myoblasts, adipocytes and neuronal cells (Götz and Montenarh, 2017). It is involved in myogenic differentiation through its phosphorylation of paired box transcription factor Pax3 where, interestingly, both phosphorylation and dephosphorylation is required (Miller *et al.,* 2008; Dietz *et al.,* 2009). S205 of Pax3 is phosphorylated in proliferating myoblasts and is rapidly removed within 15 min of differentiation, whereas S209 only becomes phosphorylated within the initial 15 min of differentiation (Dietz *et al.,* 2011). This is thought to regulate the expression of proteins needed for myogenesis. Although S205 and S209 are phosphorylated independently, S205 phosphorylation is required for downstream GSK3β phosphorylation of S201, showing that phosphorylation of residues within a protein can be linked (Dietz *et al.,* 2011). Due to the multiple cellular substrates and functions of CK2, it is possible that it also phosphorylates PCM1 in the context of myogenesis.

GFP-PCM1(1-331) WT, S93A and S93D mutants all showed similar localisation in myoblasts and myotubes, showing the phosphorylation status of S93 does not determine nMTOC formation. S93 may still be involved in nMTOC formation if effects of the phosphorylation are through transmitting information to the C-terminal portion of the protein, by changing its conformation or causing further residues to be phosphorylated. To study this, the S93 mutations should be applied to the full-length PCM1. The example of Pax3 phosphorylation by CK2 shows that identification and definition of phosphorylated residues is not straight-forward and may require simultaneous mutation of multiple residues. Of course, CK2 is only one kinase which may be involved in nMTOC formation. There are over 500 kinases in human, and although kinases required for myogenesis are being determined (Knight & Kothary 2011), it is not easy to determine which ones are involved in the relocalisation of MTOC proteins to the NE. Performing mass spectrometry on proteins thought to be phosphorylated upon myogenesis is a method to determine this, by looking for consensus phosphorylation motifs formed by phosphorylated residues. Other phosphorylation candidates include other centrosomal proteins, motor proteins and/or nesprin-1α2, all of which could be studied similarly.

5.3.4 Summary

This chapter shows that exogenously expressed nesprin-1α2 in myoblasts is not sufficient to recruit PCM1 and pericentrin to the NE. Instead, forced localisation of PCM1 to the NE in myoblasts is able to recruit pericentrin to the NE to some extent. Protein phosphorylations may be needed for centrosome disassembly and to promote full nMTOC formation, and it would be worthwhile to study this in full-length PCM1. It would be interesting to determine the minimal components required to initiate the recruitment of centrosomal proteins to the NE in a non-myotube, and to investigate if these same components are also sufficient for the formation of an active MTOC.

Chapter 6

Discussion

6.1 Summary

This study provides better understanding of nMTOC formation during myogenesis mainly by utilising a range of nesprin-1α2, PCM1 and pericentrin constructs in localisation studies, and RNAi-mediated PCM1 depletion to identify its role as a protein scaffold. It identifies the domains of nesprin-1α2, PCM1 and pericentrin involved in nMTOC formation. It also finds that PCM1 requires the same nesprin-1α2 residues as kinesin-1 for localisation at the NE, and suggests that PCM1 plays a partial role, in addition to nesprin-1α2, in recruiting pericentrin and AKAP450 to the NE. Treatment of cells with nocodazole showed that transfer and anchorage of PCM1 and pericentrin to the NE is microtubule-independent and so is unlikely to involve kinesins or dynein. Attempts to reconstitute the nMTOC in myoblasts and U2OS cells suggested that a myogenic-specific factor must accompany nesprin-1α2 receptor expression, and that this is the major barrier for nMTOC formation, as forced NE localisation of the N-terminal half of PCM1 appeared to be able to recruit pericentrin to the NE in myoblasts. Mass spectrometry of PCM1(1-331), the region containing the NE localisation domain, suggested that S93 may be phosphorylated upon myogenesis. Altogether, the results start to delineate nMTOC formation at the nesprin-1α2 interface and defines possible roles of PCM1 at the myotube NE.

6.2 The nMTOC at the nesprin-1α2 interface

Nesprin-1G and nesprin-1α2 are the nesprin-1 isoforms expressed in muscle cells and localise at the NE (Duong *et al.,* 2014). The MTOC is transferred from the centrosome to the NE to form the nMTOC upon myogenesis by nesprin-1 acting as a centrosomal protein receptor (Gimpel *et al.,* 2017; Espigat-Georger *et al.,* 2016). As a result, microtubules emanate from the NE in myotubes (Fant *et al.,* 2009; Gimpel *et al.,* 2017). This study shows that the nesprin-1α2 isoform alone is sufficient for the recruitment of centrosomal protein AKAP450 to the NE in nesprin-1 null myotubes, in agreement with Gimpel *et al.* (2017), and shows for the first time that it also recruits PCM1. Specifically, this study finds nesprin-1α2(N-AD) is sufficient for both PCM1 and AKAP450 to localise at the NE, whilst

the N-terminal isoform-specific 31 residue region acts to stabilise the localisation. The recruitment of pericentrin was not tested as no antibody was available, however later results in this study showing PCM1 is a pericentrin scaffold suggest it would indeed be recruited. Maximum association of PCM1 at the NE requires the kinesin-binding LEWD motif in nesprin-1α2, located in the AD, which is also required for kinesin-1 and dynein to interact with nesprins (Zhu *et al.,* 2017; Wilson and Holzbaur, 2015; Zhou *et al.,* 2017). As it has already been shown that kinesin-1 recruitment to the NE is reduced in PCM1 depleted myotubes (Espigat-Georger *et al.,* 2016), PCM1 and kinesin-1 may be interdependent on each other for their recruitment to the NE.

Localisation studies using protein truncations revealed that PCM1(1-331) localises at the nMTOC, whereas localisation to the centrosome instead requires the central region PCM1(544-1089). There is no physical structure at the centriolar satellite, whereas localisation at the nMTOC is through binding to the surface of the NE, which may mask PCM1 binding sites. Hence, N-terminal binding of PCM1 at the NE may be required to keep its residues responsible for protein binding exposed. The PACT domain of pericentrin is sufficient for localisation at both the centrosome and NE in myotubes though no interaction studies were performed. Forced expression of PCM1(1-1089) at the NE is able to partially recruit pericentrin and AKAP450 in myoblasts and myotubes, respectively. Thus, the N-terminal half of PCM1, which contains the region with interacts that nesprin-1α2, also recruits pericentrin and AKAP450 close to nesprin-1α2. These ideas are illustrated in Figure 6.1. In the future, interaction studies between nesprin-1α2 truncation mutants and PCM1 would further narrow down the interaction domains responsible for the localisation of PCM1 to the NE.

In non-myotube cells, exogenous expression of nesprin-1α2 at the NE does not drive nMTOC assembly even when centrosomal proteins or fragments are in the cytoplasm. It is therefore likely that myogenic factors such as protein-modifications, or another myotube-specific protein is needed for centrosomal proteins to bind to the NE.

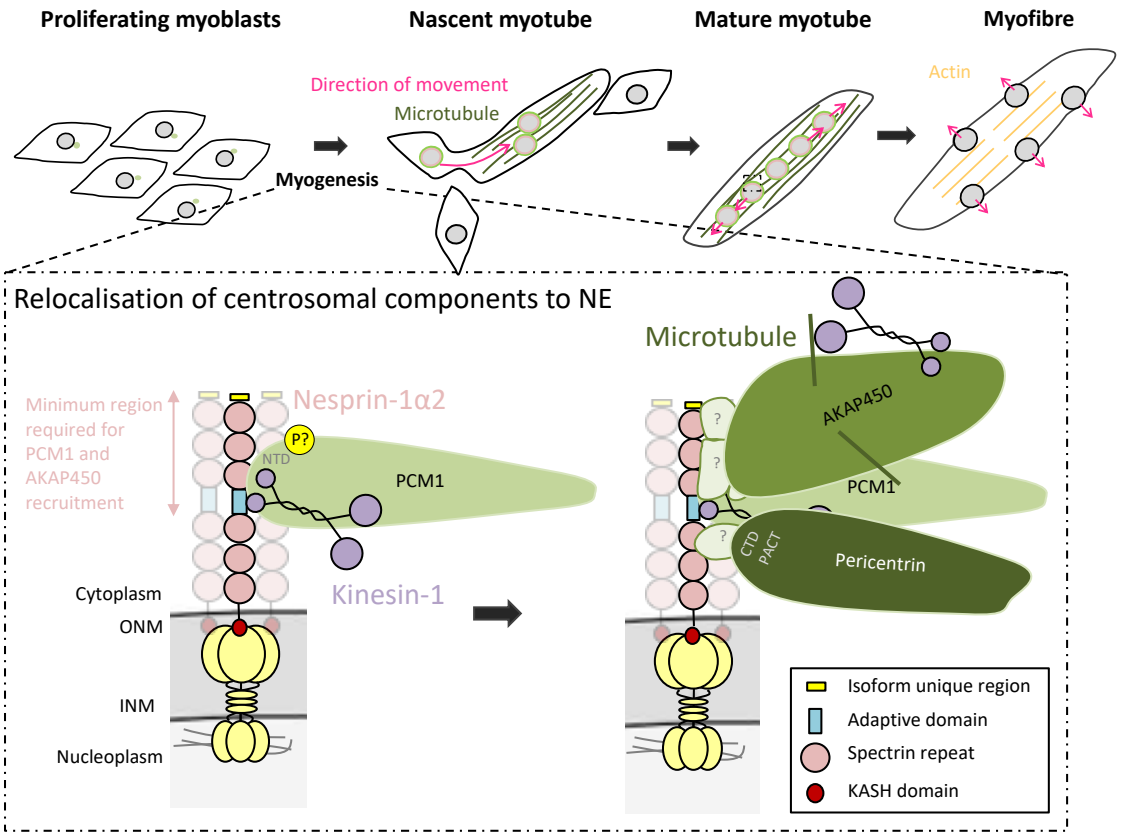


Figure 6.1 Nesprin-1α2 mediates nMTOC formation, whereas PCM1 functions as part of a multi-protein scaffold to recruit pericentrin and AKAP450 to the NE

Upon myogenesis and centrosome disassembly, centrosomal proteins relocalise to the NE. The N-terminal region of PCM1 binds to the NE by interacting with nesprin-1α2. The kinesin-1 binding region (LEWD motif) of nesprin-1α2 is required for full PCM1 recruitment, suggesting a relationship between the NE localisation of PCM1 and kinesin-1. Phosphorylation of PCM1 may also be required for its NE localisation. At the NE, PCM1 recruits other centrosomal proteins to the NE, some of which also function as a protein scaffold. This generates a multi-protein complex, where pericentrin, via its PACT domain, and AKAP450 are suggested to be recruited to the NE through a number of scaffold proteins , including PCM1. Pericentrin may be recruited to the NE by a limited number of proteins such that, when PCM1 is depleted, pericentrin recruitment to the NE is reduced. In contrast, AKAP450 may be recruited to the NE by a larger number of proteins such that, when PCM1 is depleted, full AKAP450 recruitment to the NE is maintained by the other scaffold proteins. Once at the NE, centrosomal proteins work together to control microtubule nucleation and dynamics. AKAP450 allows nucleation of microtubules from the NE (Gimpel *et al.,* 2017), which are utilised by motor proteins for nuclear positioning. Not to scale.

6.3 Ordered nMTOC assembly

Reminiscent of the PCM at the centrosome, the arrangement of centrosomal proteins at the nMTOC may be ordered. At the centrosome, the C-terminus of pericentrin and Cep152 binds to the mother centriole as the N-terminal ends extend outwards to define the area of the PCM. Other PCM proteins are arranged into ordered ring layers around the centriole (Lawo *et al.,* 2012). Centrosomal proteins are mainly coiled-coil proteins and often acts as scaffolds for each other (Graser *et al.,* 2007; Dammermann and Merdes, 2002; Keryer *et al.,* 2003). This study shows that, at the myotube NE, PCM1 is required for maximum localisation of pericentrin but not AKAP450, in agreement with findings by Gimpel *et al.* (2017). Yet, forced localisation of PCM1(1-1089) to the NE of nesprin-1 null myotubes led to minor AKAP450 recruitment to the NE. This difference in research findings is highlighted in Table 6.1. This can be explained if the nMTOC, like the centrosomal MTOC, is a multi-protein complex containing multiple protein scaffolds. PCM1 would be one of multiple scaffolding proteins at the NE, all which require nesprin-1α2 for localisation. The effect of PCM1 depletion on a downstream binding protein would then depend on the remaining scaffold proteins at the NE. The current data shows that PCM1 is a major pericentrin scaffold, therefore pericentrin localisation to the NE is reduced upon PCM1 depletion. Instead, PCM1 is only one of many AKAP450 scaffolding proteins, and therefore, upon PCM1 depletion, full localisation of AKAP450 at the NE is maintained by other scaffolding proteins. These ideas are illustrated in Figure 6.1.

Indeed, Bio-ID screening to find proteins close in proximity to nesprin-1α2 in myotubes only identified the centrosomal proteins PCM1, pericentrin, AKAP450, AKAP6 and Cep170 (Gimpel *et al.,* 2017), whereas other centrosomal proteins, such as ninein and CDK5RAP2 are also part of the nMTOC (Bugnard *et al.,* 2005; Srsen *et al.,* 2009). Although experimental reasons, such as low protein abundancy, may have led other proteins being not to be detected, it may simply be because they are further away from nesprin-1α2, due to their recruitment to the NE being from several layers of scaffolding proteins.

6.4 Myogenic factors in nMTOC assembly

Work in this study showed that the expression of GFP-nesprin-1α2 in non-myotube cells was not sufficient for the recruitment of centrosomal proteins, or their truncations to the NE, despite Gimpel *et al.* (2017) reporting that exogenous nesprin-1α2 and the pericentrin PACT domain is able to co-localise in myoblasts. This difference in research findings is highlighted in Table 6.1. Yet, nesprin-1α2 was confirmed as a centrosomal protein receptor, and full-length PCM1 and pericentrin S, expressed in myoblasts, do not require alternative isoform expression for NE localisation, as these isoforms localised to the myotube NE when exogenously expressed. This indicates that existing proteins are transferred from the centrosome to the nMTOC and indeed, the nMTOC appears to assemble at the NE surface closest to the centrosome (Srsen *et al.,* 2009). This suggests that PTMs such as phosphorylation are required for nMTOC formation. This is likely as other major centrosome reorganisation events such as cilia disassembly (Wang *et al.,* 2013) or centrosome maturation during mitosis (Lee and Rhee, 2011) also require the phosphorylation of centrosomal proteins.

This study shows that phosphorylation of centrosomal proteins may be required for centrosome disassembly, but this is still not sufficient for nMTOC formation as GFP-nesprin-1α2 expression, accompanied with centrinone-mediated release of centrosomal proteins into the cytosol, or overexpression of GFP-PCM1(1-331) which localises in the cytosol, is not sufficient for nMTOC assembly. The requirement for centrosomal proteins to be phosphorylated for nMTOC formation may be for other reasons. At the centrosome MTOC, many centrosomal proteins are trafficked towards the centrosome in a microtubule-dependent manner by the motor protein dynein (Takahashi *et al.,* 2002; Delgehyr *et al.,* 2005; Kubo *et al.,* 1999). However, this study shows the transfer and anchorage of pericentrin, in agreement with Zaal *et al.* (2001), and PCM1 to the nMTOC is microtubule-independent. Hence, phosphorylation of centrosomal proteins upon myogenesis may initiate microtubule-independent transport, for example by activating the NE localising domain, to enable the protein to undergo actin-based transport or

Table 6.1 Differences between previously published data and work from this project

Conclusions and supporting data from previous published data are stated alongside any differences found in the current work.

Published data	Data from current work
Data: depletion of PCM1 in myotubes does not lead to reduced AKAP450 localisation at the NE (Gimpel *et al.,* 2017). Conclusion: In myotubes, PCM1 is not required for the localisation of AKAP450 at the NE.	Data: As with the work of Gimpel *et al.* (2017), depleting PCM1 in myotubes did not lead to reduced AKAP450 localisation at the NE. However, artificial tethering of GFP-PCM1(1-1089)-KASH to the NE in nesprin-1 null myotubes appeared to be able to partially recruit AKAP450 to the NE. Conclusion: PCM1 plays a weak role in recruiting AKAP450 to the NE, compared to and in addition to nesprin-1α2 and possibly other proteins at the nMTOC.
Data: dsRed-PACT is at both the myoblast centrosome and NE when co-expressed with GFP-nesprin-1α (Gimpel *et al.,* 2017). Conclusion: exogenous expression of nesprin-1α in myoblasts is sufficient to drive some exogenously expressed pericentrin PACT domain to the NE, though an in external factor is required for its full recruitment to the NE.	Data: Myc-PACT remained at the myoblast centrosome upon co-expression with GFP-nesprin-1α2. Conclusion: an external factor is required in addition to nesprin-1α2 expression for the release of pericentrin from the centrosome and/or its recruitment to the NE in myotubes.

diffusion. Alternatively, PCM1 phosphorylation may be required for it to interact with nesprin-1α2 or another NE protein. This can be tested with GFP-Trap co-immunoprecipitation of exogenous HA-nesprin-1α2 and GFP-PCM1(1-331) in myotube and non-myotube cells.

Identification of myotube-specific PCM1 phosphorylations was attempted by mass spectrometry of PCM1(1-331). This identified S93 as a possible myotube-specific phosphorylation site, which was only explored in PCM1(1-331) without any definitive results. The ability to perform mass spectrometry and mutant analysis on the full-length PCM1 would be desirable to attempt to identify and define meaningful residues for nMTOC formation. In addition, mass spectrometry was only carried out twice, and a third experiment would better to pin-point interesting residues for investigation.

Equally, though unexplored during this study, nesprin-1α2 may require activation in myotubes to recruit MTOC components. This could be achieved by phosphorylation, in which case the kinase involved is still present in mature myotubes as GFP-nesprin-1α2 constructs expressed at the myotube stage are still able to recruit PCM1 and AKAP450 to the NE. Another way of activation could be through the SUN protein it binds to form the LINC complex. Like nesprins, SUN proteins also have tissue-specific isoforms and there is mRNA and protein evidence indicating the expression of additional SUN1 splice variants upon myogenesis (Göb *et al.,* 2010), or post-translational modifications. If this is true, a myotube-specific SUN1 splice variant or post-translation modification may be required to generate a LINC complex capable of nMTOC formation. Overexpressed nesprin-1α2 in non-myotubes is already able to localise to the NE, showing a muscle-specific SUN1 would not be needed for nesprin-1α2 localisation but only to mediate its function as a protein receptor. Indeed, one point mutation in SUN1 is able prevent nesprin-1 from recruiting pericentrin to the NE (Meinke *et al.,* 2014), showing its importance other than tethering nesprin-1 to the NE.

Also unexplored in this study, an unidentified myotube-specific protein may be required to bridge nesprin-1α2 and centrosomal proteins. Mass spectrometry of

nesprin-1α2 may identify such a protein. Bio-ID of nesprin-1α2 in myotubes (Gimpel *et al.,* 2017) did not identify proteins that were not MTOC or motor proteins, however a mass spectrometry approach using PCM1 as the bait may yield different and new interacting partners. An identified protein may be muscle-specific, or a protein that also functions in proliferating cells. For example, the cell cycle regulator and GTPase Cdc42 is required for the fusion of myoblasts (Vasyutina *et al.,* 2009). These proteins may also require phosphorylation for their function in myogenesis.

6.5 Co-operation of proteins in a functional nMTOC

A number of centrosomal proteins may colocalise at a particular site, but this does not necessarily mean that they are capable of microtubule nucleation. A specific combination of proteins with complementary functions is required for an active and functional MTOC. The minimal set of proteins sufficient for microtubule nucleation *in vitro* is the *C.elegans* protein SPD-5 and the *C.elegans* homologues of XMAP215 and TPX2. SPD-5 forms the PCM scaffold to localise XMAP215 and TPX2, which both concentrate tubulin and act as a microtubule polymerase and stabiliser, respectively (Woodruff *et al.,* 2017). Other proteins are required for microtubule dynamics at the human centrosome. At the interphase centrosome, the γ-TuRC is commonly the microtubule nucleator but requires activation by CDK5RAP2 (Choi *et al.,* 2010), whereas anchorage of microtubules is thought to be achieved or mediated by PCM1 (Dammermann and Merdes, 2002).

Likewise the formation of a functional nMTOC must also involve proteins capable of microtubule nucleation, anchorage and maintenance. However, functions of centrosomal proteins at the NE remain unclear. To date, PCM1 is known to be required for myonuclear positioning, though its specific role in microtubule regulation at the NE is unknown (Espigat-Georger *et al.,* 2016). Out of γ-tubulin, AKAP450, pericentrin and CDK5RAP2, only the function of γ-tubulin and AKAP450 is known at the nMTOC, both being involved in microtubule nucleation (Gimpel *et al.,* 2017; Bugnard *et al.,* 2005). Indeed there is no microtubule nucleation from the NE in nesprin-1 null myotubes due to the mislocalisation of all tested centrosomal

proteins from the NE (Gimpel *et al.,* 2017). However full nMTOC formation and microtubule nucleation from the NE is expected to be rescued upon GFP-nesprin-1α2 expression, though this has not been tested. Assuming this is correct, a major question is whether the nMTOC formed by nesprin-1α2(N-AD), which recruits at least PCM1 and AKAP450, and possibly pericentrin, is sufficient for microtubule nucleation. This is particularly interesting as AKAP450 is required for microtubule nucleation from the myotube NE (Gimpel *et al.,* 2017), and PCM1 is a protein scaffold and may promote recruitment of other proteins to the NE. Of course, other major centrosomal proteins should be stained in immunofluorescence microscopy to reveal whether they are present in the nesprin-1α2(N-AD) nMTOC. Mass spectrometry could also be used to identify the presence of additional centrosomal proteins. Should there be microtubule nucleation, the speed and extent of nucleation and anchorage could be compared to that of full-length nesprin-1α2-containing myotubes to determine the functions of the nMTOC components. It is possible that different nesprin-1α2 fragments recruit different sets of proteins to form nMTOCs with different properties. In this case the same assays could be applied to further define functions of other centrosomal proteins at the NE.

At present, most muscle-disease causing mutations are in LINC complex proteins or LINC complex associated proteins at the nucleoplasm. Mutations affecting the nucleoplasmic LINC complex component SUN1 and its associated protein lamin A/C can exert effects through the NE to affect nesprin-1 binding to pericentrin (Meinke *et al.,* 2014; Mattioli *et al.,* 2018) and though not tested, possibly other nMTOC proteins. Although the primary role of nesprin-1α2 is thought to be at the ONM (Holt *et al.,* 2019), emerin and lamin directly interact with nesprin-1α2 *in vitro* (Mislow *et al.,* 2002) and mutations which increase such interactions may cause nesprin-1α2 to localise at the INM instead of the ONM, in turn meaning centrosomal protein can no longer be recruited to the NE. Mutations in centrosomal proteins may be less extreme as they often play redundant roles. However due to the multiple roles of centrosomal proteins such as in ciliation and mitosis, mutations have the potential to give rise to severe disease in multiple tissues. For example, a patient with mutations leading to reduced pericentrin mRNA levels showed symptoms of dwarfism and insulin resistance (Huang-Doran

et al., 2011). Likewise, mutations of the pericentrin PACT domain is more likely to affect multiple cell types as it is required to bind both the centrosomal and nuclear MTOC, and multiple other proteins at the centrosome. It is mutations in regions important for muscle only such as the NE localisation region of PCM1, residues 1-331, which could potentially lead to muscle-specific disease. Mutations affecting PCM1 localisation to the NE would also affect the proteins it acts as a scaffold for, and the additional effects may affect nMTOC formation, ultimately leading to disorganised microtubule organisation.

6.6 Future perspectives

Over the years, identification of centrosome components by mass spectrometry-based studies has identified hundreds of centrosomal proteins (Paz and Lüders, 2018), some of which have been shown to be involved in nMTOC formation. How many of these proteins localise to the nMTOC, along with their roles, are unknown. One major question is whether the nesprin-1α2(N-AD) fragment, capable of recruiting at least PCM1 and AKAP450 to the NE, is also sufficient to form a nMTOC that supports microtubule nucleation and dynamics. Other than the myotube MTOC, further work could address understanding of microtubule dynamics at other cellular sites as roles of centrosomal protein are sometimes conserved between MTOCs. Indeed, investigation of the centrosome is difficult as it is a multi-functional organelle where proteins have multiple roles, such as in cilia disassembly and cell division. Instead, defining the roles of proteins at the nMTOC may be more straightforward. Defining the roles of centrosomal proteins could also identify proteins involved in hyperactive MTOCs in cancer cells (Salisbury *et al.,* 1999; Godinho *et al.,* 2014).

Another major question from this study is what phosphorylation events mediate nMTOC formation. Other questions raised from this study which closely define nMTOC formation are how PCM1 and pericentrin are recruited independently of microtubules and anchored at the NE, and what is the relationship between PCM1 and kinesin at the nMTOC. Furthermore, this study has identified the regions of nesprin-1α2, PCM1 and pericentrin responsible in nMTOC formation, allowing for

the probing of direct interactions between the proteins by bacterial expression of the proteins, and structural studies if the interactions are found to be direct. Investigating these objectives to understand the process of how the nMTOC is assembled is important to understand how microtubule nucleation from the myonuclei can be affected and ultimately lead to nuclear mispositioning and muscle disease.

6.7 Conclusion

The results of this study confirm that during the formation of multinucleated myotubes, the LINC complex component nesprin-1α2 becomes expressed and localises to the NE to function as a protein receptor for PCM1, pericentrin and AKAP450. A role for the isoform-specific N-terminal 31 residues is defined for the first time as it is required for maximum localisation of PCM1 to the NE. In turn, PCM1 promotes recruitment of pericentrin and AKAP450 to the NE to form a multi-protein complex. The transfer of centrosomal proteins from the centrosome to the NE is microtubule-independent and likely requires a myogenic factor. Further investigation of the interactions and process of nMTOC assembly will clarify how microtubules are nucleated and organised at the myonuclear NE for nuclear positioning during early myogenesis.

Chapter 7
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Appendix

Appendix A: GFP-PCM1(1-331) protein sequence

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT
LVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGD
TLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQ
LADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDEL
YKSGLRSMATGGGPFEDGMNDQDLPNWSNENVDDRLNNMDWGAQQKKANRSSEKN
KKKFGVESDKRVTNDISPESSPGVGRRRTKTPHTFPHSRYMSQMSVPEQAELEKLKQRI
NFSDLDQRSIGSDSQGRATAANNKRQLSENRKPFNFLPMQINTNKSKDASTSPPNRETI
GSAQCKELFASALSNDLLQNCQVSEEDGRGEPAMESSQIVSRLVQIRDYITKASSMREDL
VEKNERSANVERLTHLIDHLKEQEKSYMKFLKKILARDPQQEPMEEIENLKKQHDLLKR
MLQQQEQLRALQGRQAALLALQHKAEQAIAVMDDSVVAETAGSLSG

Green = GFP

Black = linker

Red = PCM1(1-331)

Appendix B: Phosphorylation status of all PCM1(1-331) S/T/Y residues in myoblasts and myotubes, as detected by mass spectrometry

	Phosphorylation status			
	Experiment 1		Experiment 2	
Residue	Myoblast	Myotube	Myoblast	Myotube
T3	x	NC	x	NC
S21	x	x	x	NC
S44	NC	NC	NC	NC
S45	NC	NC	NC	NC
S56	x	c	x	x
T61	x	x	x	x
S65	✓	✓	✓	✓
S68	✓	✓	✓	✓
S69	✓	✓	✓	✓
T77	NC	x	x	x
T79	x	x	x	x
T82	x	x	x	x
S86	x	x	x	x
Y88	x	x	✓	x
S90	x	x	x	✓
S93	x	✓	✓	✓
S110	x	x	x	x
S116	NC	NC	x	x
S119	NC	NC	x	x
S121	NC	NC	x	x
T126	NC	NC	x	x
S135	NC	x	x	NC
T150	x	x	x	x
S153	NC	x	x	x
S157	NC	✓	✓	✓
T158	NC	x	✓	✓
S159	NC	✓	✓	✓
T165	x	x	x	✓
S168	x	x	x	x
S177	x	x	x	NC
S180	x	x	x	NC
S190	x	x	x	NC
S202	x	x	x	x
S203	x	x	x	x
S207	x	x	x	x
Y215	NC	x	x	x
T217	NC	x	x	x
S220	NC	NC	x	x
S221	NC	NC	x	x
S233	NC	NC	NC	NC
T240	x	x	x	x
S252	NC	NC	NC	NC
Y253	NC	NC	NC	NC
S320	NC	NC	x	NC
T325	NC	NC	x	NC
S328	NC	x	NC	NC
S330	NC	x	NC	NC

✓ = phosphorylation

x = no phosphorylation

NC = not covered