

Mouse models of nesprin related diseases

Can Zhou^{1,2}, Li Rao¹, Derek T. Warren³, Catherine M. Shanahan², Qiuping Zhang^{2*}

¹Dept. of Cardiology, West China Hospital of Sichuan University, Chengdu 610041, China

²King's College London British Heart Foundation Centre of Research Excellence, Cardiovascular
Division, London SE5 9NU, UK

³School of Pharmacy, University of East Anglia, Norwich, NR4 7TJ, UK

*Corresponding author:

Dr. Qiuping Zhang

King's College London,

Cardiovascular Division,

James Black Centre,

125 Coldharbour Lane,

London SE5 9NU, UK

Tel: +44 20 78485222

Fax: +44 20 78485193

Email: qp.zhang@kcl.ac.uk

Abstract

Nesprins (nuclear envelope spectrin repeat proteins) are a family of multi-isomeric scaffolding proteins. Nesprins form the LInker of Nucleoskeleton-and-Cytoskeleton (LINC) complex with SUN (Sad1p/UNC84)-domain containing proteins at the nuclear envelope (NE), in association with lamin A/C and emerin, linking the nucleoskeleton to the cytoskeleton. The LINC complex serves as both a physical linker between the nuclear lamina and the cytoskeleton and a mechanosensor. The LINC complex has a broad range of functions and is involved in maintaining nuclear architecture, nuclear positioning and migration and also modulating gene expression. Over 80 disease related variants have been identified in *SYNE-1/2* (nesprin-1/2) genes, which result in muscular or central nervous system (CNS) disorders including autosomal dominant Emery–Dreifuss muscular dystrophy (EDMD), dilated cardiomyopathy (DCM) and autosomal recessive cerebellar ataxia type 1 (ARCA1). To date, 17 different nesprin mouse lines have been established to mimic these nesprin-related human diseases, which have provided valuable insights into the roles of nesprin and its scaffold LINC complex in a tissue specific manner. In this review, we summarise the existing nesprin mouse models, compare their phenotypes and discuss the potential mechanisms underlying nesprin-associated diseases.

INTRODUCTION

Nesprins (nuclear envelope spectrin repeat proteins) are the latest identified members of the spectrin repeat (SR) containing protein family [1]. To date, six genes encoding for different KASH domain containing proteins named as nesprins-1, -2, -3, -4, Lymphoid-restricted membrane protein (LRMP) and KASH5 have been identified in mammals [1-6].

Nesprin-1 and -2 are encoded by *synaptic nuclear envelope (SYNE)-1* and -2 genes. The longest isoforms of nesprin-1 and -2 are the second (~1MDa) and third (~800kDa) largest proteins in man [1]. The general structure of these proteins comprises of an evolutionarily conserved Klarsicht/ANC-1/Syne Homology (KASH) domain at the C-terminus that targets nesprin family members to the nuclear envelope (NE); a central rod domain containing multiple SRs that mediate protein-protein interactions; and paired Calponin Homology (CH) domains at the N-terminus, also known as the actin binding domain (ABD) that binds to filamentous actin (F-actin) [1, 2]. Nesprin-1/2 have diverse isoforms, which vary in length and differ in domain composition due to extensive alternative transcription initiation, termination and splicing [7] (for an extensive review of nesprin-1/2 isoforms, we refer readers to [8]). Giant nesprin-1/2 isoforms are ubiquitously expressed, in particular nesprin-1 giant is enriched in vascular smooth muscle, while smaller nesprin-1/2 isoforms, such as nesprin-1 α_2 , 2 α_1 and 2 ϵ_2 , are specifically expressed in skeletal and cardiac muscle [1, 9, 10]. Nesprin-1/2 localise at either side of the NE through their KASH domains. Smaller nesprin-1/2 isoforms interact with lamin A/C, emerin and nucleoplasmic domain of Sad1p/UNC84 (SUN) domain containing proteins SUN1/2 via their C-terminal SRs at the inner nuclear membrane (INM) [2, 11-13], however, it remains unclear how INM localisation is established. Nesprin-1/2 giant isoforms localise at the outer nuclear membrane (ONM) and form the Linker of Nucleoskeleton-and-Cytoskeleton (LINC) complex via associations between their KASH domains and the SUN-domains of SUN1/2 in the perinuclear space (PNS) [14, 15]. The LINC complex tethers the NE to cytoskeletal elements including actin filaments and microtubule (MT) network [16-19]. This molecular linking network is pivotal in regulating nuclear integrity, maintaining nuclear-cytoskeleton coupling, participating in mechanotransduction, nuclear migration and positioning especially in muscle cell differentiation [2, 8, 17, 18, 20, 21].

Nesprin-3, -4, LRMP and KASH5 are encoded by *SYNE-3*, *-4*, *LRMP* and *KASH5* genes and are much smaller than giant nesprin-1/2. They contain the KASH domain at the C-terminus and lack the N-terminal CH domains [3, 4, 6]. Nesprin-3 links the nucleus to intermediate filaments (IFs) via an interaction with plectin and regulates cell morphology, perinuclear cytoskeletal architecture and cell polarisation [3, 22, 23]. Differing from the ubiquitously expressed nesprin-1/2/3, nesprin-4 is predominately expressed in epithelial cells, KASH5 is limited to meiotic cells while LRMP is rich in zebrafish zygotes and also expressed in the taste bud cells of the tongue in mammals [4, 6, 24, 25]. Nesprin-4, KASH5 and LRMP are involved in the MT associated LINC complex formation and function. Nesprin-4 recruits kinesin-1 to the NE and is involved in MT-dependent nuclear positioning [4]. KASH5 participates in MT-driven telomere movement and chromosome pairing during mammalian meiosis, whereas LRMP mediates centrosome-nuclear attachment in zebrafish zygotes by associating with the MT-dynein/dynactin complex [24, 26].

The ability of nesprin family members to link the nucleus with different cytoskeletal elements has led to the assumption that they primarily function as nucleoskeleton and cytoskeleton linkers. Intriguingly, many nesprin-1/2 isoforms that lack KASH domains exist and localise to various sub-cellular compartments, such as the sarcomere, sarcoplasmic reticulum, mitochondria, focal adhesions, Golgi and promyelocytic leukemia (PML) bodies, suggesting that nesprins possess additional functions [2, 7, 27, 28]. So far, over 80 disease associated variants, have been identified in *SYNE-1/2* genes, which result in muscular or central nervous system (CNS) disorders including autosomal dominant Emery–Dreifuss muscular dystrophy (EDMD), dilated cardiomyopathy (DCM) and autosomal recessive cerebellar ataxia type 1 (ARCA1) [12, 29-37].

To elucidate the roles of nesprin mutations in pathogenesis of these diseases, 17 different nesprin mouse lines have been established and characterised, providing valuable insights into the

roles of nesprins and the LINC complex in different tissues (Figure 1, Table 1). In this review, we summarise all the existing nesprin mouse models into two categories: 1) nesprin-1/2 mouse models with skeletal and cardiac muscle phenotypes and 2) all other nesprin mouse models. We specifically focus on the cardiac and skeletal muscle defects in nesprin-1/2 mouse models and discuss the potential mechanisms underlying nesprin-associated diseases.

NESPRIN-1/2 MOUSE MODELS WITH MUSCLE DISORDERS

A nesprin mouse model with both skeletal and cardiac muscle disorders

Nesprin-1 KASH Knockout (KO)^①

The only nesprin mouse model that demonstrated both EDMD and DCM-like phenotypes was generated via global targeted deletion of the last exon of *Syne-1* gene leading to removal of the last two exons that included the KASH domain in Prof. McNally's lab, we renamed this mouse model as Nesprin-1 KASH KO^①[29, 38]. The KASH1 KO^① pups had a high perinatal mortality (49%) due to un-inflated lungs causing respiratory failure [38]. Surviving litters exhibited EDMD and DCM-like phenotypes, including hind limb weakness, abnormal gaits, kyphoscoliosis, muscle pathology, conduction defects (CD) and left ventricle (LV) systolic dysfunction [38]. The surface electrocardiogram (ECG) showed CD presented earlier in atria than ventricle and reduced LV fractional shortening, which was similar to observations described in patients with DCM and conduction system defects [29, 39].

Cardiomyocytes (CMs) derived from KASH1 KO^① mice displayed elongated nuclear morphology, large invaginations of the NE and reduced levels of heterochromatin [29]. Reduced

muscle fibre size accompanied with centralised nuclei was also evident in skeletal muscle biopsies, indicating defective nuclear positioning [38]. Interactions between nesprin-1 and SUN2 were disrupted, although lamin A/C, emerin and SUN2 remained staining at the NE [38]. The major limitation for this mouse model was that the additional 61 amino acid insertion replaced the KASH domain in KASH-containing nesprin-1 mutants. This fusion potentially created dominant negative (DN) nesprin-1 constructs that might contribute to the muscle phenotypes.

Nesprin-1/2 mouse model with cardiac muscle disorders

Nesprin-1/2 C-terminal double KO (DKO)

The nesprin-1/2 C-terminal DKO mouse model was independently generated in Prof. Chen's lab, by crossing two previously established mouse lines: cardiac specific nesprin-1 C-terminal KO mice and global nesprin-2 C-terminal KO mice [40]. DKO hearts were theoretically ablated of all nesprin-1 and -2 isoforms containing the C-terminal SR region with or without the KASH domain. This included the muscle specific isoforms nesprin-1 α_2 , nesprin-2 α_1 and nesprin-2 α_2 . No overt cardiac and skeletal muscle dysfunction phenotypes were reported in either cardiac specific nesprin-1 deletion or global nesprin-2 deletion mice previously [40]. However, DKO mice displayed early onset cardiomyopathy: LV wall thickness and fractional shortening was decreased at 10 weeks and deteriorated over time [40]. Increased fibrosis and apoptosis was detected in DKO heart tissue and expression of foetal genes ANP, β MHC and profibrotic genes procollagen 1 α_1 , 3 α_1 were also increased [40].

Similar to the nesprin-1 KASH KO^① mice, the CMs derived from this DKO mice also showed morphologically altered nuclei [40]. However, the LINC complex associated components,

lamin A/C and emerin, were mis-localised from the NE, although their expression levels remained unchanged in the DKO mice, indicating the compromised LINC complex was caused by defects in the localisation of these proteins rather than changes in expression levels [40]. Furthermore, when mechanical load was applied to DKO and WT CMs, there were dramatically upregulated biomechanical responsive genes including *egr-1*, *iex-1*, *c-jun*, *c-fos* and *c-myc* in WT CMs, while expression of these genes was abrogated in DKO CMs [40]. These data demonstrate that nesprin-1/2 play essential roles in cardiac physiology, maintenance and responsiveness to biomechanical load.

Nesprin-1/2 mouse models with skeletal muscle disorders

Nesprin-1 KASH DN/KO^②, Nesprin-2 KASH DN/KO and Nesprin-1/2 KASH DKO

Five KASH1 and/or KASH2 domain mouse lines were generated in Professor Han's lab to investigate nesprin functions in striated muscle [41, 42]. A striated muscle specific nesprin-1 KASH DN mouse line was generated by using a muscle creatine kinase (MCK) promoter that enabled highly preferential expression of nesprin-1 C-terminal 344 amino acids including the last SR and KASH domain in skeletal and cardiac muscle [41, 43, 44]. Utilising the same strategy, striated muscle specific nesprin-2 KASH DN mice were also established [42]. Of note, although this strategy was designed to target skeletal and cardiac muscle, smooth muscle may also have been affected and contribute to this phenotype. However, this remains to be tested. In addition, global KASH1 KO^② and KASH2 KO mice were generated via targeted deletion of the last one (*Syne-1*) and two (*Syne-2*) exons respectively, which encode for the KASH domains of *Syne-1* and

Syne-2 genes [42]. The global nesprin-1/2 KASH DKO line was generated by crossing the KASH1 KO² with KASH2 KO [42].

All KO/DN strains were viable, with the exception of the KASH1/2 DKO litters that died after birth due to respiratory failure, which was suggested to be likely secondary to the CNS development defect [42, 45]. Defects in other organs/tissues that are rich in nesprin-1/2 isoforms could also potentially contribute to this lethality. For example: the function of smooth muscle (containing mainly nesprin-1 giant isoform) and/or diaphragm (skeletal muscle containing mainly nesprin-1 α_2 and nesprin-2 α_1) could be perturbed and lead to impaired dilation of the bronchi and abnormal volume/pressure changes of thoracic cavity during respiration [9, 46, 47]. However, no overt cardiac or skeletal muscular-related symptoms were reported in the KASH1/2 DKO mice [41, 42].

Nesprin-1 plays major roles in nuclear migration and myogenesis. Nuclear positioning defects in muscle fibres were also prevalent in skeletal muscle biopsies derived from these mice. The KASH1 KO² and KASH1/2 DKO displayed the most severe nuclear positioning phenotypes with the total loss of synaptic nuclei and clustered non-synaptic nuclei [41, 42]. The difference in severity between the strains is potentially explained because a low level of endogenous nesprin-1 remained at the NE in KASH1/KASH2 DN mice, whereas nesprin-1 is absent from the NE in the cells derived from the KASH1 KO² or KASH1/2 DKO. [41, 42]. In contrast, nuclei were properly anchored in skeletal muscle derived from KASH2 KO mice, suggesting nesprin-2 KASH containing isoforms do not participate in myonuclear positioning [42]. Nonetheless, KASH2 may be more critical in retinal and neuronal development as there were retina and learning/memory defects in KASH2 KO mice (further discussion refer to the section of ‘OTHER NESPRIN MOUSE MODELS’) [45, 48].

Nesprin-1 C-terminal KO

Global Nesprin-1 C-terminal KO line was established by floxed deletion of the 16th exon of *Syne-1* gene and resulted in a premature stop codon at the 13th exon (counted backward from the last exon) in Prof. Chen's lab [49]. This KO mouse would theoretically have ablated all nesprin-1 isoforms containing the C-terminal SR region, including nesprin-1 α_1 , nesprin-1 α_2 and nesprin-1 β . KO litters exhibited over 60% lethality due to muscle weakness leading to feeding insufficiency [49]. The surviving KO pups displayed growth retardation and reduced exercise capacity, although their heart function was normal [49]. Furthermore, centralised nuclei were observed in the KO mouse muscle fibres [49]. *Ex vivo* analysis showed the expression levels of SUN1 and SUN2 were upregulated in heart and skeletal muscle respectively, further confirming that nesprin-1 and the LINC complex play essential roles in nuclear positioning [49].

Nesprin-1 α_2 KO

Global nesprin-1 α_2 KO mice were also generated in Prof. Chen's lab by floxing the first unique exon of nesprin-1 α_2 and then crossed with Sox2Cre mouse to knockout nesprin-1 α_2 isoform that is highly expressed in skeletal and cardiac muscle [50]. The lethality was high as only 2 out of 17 litters survived. At embryonic day 18.5 (E18.5), KO mice remained cyanotic and died within 5 minutes while WT turned pink and started to breath [50]. Those findings strongly indicate the nesprin-1 α_2 is indispensable for embryonic development.

Viable KO mice were small with reduced body weight, and developed kyphosis after postnatal day 21 (P21), indicating EDMD-like skeletal muscle dysfunction [50]. Nuclei clustering was observed in the tibialis anterior (TA) muscle derived from these nesprin-1 α_2 KO mice at E18.5,

which was akin to KASH1 KO^② and nesprin-1 C-terminal KO mice [42, 49, 50]. Nesprin-1 α_2 KO samples displayed reduced SUN1 expression and mis-localisation of SUN1 from the NE [50]. Surprisingly, the MT motor proteins kinesin-1 including its subunits kinesin light chain-1/2 (KLC-1/2) and kinesin heavy chain kif5b were also displaced from the NE, supported the notion of dynamic myonuclear positioning was governed by muscle specific nesprin isoform nesprin-1 α_2 and its association with kinesin-1 *in vivo* [50].

OTHER NESPRIN MOUSE MODELS

Nesprin-1/2 mouse models with skin/retina/brain phenotypes

Nesprin-1 CH KO and Nesprin-2 CH KO

Two global nesprin-1/2 N-terminal ABD KO models exist [17, 50]. Nesprin-2 ABD was knocked out by deletion of the 2nd - 4th exons of *Syne-2* gene encoded for first CH domain in nesprin-2 CH KO mice [17]. These mice were well developed, and did not exhibit any skeletal muscle or heart defects [17]. However, the epidermis from these global KO mice was thicker but did not reveal gross abnormalities [17]. Isolated primary dermal fibroblasts and keratinocytes from the KO mice possessed deformed nuclei with unevenly distributed emerin, indicating disrupted NE organisation [17]. Unlike nesprin-2 CH KO mice, nesprin-1 CH KO mice were generated by floxing the 9th exon, which encodes for the second CH domain of nesprin-1 [50]. These mice developed normally without any phenotypes, and the expression levels and localisation of LINC complex components were also unchanged [50]. These two different phenotypes could be caused by the distinct functions of two CH domains. Nesprin-1 and -2 contain two CH domains (CH1 and CH2) juxtaposed at the N-terminus. A single CH2 domain is not able to bind to actin filaments,

but it acts as a CH1 enhancer to strengthen the binding with F-actin [51, 52]. Therefore, the function of ABD may be partially present in nesprin-1 CH KO, while ABD function was abolished in nesprin-2 CH KO mice via depletion of the CH1 domain.

Nesprin-2 KASH KO and Nesprin-1/2 KASH DKO

The global KASH2 KO mouse line generated by Professor Han's lab was initially used to investigate nuclear positioning in muscle as discussed above [42]. There were no muscle defects observed, but a severe reduction of the thickness of the outer nuclear layer in the retina, resulted from the mis-localisation of photoreceptor nuclei [48]. Moreover, KASH2 KO mice showed reduced response to a maze test and abnormally active responses to new environments, indicating a memory and learning defect [45]. In addition, homozygous/heterozygous global nesprin-1/2 KASH DKO mouse line generated by the same group showed more severe brain phenotypes: a smaller brain with enlarged lateral ventricles, inverted layers, loss of other specific cell layers and disrupted laminary structure [45]. It is intriguing that similar retinal and neuronal phenotypes were also observed in the mice with deletion of both SUN1 and SUN2 [45, 48].

The three mouse lines above revealed the crucial roles of the LINC complex in retinogenesis and neurogenesis: nesprin-1/2, in particular nesprin-2 interacts with SUN1/2 to form the LINC complex, connecting nucleus to the centrosome through interactions with MT motor proteins dynein/dynactin and kinesin, which regulates dramatic nuclear movement during interkinetic nuclear migration and/or nucleokinesis processes that are necessary for proper brain and retina development [53-55].

Nesprin-2 (GFP) KASH DN

In addition to the 13 nesprin-1/2 mouse models discussed above, Hodzic's lab generated a KASH2 DN mouse strain with insertion of GFP fused to the last 65 amino acids of nesprin-2 KASH domain [56-58]. It was generated using the Cre/Lox system, which allows targeted disruption of endogenous SUN/KASH interactions through the inducible expression of a recombinant KASH domain. To date, using this GFP-KASH2 DN mouse line as a genetic tool, several LINC complex disruption models were successfully established in a tissue specific manner such as cerebellum, retina and skeletal muscle. This strategy bypasses the perinatal lethality and potential cell non-autonomous effects of other existing global nesprin-1/2 KO mouse models [38, 42, 49, 50]. These mice exhibited efficient exogenous KASH2 overexpression, mis-localisation of the endogenous nesprin to the endoplasmic reticulum (ER), leading to uncouple the nucleoskeleton from the cytoskeleton, which further our understanding of physiological relevance of LINC complexes during development and homeostasis in a wide variety of mammalian tissues [57, 58].

Nesprin-3, Nesprin-4 and KASH5 mouse models

Nesprin-3 KO mice were generated via targeted deletion of the 2nd exon, which contains the translation start site for both nesprin-3 α and nesprin-3 β isoforms [59]. These KO mice were indistinguishable from WT littermates. There were no overt phenotypes reported [59].

Nesprin-4 KO mice were established by replacing the 2nd to 6th exons with an IRES β -gal cassette, resulting in an effectively null allele [60]. Loss of nesprin-4 impaired localisation of nuclei from the basal to apex in outer hair cells (OHC), leading to progressive hearing loss, reproducing phenotype found in deaf patients [60].

KASH5 KO mice were generated via targeted deletion of exons 5th-8th, which resulted in a premature stop codon, leading to a complete absence of KASH5 [61]. The KASH5 KO mice were

phenotypically normal except both male and female were infertile. Testis were much smaller in males, whilst the ovaries were barely visible in females [61]. A potential explanation is that KASH5 associates with SUN1, indirectly coupling telomeres with the MT motor protein dynein, facilitating efficient homologous chromosome pairing during spermatogenesis [61], thus the deficiency of KASH5 would cause the arrest of meiosis, and subsequent infertility.

POSSIBLE MECHANISMS UNDERLYING THE PHENOTYPES OBSERVED IN NESPRIN MOUSE MODELS

To date, majority of nesprin variants identified in *SYNE-1* and *SYNE-2* genes have been shown to contribute to pathogenesis of muscle diseases including EDMD, DCM, congenital muscular dystrophy (CMD), arthrogryposis multiplex congenita (AMC) [12, 29-37, 62] and CNS disorders such as ARCA1, autism spectrum disorder and bipolar disorder respectively [63-69]. Nesprin-1/2 variants associated with skeletal muscle or cardiac muscle defects are heterozygous missense mutations causing amino acid substitutions and mainly localise at the C-terminus of *SYNE-1/2*. In this region, there is a highly conserved fragment with little secondary structure named ‘adaptive domain’ (AD). The AD together with SRs in this region help to mediate homodimerisation of nesprin proteins and bind to other LINC complex components, including lamin A/C and emerin, with high affinity [70, 71]. The AD also plays roles in maintaining structural and thermodynamic properties of the nesprin proteins, which has confirmed by thermal unfolding tests using circular dichroism and dynamic light scattering [70]. Therefore, these nesprin mutations potentially cause defects in structure/flexibility of the functional domains binding to lamin A/C, emerin and SUN1/2. In contrast, nesprin-1/2 variants associated with CNS disorders are homozygous nonsense mutations, which scatter along *SYNE-1/2* genes, especially *SYNE-1*,

resulting in truncation of multiple nesprin isoforms (further details please refer to a recent review [72]). These two distinct types of nesprin-1/2 variants may affect tissue specific isoforms of nesprin-1 and/or -2 and their associated LINC complexes, leading to tissue specific diseases. In addition, only one *SYNE-4* mutation causing high-frequency hearing loss was reported in two families, which phenotype has been recapitulated in nesprin-4 KO mice [60]. Therefore, most of the nesprin mouse models were generated for investigating nesprin-1/2 functions.

10 out of 14 nesprin-1/2 mouse models exhibited skeletal muscle or cardiac defects whereas 2 strains displayed CNS disorders. Although the severity of the phenotypes in affected organs/tissues are variable, they all exhibit similar cellular and molecular changes: defects in nuclear morphology, nuclear positioning and migration, abnormal localisation and binding of the LINC complex proteins SUN1/2, lamin A/C and emerin with nesprin-1/2, indicating a perturbation of the LINC complex, similar to those caused by nesprin mutations reported in the patient cells with muscle or CNS disorders. These support two principle hypotheses underlying nesprin-related diseases: structural disruption and gene dysregulation.

Evidence for structural hypothesis

Studies on patient tissues/cells carrying nesprin-1/2 mutations revealed structural pathological changes. Muscle biopsies derived from EDMD patients carrying nesprin-1/2 mutations exhibited centralised nuclei with increased variability of fibre size [31, 34]. Further studies demonstrated cellular changes with misshapen nuclei, invaginated and detached NE and reduced heterochromatin density in EDMD and AMC patient cells carrying nesprin-1/2 mutations [12, 37]. In agreement with these observed changes in patient samples, 10 nesprin-1/2 mouse models exhibited either mis-positioning of non-synaptic and synaptic nuclei in skeletal muscle or

reduced distance between adjacent nuclei, or elongated nuclei in cardiac muscle, whilst 2 strains (nesprin-2 KASH KO and nesprin-1/2 KASH DKO) revealed the defect in neuronal migration [29, 38, 40-42, 49, 50]. Furthermore, LINC complex components lamin A/C, emerin and/or SUN1/2 were mis-localised from the NE and their interactions with nesprin-1/2 were altered [12, 30, 38, 40, 50]. The findings strongly suggested nesprin-1/2 mutations cause major disruption in integrity of the LINC complex, uncouple the nucleoskeleton from the cytoskeleton, resulted in fragile nuclei. This was particularly striking in skeletal and cardiac muscle when subjected to mechanical strain, which consequently affect nuclear migration and positioning as these process required substantial cytoskeletal forces acting on the nucleus via an intact LINC complex.

Pioneering insights into function of the LINC complex in nuclear migration and positioning came from studying nesprin-1/2 in lower organisms. Depletion of *Drosophila* nesprin orthologue MSP-300 resulted in defective muscle/tendon connections and mis-localised nuclei in oocyte cells [73]. Mutations in *C. elegans* ANC-1, a single giant ONM nesprin orthologue, also led to a muscle defect due to disrupted myonuclear migration and decoupled mitochondria from the actin cytoskeleton [74]. Indeed, emerging evidence has further revealed that functions of the LINC complex in nuclear migration are evolutionary conserved in mammals. In both muscle and CNS, nesprin-1/2 act as a hub to assemble the system required for nuclear movement through their interactions with SUN1/2 via their KASH domains to form the LINC complex at the NE, and also bind to the MT motor proteins dynein/dynactin and kinesin via their SRs [18, 45, 75]. Recently, a 'LEWD' motif at C-terminus of nesprin-1/2 has been identified as a binding region for motor proteins kinesin-1 and C-terminal SRs of *C. elegans* UNC-83, nesprin orthologue, for dynein [18, 30, 76]. Therefore, nuclei can 'walk' bi-directionally along the MT-LINC complex that surrounds the NE to achieve precisely nuclear migration and positioning in myoblasts/myotubes and neuronal

progenitors/neurons and promote muscle and CNS development [18, 45]. In addition, centrosomal proteins Akap450, pericentriolar material-1 (PCM-1) and pericentrin, components of the microtubule-organising centre (MTOC), have recently been shown to relocalise to the NE, participating in MT nucleation at the initial stage of muscle cell differentiation in a nesprin-1 α dependent manner [19, 77].

Evidence for gene dysregulation hypothesis

LINC complex mediates mechanotransduction events, translating biophysical forces into biochemical signalling that regulates gene expression. Several studies have now confirmed the nesprin giant proteins are subjected to mechanical tension and that physical forces transmitted across the LINC complex regulate recruitment of lamin A/C to the INM and emerin phosphorylation [78, 79]. These two LINC complex associated components have also been shown to anchor chromatin via direct interactions with histones or other chromatin-associated proteins, such as heterochromatin protein 1 (HP1) and lamin B receptor (LBR) for lamin A/C and barrier-to-autointegration factor (BAF) for emerin. These interactions are essential for genome arrangement and gene expression [80-83]. Moreover, various transcriptional factors have been reported as binding partners for the LINC complex components including extracellular signal-regulated kinase (ERK) 1/2 and α -catenin for nesprin-2, retinoblastoma protein (pRb), c-fos and ERK1/2 for lamin A/C [84-87]. Interestingly, several recent studies demonstrated nesprin mutations result in gene dysregulation. Firstly, enhanced ERK1/2 activity was observed in nesprin-1 KASH KO^① heart tissue, fibroblasts derived from EDMD-DCM patients and nesprin-1 mutant transfected cells, which might contribute to aberrant activation and expression of downstream genes encoding for the components of muscle fibre and sarcomere, thus contributing to muscle

dysfunction [30]. Secondly, impaired response to mechanical stress was reported in CMs isolated from KASH1/2 DKO mice, which showed completely blunted response in biomechanical genes [40]. Finally, either myoblasts derived from EDMD patients or C2C12 myoblasts infected with nesprin-1 mutations identified in DCM patients exhibited perturbed myogenesis with less-defined myotube structure or reduced expression of myogenic transcriptional factors, such as myoD, myogenin and myosin heavy chain [12, 30]. Further investigation is required to elucidate the mechanisms between LINC complex disruption and the observed gene dysregulation.

In summary, both structural and gene regulation hypotheses are not mutually exclusive. It is not clear which, if either, is the key trigger. We propose a potential mechanism whereby nesprin mutations may fail to build a functional scaffold and/or to maintain chromatin compartmentalisation with other LINC complex components such as SUN1/2, emerin and lamin A/C, leading to reduced heterochromatin and defective compartmentation; potentially it causes defects in initiation of the gene transcription process resulting in decreased or delayed expression of the genes encoding proteins that are critical for structural integrity or participating in regulating cell activities such as cell differentiation, migration and division, thus accelerating pathogenesis and driving the onset of diseases.

CONCLUDING REMARKS

17 various nesprin mouse models have generated valuable information regarding the roles of nesprins and the LINC complex in anchoring the nucleus to cytoskeletal networks, nuclear positioning and migration, especially in muscle cells. However, questions regarding how disruption of the LINC complex caused by nesprin mutants lead to changes in structural, cell

signalling and gene expression, contributing to pathogenesis of nesprin-related diseases remain unanswered.

Further knowledge is required to better understand the diverse spectrum of nesprin isoforms. Moreover, full characterisation of tissues or cells derived from patients with EDMD, DCM or ARCA1 etc., and introduced pluripotent stem cells (iPSC) will help to decipher the roles of nesprins in developmental cell fate and physiological functions of disease-relevant cells [88]. Furthermore, in contrast to current nesprin mouse models with deletion or overexpression of functional domains (KASH domains, C-terminal SRs and CH domains), more clinically relevant mouse models could be generated to recapitulate the human conditions via CRISPR Cas9 genome edited knock-in mouse lines, or focus on functional studies on tissue specific nesprin isoforms such as nesprin-1 α_2 , 2 α_1 and 2 ϵ_2 in heart and skeletal muscle, and two nesprin-1 KASH-less isoforms in brain [9, 49, 89]. In addition, whole genome or single cell microarray analysis could be performed on cells isolated from these models to explore the expression profiles of dysregulated genes and associated signalling pathways. These approaches would potentially help uncover how dysfunction of nesprins and the LINC complex contribute to pathogenesis of nesprin-related diseases and provide information on establishing therapeutic targets for these disorders.

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FIGURE LEGENDS

Figure 1. Nesprin-1/2 mouse models

Various nesprin-1/2 mouse models were generated via targeting to different regions of *Syne-1* and *Syne-2* genes. The colours indicate different research groups who generated the models (Red - Noegel A. [17]; Blue - Han M.[41, 42]; Violet - McNally, E. M. [38]; Green - Chen J. [40, 49, 50]; Grey – Hodzic D. [57]. Nesprin-1/2 KASH DKO mice were generated via breeding nesprin-1 KASH KO^② with nesprin-2 KASH KO (Han group) [42]. Nesprin-1/2 C-terminal DKO mice were independently generated via crossing two established mouse lines: cardiac specific nesprin-1 C-terminal KO and nesprin-2 C-terminal KO (Chen group) [40]. KO: knockout; DKO: double knockout; DN: dominant negative; csNesprin-1 C-terminal KO: cardiac specific Nesprin-1 C-terminal KO.

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Table 1: Summarisation of nesprin mouse models

Gene	Mice	Targeted region	Targeted tissue	Strategy	Affected organs	Phenotypes and/or cellular changes	Ref.
<i>Syne-1</i>	Nesprin-1 KASH KO ^①	KASH domain	Global	Floxed deletion of the last exon of <i>Syne-1</i> resulting in the deletion of last two exons (~100 residues, including KASH domain) and inserted additional 61 amino acids without homology to any known proteins	Heart Skeletal muscle	<ul style="list-style-type: none"> 49% new born litters died due to respiratory failure; Rest of pups survived up to 1 year and developed heart conduction defect with left ventricle (LV) systolic dysfunction; Smaller muscle fibres with mis-positioned nuclei; Kyphoscoliosis. 	[29, 38]
	Nesprin-1 KASH DN		Skeletal and cardiac	Overexpression of the last 344 amino acid (SR74 to KASH domain) of <i>Syne-1</i> under a muscle creatine kinase (MCK) promoter	Skeletal muscle	<ul style="list-style-type: none"> No overt phenotype reported; In muscle fibre, synaptic nuclei failed to aggregate at the neuromuscular junction (NMJ). 	[41]
	Nesprin-1 KASH KO ^②		Global	Targeted deletion of the last exon of <i>Syne-1</i> , including KASH domain	Skeletal muscle	<ul style="list-style-type: none"> No external phenotype reported; In muscle fibre, synaptic nuclei completely lost at the NMJ, non-synaptic nuclei formed clusters or arrays. 	[42]
	Nesprin-1 C-terminal KO	C-terminus	Global	Floxed deletion of the 16 th exon of <i>Syne-1</i> resulting in a premature stop codon at the 13 th exon (counted backward from the last exon), which ablated all nesprin-1 isoforms containing the C-terminal SRs region with or without KASH domain	Skeletal muscle	<ul style="list-style-type: none"> Over 60% mice died prenatally due to feeding insufficiency caused by muscle weakness; In muscle fibres: mis-positioned nuclei and disrupted nuclei anchorage; Reduced exercise capacity; Normal heart function. 	[49]
	Cardiac Nesprin-1 C-terminal KO		Cardiac	Crossing the <i>Syne-1</i> KO mice [48] with Nkx2.5Cre to KO nesprin-1 C-terminus containing isoforms in heart	Heart	<ul style="list-style-type: none"> No overt cardiac functional defect; Isolated cardiomyocytes showed changed nuclear morphology and perturbed mechanotransduction response. 	[40]
	Nesprin-1 CH KO	CH domain	Global	Floxed exon 9, encoding for the 2 nd CH domain in <i>Syne-1</i> and crossed with Sox2Cre mice to globally ablate expression of nesprin-1 CH domain containing isoforms	None	<ul style="list-style-type: none"> Survived up to 18 months; No overt phenotypes reported. 	[50]
	Nesprin-1 α_2 KO	Isoform specific	Global	Floxed first exon which is unique to nesprin-1 α_2 , then crossed with Sox2Cre mice to globally ablate expression of nesprin-1 α_2	Skeletal muscle	<ul style="list-style-type: none"> 12% of litters survived; In muscle fibres: nuclei were mis-positioned due to loss the interaction between nesprin-1α_2 and microtubule motor protein kinesin-1. 	[50]
<i>Syne-2</i>	Nesprin-2 KASH DN	KASH domain	Skeletal and cardiac	Overexpression of 183 amino acids of KASH containing domain of <i>Syne-2</i> under the MCK promoter	Skeletal muscle	<ul style="list-style-type: none"> No external phenotype reported; In muscle fibres, synaptic nuclei failed to aggregate at the NMJ. 	[42]
	Nesprin-2 (GFP) KASH DN		Reporter mouse to be induced by crossing with tissue specific Cre	Overexpression of GFP fused to last 65 amino acids of KASH containing domain of <i>Syne-2</i> using the Cre/Lox system, thus DN KASH2 can be expressed under a tissue specific promoter	Retina Brain Skeletal muscle (due to tissue specific expression)	<ul style="list-style-type: none"> In retina: nuclei mis-localised on the basal side of the outer nuclear layer In Purkinje cells and skeletal muscle: exogenous EGFP-KASH2 was efficiently expressed, leading to mis-localised endogenous nesprin-1 and -2. 	[56-58]

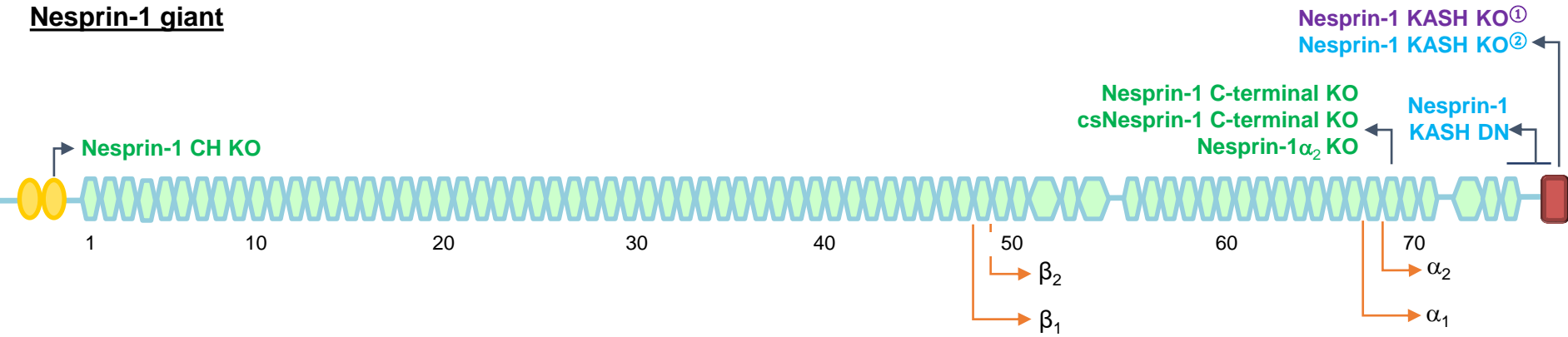
	Nesprin-2 KASH KO		Global	Targeted deletion of the last two exons of <i>Syne-2</i> including KASH domain	Retina Brain	<ul style="list-style-type: none"> In retina: thinner outer nuclear layer, electrophysiological dysfunction, mis-localisation of photoreceptor nuclei and defects in photoreceptor cell migration; Defects in learning and memory due to the disrupted laminary structures. 	[42, 45, 48]
	Nesprin-2 C-terminal KO	C-terminus	Global	Floxed deletion of the 7 th exon of <i>Syne-2</i> (counted backward from the last exon), targeted to delete all nesprin-2 isoforms containing the C-terminal SRs region with or without KASH domain	Heart	<ul style="list-style-type: none"> Survived up to 18 months; Isolated cardiomyocytes showed changed nuclear morphology and perturbed mechanotransduction response. 	[40]
	Nesprin-2 CH KO	CH domain	Global	Targeted deletion of the 2 nd - 4 th exons of <i>Syne-2</i> gene encoding the first CH domain	Skin	<ul style="list-style-type: none"> In skin: thicker epidermis, increased epithelial nuclear size and heavily misshaped nuclei 	[17]
<i>Syne-1/2</i>	Nesprin-1/2 C-terminal DKO	C-terminus	Global (nesprin-2 KO) & Cardiac (nesprin-1 KO)	Crossing the cardiac specific nesprin-1 C-terminal KO mice with the global nesprin-2 C-terminal KO mice	Heart	<ul style="list-style-type: none"> Reduced LV wall thickness, LV systolic dysfunction, increased fibrosis and apoptosis; Changed nuclear morphology, reduced density of heterochromatin, altered nuclear positioning and impaired mechanotransduction response. 	[40]
	Nesprin-1/2 KASH DKO	KASH domain	Global	Crossing the KASH1 KO ² mice (deletion of the last exon of <i>Syne-1</i>) with KASH2 KO mice (deletion of the last two exons of <i>Syne-2</i>)	Skeletal muscle Brain	<ul style="list-style-type: none"> New born litters failed to breath and died shortly after birth; In muscle fibres: the synaptic-nuclei number were absent in the NMJ; In brain: smaller brain with enlarged lateral ventricles, inverted layers, loss of other specific cell layers and disrupted laminary structure 	[42, 45]
<i>Syne-3/4 & KASH5</i>	Nesprin-3 KO	Nesprin-3	Global	Targeted deletion of the 2 nd exon of <i>Syne-3</i> , which contains the translation starting site for both nesprin-3 α and nesprin-3 β isoforms	None	<ul style="list-style-type: none"> No overt phenotype reported. 	[59]
	Nesprin-4 KO	Nesprin-4	Global	Replacement of the 2 nd to 6 th exons with an IRES β -gal cassette, resulting in an effectively null allele	Ear	<ul style="list-style-type: none"> Impaired the localisation of nuclei from the basal to apex in outer hair cells, leading to progressed hearing loss. 	[60]
	KASH5 KO	KASH5	Global	Targeted deletion of 5 th - 8 th exons which resulted in a premature stop codon, leading to a complete absence of KASH5	Reproductive system	<ul style="list-style-type: none"> Defects of homologous chromosome pairing during spermatogenesis, resulting in infertility. 	[61]

KO: knockout; DKO: double knockout; DN: dominant negative.

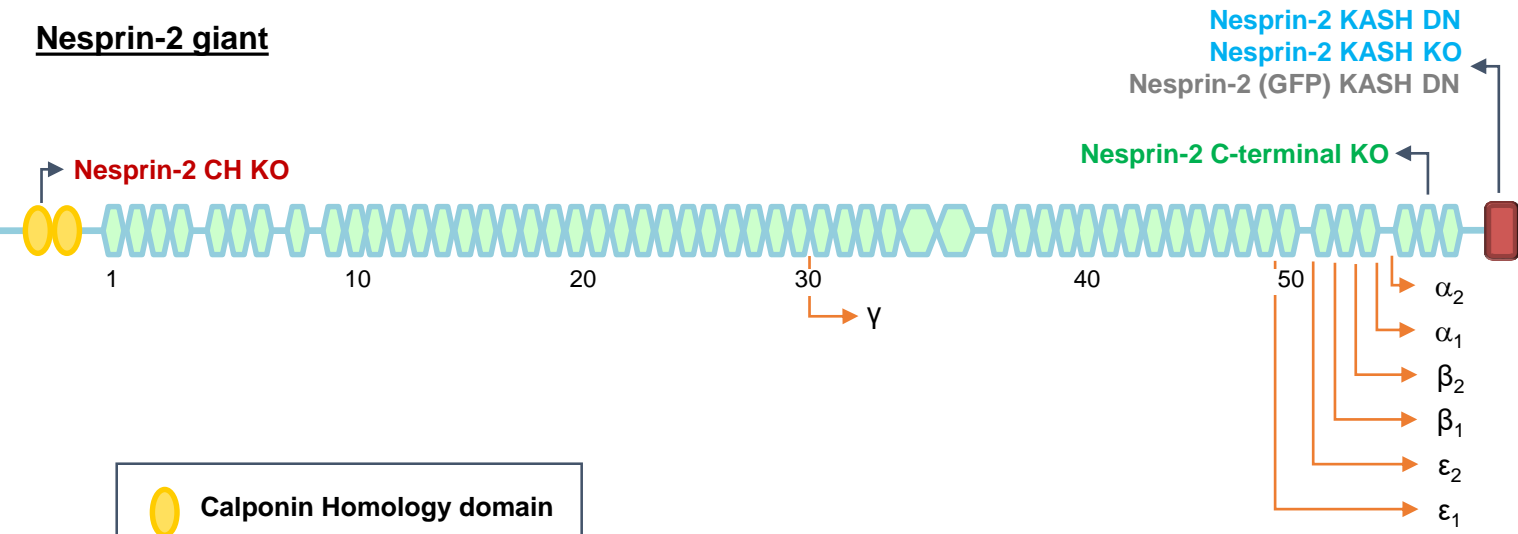
Nesprin-1 KASH KO¹: the nesprin-1 KASH KO mouse model generated in Prof. McNally's lab;


Nesprin-1 KASH KO²: the nesprin-1 KASH KO mouse model generated in Prof. Han's lab.


Nesprin-1 giant




Nesprin-2 giant



**Calponin Homology domain**

**Spectrin Repeat**

**KASH domain**