# TABLE OF CONTENTS

Table of Contents .......................................................................................................................... 2
Abstract ........................................................................................................................................ 4
Declaration ..................................................................................................................................... 5
Copyright statement ...................................................................................................................... 5
Acknowledgements ......................................................................................................................... 6

I. Introduction ................................................................................................................................. 7

1. Nuclear Envelope and the LINC complex ................................................................................. 7
   1.1. The first LINC component: the SUN ................................................................................ 8
   1.2. The second LINC component: the KASH ........................................................................ 8
   1.1. Structure and function of the LINC complex ..................................................................... 9
   1.1. Nuclear envelopeopathies and dystonia ......................................................................... 11

2. TorsinA ....................................................................................................................................... 12
   2.1. Alterations to the activity of TorsinA .............................................................................. 13
   2.2. Function and binding partners of TorsinA ...................................................................... 14
   2.3. Evidence for a role of TorsinA in the Nuclear envelope .................................................. 17

3. Nuclear envelope breakdown during mitosis ............................................................................ 18

4. Aims and objectives ..................................................................................................................... 20

II. Materials and methods .............................................................................................................. 21

1. Methods .................................................................................................................................... 21
   1.1. Nucleic acid procedures .................................................................................................... 21
   1.2. Tissue culture procedures ............................................................................................... 27
   1.3. Protein procedures ........................................................................................................... 29
   1.4. Fluorescence microscopy ................................................................................................ 36
   1.5. Quantification of immunofluorescence images ................................................................. 37

2. Materials .................................................................................................................................... 39

III. The LINC Complex during mitosis ............................................................................................ 42

1. Introduction ............................................................................................................................... 42

2. Overlap between endogenous LINC components during cell division ..................................... 42
   2.1. SUN1 and Nup153 have different distributions during mitosis ....................................... 43
   2.1. SUN1 and SUN2 mostly overlap but distribute differently during telophase .................... 45
   2.2. SUN1 and nesprin-2 overlap during the whole mitotic cycle ......................................... 48
   2.3. SUN1 and nesprin-2 but not SUN2 overlap in clusters during telophase ......................... 50

3. Nesprin-2 does not have the exact same timing as neither SUN2 nor SUN1 in returning to the NE

3.1. Developing cell lines expressing GFP-nesprin-2 ................................................................. 50
3.2. SUN1 and GFP-nesprin-2 overlap in telophase clusters free of SUN2 ............................... 52
3.3. SUN1 leaves the cleavage furrow sooner than GFP-nesprin-2 and SUN2 ......................... 53
3.4. Clusters of SUN1/nesprin-2 are located in the ER .............................................................. 54
3.5. SUN1 and GFP-nesprin-2 clusters persist in early G1 ......................................................... 54

4. Effect of TorsinA depletion on the LINC complex during cell division .................................... 55
   4.1. Optimisation for TorsinA knock-down ............................................................................. 56
   4.2. GFP-nesprin-2 during the mitotic cycle when TorsinA is reduced .................................... 58

5. Effect of the exogenous LAP1-V5-BioID on the LINC complex during cell division ............ 59
III. 5.1. Overexpressing LAP1-V5-BioID causes an increase in the numbers of GFP-nesprin-2 clusters

Error! Bookmark not defined.

III. 6. Discussion ................................................................................................................................. 62

IV. LINC complex disassembly and degradation .............................................................................. 66

IV. 1. Introduction .................................................................................................................................. 66

IV. 2. Nesprin-2 is subjected to proteasomal degradation ................................................................. 66

IV. 3. Displacement of GFP-nesprin-2 from the NE ................................................................................ 70

IV. 3.1. KASH4 recombinant protein does not affect GFP-nesprin-2 .............................................. 71

IV. 3.1. SUN1 knock-down decreases GFP-nesprin-2 levels ............................................................ 72

IV. 3.2. ER-localised SUN1 recombinant protein displaces GFP-nesprin-2 to the ER and decreases GFP-nesprin-2 levels ................................................................................................................. 79

IV. 4. Displacement of nesprin-2 from the NE .................................................................................... 85

IV. 4.1. SUN1 knock-down does not affect endogenous nesprin-2 levels ........................................ 85

IV. 4.2. KASH4 recombinant protein displaces nesprin-2 to the ER and increases nesprin-2 levels .............................................................................................................................................. 87

IV. 4.3. ER-localised soluble SUN1 recombinant protein displaces nesprin-2 to the ER and increases nesprin-2 levels ............................................................................................................................. 89

IV. 5. Effect of TorsinA and LAP1 knock-down on the LINC complex .................................................. 91

IV. 5.1. Optimisation of LAP1 knock-down ......................................................................................... 91

IV. 5.2. Disrupting TorsinA decreases the levels of GFP-nesprin-2 .................................................... 92

IV. 5.3. TorsinA knock-down increases the levels of endogenous LINC components ................. 94

IV. 5.4. TorsinA knock-down increases the mobility of GFP-nesprin-2 .............................................. 97

IV. 6. Discussion .................................................................................................................................... 98

V. Interactome network with proximity labelling .............................................................................. 103

V. 1. Introduction ..................................................................................................................................... 103

V. 2. Generation of cells stably expressing the baits ........................................................................ 103

V. 3. Characterisation of the BioID biotin ligase ............................................................................... 107

V. 4. Pull-down and biotinylation optimisation .................................................................................. 110

V. 5. Analysis of the pull-downs ......................................................................................................... 115

V. 6. Discussion ...................................................................................................................................... 127

VI. General discussion and future work ............................................................................................ 131

References ........................................................................................................................................... 134

Word count: 47,583
The LINC complex is composed of SUN and KASH domain proteins spanning the nuclear envelope (NE), allowing communication between the cytoskeleton and the nucleoskeleton. Mutations in these proteins can result in diseases known as nuclear envelopathies. Early Onset Torsion Dystonia (EOTD) is caused by a mutation in a protein called TorsinA and is associated with disruption of the NE similar to some nuclear envelopathies. TorsinA is an AAA ATPase located in the endoplasmic reticulum (ER) and the NE. Although the function of TorsinA is unknown, a potential target for TorsinA is the LINC complex. This study aimed to examine the role of TorsinA in regulating LINC complex assembly/disassembly in mitosis and interphase. Localisation and levels of the relevant proteins were studied using microscopy experiments and biochemical assays. As an alternative approach to study TorsinA function, biotin ligase proximity labelling (BioID) was used to biotinylate near neighbours of TorsinA and its cofactors LAP1 and LULL1, with the aim of identifying TorsinA interacting proteins. The results suggest that LINC components do not always interact throughout the cell cycle, and a population of SUN1 and the KASH domain protein nesprin-2 remain trapped in ER clusters after the NE reseals at the end of mitosis. Nesprin-2 trapped in the ER is likely targeted for degradation, as evidenced by the effect of displacing nesprin-2 to the ER on levels of exogenously expressed nesprin-2. Finally, TorsinA was suggested to affect the LINC complex by strengthening the interaction between LINC components as TorsinA depletion increased mobility and degradation rate of nesprin-2. BioID provided a few candidate proteins that may be interesting TorsinA interactors, including Lamin-B1 and emerin.
DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

COPYRIGHT STATEMENT

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/display.aspx?DocID=24420), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.library.manchester.ac.uk/about/regulations/) and in The University’s policy on Presentation of Theses.
ACKNOWLEDGEMENTS

I would like to express my very great appreciation to Dr. Lisa Swanton for the valuable suggestions and guidance during the development and writing of this research work. I would also like to thank Dr Brian Burke, Prof. Viki Allan, Dr Christoph Ballestrem, Prof. Dean Jackson, Dr Sue Shackleton, Jessica Bowler, Dr Lydia Wunderley, Arnette Wong, Dr Xie Wei, Dr Anna Salter, Dr Rajkumar Ramalingan, Dr Peter March, Dr Steven Marsden, Roger Meadows, Dr Graham Smith, Sarah Binte and Dr Liu Zhongliang Jaron. Assistance provided by Dr Carolina Uggenti, Dr Kit Briant, Dr Paul Atherton, Dr Martina Maric, Ye Xiaqian, Dr Alexandre Chojnowski, Jack Clews and Dr Yee Hui Koay was greatly appreciated.
I. **INTRODUCTION**

I. 1. **Nuclear Envelope and the LINC complex**

The nucleus is a compartment in which the genome is confined. The nuclear membrane defining the outer bounds of the nucleus is called the nuclear envelope (NE). The NE is composed of two lipid bilayers. The external lipid bilayer of the NE is the outer nuclear membrane (ONM), and the internal lipid bilayer is the inner nuclear membrane (INM). The lumen between these two layers is called the PNS (perinuclear space). The ONM is continuous with the endoplasmic reticulum (ER) membrane and is connected to the INM by NPCs (nuclear pore complexes), which span the entire NE, perforating it and controlling the traffic of molecules between the cytosol and nucleoplasm (Fig. 1.1). Lipid layers alone are not enough to hold in place and shape such a large structure, and thus additional scaffolds are needed. The cytoskeleton, including microtubules, actin and intermediate filaments, is organized around the nucleus in the cytoplasm while the nucleoskeleton, including the nuclear lamina composed of lamin intermediate filaments, is organized on the inner side of the NE (Alberts et al., 2002).

Spanning across the NE and linking together the nucleus and the cytoskeleton, there are protein complexes that form structures similar to bridges. They are important for the scaffolding of the nucleus and regulate forces between each side of the NE (Razafsky et al., 2014). These bridges are called Linker of nucleoskeleton and cytoskeleton (LINC) complexes (Fig. 1.1), and are composed of proteins containing Klaricht, ANC-1, Syne homology (KASH) and Sad1 and UNC-84 (SUN) domains (Crisp et al. 2006). The different binding partners of these proteins on either side of the NE allow for various elements of the cytoskeleton and nucleoskeleton to be physically connected (Kracklauer et al., 2013).

---

**Figure 1.1 LINC complexes form at the nuclear envelope.** Cartoon representing the nuclear envelope and LINC complex interacting partners. NE: Nuclear envelope; ER: Endoplasmic reticulum. KASH domain of nesprins (red) interact with SUN domains of SUN proteins (orange) to form LINC complexes. LINC complexes interact with lamins and DNA binding proteins in the nucleoplasm, and with dyneins, microtubules, actin, plectin and intermediate filaments in the cytoplasm.
I. 1.1. The first LINC component: the SUN

The SUN domain is a 200 amino-acids sequence which defines the SUN family and is much conserved across evolution, including lower eukaryotes such as yeast. The SUN proteins are targeted to the NE by a predicted inner nuclear membrane-sorting motif (INM-SM), a SUN-nuclear envelope localisation signal (SUN-NELS), and a classic nuclear localisation signal (cNLS) (reviewed by Tapley and Starr, 2013). A-type lamins on the nuclear face of the INM then interact with the N-terminus of SUN proteins (Crisp et al., 2006). Unlike SUN2, SUN1 localisation is not dependent on the lamins (Hasan et al., 2007; Ostlund et al., 2009), however SUN1 seems to associate with and localise to NPCs (Liu et al., 2007). The SUN proteins are single pass transmembrane proteins of the INM with an N-terminal region in the nucleoplasm and a C-terminal region in the PNS. The SUN domain is at the C-terminus of the protein, and forms a binding site for proteins from the KASH family (reviewed in Burke, 2012). In mammals, there are five SUN proteins (SUN1-5) (Fig. 1.2) (Table 1.1). SUN1 and SUN2 are expressed ubiquitously while other SUN proteins seem to be expressed in the testis only (reviewed in Kracklauer et al., 2013). SUN1 or SUN2 knockout mice are viable despite some phenotypes such as hearing loss and sterility for SUN1 knockout (Horn et al., 2013). However, a SUN1 SUN2 double knockout is lethal, suggesting that their roles may be partially redundant (Lei et al., 2009).

![Figure 1.2 The SUN family.](image)

The SUN family is composed of five members in mice, all of which contain a C-terminal SUN domain, transmembrane domains and coiled-coil domains. Molecular weights range from ~38 to ~110 kDa. Green: predicted hydrophobic regions including presumptive transmembrane domains (TM); blue: coiled-coil domains; yellow: SUN domains (Kracklauer et al., 2013).

I. 1.2. The second LINC component: the KASH

The KASH family of proteins is defined by the presence of a KASH domain, a 30 amino-acids sequence also conserved among eukaryotes (Starr and Han, 2002). This sequence, positioned in C-terminal position of the protein, is necessary and sufficient to target a KASH protein to the ONM as the interaction with SUN is the driving factor of KASH protein localisation (Ostlund et al., 2009). A single pass transmembrane segment follows the KASH domain, another signature element of a KASH protein. The cytoplasmic domain contains a variable number of spectrin repeats (SRs), ending in N-terminal domain that interacts with elements of the cytoskeleton (reviewed in Starr and Fridolfsson, 2010). SRs provide docking sites for proteins and higher order complexes (Parry et al., 1992; Yan et al., 1993). In vertebrates, there are at least four different KASH proteins. Nuclear envelope spectrin-repeat proteins...
(Nesprins) -1 to -4 (Fig. 1.3). On the N-terminal double CH domain, nesprin-1 and nesprin-2 bind to actin (Zhen et al., 2002; Padmakumar et al., 2004). Lacking N-terminal CH domains, nesprin-3 instead binds to plectin (Wihelmsen et al., 2005) and nesprin-4 binds to kinesin-1, a molecular motor interacting with microtubules (Roux et al., 2009) (Table 1.1).

Nesprin-1 or nesprin-2 knockout mice are viable despite some phenotypes such as perturbation of synaptic nuclear anchorage for nesprin-1 knockout. However, a nesprin-1 nesprin-2 double knockout causes lethal respiratory failure, suggesting that their roles may be partially redundant (Zhang et al., 2007). Nesprin-1 and nesprin-2 isoforms exist with varying numbers of SRs and sizes (Fig. 1.4).

The isoforms are tissue-specific and generated through alternative transcription (Rajgor et al., 2012). Small nesprin-1 and nesprin-2 KASH variants are potentially capable of NE entry and localise to the INM, where they supposedly interact with the lamina and emerin (Mislow et al., 2002; Wheeler et al., 2007), as well as with SUN proteins. However, instead of binding the C-terminal SUN domain, these short KASH variants might interact with the N-terminal domain of SUN proteins (Haque et al., 2010). There exist tissue-specific alternate transcripts lacking the KASH domain that locate to other compartments than the NE (reviewed in Rajgor and Shanahan, 2013).

Figure 1.3 The nesprin family. The nesprin family is composed of four members, all of which contain a C-terminal KASH domain, and one or more SRs. Nesprin-1 and nesprin-2 have molecular weights of ~1000 and ~800 kDa, respectively, and present N-terminal CH domains. Nesprin-3 and nesprin-4 have molecular weights of ~110 and ~42 kDa, respectively, and lack the CH domains (reviewed in Rajgor and Shanahan, 2013).

I. 1.1. Structure and function of the LINC complex

SUN proteins in the INM serve to tether the KASH proteins in the ONM (Ostlund et al., 2009). The solved crystal structure of the association between SUN2 and nesprin-1 or nesprin-2 indicates that they interact with a 3:3 stoichiometry (Sosa et al., 2012). A trimer of SUN proteins, forming a coiled-coil triple helix, binds three KASH domains in the SUN trimeric clover-like structure (Fig. 1.5). Furthermore, a conserved cysteine in the SUN domain is ideally positioned relative to a conserved cysteine in the KASH domain, potentially creating an intermolecular disulphide bond stabilizing the interaction even more. It is to be noted that the disulphide bond is not necessary for the domains to interact (Sosa et al., 2012). Due to their structure, LINC complexes form molecular spacers that set the distance between the ONM and the INM to about 45 nm (Sosa et al., 2012). Indeed, depletion of the SUN proteins disrupts the spacing between the nuclear membranes (Crisp et al., 2006). Furthermore, the LINC complex plays an important role in force transmission between the cytoskeleton and the nucleus. Hence, disrupting the LINC complexes reduces the propagation of mechanical stress in cell (Lombardi et al., 2011). Functions of
By combining all the identified 5'UTRs with the nesprin-1 giant and nesprin-2 giant 3'UTRs, up to 16 different nesprin-1 (from ~43 to ~1000 kDa) and 12 different nesprin-2 (from ~48 to 800 kDa) KASH domain-containing variants can be created. Although many of the large variants are hypothetical and yet to be validated at the mRNA and protein level, the tissue specific expression of the 5'UTRs suggests that they are likely to be highly tissue specific. Furthermore, the exposure of unique N-terminal binding motifs generated through alternative initiation suggests that each variant may scaffold unique protein complexes to the NE (reviewed in Rajgor and Shanahan, 2013).

The LINC complex involve mechanical action and crosstalk between the two side of the NE, such as during nuclear anchorage and migration (Khatau et al., 2012), position of the nuclei in sensory epithelial cells (Horn et al., 2013), maintaining the centrosome-nucleus connection, shaping the nucleus and meiotic chromosome movements (Burke and Roux, 2009; Fridkin et al., 2009; Starr and Fridolfsson, 2010). Strikingly, neurons have to undergo nuclear movement and migration during development and are therefore dependent on the LINC complex as well (reviewed in Razafsky et al., 2011). Furthermore, perturbations in mechanical stimuli can influence DNA repair and epigenetics through the LINC complex (González et al., 2011).
<table>
<thead>
<tr>
<th>SUN protein</th>
<th>KASH protein</th>
<th>Cytoskeletal partner</th>
<th>Function of the LINC complex</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUN1</td>
<td>Nesprin-1</td>
<td>Actin</td>
<td>Anchorage of nuclei, maintenance of NE integrity</td>
<td>Crisp et al., 2006</td>
</tr>
<tr>
<td>SUN2</td>
<td>Nesprin-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUN1</td>
<td>KASH5</td>
<td>Microtubules</td>
<td>Meiotic telomere attachment to the NE</td>
<td>Ding et al., 2007; Schmitt et al., 2007; Morimoto et al., 2012</td>
</tr>
<tr>
<td>SUN2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUN1</td>
<td>Nesprin-3</td>
<td>Plectin</td>
<td>Intermediate filament network connection</td>
<td>Wilhelmsen et al., 2005</td>
</tr>
<tr>
<td>SUN2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUN1</td>
<td>Nesprin-4</td>
<td>Microtubule motor</td>
<td>Centrosome and Golgi apparatus attachment</td>
<td>Roux et al., 2009</td>
</tr>
<tr>
<td>SUN2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUN1η</td>
<td>Nesprin-3</td>
<td>Actin (?)</td>
<td>Nuclear remodelling in spermiogenesis</td>
<td>Göb et al., 2010</td>
</tr>
<tr>
<td>SUN3</td>
<td>Nesprin-1</td>
<td>Microtubules (?)</td>
<td>Nuclear remodelling in spermiogenesis</td>
<td>Göb et al., 2010</td>
</tr>
<tr>
<td>SUN4</td>
<td>?</td>
<td>?</td>
<td>Spermiogenesis-specific expression, function unknown</td>
<td>Shao et al., 1999</td>
</tr>
<tr>
<td>SUN5</td>
<td>?</td>
<td>?</td>
<td>Spermiogenesis-specific expression, function unknown</td>
<td>Frohnert et al., 2011</td>
</tr>
</tbody>
</table>

Table 1.1 Proposed function of SUN mammalian proteins and their interacting partner in LINC complexes. SUN proteins interact with nesprins bearing the KASH domain to form LINC complexes (Kracklauer et al., 2013).

I. 1.1. Nuclear envelopathies and dystonia

The diseases affecting the NE are mostly known as nuclear envelopathies. The most studied mutations in this group of diseases are mutations linked to proteins associated with the nuclear lamina, the laminopathies. Very often, structural abnormalities of the nucleus can be observed and result in muscular or neurological symptoms (reviewed in Burke and Stewart, 2002). Mutations in LINC components (reviewed by Starr, 2009) and associated proteins such as lamins and emerin likely result in the same phenotype because all these proteins interact together (Haque et al., 2010). Another disease, the EOTD (early-onset torsion dystonia), exhibits defects in the spacing of the NE membrane similar to lamin mutations although only neurons seem to be affected. More precisely, this phenotype is seen in postmigratory/postmitotic neurons, and leads to motor dysfunction with irregular tremors that start in a muscular region and spread to other body regions. The gene DYT1 encoding for the protein TorsinA is responsible for the disease (reviewed in Breakefield et al., 2001 and Gerace, 2004), and a number of observations suggest that TorsinA functions within the NE (Naismith et al., 2004).
Figure 1.5 Model for the LINC complex bridging the nuclear envelope. The N-terminal, nucleoplasmic domains of SUN proteins anchor the LINC complex to the nucleoskeleton by interacting with the lamina. The lumenal region of SUN proteins forms a triple helical coiled-coil, allowing trimerization of their SUN domains. KASH peptides interact with the SUN domains with a 3:3 stoichiometry by forming a KASH-lid of the SUN domain. The C-terminal, cytoplasmic domain of the nesprins bearing the KASH domain interact with the cytoskeleton. INM, inner nuclear membrane; ONM, outer nuclear membrane (Chang et al., 2015).

I. 2.  

TorsinA was first discovered as the product of the mutated gene responsible for the movement disorder EOTD (Ozelius et al., 1997). TorsinA is an essential protein in mice as its deletion resulted in death within 48 hours of birth (Goodchild et al., 2005). In mammals, TorsinA is part of the torsin family, along with TorsinB, Torsin2 and Torsin3 (Jungwirth et al., 2010) which are not well characterized yet besides their common ability to form heterohexamers. In the course of evolution, the torsins appeared with metazoans, and are not present in prokaryotes or lower eukaryotes such as yeast (Breakefield et al., 2001). The torsins belong to the AAA+ ATPase family (ATPases associated with diverse cellular activities) (Ozelius et al., 1997) and they are their only known members to localise to the lumen of the NE and ER. The biological functions of AAA+ ATPases are diverse and include protein folding and
unfolding, disassembly of stable protein-protein interactions (including protein complexes), degradation of proteins by proteolysis, and intracellular motility (reviewed in Hanson and Whiteheart, 2005).

A difference of note between TorsinA and B is in their expression pattern. Whereas TorsinA seems to be expressed in most tissues, TorsinB is notably absent from the brain (Jungwirth et al., 2010). This discrepancy could explain why the brain is selectively affected by a TorsinA mutation responsible for EOTD (Atai et al., 2012), as TorsinB could compensate for the function of TorsinA in other tissues (Zhao et al., 2013).

I. 2.1. Alterations to the activity of TorsinA

Exploring the consequences of the TorsinA mutation responsible for EOTD disease as well as TorsinA activity mutants such as those found in other AAA+ ATPases may give more insight to the function of TorsinA. Responsible for most cases of EOTD is the deletion of a glutamic acid residue at position 302 or 303 of the TorsinA sequence (Ozelius et al., 1997) in the Sensor-2 motif, probably responsible for regulating the activity of the active site by means of conformational change (Lindquist et al., 2002) (Table 1.2, Fig. 1.6). The precise effect of the ΔE mutation on TorsinA is not clear, but it is a loss of function allele as expressing TorsinΔΔE does not rescue TorsinA−/− mice viability (Goodchild et al., 2005), and the ΔE mutation biochemically appears to disrupt interaction with many binding partners (Zhu et al., 2010), also preventing the ATPase activity to occur (Zhao et al., 2013). Mice expressing TorsinΔΔE on a TorsinA−/− background feature abnormal nuclear membranes in postmigratory embryonic neurons such as membranous vesicle-appending structures (termed “blebs”) in the perinuclear space (Goodchild et al., 2005). When overexpressed, TorsinΔΔE concentrates in the perinuclear space of the NE, forming inclusions (Hewett et al., 2000). TorsinΔΔE has the ability to form heterooligomers with the wild-type protein, which is suggested to result in the dominant phenotype of the mutation (Goodchild et al., 2004).

<table>
<thead>
<tr>
<th>AAA+ module motif</th>
<th>Key residues</th>
<th>Typical mutation</th>
<th>Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walker A</td>
<td>K in GXXXXGK(T/S)</td>
<td>K→A</td>
<td>Inhibits nucleotide binding</td>
<td>Babst et al., 1998; Matveeva et al., 1997</td>
</tr>
<tr>
<td>Walker B</td>
<td>E in hhhhDE</td>
<td>E→Q</td>
<td>Impairs ATP hydrolysis, functions as a ‘substrate trap’</td>
<td>Weibezahn et al., 2003; Dalal et al., 2004; Babst et al., 1998</td>
</tr>
<tr>
<td>Sensor-2</td>
<td>R in the sequence GAR near the N terminus</td>
<td>R→A/M</td>
<td>Impairs ATP hydrolysis, sometimes also ATP binding</td>
<td>Ogura et al., 2004</td>
</tr>
</tbody>
</table>

Table 1.2 Common AAA+ mutants manipulating activity to understand AAA+ protein function (Hanson et al., 2013).

Typically, mutations in the Walker A motif of AAA+ ATPases prevents nucleotide binding, and supposedly the mutated protein cannot bind to a substrate either (Babst et al., 1998; Matveeva et al., 1997) (Table 1.2, Fig. 1.6). Mutations in the Walker B motif are known to prevent AAA+ ATPase from
being able to hydrolyse ATP. In consequence, they bind tightly to their substrate, thus the nickname ‘substrate trap’ (Babst et al., 1998; Weibezahn et al., 2003; Dalal et al., 2004) (Table 1.2, Fig. 1.6).

I. 2.2. Function and binding partners of TorsinA

Despite studies establishing binding partners for TorsinA (Table 1.3), no clear function has yet been elucidated yet for TorsinA. However, early studies have shown that the TorsinAΔE and the TorsinAEQ ‘substrate trap’ mutant are concentrated in the NE, indicating a role in the NE (Naismith et al., 2004). The phenotype of EOTD bears some similarities with some nuclear envelopathies with NE membrane disruption, and the fact that TorsinA binds to some members of the LINC complex indicates that one of the functions of TorsinA may be to regulate LINC complexes at the NE (see below).

The most documented proteins interacting with TorsinA are two of its protein cofactors, LAP1 (Lamina associated polypeptide 1) and LULL1 (Luminal domain like LAP1, with a 60% similarity in their amino-acids sequence). Both appear to be involved in the localisation and the ATPase activity of TorsinA. LAP1 binds the luminal domain of TorsinA, with greatly increased affinity towards the ATP-bound E171Q ‘trap’ mutant and reduced affinity towards the TorsinAΔE mutant, and recruits TorsinA to the NE. LAP1 is composed of a nucleoplasmic and a transmembrane N-terminal domain, and a C-terminal luminal domain which binds to TorsinA (Goodchild et al., 2005, Naismith et al., 2009) (Fig. 1.7).

![Figure 1.6 TorsinA, LAP1 and LULL1 domains. Cartoon illustrating the domains and localisation of TorsinA, LAP1 and LULL1. Known TorsinA mutants are indicated above the domain with mutated residues. (A) TorsinA and the mutants TorsinAKA, TorsinAEQ and TorsinAΔE. (C) LAP1. (D) LULL1. SS: Signal sequence; KDEL: KDEL retention signal; ER: endoplasmic reticulum; C-ter: C-terminus; N-ter: N-terminus.](image)

LULL1 is a protein whose gene is on human chromosome 1q24, located adjacent to the LAP1 gene from which it was likely duplicated. LULL1 also binds the luminal domain of TorsinA (with greatly increased affinity towards the ATP-bound E171Q ‘trap’ mutant and reduced affinity towards the TorsinAΔE mutant) and recruits it to the NE. LULL1 is composed of a cytoplasmic and a transmembrane

---

**Figure 1.6 TorsinA, LAP1 and LULL1 domains.** Cartoon illustrating the domains and localisation of TorsinA, LAP1 and LULL1. Known TorsinA mutants are indicated above the domain with mutated residues. (A) TorsinA and the mutants TorsinAKA, TorsinAEQ and TorsinAΔE. (C) LAP1. (D) LULL1. SS: Signal sequence; KDEL: KDEL retention signal; ER: endoplasmic reticulum; C-ter: C-terminus; N-ter: N-terminus.
N-terminal domain, concentrating it in the ER, and a lumenal C-terminal domain which binds to TorsinA (Goodchild et al., 2005, Naismith et al., 2009) (Fig. 1.7).

Considering that LAP1 and LULL1 coimmunoprecipitate with TorsinA, an early hypothesis proposed that LULL1 and LAP1 are substrates for TorsinA (Goodchild et al., 2005). However, neither LAP1 nor LULL1 are relocated by TorsinA, nor do they change their conformation subsequently to TorsinA binding. Recent studies show that they are positive coregulators. While TorsinA alone is catalytically inactive, LAP1 and LULL1 were directly required for TorsinA to have an ATPase activity in vitro (Zhao et al. 2013). They have a AAA+ ATPase-like fold, but lack the canonical nucleotide binding motifs of a AAA+ ATPase. However, both LAP1 and LULL1 feature a strictly conserved arginine (R563 and R449 respectively) exactly where the ‘arginine finger’ of canonical AAA+ ATPases is present (Fig. 1.8), whereas TorsinA does not have that conserved arginine. A conserved ‘arginine finger’ positioned to reach the phosphate binding site is often required in the neighboring protomer of the AAA+ ATPase hexameric ring assembly to permit the ATPase activity (Wendler et al., 2012). TorsinA bound to its cofactors whose conserved arginine has been deleted is not redirected to the NE and loses its ATPase function as well as the ability to separate from the cofactors, showing that LAP1 and LULL1 provide the ‘arginine finger’ to the active site of TorsinA and together they permit the ATPase activity of the protein complex. They form heterohexameric rings (LAP1-TorsinA)₃ or LULL1-TorsinA)₃. (Sosa et al., 2014; Goodchild et al., 2015) (Fig. 1.8). The interaction with TorsinAΔE is severely impaired for both of them (Zhao et al. 2013), likely because the sensor-2 conformation of TorsinAΔE does not correlate with the ATP-bound state of TorsinA.

Figure 1.7 TorsinA and its cofactors. Cartoon representing the nuclear envelope, LINC complex interacting partners (nesprins and SUN proteins) and TorsinA and its cofactors. NE: Nuclear envelope; ER: Endoplasmic reticulum. LULL1 interacts with TorsinA in the ER and LAP1 interacts with TorsinA at the NE.

LAP1 and LULL1 are expressed in all tissues (Goodchild et al., 2005). Overexpressing LULL1 (Naismith et al., 2009) or deleting LULL1 in mice does not affect the NE, however deleting LAP1 in mice triggers the same NE phenotype as deleting TorsinA except that the phenotype is observed in all tissues and not restricted to neuronal tissues. TorsinA /-/- mice (as well as TorsinAΔE/ΔE mice) exhibit perinatal
lethality and morphological abnormalities in the perinuclear space of neuronal nuclear membranes (membranous vesicle-appearing structures, “blebs”) (Goodchild et al., 2005), whereas that phenotype extends to all nonneuronal cell types in LAP1−/− mice (Kim et al., 2010; Shin et al., 2014). This finding suggests that a LAP1 and TorsinA malfunction may also be a potential suspect for the EOTD disease, and the observation that the EOTD phenotype is localized in the central nervous system (CNS) may be explained by TorsinB interacting correctly with LAP1 and filling the same role as TorsinA in the CNS, but in other tissues (Zhao et al., 2013). TorsinA being targeted to the NE by its cofactors LULL1 and LAP1 suggests that TorsinA has a hypothetical function to accomplish at that location.

In addition, different roles for TorsinA have been proposed by a number of studies (Table 1.3). One of these is as a chaperone involved in the quality control of protein folding in the ER, associated with the ERAD (endoplasmic reticulum-associated degradation) pathway (Esapa et al., 2007). Indeed, TorsinA binds to Derlin-1, an ERAD factor implicated in the retro-translocation of abnormal proteins out of the ER. Furthermore, increasing levels of TorsinA facilitated degradation of misfolded proteins, and TorsinAΔE is impaired in this function resulting in an increased sensitivity to stress in the cells (Nery et al., 2011).

Figure 1.7 Heterohexameric ring assembly of TorsinA and LAP1 domains. Schematic diagram of a heterohexameric model of alternating LAP1 (light blue) and TorsinA (light green), based on the hexameric ring of ClpC-D2 AAA ATPase domains (Wang et al., 2011). The C-terminal domain of Torsin is colored bright green. An ATP molecule (red) is modeled into the TorsinA nucleotide binding pocket. The conserved arginine finger in LAP1 is in blue. The disulfide bridge within LAP1 is in yellow. Interaction with LULL1 is predicted to (Sosa et al., 2014)
<table>
<thead>
<tr>
<th>Protein</th>
<th>Location/role/binding domains</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAP1</td>
<td>NE, cofactor, luminal domain bound to N-ter of TorsinA</td>
<td>Goodchild et al., 2005; Zhao et al., 2013</td>
</tr>
<tr>
<td>LULL1</td>
<td>ER, cofactor, luminal domain bound to N-ter of TorsinA</td>
<td>Goodchild et al., 2005; Zhao et al., 2013</td>
</tr>
<tr>
<td>Nesprins 1-2-3</td>
<td>NE, substrate, Kash domain bound to C-ter of TorsinA</td>
<td>Nery et al., 2008</td>
</tr>
<tr>
<td>SUN2</td>
<td>NE, substrate, SUN domain</td>
<td>Vander Heyden et al., 2009</td>
</tr>
<tr>
<td>SUN1</td>
<td>NE, potential substrate, SUN domain</td>
<td>Jungwirth et al., 2011</td>
</tr>
<tr>
<td>Derlin-1</td>
<td>ER, bridge to bind p53 and VIMP in ERAD</td>
<td>Nery et al., 2011</td>
</tr>
<tr>
<td>misfolded ε-sarcoglycan</td>
<td>ER, ERAD substrate</td>
<td>Esapa et al., 2007</td>
</tr>
<tr>
<td>DAT</td>
<td>Cell surface, substrate</td>
<td>Torres et al., 2004</td>
</tr>
<tr>
<td>Calnexin</td>
<td>ER</td>
<td>Naismith et al., 2009</td>
</tr>
</tbody>
</table>

Table 1.3 Proteins binding to TorsinA. NE, nuclear envelope; ER, endoplasmic reticulum; ERAD: Endoplasmic-reticulum-associated protein degradation.

I. 2.3. Evidence for a role of TorsinA in the Nuclear envelope

Despite studies establishing binding partners for TorsinA, no clear function has been elucidated yet for TorsinA. A number of diverse functions have been proposed including roles in processing and degradation in the ER/secretory pathway (Nery et al., 2011) or protection from oxidative stress (Breakefield et al., 2011). However, early studies showed that the TorsinAΔE and the TorsinAEQ ‘substrate trap’ mutant are concentrated in the NE, indicating a role in the NE (Naismith et al., 2004). Other lines of evidence supporting a role for TorsinA in the NE include the disruption of NE structure in TorsinA-/ mice (Goodchild et al., 2005) and discovery of binding partners and potential substrates of TorsinA at the NE. Indeed, TorsinA and TorsinB associate with nesprin-1, nesprin-2 and nesprin-3 via their C-terminal domains. TorsinA knockout or overexpressed TorsinAΔE triggers nesprin-3 to be found predominantly in the ER. TorsinA knockout has a negative effect on nuclear polarisation and positioning in migrating fibroblasts, suggesting a role for TorsinA in these LINC complex dependent processes (Nery et al., 2008).

A separate study showed that overexpression of the ER located TorsinA binding partner LULL1 causes redistribution of TorsinA to the NE, and this resulted in loss of nesprin-2, nesprin-3 and SUN2 from the NE. Under these conditions, the overall levels of SUN2 proteins appeared to be decreased (Vander Heyden et al., 2009). These findings suggest that the LINC complex could be destabilised and/or degraded by TorsinA within the NE. In addition, SUN1 was found to concentrate TorsinAΔE in the NE, an effect that is dependent on a tyrosine residue at position Y147 of the TorsinA sequence. This finding also hints at the LINC complex being a substrate for TorsinA (Jungwirth et al., 2011). Finally, a recent
study has shown that TorsinA and LAP1 are required for Transmembrane actin-associated nuclear (TAN) line assembly and persistence (Saunders et al., 2017). TAN arrays are composed of SUN2 and nesprin-2G, they couple the nucleus with moving actin cables to move it to the rear of the cell during fibroblasts migration (Luxton et al., 2011).

### Evidence pointing to a role of TorsinA with the LINC complex

<table>
<thead>
<tr>
<th>Evidence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TorsinA directly interacts with the KASH domain of several nesprins</td>
<td>Nery et al., 2008</td>
</tr>
<tr>
<td>TorsinA is required for the localisation of nesprin-3α to the NE</td>
<td>Nery et al., 2008</td>
</tr>
<tr>
<td>LULL1-mediated concentration of TorsinA within the NE displaces nesprin-2G, nesprin-3 and SUN2</td>
<td>Vander Heyden et al., 2009</td>
</tr>
<tr>
<td>SUN1 is involved in the proper localisation of TorsinA to the NE</td>
<td>Jungwirth et al., 2011</td>
</tr>
<tr>
<td>TorsinA and LAP1 are required for TAN line assembly and persistence</td>
<td>Saunders et al., 2017</td>
</tr>
</tbody>
</table>

**Table 1.4 Evidence that TorsinA interacts with the LINC complex.** NE, nuclear envelope; ER, endoplasmic reticulum; TAN lines, transmembrane actin-associated nuclear lines. Adapted from Saunders and Luxton, 2016.

### I. 3. Nuclear envelope breakdown during mitosis

During the cell cycle, a parental cell duplicates its genetic material then separates it equally between two daughter cells. This process of separation and division into two identical cells is called mitosis and consists of several phases: interphase, prophase, prometaphase, metaphase, anaphase and finally telophase. The NE of lower eukaryotes remains intact during this process, which is termed ‘closed’ mitosis. However, the NE of metazoans is completely disassembled and removed from chromatin (reviewed in Güttiger et al., 2009). During ‘open’ mitosis, nuclear envelope breakdown (NEBD) takes place at the transition between prophase and prometaphase as the NE is broken and subsequently merged to the ER membrane (Fig. 1.8) (Table 1.5). To prepare for this event, during prophase chromatin condenses and the duplicated centrosomes separate. Some INM proteins such as SUN1 are phosphorylated (Patel et al., 2014) and lose their interaction with the chromatin and the lamins which are themselves phosphorylated (Ottaviano et al., 1985). The nuclear lamina and the NPCs disassemble. NE proteins are incorporated into the interconnected ER. NEBD marks the end of prophase and the start of prometaphase. The mitotic spindle forms and all the chromosomes are attached to microtubules and organised at the metaphase plate. Sister chromatids are separated during anaphase, and NE reformation starts around the surface of chromatin with the binding of membranes. During telophase, the NE is fully formed and closed, then cytokinesis separates the two daughter cells.
**Figure 1.8 Nuclear envelope breakdown during mitosis.** Cartoon illustrating the breakdown of the nuclear envelope and its incorporation to the ER during mitosis. Lamins: pink; chromosomes: dark blue; microtubules: green; ER/NE membranes: blue (Ungricht et al., 2017).

<table>
<thead>
<tr>
<th>NE breakdown and reassembly steps during mitosis</th>
<th>Phase</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamina phosphorylation and depolymerisation</td>
<td>Early to late prophase</td>
<td>Gerace et al., 1980</td>
</tr>
<tr>
<td>INM proteins phosphorylation (including SUN1), loss of interaction and retraction into the ER</td>
<td>Prophase</td>
<td>Ellenberg et al., 1997; Yang et al., 1997; Patel et al., 2014</td>
</tr>
<tr>
<td>NPC disassembly</td>
<td>Late prophase</td>
<td>Dultz et al., 2008</td>
</tr>
<tr>
<td>Holes in the NE caused by microtubules around the centrosomes</td>
<td>Late prophase</td>
<td>Beaudouin et al., 2002</td>
</tr>
<tr>
<td>Removal of NE membranes from chromatin, fusion with tubulated ER</td>
<td>Prometaphase (NEBD)</td>
<td>Salina et al., 2002; Beaudouin et al., 2002; Puhka et al., 2007</td>
</tr>
<tr>
<td>Chromatin recruitment of NPC ‘prepores’</td>
<td>Late anaphase</td>
<td>Dultz et al., 2008; Harel et al., 2003; Walther et al., 2003</td>
</tr>
<tr>
<td>ER membrane tubules bind to the chromatin surface</td>
<td>Late anaphase</td>
<td>Anderson et al., 2007</td>
</tr>
<tr>
<td>Dephosphorylation of INM proteins (including SUN1) leading to chromatin binding</td>
<td>Early telophase</td>
<td>Wilson et al., 1988; Pyrpasopoulou et al., 1996; Liu et al., 2007</td>
</tr>
<tr>
<td>Attachment of membrane sheets to chromatin and INM proteins, remodelling into the NE double membrane</td>
<td>Telophase</td>
<td>Wiese et al., 1997; Anderson et al., 2007</td>
</tr>
<tr>
<td>Assembly of NPCs</td>
<td>Telophase</td>
<td>Dultz et al., 2008</td>
</tr>
<tr>
<td>Reformation of the lamina after import through NPCs</td>
<td>Telophase</td>
<td>Newport et al., 1990</td>
</tr>
</tbody>
</table>

**Table 1.5 Nuclear envelope breakdown and reformation during mitosis.** NE, nuclear envelope; ER, endoplasmic reticulum; NEBD, nuclear envelope breakdown; INM, inner nuclear membrane; NPC, nuclear pore complex.
I. 4. Aims and objectives

SUN and KASH interaction has been documented (Sosa et al., 2012), however key questions that remain unanswered include what controls the association of SUN and KASH proteins, and in particular whether LINC complexes are rearranged during key cellular processes such as mitosis when the NE membrane is rearranged (Turgay et al., 2014). A previous study (Patel et al., 2014) suggested that the LINC complex remains intact during mitosis, however this was observed with biochemical assays and only with cells arrested prometaphase. An imaging-based approach could provide insight on the behaviour and localisation of LINC components during all phases of the cell cycle. Therefore, the first aim was to directly observe whether the LINC components overlap during each phase of mitosis and if they have a similar distribution, before perturbing TorsinA to potentially affect the LINC complex.

TorsinA, a protein belonging to the AAA+ ATPase family of proteins, has recently been observed to interact with LINC components by coimmunoprecipitation with nesprin-3 (Nery et al., 2008). Overexpression of the ER located TorsinA binding partner LULL1 causes redistribution of TorsinA to the NE, and this resulted in loss of nesprin-2, nesprin-3 and SUN2 from the NE. Under these conditions, the overall levels of SUN2 proteins appeared to be decreased (Vander Heyden et al., 2009). Furthermore, the mutant TorsinAΔE responsible for EOTD requires SUN1 to be enriched in the NE, therefore the LINC complex was suspected to be a substrate for TorsinA (Jungwirth et al., 2011). AAA+ ATPases are known to be involved in protein folding and unfolding, disassembly of protein complexes and degradation of proteins by proteolysis (reviewed in Hanson and Whiteheart, 2005). By analogy with other AAA+ ATPases, TorsinA could either target LINC complex components for degradation, or separate LINC complexes from each other, or assemble LINC components together. Hence, the aim of this project is to test the hypothesis that the role of TorsinA is regulating the LINC complex. In order to address this, the LINC complex will be examined during mitosis to observe whether the proteins assemble and disassemble, and the LINC complex will be disrupted in several manners to prevent interaction between LINC components and establish whether there is a correlation with the effect of TorsinA and its potential role.

Finally, the use of a novel technique of biotin proximity ligation is proposed to identify novel interacting partners of TorsinA and its coactivators LAP1 and LULL1, potentially giving insight into the function of TorsinA. Therefore, the aims of the project were:

- To study potential disassembly and reassembly of LINC complexes during mitosis using an imaging-based approach.

- To disrupt LINC complex interaction with dominant negative recombinant LINC proteins in order to establish a model for LINC components binding and localisation.

- To examine of the role of TorsinA relative to LINC components interaction and localisation.

- To identify TorsinA, LAP1 and LULL1 interactors with proximity labelling.
II. MATERIALS AND METHODS

II. 1. Methods

II. 1.1. Nucleic acid procedures

II. 1.1.1. Plasmid transformation, extraction and purification

Chemically competent *E. coli* (DH5α, strain Invitrogen) were thawed on ice. In a 1.5 ml eppendorf tube, 1 μl of plasmids containing the DNA of interest were added to 50 μl competent cells. Cells were left on ice for 10 minutes then heat-shocked for 45 seconds at 42°C and placed in ice for 2 minutes. 1 ml of pre-warmed antibiotic-free LB broth was added and cells were incubated for 1 hour at 37°C with vigorous shaking (200 rpm). 100 μl of cells were spread on pre-warmed selective LB-agar plates with the appropriate antibiotic and incubated overnight at 37°C.

II. 1.1.2. Plasmid extraction and purification

Individual colonies were selected from the LB-agar plates and inoculated in LB broth supplemented with 100 μg/μl ampicillin for overnight growth at 37°C with vigorous shaking (200 rpm). Plasmid DNA was extracted from 5 ml cell cultures with the QIAprep mini kit® (Qiagen) and from 50 ml with the Nucleobond Xtra Midi EF® kit (Machery-Nagel) according to the manufacturer’s protocol. The purified DNA concentration was measured using a Nanodrop spectrophotometer.

II. 1.1.3. DNA sequencing

Sequencing analysis of plasmid DNA was performed by GATC biotech in Manchester and 1st Base in Singapore. Plasmids on a pcDNA3.1 or pcDNA5 backbone vector were sequenced with CMV forward and/or BGH reverse primers, plasmids on a pTRIPZ backbone vector were sequenced with 143A forward and/or 129B reverse primers (Table 2.1).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>143A Forward</td>
<td>5’-GGCCCTCTCTATAGTGGAGCTATTATA-3’</td>
</tr>
<tr>
<td>129B Reverse</td>
<td>5’-CGCTCTCTGGAAAGAAAACCA-3’</td>
</tr>
</tbody>
</table>

Table 2.1 Primers used to sequence genes of interest in pTRIPZ.

II. 1.1.4. Polymerase chain reaction (PCR)

Genes coding for the recombinant LINC proteins were provided by Brian Burke (IMB, Singapore) in pcDNA3.1 vectors (Invitrogen). The restriction sites flanking the genes of interest in pcDNA3.1 and pcDNA5 are incompatible with the restriction sites *AgeI* and *EcoR*I in pTRIPZ (Dharmacon), the lentiviral inducible vector required to make stably transfected cell lines. Compatible sites were added to the genes using PCR. PCRs were prepared using the Phusion Flash High-Fidelity Master Mix (Thermo Fisher Scientific) in 0.2 thin-walled PCR tubes. Reaction mix included: Phusion Flash Master Mix; 0.5 μM forward and reverse primers (Table 2.2); 200 ng DNA; 16% DMSO (v/v); MQ H2O. An MJ Research Peltier Thermal Cycler PTC-225 was used to perform the reactions with the chosen conditions (Table
50 μl of the PCR products were subsequently cleaned with a QIAGen Clean PCR spin column (Qiagen) using the manufacturer’s protocol. Cleaned PCR products were digested in CutSmart, Agel-HF and EcoRI-HF (all from NEB).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgeI LINC Forward</td>
<td>5’-CCGATCCAGCCTCCGGACTCTAGCGTTTAACCTAAGCTTAGGCGGCTAGC-3’</td>
</tr>
<tr>
<td>EcoRI LINC Reverse</td>
<td>5’- ATCATAGAATTCCTAGACTCGAGCGGCCGCGCCG -3’</td>
</tr>
</tbody>
</table>

**Table 2.2** Primers used to add AgeI and EcoRI flanking sites to the LINC components genes of interest in pcDNA3.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10 s</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>69</td>
<td>30 s</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>60 s</td>
<td>35</td>
</tr>
<tr>
<td>Finish extension</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 2.3** PCR reaction conditions to amplify the genes of interest in pcDNA3, using the primers in Table 2.2.

A gene coding for the biotin ligase second generation BioID was provided by Brian Burke (IMB, Singapore) in a pcDNA3.1 vector (Invitrogen). Genes coding for the recombinant TorsinA proteins and the V5 tag were provided by Lydia Wunderley (University of Manchester) in pcDNA5/FRT/TO vectors (Invitrogen). An overlap PCR was performed to combine the TorsinA proteins with a V5 tag and a biotin ligase to incorporate the recombinant final product flanked by the restriction sites AgeI and MluI in pTRIPZ. PCRs were prepared using the Phusion Flash High-Fidelity Master Mix (Thermo Fisher Scientific) in 0.2 thin-walled PCR tubes. Reaction mix included: Phusion Flash Master Mix; 0.5 μM forward and reverse primers (Table 2.4); 200 ng DNA; 16% DMSO (v/v); MQ H2O. The reactions were incubated using the chosen cycle conditions (Table 2.5) and the samples were run on a 1% (w/v) ultrapure agarose-TAE gel with Ethidium Bromide next to a size marker Gene Ruler 1 kb. Bands of the right sizes were cut with surgical blades and cleaned with a QIAquick Gel Extraction Kit (QIAGen) using the manufacturer’s protocol. 8 μl of the purified PCR products were subsequently combined in a new 50 μl reaction mix including Phusion Flash Master Mix and MQ H2O. The reactions were incubated using the chosen cycle conditions (Table 2.6) then 0.5 μM of the first forward and the last reverse primers (Table 2.4) were added to the reaction tube and incubated using the chosen cycle conditions (Table 2.7). A band of the right size was excised from the gel on an Ultraviolet Transilluminator and cleaned with a QIAquick Gel Extraction Kit. 50 μl of the PCR products were subsequently cleaned with a QIAGen Clean PCR spin column. Cleaned PCR products were digested in FastDigest Buffer, BshTI and MluI (all from Thermo Fisher Scientific).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgeI TorsinA Forward</td>
<td>5’-ATCGCACCGGTATGAAGCTGGCC-3’</td>
</tr>
<tr>
<td>Ascl TorsinA Reverse</td>
<td>5’-ATTGGCTTACCTGGGCGCCACCATCGTAGTAATAA-3’</td>
</tr>
<tr>
<td>Ascl V5 Forward</td>
<td>5’-TTATTAATCAGATGGCCGAGGTAAGCCAAT-3’</td>
</tr>
<tr>
<td>PacI V5 Reverse</td>
<td>5’-ACCTCCTCAGCCAGATCGTTCTGAATTTAATAGGTAGTGCTA-3’</td>
</tr>
<tr>
<td>PacI BioID Forward</td>
<td>5’-TAGACTCTACCTAAATTTAGAAGAACCTGCTGGCTGAAGGAGGT-3’</td>
</tr>
<tr>
<td>MluI BioID Reverse</td>
<td>5’-GGCCACGCGTTTTAGATTTTCTTTCTCGAGGCT-3’</td>
</tr>
</tbody>
</table>

Table 2.4 Primers used to overlap into recombinant gene TorsinA-V5-BioID.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10 s</td>
<td>25</td>
</tr>
<tr>
<td>Annealing</td>
<td>64</td>
<td>30 s</td>
<td>25</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>22 s</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 2.5 PCR reaction conditions to amplify the different parts needed for the construction of TorsinA-V5-BioID, using the primers in Table 2.4.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10 s</td>
<td>15</td>
</tr>
<tr>
<td>Annealing</td>
<td>60</td>
<td>50 s</td>
<td>15</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>22 s</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 2.6 PCR reaction conditions to perform an overlap PCR TorsinA-V5-BioID, using the purified PCR fragments obtained from the primers in Table 2.4 and the reaction conditions in Table 2.5.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10 s</td>
<td>20</td>
</tr>
<tr>
<td>Annealing</td>
<td>72</td>
<td>30 s</td>
<td>20</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>50 s</td>
<td>20</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.7 PCR reaction conditions to amplify the overlap PCR TorsinA-V5-BioID, using the overlapped product of the reaction conditions in Table 2.6.
A gene coding for the biotin ligase BirA was provided by Alexandre Chojnowski (IMB, Singapore) in a pcDNA3.1 vector. To introduce the BirA instead of BioID in the TorsinA-V5-BioID construct on a pTRIPZ backbone, compatible sites PacI and MluI as well as a stop codon were added to the sequence of the gene using PCR. PCRs were prepared using the Phusion Flash High-Fidelity Master Mix (Thermo Fisher Scientific) in 0.2 thin-walled PCR tubes. Reaction mix included: Phusion Flash Master Mix; 0.5 μM forward and reverse primers (Table 2.8); 200 ng DNA; 16% DMSO (v/v); MQ H₂O. The reactions were incubated using the chosen cycle conditions (Table 2.9). 50 μl of the PCR products were subsequently cleaned with a QIAgen Clean PCR spin. Cleaned PCR products were digested in CutSmart and PacI (NEB), purified, then digested in FastDigest Buffer and MluI (Thermo Fisher Scientific).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PacI BirA</td>
<td>5’-AAAAATTAATTTAAAAAGGACAACACCCTGCCCCTG-3’</td>
</tr>
<tr>
<td>MluI BirA</td>
<td>5’-TATTTAGAATTCTTTACTTCTTCTCGCTTCTCAGGGAG-3’</td>
</tr>
</tbody>
</table>

Table 2.8 Primers used to add PacI and MluI flanking sites to the BirA gene.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10 s</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>65</td>
<td>30 s</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 s</td>
<td>35</td>
</tr>
<tr>
<td>Finish extension</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.9 PCR reaction conditions to amplify the BirA gene, using the primers in Table 2.8.

TorsinA-V5-BioID was used as a template to create V5-BioID-KDEL and combine it with overlapping sequences to create the prolactin signal peptide and obtain SS-V5-BioID-KDEL, flanked by the restriction sites AgeI and MluI in pTRIPZ. PCRs were prepared using the Phusion Flash High-Fidelity Master Mix (Thermo Fisher Scientific) in 0.2 thin-walled PCR tubes. Reaction mix included: Phusion Flash Master Mix; 0.5 μM forward and reverse primers (Table 2.10); 200 ng DNA; 16% DMSO (v/v); MQ H₂O. The reactions were incubated using the chosen cycle conditions (Table 2.11; Table 2.12) and the samples were run on a 1% (w/v) ultrapure agarose-TAE gel with Ethidum Bromide next to a size marker Gene Ruler 1 kb. Bands of the right sizes were cut with surgical blades and cleaned with a QIAquick Gel Extraction Kit (QIAgen) using the manufacturer’s protocol. 8 μl of the purified PCR products were subsequently combined in a new 50 μl reaction mix including Phusion Flash Master Mix and MQ H₂O. The reactions were incubated using the chosen cycle conditions (Table 2.13) then 0.5 μM of the first forward and the last reverse primers (Table 2.10) were added to the reaction tube and incubated using the chosen cycle conditions (Table 2.14). A band of the right size was excised from the gel on an Ultraviolet Transilluminator and cleaned with a QIAquick Gel Extraction Kit. 50 μl of the PCR
products were subsequently cleaned with a QIAgen Clean PCR spin column. Cleaned PCR products were digested in CutSmart, Agel and EcoRI (NEB).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgeI SS Forward</td>
<td>5’-ATTAACCCGTATGAACATCAAGGATCGCCATGGAAAGGTCCCTCCTCC-3’</td>
</tr>
<tr>
<td>SS Reverse</td>
<td>5’-CTCTGGGCAACAGCAGCAGTTTGAACACCAGCAGGAGGAGGGACCCCTT-3’</td>
</tr>
<tr>
<td>SS V5 Forward</td>
<td>5’-CCTGCTGCTGTCAGAGCGTGCCCAGGTAAGCCAAATCCCTAATCCGC-3’</td>
</tr>
<tr>
<td>EcoRI BioID KDEL Reverse</td>
<td>5’-TTATGAATTTACAAACTCATCTTTGCTTTTCTCAGGCTGAACCTCAGGCG-3’</td>
</tr>
</tbody>
</table>

Table 2.10 Primers used to overlap into recombinant gene SS-V5-BioID-KDEL.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10 s</td>
<td>25</td>
</tr>
<tr>
<td>Annealing</td>
<td>75</td>
<td>30 s</td>
<td>25</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>10 s</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 2.11 PCR reaction conditions to amplify the signal sequence with no template DNA, using the relevant primers in Table 2.4.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10 s</td>
<td>25</td>
</tr>
<tr>
<td>Annealing</td>
<td>75</td>
<td>30 s</td>
<td>25</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 s</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 2.12 PCR reaction conditions to amplify V5-BioID-KDEL from TorsinA-V5-BioID, using the relevant primers in Table 2.10.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10 s</td>
<td>15</td>
</tr>
<tr>
<td>Annealing</td>
<td>60</td>
<td>30 s</td>
<td>15</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>40 s</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 2.13 PCR reaction conditions to perform an overlap PCR SS-V5-BioID-KDEL, using the purified PCR fragments obtained from the primers in Table 2.10 and the reaction conditions in Table 2.11 and Table 2.12.

A gene coding for the LAP1 gene was extracted from DNA and provided by Martina Maric (IMB, Singapore). To introduce LAP1 instead of TorsinA in the TorsinA-V5-BioID construct on a pTRIPZ backbone, compatible sites XmaI and Ascl were added to the sequence of the gene using PCR. PCRs
<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10 s</td>
<td>20</td>
</tr>
<tr>
<td>Annealing</td>
<td>75</td>
<td>30 s</td>
<td>20</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>40 s</td>
<td>20</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.14 PCR reaction conditions to amplify the overlap PCR SS-V5-BioID-KDEL, using the overlapped product of the reaction conditions in Table 2.13.

were prepared using the Phusion Flash High-Fidelity Master Mix (Thermo Fisher Scientific) in 0.2 thin-walled PCR tubes. Reaction mix included: Phusion Flash Master Mix; 0.5 μM forward and reverse primers (Table 2.15); 200 ng DNA; 16% DMSO (v/v); MQ H₂O. The reactions were incubated using the chosen cycle conditions (Table 2.16). 50 μl of the PCR products were subsequently cleaned with a QiAgen Clean PCR spin column. Cleaned PCR products were digested in CutSmart, Xmal and Ascl (NEB).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xmal LAP1</td>
<td>5’-AAAAACCCGGATGGCGGCAGGCGGCGGCGGAGAG-3’</td>
</tr>
<tr>
<td>Ascl LAP1</td>
<td>5’-AAAAAGGCAGCGCTAAGCAGATGCCCTTTTCAGGGC-3’</td>
</tr>
</tbody>
</table>

Table 2.15 Primers used to add Agel and Ascl flanking sites to the LAP1 gene.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10 s</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>6</td>
<td>30 s</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>50 s</td>
<td>35</td>
</tr>
<tr>
<td>Finish extension</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.16 PCR reaction conditions to amplify the LAP1, using the primers in Table 2.15.

A gene coding for the LULL1 gene obtained from an ORF library (Transomic). To introduce LULL1 instead of TorsinA in the TorsinA-V5-BirA construct on a pTRIPZ backbone, compatible sites *Agel* and *Ascl* were added to the sequence of the gene using PCR. PCRs were prepared using the Phusion Flash High-Fidelity Master Mix (Thermo Fisher Scientific) in 0.2 thin-walled PCR tubes. Reaction mix included: Phusion Flash Master Mix; 0.5 μM forward and reverse primers (Table 2.17); 200 ng DNA; 16% DMSO (v/v); MQ H₂O. The reactions were incubated using the chosen cycle conditions (Table 2.18). 50 μl of the PCR products were subsequently cleaned with a QiAgen Clean PCR spin column. Cleaned PCR products were digested in CutSmart, Agel and Ascl (NEB).
### Table 2.17 Primers used to add AgeI and Ascl flanking sites to the LAP1 gene.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgeI LULL1</td>
<td>5'-TAATCTACCGGTATGGCGACAGTGG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-ATAGAAGGCGCGCGAAAGGCAC-3'</td>
</tr>
</tbody>
</table>

### Table 2.18 PCR reaction conditions to amplify the LAP1, using the primers in Table 2.17.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10 s</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>65</td>
<td>30 s</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>45 s</td>
<td>35</td>
</tr>
<tr>
<td>Finish extension</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
</tbody>
</table>

II. 1.1.5. Preparing the target vector for the PCR fragments

In parallel to the PCR reaction, the pTRIPZ vectors were cut with the compatible restriction sites. DNA loading dye was added to the digested mix and the sample was run on a 1% (w/v) ultrapure agarose-TAE gel (Invitrogen) with Ethidium Bromide next to a size marker Gene Ruler 1 kb at 90V. The desired band was excised from the gel on an Ultraviolet Transilluminator and cleaned with a QIAquick Gel Extraction Kit. To minimize self-ligation of the vector, cleaned vector was dephosphorylated with Shrimp Alkaline Phosphatase (Roche).

II. 1.1.6. Ligation of the PCR products into the vectors and verification

Digested PCR products were ligated into the dephosphorylated pTRIPZ vector cut at the compatible sites at a ratio of 5:1 (PCR product:vector) using the Quick Ligation kit (NEB). 5 μl of each ligation mix were transformed in DH5α. Clones were screened by restriction digest and those containing insert of correct size were sequenced. SerialCloner2.6.1 (SerialBasics) and BLAST (NCBI) were used to confirm DNA sequences.

II. 1.2. Tissue culture procedures

1.1.1. Cell culture and maintenance

HeLa-M cells provided by Viki Allan (University of Manchester) were cultured in DMEM: Dulbecco’s modified Eagle’s Medium D 6429 (Sigma) supplemented with 2 mM L-Glutamine (Sigma), 0.1 mM MEM non-essential amino acids (Sigma) and 10% (v/v) heat-inactivated FBS (Foetal bovine serum, Sigma). Cells were incubated at 37°C, 5% CO₂. After reaching 90-100% confluency, cells were washed in PBS (Dulbecco’s Phosphate Buffered Saline D8537, Sigma) and split using 1x trypsin-EDTA solution (Sigma) into cell culture T75 flasks; 10 cm dishes; 15 cm dishes; 6 well dishes and 12 well dishes; 24 well dishes;
96 well dishes (Corning). 293T human embryonic kidney derived cells provided by Martina Maric (IMB, Singapore) were cultured in the exact same conditions.

1.1.2. **Cell lines transfection and selection for stable expression**

293T cells were split 24 hours before transfection to reach 80% confluency during transfection. 2.9 μg of GAG/POL and 1.4 μg of VSVG both provided by Alexandre Chojnowski (IMB, Singapore) were added in 500 μl Opti-MEM (GIBCO) to 5.7 μg of a pTRIPZ vector containing the recombinant gene of interest (Table 2.19). The mixture was added to a tube containing 500 μl Opti-MEM and 50 μl Polyethylenimine (PEI). After vortexing the tube and a 10 minutes incubation at room temperature, 1 ml of the solution was transfected to the 293T cells. After 6-8 hours, the media was replaced with 10 ml fresh DMEM. The following day, the supernatant containing the virus was collected and filtered through a 0.45 μm filter. 5 ml of the filtered supernatant was added to HeLa-M cells at 70% confluency. 3-4 hours after infection, the media was replaced with fresh DMEM. The following day, the media was replaced with DMEM containing 2 mg/ml puromycin. Media was changed every few days until the cells were spreading over the dish. When the cells were confluent, they were split in a 15 cm dish until confluency then trypsinised and harvested in 50 mL PBS at 2000 rpm for 3 min. Cell pellets were resuspended in DMEM containing 10% DMSO (Sigma) and transferred to cryotubes stored at -80°C using isopropanol tanks overnight. The cryotubes were subsequently moved to liquid nitrogen tanks for long term storage.

Cells stable expressing GFP-nesprin-2 were further selected from single clones. 200 μl of 20,000 cells/ml were inoculated in the first well of a 96 well dish. The wells in the column were subsequently diluted 1:2 in series, then the wells in each row were subsequently diluted 1:2 in series. The final volume of the wells was brought to 200 μl and the cells were grown for 4-7 days. Wells containing a single colony were trypsinised and inoculated in a 24 well dish, and the volume of the wells was brought to 500 μl. Once confluent, the cells were trypsinised and inoculated in a T75 flask for use and for storage as described previously. Doxycycline at 1 μg/ml was added to induce the expression of the gene of interest stably integrated in the genome.

II. 1.2.1. **Transient expression**

Cells were split 24 hours prior to transfection and 60,000 to 100,000 cells were seeded in 1 mL DMEM in 12 well plates. Cells were then transfected with 1.6 μg of vector DNA (Table 2.19) and Lipofectamine 2000 (Invitrogen) for SDS-PAGE analysis, or 1 μg of vector DNA and FuGENE 6 (Promega) for immunofluorescence, according to the manufacturer's protocol. Briefly, DNA and Lipofectamine 2000 were added to separate tubes with 100 μl Opti-MEM and then combined prior to transfection, while DNA and FuGENE 6 were mixed in 100 μl Opti-MEM. Mixtures were then added dropwise to the wells. When the vector DNA was pTRIPZ, 1 μg/mL doxycycline was added to induce the expression. 24h post-transfection, the cells were fixed for immunofluorescence if they were on coverslips, or harvested in SDS-PAGE Sample buffer with DTT 100 mM.
II. 1.2.2. siRNA silencing

24 hours prior to transfection, 60,000 cells were seeded in 1mL DMEM in 12 well plates. For immunofluorescence, the cells were seeded on coverslips. Cells were then transfected with 10 nm siRNA duplex (Table 2.20) (Dharmacon) using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer’s protocol. Briefly, siRNA and Lipofectamine RNAiMAX were mixed in 100 μl DMEM prior to transfection. Scrambled siRNA was used as a negative control. Mixtures were then then added dropwise to the wells. 48h post-transfection, the cells were fixed for immunofluorescence if they were on coverslips, and harvested in SDS-PAGE Sample buffer with DTT 100 mM.

II. 1.2.3. Proteasome inhibition

Cells in 12-well dishes had their media replaced with fresh DMEM supplemented with proteasome inhibitor II (PSII) (Calbiochem) 10 μM for 6 hours and then fixed if grown on coverslips and/or harvested in SDS-PAGE sample buffer.

II. 1.3. Protein procedures

1.1.1. SDS-PAGE and Western Blot protein analysis

Protein samples harvested in SDS-PAGE sample buffer (0.02% bromophenol blue; 62.5 mM Tris; 4% (w/v) Sodium dodecyl sulfate; 10% (v/v) glycerol; pH 7.6) with DTT 100 mM were heated for 10 minutes at 70°C then sonicated with a Bioruptor (Diagenode) at high intensity with an interval of 30 s for 5 min, then loaded alongside ColorPlus prestained protein marker, broad range 7-175 kDa (NEB), onto a 10% or 12% Tris-glycine polyacrylamide gel for approximately 1 hour at 30 mA in running buffer (25 mM Tris; 190 mM glycine; 0.08% (w/v) SDS). SDS-PAGE gels were transferred onto nitrocellulose membranes (LI-COR) by wet transfer at 300 mA for 1h in transfer buffer (20 mM Tris; 150 mM glycine, 20% (v/v) methanol). Membranes were stained with Revert solution (LI-COR) according to the manufacturer’s protocol, scanned on an Odyssey CLx Infrared Imaging System (LI-COR) and de-stained according to the manufacturer’s protocol. Membranes were blocked for 20 min in 5% milk (Marvel) in TBS (Tris-Buffered Saline: 50 mM Tris, 150 mM NaCl; pH7.4). Membranes were subsequently incubated with primary antibodies (Table 2.21) in 2% milk-TBS solution overnight at 4°C with constant mixing. Membranes were washed 5 min in TBS three times then incubated with fluorescently labelled secondary antibodies (Table 2.22) in 2% milk-TBS for 1h at room temperature. Membranes were scanned on an Odyssey CLx and quantified using ImageStudio Lite 3.1 software (LI-COR). Intensities of the bands of interest were normalized to the intensities of their respective actin loading control bands in Excel software (Microsoft), then graphics were drawn in GraphPad Prism software (GraphPad Software).

SDS-PAGE gels were alternatively directly scanned in a ChemiDoc Imaging System (Bio-Rad) in the GFP channel then subsequently stained with Coomassie blue InstantBlue Protein Stain (Expedeon) according to the manufacturer’s protocol and scanned again in a ChemiDoc Imaging System with white light exposure.

Main antibodies and solutions used for quantification were tested to check their most effective range relative to the amount of proteins used in this study, from 12-well dishes. After a certain quantity of
proteins loaded on the gel, the proportionality to the fluorescence intensity of the detected signal may vary.

**Figure 2.1 Range of the anti-TorsinA antibody.** (A) HeLa-M cells were grown on 12-well plates for 24h, harvested with SDS-PAGE sample buffer and a titration of the sample was run on an SDS-PAGE gel. After transfer, the membrane was blotted with anti-TorsinA and scanned. Black arrow: TorsinA (about 37 kDa). (B) Fluorescence intensity from the membrane described in A. A.U.: arbitrary units.

Fluorescence intensity of the anti-TorsinA antibody (Fig. 2.1A) was proportional to the volume of sample loaded across the whole range (Fig. 2.1B).

Fluorescence intensity of the anti-β-tubulin antibody (Fig. 2.2A) was only proportional up to 2.5 µl sample loaded, and then the value of the fluorescence intensity did not accurately reflect the amount of proteins (Fig. 2.2B). Due to the small range of this antibody, it was later replaced by actin and Revert for more accurate quantifications.

Fluorescence intensity of the anti-β-actin antibody (Fig. 2.3A) was proportional to the volume of sample loaded up until 20 µl (Fig. 2.3B). Higher amounts of proteins may be out of range.
Figure 2.2 Range of the anti-β-tubulin antibody. (A) HeLa-M cells were grown on 12-well plates for 24h, harvested with SDS-PAGE sample buffer and a titration of the sample was run on an SDS-PAGE gel. After transfer, the membrane was blotted with anti-β-tubulin and scanned. β-tubulin: about 55 kDa. (B) Fluorescence intensity from the membrane described in A. A.U.: arbitrary units.

Fluorescence intensity of the Revert solution (Fig. 2.4A) was proportional to the volume of sample loaded up until 20 µl (Fig. 2.4B). Higher amounts of proteins were extremely out of range (Fig. 2.4C).

Signal for GFP in a ChemiDoc Imaging System (Fig. 2.5A) was proportional to the volume loaded until 5 µl but not after (Fig. 2.5B).

Signal for Coomassie stain in a ChemiDoc Imaging System (Fig. 2.6A) was proportional to the volume loaded until 5 µl but not after (Fig. 2.6).

In the rest of this study, 20 µl of sample were loaded for membranes (Revert; anti-TorsinA; anti-β-actin) and 5 µL of sample were loaded for gel scans (GFP; Coomassie) unless stated otherwise.
Figure 2.3 Range of the anti-β-actin antibody. (A) HeLa-M cells were grown on 12-well plates for 24h, harvested with SDS-PAGE sample buffer and a titration of the sample was run on an SDS-PAGE gel. After transfer, the membrane was blotted with anti-β-actin and scanned. Actin: about 42 kDa. (B) Fluorescence intensity from the membrane described in A. A.U.: arbitrary units.

1.1.1. Protein precipitation with trichloroacetic acid

Cell lysates were incubated 10 minutes on ice with 10 µl trichloroacetic acid (TCA) then centrifuged for 15 minutes at 14000 rpm in a microcentrifuge at 4°C. The supernatant was discarded and the pellet was washed with 200 µl acetone then centrifuged for 5 minutes at 14000 rpm. The supernatant was discarded and the washing step was repeated. The pellet was heated 5 to 10 minutes at 95°C to drive off the acetone, then resuspended in µl SDS-PAGE sample buffer, heated for 5-10 minutes at 95°C and finally loaded on a SDS-PAGE gel.

II. 1.3.1. PNGase F treatment

30 µl of proteins in SDS-SB was combined with 10 µl nonyl phenoxypolyethoxylethanol (NP40) and 1 µl PNGase F (NEB) and left overnight at room temperature. The enzyme was deactivated by heating for 10 minutes at 75°C and the sample was loaded on a SDS-PAGE gel.
Figure 2.4 Range of the Revert solution. (A) HeLa-M cells were grown on 12-well plates for 24h, harvested with SDS-PAGE sample buffer and a titration of the sample was run on an SDS-PAGE gel. After transfer, the membrane was stained with Revert and scanned. (B) Fluorescence intensity from the membrane described in A. A.U.: arbitrary units. (C) Fluorescence intensity from the membrane described in A while omitting the last value. A.U.: arbitrary units.

1.1.1. Protease protection assay

Cells in a 24-well dish were incubated on ice for 5 minutes with 300 µl of ice-cold permeabilisation buffer (50 mM Tris; 250 mM sucrose; 5 mM MgCl₂; pH 7.5) supplemented with 0.002% (w/v) digitonin and 75 mM NaCl or 0.2% (v/v) Triton and. The buffer was replaced by 300 µl PBS with 10 µl proteinase K (QIAGen) for 5 minutes with gentle shaking. 60 µl phenylmethylsulfonyl fluoride PMSF was added to the well and the supernatant was moved to a microfuge tube. The tube was centrifuged for 2 minutes at 2000 rpm and the supernatant was discarded. The pellet was precipitated with TCA and loaded on a SDS-PAGE gel.

1.1.1. Proximity labelling with a biotin ligase

Proximity labelling was performed according to a protocol designed by Kyle Roux (Burke et al., 2012) and modified by Alexandre Chojnowski (IMB, Singapore). Cells were split 24h before induction in T75 flasks to be 80% confluent on the day of induction. Fresh media was supplemented with 1 µg/ml doxycycline for 24 hours to induce expression of the gene of interest. Media was replaced with fresh
media supplemented with 1 µg/ml doxycycline and 50 µM biotin for 24 hours. The cells were trypsinised, resuspended in 10 ml DMEM and washed twice with PBS by centrifugation at 2000 rpm for 3 minutes. Pellets were lysed with lysis buffer (50 mM Tris pH 7.4; 500 mM NaCl; 0.4% (w/v) SDS; 5 mM EDTA, 1 mM DTT; protease inhibitor (Roche)) on ice, sonicated and transferred to 2 ml microfuge tubes. The tubes were centrifugated for 10 minutes at 16000 rpm at 4°C and the supernatant was transferred to new 2 ml microfuge tubes. Triton X-100 was added for a final concentration of 2% (v/v). 100 µl of washed Dynabeads MyOne streptavidin beads (Invitrogen) were added to the tubes and they were incubated for 2 hours at room temperature with mild shaking and flipping. The tubes were transferred on a magnet to collect the beads and the buffer was removed. A first wash buffer (0.5% (w/v) sodium deoxycholate; 150 mM NaCl; 1% (v/v) NP-40; 0.1% (w/v) SDS; 50 mM Tris pH 7.4; protease inhibitor (Roche)) was used to wash the beads two times, then a second wash buffer (20 mM TrisHCl pH 7.4; protease inhibitor (Roche)) to wash the beads two times again. For western-blot analysis, the pellets

Figure 2.5 Range of GFP-nesprin-2. (A) GFP-nesprin-2 cells were grown on 12-well plates for 24h, induced with doxycycline for 24h, harvested with SDS-PAGE sample buffer and a titration of the sample was run on an SDS-PAGE gel. The gel was scanned in the GFP channel. (B) Fluorescence intensity from the gel described in A. A.U.: arbitrary units.
Figure 2.6 Range of Coomassie. (A) GFP-nesprin-2 cells were grown on 12-well plates for 24h, induced with doxycycline for 24h, harvested with SDS-PAGE sample buffer and a titration of the sample was run on an SDS-PAGE gel. The gel was incubated in Coomassie blue then scanned in the visible range. (B) Fluorescence intensity from the gel described in A. A.U.: arbitrary units.

were resuspended in 50 µL SDS-PAGE sample buffer, transferred to a magnet and the supernatant was used for analysis.

To prepare the samples for mass spectrometry, the beads were resuspended in 50 µl preparation buffer (50 mM triethylammonium bicarbonate pH 8.5; 50% (v/v) 2,2,2-trifluoroethanol). 2 µl of 0.5 M Tris(2-carboxyethyl) phosphine hydrochloride solution was added to the tubes and they were stirred by vortexing before a 20 minutes incubation at 55°C with mild shaking. 5.2 µl of 2-chloroacetamide 550 mM was added before a 30 minutes incubation at room temperature in the dark. 542.8 µl of 100 mM triethylammonium bicarbonate buffer pH 8.5 and 20 µl of LysC 0.5 µg/µl were added before a 3 to 4 hours incubation at 37°C with shaking. 130 µl of 100 mM triethylammonium bicarbonate buffer pH 8.5 and 10 µL of trypsin 0.1 µg/µL were added before an overnight incubation at 37°C with shaking. The
tubes were transferred to a magnet and the supernatant was transferred into a new microfuge tube before a centrifugation of 5 minutes at 16000 rpm. The supernatant was transferred into a 15 ml tube and 84.4 µl of trifluoro acetic acid 10% were added. To desalt the samples, 4 ml of desalting buffer (0.5 % (v/v) LC-MS grade acetic acid) was added and the sample was applied to a conditioned C18 Sep-Pak cartridge (Waters) twice. The cartridge was washed twice with desalting buffer and the sample was eluted in elution buffer (0.5 % (v/v) LC-MS grade acetic acid; 80% (v/v) LC-MS grade acetonitrile). The samples were concentrated using SpeedVac (Thermo Fisher) for 3 hours at 45°C. OrbiTrap Fusion (Thermo Fisher) was used to perform tandem mass spectrometry (MS/MS) on the samples.

II. 1.3.2. Data analysis after mass spectrometry

Progenesis Qi (Nonlinear Dynamics) was used to quantify the abundance ratio of each ion by aligning common “fingerprints”. Mascot (Matrix Science) was used to identify peptides. Universal Protein Resource (UniProt) and DAVID Bioinformatics Database (Laboratory of Human Retrovirology and Immunoinformatics) were used to consolidate the identifiers of each peptide in a custom Excel file designed by Alexandre Chojnowski (IMB, Singapore). The Excel file also allowed to group triplicates together and calculate a mean for the abundance ratio of each protein. The ratio for each protein was scored against known background contaminants from the Contaminant Repository for Affinity Purification (CRAPome) (Alexey Nesvizhskii and Anne-Claude Gingras Labs) CRAPome and from proteins found in the parental cell line. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was used to cluster the proteins into networks. Cytoscape (Institute for Systems Biology) was used to visualise and filter the data.

II. 1.4. Fluorescence microscopy

1.1.1. Immunofluorescence

Coverslips were rinsed in PBS (Phosphate-Buffered Saline: 137mM NaCl; 2.7mM KCl; 10mM Na₂HPO₄; 1.8mM KH₂PO₄) then transferred to the fixation of choice. For methanol fixation, cells were fixed in -20°C methanol for 4-10 minutes then transferred to PBS before staining. For formaldehyde fixation, cells were fixed in 3% (v/v) formaldehyde for 15 minutes, washed twice in PBS for 5 minutes, quenched with 1 ml PBS with a few drops of 1 M glycine, pH 8.5 for 5 minutes, washed in PBS for 5 minutes, permeabilised in 0.2% (v/v) Triton X-100 (Sigma), and washed in PBS for 5 minutes twice before staining. After fixation, cells were incubated face-down on a 25 µl drop of the appropriate primary antibody (Table 2.21) in PBS for 30 minutes to 1 hour and washed twice in PBS for 5 minutes, then were protected from light and incubated face-down on a 25 µl drop of the appropriate secondary antibodies (Table 2.22) and 100 ng/ml 4,6-diaminido-2-phenylindoledihydrochloride (DAPI) in PBS for 30 minutes and washed twice in PBS for 5 min. After staining, the coverslips were mounted onto microscope slides with 7 µl of ProLong Diamond and kept protected from light overnight at room temperature before being stored at 4°C.

For widefield microscopy, images were collected on an Olympus BX51 upright microscope using a 60x / 1.25 UPlan FL objective and captured using a Coolsnap ES2 camera (Photometrics) through Metavue v7.8.4.0 software (Molecular Devices). Specific band pass filter sets for DAPI, FITC and Texas red were
used to prevent bleed through from one channel to the next. Images were then processed and analysed using Fiji ImageJ.

For deltavision high-end widefield microscopy, images were acquired on an Olympus IX83 Inverted microscope using Lumencor LED excitation, a 100x/1.3 Plan Apo objective and the Sedat filter set (Chroma). The images were collected using a Retiga R6 (Q-Imaging) camera. Raw images were then deconvolved using the Huygens Pro software (SVI) and slices or maximum intensity projections of these deconvolved images are shown in the results.

II. 1.4.1. Fluorescence recovery after photobleaching (FRAP)

Cells were grown on 35 mm µ-Dish (ibidi) in Leibovitz’s L-15 media with no phenol red (Thermo Fisher Scientific) supplemented with FBS. Images where collected on a 3I spinning disk inverted confocal microscope with a Zeiss ObserverZ microscope frame) and using a 63x/1.14 Plan Apochromat objective. Samples where excited using 488 nm (100%) diode lasers via a Zeiss GFP filter cube. Images where collected using a Photometrics Evolve EMCCD camera with a camera gain of 2x with the software Slidebook 6.0.3 (3I) which also measured the fluorescence signal. The value of signal intensity from each area of interest was corrected for decay over time and fluorescence intensity of each timepoint was normalised to the initial timepoint. GraphPad Prism 7 was used to apply a nonlinear regression for all areas of interest over time (only including values after photobleaching) according to a formula provided by Paul Atherton (University of Manchester): \( Y = a - a \exp(-b \times x) \) with initial values \( a = 0.5 \) and \( b = 0.2 \); half-time = \( \ln(0.5)/-b \).

II. 1.4.2. Exposure time and fluorescence intensity

To determine whether the intensity of the signal is proportional to the exposure time in the range used for this study, several images of coverslips stained with antibodies recognising nesprin-2 and SUN1 were captured on the Olympus BX51 with a Coolsnap ES2 camera on a single field of view. The mean intensity of each field of view was calculated with ImageJ and plotted over time (Fig. 2.7). Both anti-Nesprin2 (Fig. 2.7A) and anti-SUN1 (Fig. 2.7A) show a proportional relationship between the exposure time and the fluorescence intensity. 1 second of exposure time was used for all subsequent experiments.

II. 1.5. Quantification of immunofluorescence images

To determine how to best quantify fluorescent signal intensity in the microscopy images (Fig. 2.8A) with ImageJ two methods were tested: whole group analysis, and thresholding to measure individual cells. The starting point for both methods was to remove from the intensity of each pixel the mean value of the background. Then for the first method a whole group of cells to quantify was selected (Fig. 2.8B). They were chosen because their nuclei are shown in full and well-spaced, which is ideal for the second method, and they can be all selected together without including too much of other cells. The total intensity of the selected area was measured then divided by the number of cells. The obtained intensity was 603499 pixel values in arbitrary units (A.U.) per cell in the selected area. For the second method,
Figure 2.7 Fluorescence intensity is proportional to exposure time. HeLa-M cells were grown on glass coverslips for 48h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, anti-nesprin-2 and anti-SUN1. A single field of view was captured on a snapshot widefield microscope using Metamorph and different exposure times for the camera. ImageJ was used to measure the mean fluorescence intensity of the field of view at each exposure time for (A) anti-nesprin-2 (B) anti-SUN1.

Figure 2.8 Methods of measuring the fluorescence intensity of GFP-nesprin-2 in cells. Cells were grown on glass coverslips for 24h, induced with doxycycline for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with GFP-booster. Representative images were captured on a snapshot widefield microscope using Metamorph then processed using ImageJ to show (A) Cells stably expressing GFP-nesprin-2, (B) an area of complete cells selected for intensity quantification, (C) Fiji ImageJ thresholding to separate the cells as individual quantifiable objects, (D) particle analysis of the cells after thresholding, with six cells selected as corresponding to the area in B. Scale bar: 10 μm.

Thresholding was used to differentiate each cell individually (Fig. 2.8C) then particle analysis (Fig. 2.8D) was applied to determine the fluorescence intensity of individual cells (labelled 1 to 6 in). The mean intensity was 427638 A. U. ± 13486. The difference between the result of the first method and the second
method could be explained by the presence of part of other cells in the selected area of the first method (Fig. 2.8B) and the exclusion of some ER regions from the particle analysis of the second method (Fig. 2.8D). The first method is less likely to miss regions of ER, an important consideration since LINC components may be displaced into the ER. Therefore, the first method of analysing a whole group was chosen as the method for measuring fluorescent intensity.

II. 2. Materials

<table>
<thead>
<tr>
<th>Construct</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1 GFP-KASH4</td>
<td>Brian Burke (IMB, Singapoe)</td>
</tr>
<tr>
<td>pcDNA3.1 HA-SUN1-KDEL</td>
<td>Brian Burke (IMB, Singapoe)</td>
</tr>
<tr>
<td>pcDNA3.1 GFP-nesprin-2</td>
<td>Brian Burke (IMB, Singapoe)</td>
</tr>
<tr>
<td>pcDNA5/FRT/TO TorsinA-V5</td>
<td>Lydia Wunderley (University Of Manchester)</td>
</tr>
<tr>
<td>pcDNA5/FRT/TO TorsinAΔE-V5</td>
<td>Lydia Wunderley (University Of Manchester)</td>
</tr>
<tr>
<td>pcDNA5/FRT/TO TorsinAEQ-V5</td>
<td>Lydia Wunderley (University Of Manchester)</td>
</tr>
<tr>
<td>pcDNA5/FRT/TO TorsinAKA-V5</td>
<td>Lydia Wunderley (University Of Manchester)</td>
</tr>
<tr>
<td>pTRIPZ GFP-nesprin-2</td>
<td>Constructed during this study</td>
</tr>
<tr>
<td>pTRIPZ TorsinA-V5-BiolD</td>
<td>Constructed during this study</td>
</tr>
<tr>
<td>pTRIPZ TorsinAΔE-V5-BiolD</td>
<td>Constructed during this study</td>
</tr>
<tr>
<td>pTRIPZ TorsinAEQ-V5-BiolD</td>
<td>Constructed during this study</td>
</tr>
<tr>
<td>pTRIPZ TorsinAKA-V5-BiolD</td>
<td>Constructed during this study</td>
</tr>
<tr>
<td>pTRIPZ SS-BiolD-V5-KDEL</td>
<td>Constructed during this study</td>
</tr>
<tr>
<td>pTRIPZ LAP1-V5-BiolD</td>
<td>Constructed during this study</td>
</tr>
<tr>
<td>pTRIPZ TorsinA-V5-BirA</td>
<td>Constructed during this study</td>
</tr>
<tr>
<td>pTRIPZ TorsinAΔE-V5-BirA</td>
<td>Constructed during this study</td>
</tr>
<tr>
<td>pTRIPZ TorsinAEQ-V5-BirA</td>
<td>Constructed during this study</td>
</tr>
<tr>
<td>pTRIPZ TorsinAKA-V5-BirA</td>
<td>Constructed during this study</td>
</tr>
<tr>
<td>pTRIPZ LULL1-V5-BirA</td>
<td>Constructed during this study</td>
</tr>
</tbody>
</table>

Table 2.19 Constructs used in this study.

<table>
<thead>
<tr>
<th>siRNA sequence</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAUCUUAAUGCCGUGUUUUU</td>
<td>TorsinA</td>
</tr>
<tr>
<td>UGAGAGAAAGCGGUACUA</td>
<td>LAP1 1</td>
</tr>
<tr>
<td>GGUCCGAUUCUGCGAAAGA</td>
<td>LAP1 2</td>
</tr>
<tr>
<td>GAGGAAUUGAAGACCGGAAA</td>
<td>LAP1 3</td>
</tr>
<tr>
<td>CCCGUGUGAGAGUUUAUUCUA</td>
<td>SUN1</td>
</tr>
</tbody>
</table>

Table 2.20 siRNA sequences used in this study.
<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Host Species</th>
<th>Dilution used</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>mouse</td>
<td>1:2000 (WB)</td>
<td>Abcam</td>
</tr>
<tr>
<td>GFP</td>
<td>mouse</td>
<td>1:1000 (GFP)</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>HA</td>
<td>rabbit</td>
<td>1:1000 (WB)</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:300 (HA)</td>
<td></td>
</tr>
<tr>
<td>SUN1</td>
<td>rabbit</td>
<td>1:500 (WB)</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:350 (IF)</td>
<td></td>
</tr>
<tr>
<td>V5</td>
<td>mouse</td>
<td>1:5000 (WB)</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:200 (IF)</td>
<td></td>
</tr>
<tr>
<td>SUN1</td>
<td>rabbit</td>
<td>1:1000 (WB)</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:350 (IF)</td>
<td></td>
</tr>
<tr>
<td>β-tubulin</td>
<td>mouse</td>
<td>1:10,000 (WB)</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1000 (IF)</td>
<td>Technology</td>
</tr>
<tr>
<td>Nesprin-2</td>
<td>mouse</td>
<td>1:100 (IF)</td>
<td>Burke Lab</td>
</tr>
<tr>
<td>Nup153</td>
<td>mouse</td>
<td>1:200 (IF)</td>
<td>Burke Lab</td>
</tr>
<tr>
<td>SUN1</td>
<td>mouse</td>
<td>1:1000 (WB)</td>
<td>Burke Lab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:300 (IF)</td>
<td></td>
</tr>
<tr>
<td>SUN2</td>
<td>mouse</td>
<td>1:1000 (WB)</td>
<td>Burke Lab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:300 (IF)</td>
<td></td>
</tr>
<tr>
<td>Calreticulin</td>
<td>mouse</td>
<td>1:300 (IF)</td>
<td>Abcam</td>
</tr>
<tr>
<td>KIF5B</td>
<td>rabbit</td>
<td>1:1000 (WB)</td>
<td>Abcam</td>
</tr>
<tr>
<td>PDI</td>
<td>mouse</td>
<td>1:100 (IF)</td>
<td>Abcam</td>
</tr>
<tr>
<td>TorsinA</td>
<td>rabbit</td>
<td>1:1000 (WB)</td>
<td>Swanton Lab</td>
</tr>
</tbody>
</table>

Table 2.1 Primary antibodies used in this study. IF = immunofluorescence microscopy; WB = western blotting. The anti-nesprin-2 antibody used was raised against an antigen with a sequence encompassing the first spectrin repeat, the linker site and the coiled-coil domain right before the KASH domain of nesprin-2 and should therefore be able to recognise all KASH isoform variants of nesprin-2 (Fig. 1.4).
<table>
<thead>
<tr>
<th>Antibody</th>
<th>For primary host species</th>
<th>Host species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRDye 800CW(G)</td>
<td>rabbit</td>
<td>donkey</td>
<td>1:5000 (WB)</td>
<td>LI-COR</td>
</tr>
<tr>
<td>IRDye 680CW(R)</td>
<td>mouse</td>
<td>donkey</td>
<td>1:5000 (WB)</td>
<td>LI-COR</td>
</tr>
<tr>
<td>IRDye 800CW(G)</td>
<td>mouse</td>
<td>donkey</td>
<td>1:5000 (WB)</td>
<td>LI-COR</td>
</tr>
<tr>
<td>IRDye 680CW(R)</td>
<td>rabbit</td>
<td>goat</td>
<td>1:5000 (WB)</td>
<td>LI-COR</td>
</tr>
<tr>
<td>DyLight 488(G)</td>
<td>rabbit</td>
<td>donkey</td>
<td>1:400 (IF)</td>
<td>Jackson</td>
</tr>
<tr>
<td>DyLight 594(R)</td>
<td>mouse</td>
<td>donkey</td>
<td>1:200 (IF)</td>
<td>Jackson</td>
</tr>
<tr>
<td>Alexa Fluor 750(C)</td>
<td>mouse</td>
<td>goat</td>
<td>1:200 (IF)</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Alexa Fluor 750(C)</td>
<td>rabbit</td>
<td>goat</td>
<td>1:200 (IF)</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Streptavidin IRDye 800CW(G)</td>
<td>n/a</td>
<td>n/a</td>
<td>1:5000 (WB)</td>
<td>LI-COR</td>
</tr>
<tr>
<td>Streptavidin 647(FR)</td>
<td>n/a</td>
<td>n/a</td>
<td>1:400 (IF)</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>GFP-booster</td>
<td>n/a</td>
<td>n/a</td>
<td>1:200 (IF)</td>
<td>ChromoTek</td>
</tr>
</tbody>
</table>

**Table 2.22** Secondary antibodies and fluorophore conjugates used in this study. IF = immunofluorescence microscopy; WB = western blotting; G = green; R = red; FR = far red; C = cyan.
III. THE LINC COMPLEX DURING MITOSIS

III. 1. Introduction

The overall aim of this PhD was to examine the potential relationship of TorsinA to the LINC complex. TorsinA interacts with the KASH domain of several nesprins (Nery et al., 2008), SUN1 is involved in the proper localisation of TorsinA to the NE (Jungwirth et al., 2011) suggesting that the LINC complex is a substrate for TorsinA. AAA+ ATPases are usually molecular machines that drive conformational rearrangements of protein complexes (reviewed in Hanson and Whiteheart, 2005), and thus the assembly or disassembly of the LINC components could be a potential target of the AAA+ ATPase TorsinA. A good starting point was to determine whether there are natural occurrences of the LINC complex undergoing assembly or disassembly in live cells.

During mitosis, nuclear envelope breakdown (NEBD) takes place at the transition between prophase and prometaphase (reviewed in Güttinger et al., 2009; Ungricht et al., 2017) as the NE is broken and subsequently merged to the ER membrane (Fig. 1.8) (Table 1.5). These rearrangements of the membrane raise the question of whether the LINC complex, a bridge spanning the NE and connecting the INM to the ONM, remains assembled during the whole process. A previous study (Patel et al., 2014) suggested that the LINC complex remains intact during mitosis. However, this was determined biochemically and the cells were arrested in prometaphase with nocodazole. Therefore, an imaging-based approach was used here to directly observe whether the LINC components overlap during each phase of mitosis and if they have a similar distribution, before perturbing TorsinA to potentially affect the LINC complex.

The first cell line considered was HeLa with a T-REx Flp-In™ system (Ward et al., 2011) to generate isogenic cell lines stably expressing LINC components under the control of a tetracycline inducible promoter. However, a large proportion of the T-REx cells had irregular cell and nuclei morphology, and thus HeLa-M cells were chosen instead. An advantage of HeLa-M cells is their bigger nuclei which makes their observation ideal for microscopy.

Several microscopy systems were used. Widefield microscopes with a snapshot camera to capture larger number of cells for fluorescence quantification and a deltavision high-end widefield microscope to generate sharp images at a higher resolution.

III. 2. Overlap between endogenous LINC components during cell division

A starting point to investigate whether the LINC components remain assembled during mitosis is to follow them through the different phases of the cell cycle. A commercial SUN1 antibody was used (SIGMA) whereas antibodies for other LINC components SUN2 and nesprin-2, as well as Nup153, had been produced in mice in Brian Burke’s lab (IMB, Singapore). GFP-booster (nanobodies targeted to GFP) were used to amplify GFP signal (ChromoTek).
NPCs disassemble and solubilise during mitosis, they are not incorporated in the ER/NE membrane after NEBD (Ungricht et al., 2017) and thus their fate should follow a different pattern than components of the LINC complex. Demonstrating a different localisation between NPCs and LINC components would prove that the separation of NE proteins can be confirmed with an imaging approach. Nucleoporin153 (Nup153) is expected to localise for the most part to the nuclear rim during mitosis, but not to the membranes (Mackay et al., 2009). If the SUN proteins and the nucleoporins have ceased to interact during mitosis, the localisation of Nup153 should differ from LINC components that are distributed to the ER/NE membranes.

To examine separation of SUN1 from Nup153, HeLa-M cells were grown on glass coverslips, fixed in paraformaldehyde and stained with antibodies recognising SUN1 and Nup153. All phases of the mitotic cycle were captured on a widefield microscope (Fig. 3.1). During interphase, Nup153 and SUN1 overlapped and spanned the entirety of the NE. Then as prophase began, Nup153 proteins were lost from the NE and were present at the surface of the chromatin. Conversely, SUN1 stayed at the edge of the chromatin and did not overlap with it. The difference in SUN1 and Nup153 localisation was most striking during metaphase and anaphase as Nup153 is concentrated towards the centre of the former nucleus while SUN1 appears most enriched at the periphery and the fluorophores show very little overlap on the merge images. During telophase both SUN1 and Nup153 were recruited to the newly formed NE where they overlapped once again. The only difference between SUN1 and Nup153 in telophase seemed to be clusters of SUN1 that had not been fully dispersed through the NE (Fig. 3.1, arrows) suggesting that SUN1 returns to the NE more slowly than Nup153.

In conclusion, the observed localisation of Nup153 was consistent with the literature (Mackay et al., 2009) while the LINC complex is redistributed to the ER/NE membrane (reviewed in Ungricht et al., 2016). Both patterns were successfully imaged and the imaging showed only strong overlap between the fluorophores during interphase and telophase. The same technique will be used to see whether different localisations can be observed between LINC components.

III. 2.1. **SUN1 and Nup153 have different distributions during mitosis**

Once the dynamic localisation of SUN1 during the cell cycle had been established, the localisation of SUN2 also needed to be examined for comparison. SUN1 and SUN2 are often considered as interchangeable in terms of function (Lei et al. 2009), however they have different interacting partners to target them to the NE as SUN2 is dependent on the lamins to localise to the NE (Hasan et al., 2007; Ostlund et al., 2009) while SUN1 is solely dependent on the NPCs to localise to the NE (Liu et al., 2007). Although SUN1 did not seem to follow the localisation of Nup153 during mitosis (Fig. 3.1), these preferential interactions may translocate to a difference in localisation between SUN1 and SUN2 during mitosis.
Figure 3.1 Distribution of SUN1 and Nup153 during mitosis. Representative widefield fluorescence images of HeLa-M cells undergoing mitosis. Cells were grown on glass coverslips for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with anti-SUN1 and anti-Nup153 followed by fluorescently labelled secondary antibodies, and DAPI. Cells undergoing mitosis were identified by the appearance of their DNA and representative images were captured using Metamorph then processed using Fiji ImageJ. Arrows: SUN1 clusters. Scale bar: 10 μm.
When observed alongside SUN2, SUN1 had the same distribution as seen previously (Fig. 3.1). Together with SUN2, both LINC proteins were present in the ‘ring shaped’ NE during interphase and the fluorophores overlapped (Fig. 3.2). As prophase began, they were lost from the NE following NEBD and dispersed around the chromatin. All throughout the following prometaphase, metaphase and anaphase, both proteins remained at the periphery of the chromatin in what seemed to be ER/NE network and the fluorophores overlapped. During anaphase SUN1 and SUN2 were recruited to the reforming NE at the surface of the chromatin. As seen previously (Fig 3.2, arrows), SUN1 seemed to be partially delayed in its return to the NE compared to SUN2, with clusters of SUN1 staining evident around the NE in telophase that did not appear to be enriched in SUN2 (Fig 3.3, arrows). This result suggests that SUN1 and SUN2 do not behave in precisely the same way during NE reassembly following mitosis, and opens the question whether nesprins interact with SUN1 and/or SUN2 during mitosis.

III. 2.2. SUN1 and nesprin-2 overlap during the whole mitotic cycle

The next set of experiments addressed the question of whether the SUN domain proteins and the KASH domain proteins of the LINC complex remained associated throughout mitosis, and thus nesprin-2 was observed alongside SUN1 for all phases of the cell cycle (Fig. 3.3). The anti-nesprin-2 antibody used was raised against an antigen with a sequence encompassing the first spectrin repeat, the linker site and the coiled-coil domain right before the KASH domain of nesprin-2 and should therefore be able to recognise all KASH isoform variants of nesprin-2 (Fig. 1.4). SUN1 had the same distribution as seen previously (Fig. 3.1; Fig. 3.2). Following NEBD during prophase, there was a loss of the protein at the NE and then it stayed at the periphery of the chromatin until anaphase when it was recruited to the newly formed NE at the surface of the chromatin. Some late clusters can be seen in telophase. Nesprin-2 overlapped with SUN1 perfectly during interphase (Fig. 3.3), then followed the same pattern during the following stages of the cell cycle. However, in contrast to SUN1 which was restricted to the NE in interphase cells, a low level of nesprin-2 staining was observed on the surface of chromatin during several phases (Fig. 3.3 interphase, prophase and telophase). Considering that nesprin-2 could not be present inside the nucleus during interphase, this observed phenotype could be due to the antibody recognising nesprin-2 not giving a clear and sharp staining like the antibodies recognising SUN1 and SUN2 did. Despite this, SUN1 and nesprin-2 seemed to show a very high degree of overlapping during all phases of the cell cycle. Interestingly, nesprin-2 staining was also evident in the SUN1 telophase clusters observed in telophase cells (Fig. 3.3, arrows). Higher resolution microscopy needs to be performed to confirm whether this was a visual artefact or if nesprin-2 is present inside the nucleus. Despite this, SUN1 and nesprin-2 seemed to mostly overlap during all phases of the cell cycle, including in the telophase clusters in which both proteins could be found.

These data suggest that nesprin-2 could still be physically interacting with SUN1 throughout mitosis. However, since SUN2 was not present in the SUN1 and nesprin-2 positive clusters seen in telophase, this indicates that SUN2 may not be interacting with other LINC components at that point of the cell cycle. Further observation using a microscope allowing for sharper pictures and a better resolution was required to address this possibility.
Figure 3.2 Panel of SUN1 and SUN2 during the cell cycle. Representative widefield fluorescence images of HeLa-M cells undergoing mitosis. Cells were grown on glass coverslips for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with anti-SUN1 and anti-SUN2 followed by fluorescently labelled secondary antibodies, and DAPI. Cells undergoing mitosis were identified by the appearance of their DNA and representative images were captured using Metamorph then processed using Fiji ImageJ. Arrows: SUN1 clusters. Scale bar: 10 μm.
Figure 3.3 Panel of SUN1 and nesprin-2 during the cell cycle. Representative widefield fluorescence images of HeLa-M cells undergoing mitosis. Cells were grown on glass coverslips for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with anti-SUN1 and anti-nesprin-2 followed by fluorescently labelled secondary antibodies, and DAPI. Cells undergoing mitosis were identified by the appearance of their DNA and representative images were captured using Metamorph then processed using Fiji ImageJ. Arrows: LINC component clusters. Scale bar: 10 μm.
III. 2.3.  **SUN1 and nesprin-2 but not SUN2 overlap in clusters during telophase**

The deltavision microscope has a better spatial resolution than a standard widefield microscope, and the subsequent deconvolution of the raw images further improves the contrast and resolution by hiding the diffracted light and pinpointing the source of the emitted light. Due to the microscope taking a series of pictures in the z-axis, it is possible to reconstitute in 3D the source of the light and precisely select a certain slice in the vertical space to see how different SUN2 behaves from the other LINC components during telophase and if it is separated from nesprin-2.

Cells were grown on glass coverslips, fixed in paraformaldehyde and stained with antibodies recognising SUN2 and SUN1 (Fig. 3.4A) or nesprin-2 (Fig. 3.4B). Cells in telophase were captured on a deltavision microscope, and slices representative of the cells observed were selected after deconvolution (Fig. 3.4). As observed on the standard widefield microscope (Fig. 3.2), SUN2 was concentrated in the reforming NE of telophase cells (Fig. 3.4A). SUN1 was partially localised to the NE in these cells, but was also evident in clusters that were distinct from the NE and lacked SUN2 (Fig. 3.4A, arrows). In addition, SUN1 present in the NE appeared to be more punctate than SUN2 which already fully spanned the whole NE. Nesprin-2 exhibited a very similar distribution to SUN1 in telophase, it was also partially localised to the NE (Fig. 3.4B) and enriched in clusters separate from the NE that lacked SUN2 (Fig. 3.4A, arrows). In addition, nesprin-2 was never overlapping with chromatin, it was diffuse at the NE (Fig. 3.4A) and present in clusters without SUN2. Finally, contrary to the observation with a widefield microscope (Fig. 3.3), nesprin-2 staining was not observed on the surface of chromatin during telophase (Fig. 3.4B) indicating that the staining was likely to be noise that disappeared with the deltavision system.

In summary, these results suggest that SUN1 and nesprin-2 have a similar localisation during NE breakdown and reassembly. Relative to SUN2, SUN1 and nesprin-2 appear to be delayed in being recruited to the NE during telophase and are retained in clusters that are distinct from the NE.

On the other hand, SUN2 is wholly absent from the bigger clusters and is recruited to the NE earlier and more completely than SUN1 and nesprin-2 during telophase. The observation that SUN2 is not present in clusters with nesprin-2 indicates that disassembly of these LINC complex components may have occurred during mitosis.
Figure 3.4 SUN1 and nesprin-2 have a different distribution than SUN2 in telophase. Representative deltatiation fluorescence images of HeLa-M cells during telophase. Cells were grown on glass coverslips for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, anti-SUN2, anti-SUN1 (A) or anti-nesprin-2 (B) followed by fluorescently labelled secondary antibodies. Cells undergoing mitosis were identified by the appearance of their DNA and representative images were captured using Metamorph, deconvolved using Huygens Professional then some Z-stacks were selected using Fiji ImageJ. Arrows: LINC component clusters. Scale bar: 10 μm.
III. 3. Nesprin-2 does not have the exact same timing as neither SUN2 nor SUN1 in returning to the NE

III. 3.1. Developing cell lines expressing GFP-nesprin-2

The different LINC components were observed in pairs. The ability to examine all three proteins together would help reveal more details on their relative timings. A range of isotype-specific secondary antibodies were tested to visualize all three LINC components simultaneously, however the staining was not good enough and therefore stably transfected cell lines expressing inducible GFP-nesprin-2 were generated. Nesprin-2 was chosen because out of the three LINC components studied, the antibody for endogenous nesprin-2 did not give a clear stain nor did it detect nesprin-2 in western blot and thus it would be helpful to replace its use. Furthermore, nesprin-2 relies on the SUN domain to be located at the NE and has a higher mobility than the SUN proteins at the NE (Ostlund et al., 2009) which would be useful for live experiments such as FRAP.

The GFP tag was located at the N-terminus of nesprin-2 (Fig. 3.5A), as placing it in C-terminus would have been likely to disrupt the interaction with the SUN proteins. The N-terminal location may potentially disrupt some of the nesprin-2 interactions in the cytoplasm, but the spectrin repeats (SRs) that are most important with the interaction to the cytoskeleton (Djinovic-Carugo et al., 2002) are retained. The cDNA was coding for the nesprin-2α1 isoform, which features three SRs and lacks an N-terminal CH domain (Fig. 1.3).

The human nesprin-2α1 cDNA with a GFP coding sequence was sub-cloned in a pTRIPZ lentiviral vector with tet-ON inducible expression (Das et al., 2004). The recombinant lentiviral vector was cultivated in mouse 293T cells before infecting HeLa-M cells. Stably transfected cells were selected with puromycin and single cell clones were selected after serial dilutions. Overexpression of nesprin-2 caused it to localise to the ER due to saturation of SUN domain binding partners needed for NE retention (Ostlund et al., 2009). Therefore, a number of clonal cell lines were screened in order to identify one that expressed GFP-nesprin-2 at a level where it was localised solely to the NE. Staining with the nesprin-2 antibody (Fig. 3.5B) was used to quantify the total nesprin-2 expression level (endogenous plus GFP-nesprin-2) in stable GFP-nesprin-2 cells and parental HeLa-M (Fig. 3.5C). GFP-booster (nanobodies targeted to GFP) was used to enhance an otherwise weak GFP signal and revealed the GFP-nesprin-2 protein correctly localised to the NE without being present in the ER (Fig. 3.5B). Similarly, endogenous nesprin-2 was detected at the NE and there was no ER presence. Using ImageJ, the fluorescence intensity emitted by individual cells was measured. Anti-nesprin-2 staining in HeLa cells had an average fluorescence of 325.1 A.U. ± 17.7 per cell. Cells stably expressing GFP-nesprin-2 had an average fluorescence of 830 A.U. ± 35.9 (Fig. 3.5C). Thus, it was concluded that GFP-nesprin-2 is expressed 2.6-fold higher levels than the endogenous nesprin-2, which is relatively close to endogenous levels.

The anti-nesprin-2 antibody does not work for western-blot, but it was possible to use an anti-GFP antibody to visualise GFP-nesprin-2 in cell extracts. Cells were grown in the presence or absence of doxycycline and lysates were analysed by western blotting with anti-GFP (Fig. 3.5D). This confirmed
Figure 3.5 Cell lines stably expressing GFP-nesprin-2. (A) Cartoon illustrating the recombinant nesprin-2 protein used in this study and its estimated molecular weight. GFP: eukaryotic green fluorescent protein; TM: transmembrane domain; CC: coiled-coil domain; SRs: spectrin repeats. (B) Representative widefield fluorescence images of HeLa-M and stable GFP-nesprin-2 grown on glass coverslips for 24h, treated with doxycycline for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with anti-nesprin-2 followed by a fluorescently labelled secondary antibody, GFP-booster and DAPI. Several representative images of groups of cells were captured using MetaMorph then processed using Fiji ImageJ. Scale bar: 10 μm. (C) Average fluorescence intensity of nesprin-2 from the experiments described in B. Errors bars show SEM. n ≥ 17 cells over 3 independent experiments. A.U., arbitrary unit. (D) Western-blot analysis for GFP and tubulin in GFP-nesprin-2 cells grown for 24h then treated with doxycycline for 24h or untreated then harvested in SDS-PAGE sample buffer, run on an SDS-PAGE gel then transferred on a membrane. The membrane was incubated with mouse monoclonal anti-GFP and anti-tubulin antibodies followed by fluorescently labelled secondary antibodies. GFP-nesprin-2: about 85 kDa. Tubulin: about 55 kDa. Dox, doxycycline.

the presence of GFP-nesprin-2 at the expected molecular weight (about 85 kDa) in the lysates of doxycycline-induced cells

Finally, to observe the location of GFP-nesprin-2 in combination with SUN proteins at a higher resolution, cells stably expressing GFP-nesprin-2 were induced and stained with antibodies recognising SUN1 and SUN2. Cells in interphase were captured on a deltatvision microscope, and a maximum intensity Z-
projection was performed after deconvolution to have a sharp view of a flattened cell in a 3D space (Fig. 3.6). This revealed that GFP-nesprin-2 was distributed to the NE and overlapped perfectly with SUN1 and SUN2.

**III. 3.2. SUN1 and GFP-nesprin-2 overlap in telophase clusters free of SUN2**

In order to verify that GFP-nesprin-2 had the same distribution as nesprin-2 during telophase (Fig. 3.4A), cells stably expressing GFP-nesprin-2 were stained with antibodies recognising SUN1 and SUN2. Images of cells in telophase were captured on a deltavision microscope (Fig. 3.7A). As observed with endogenous nesprin-2, GFP-nesprin-2 appeared in the NE and in clusters that also contained SUN1 (Fig. 3.7B). In contrast, SUN2 was in the NE but could not be detected in the clusters (Fig. 3.7B). This observation suggests that nesprin-2 and SUN2 may not all be physically associated at this point in mitosis. In addition, it indicates that nesprin-2 may associate preferentially with SUN1 during telophase. This is interesting because SUN1 or SUN2 knockout mice are viable (Horn et al., 2013), however a SUN1 SUN2 double knockout is lethal (Lei et al., 2009) which was suggesting that the nesprins are not selective as to which SUN protein they bind to.
III. 3.3. SUN1 leaves the cleavage furrow sooner than GFP-nesprin-2 and SUN2

The higher resolution of the deltavision microscope combined with the GFP tag on nesprin-2 allows for a much clearer view of the timing of events around telophase. An event occasionally occurring during mitosis is an ‘anaphase lag’ (Pampalona et al., 2016) in which a lagging chromosome fails to be included in the reformed nucleus and forms a junction between daughter nuclei at the cleavage furrow. This event is not uncommon in HeLa cells and the distribution of LINC components was observed in these cells.

Cells stably expressing GFP-nesprin-2 were stained with antibodies recognising SUN1 and SUN2 and images of cells with a lagging chromosome were captured on a deltavision microscope (Fig. 3.8). GFP-nesprin-2 and SUN2 were associated with the lagging chromosome while SUN1 was not (Fig. 3.8, Figure 3.7 SUN1 and GFP-nesprin-2 overlap in telophase clusters without SUN2. (A) Representative deltavision fluorescence images of the telophase cleavage furrow of cells stably expressing GFP-nesprin-2. Cells were grown on glass coverslips for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster, anti-SUN2 and anti-SUN1 followed by fluorescently labelled secondary antibodies. Representative images of cells in telophase were captured using Metamorph, deconvolved using Huygens Professional then a maximum intensity Z-projection was performed using Fiji ImageJ. Scale bar: 10 μm. (B) Magnified images of the regions selected in A.
Figure 3.8 SUN1 leaves the cleavage furrow earlier than SUN2 and GFP-nesprin-2. Representative deltavision fluorescence images of the telophase cleavage furrow of cells stably expressing GFP-nesprin-2. Cells were grown on glass coverslips for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster, anti-SUN2 and anti-SUN1 followed by fluorescently labelled secondary antibodies. Representative images of the telophase cleavage furrow were captured using Metamorph, deconvolved using Huygens Professional then a maximum intensity Z-projection was performed using Fiji ImageJ. Arrow: the cleavage furrow. Scale bar: 10 μm.

arrows). Only approximately a dozen cells were seen with a lagging chromosome, but they all showed this clear pattern. These observations indicate that SUN1 and nesprin-2 also do not always interact throughout the whole process of cell division.

III. 3.4. Clusters of SUN1/nesprin-2 are located in the ER

Given that NE membrane proteins are redistributed into the ER upon NE breakdown, the clusters of SUN1 and nesprin-2 that were outside the NE during telophase could be in the ER (Fig. 3.7). In order to address this issue, cells stably expressing GFP-nesprin-2 were stained with antibodies recognising calreticulin, an ER localised protein. Images of cells in telophase were captured on a deltavision microscope (Fig. 3.9A). GFP-nesprin-2 in the NE or outside the NE was consistently continuous to regions enriched in calreticulin (Fig. 3.9B), confirming that the LINC components are bound to ER or NE membranes and not aggregating in the cytoplasm.

III. 3.5. SUN1 and GFP-nesprin-2 clusters persist in early G1

The clusters of nesprin-2 and SUN1 seen in telophase were not observed in interphase cell (Fig. 3.6), suggesting that the LINC components in these clusters (Fig. 3.7) may be fully incorporated back in the NE at some point between telophase and interphase. However, examination of cells in early G1 which follows telophase (Fig. 3.10A) revealed that distinct clusters of SUN1 and GFP-nesprin-2 were still
present whilst SUN2 had fully returned to the NE (Fig. 3.10B). This is interesting because by early G1, the daughter cells have separated and fully sealed their NE (Schooley et al., 2012). Thus, INM proteins such as SUN1 remaining in the ER would have to traverse the nuclear pores in order to regain NE localisation. The NPC barrier is usually believed to restricts free diffusion for domains larger than 60 kDa, although this view has been challenged and the barrier can be less restrictive during nuclear envelope reformation (Shimozomo et al., 2009). This means that if SUN1 and nesprin-2 proteins are present in the clusters when the NE has already finished its assembly, then they could potentially be trapped in the ER and unable to return to the NE unless they undergo disassembly.

III. 4. Effect of TorsinA depletion on the LINC complex during cell division

The results above provided evidence that GFP-nesprin-2 is not always associated with SUN domain proteins during late mitosis (Fig. 3.7; Fig. 3.8; Fig. 3.10). This raises the possibility that a mechanism
exists for the assembly and/or disassembly of the LINC complex during mitosis. The goal of the next experiments is to examine whether TorsinA plays a role in LINC assembly/disassembly during mitosis.

![Figure 3.10 Telophase clusters of SUN1 and GFP-nesprin-2 without SUN2 persist in early G1. (A) Representative deltavision fluorescence images of cells stably expressing GFP-nesprin-2 in early G1. Cells were grown on glass coverslips for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster, anti-SUN2 and anti-SUN1 followed by fluorescently labelled secondary antibodies. Cells in early G1 were identified by the appearance of their DNA, dimmer and larger than in telophase although smaller than late G1. Representative images of cells were captured using Metamorph, deconvolved using Huygens Professional then a maximum intensity Z-projection was performed using Fiji ImageJ. Scale bar: 10 μm. (B) Magnified images of the regions selected in A.]

A usual way to abolish a protein’s function is to perform a knock-down, thus a TorsinA knock-down was developed.

III. 4.1. Optimisation for TorsinA knock-down

In order to determine whether TorsinA plays a role in the LINC complex function or dynamics, siRNA was used to knock-down endogenous TorsinA. First, it was important to optimize the conditions for knocking down TorsinA in HeLa cells. Cells were plated at different densities and after 24 hours were
treated with 10 nM or 20 nM TorsinA siRNA or scrambled siRNA as a control or they were transfected with a vector expressing TorsinA. After 48h, cells were harvested and subjected to western blotting with anti-TorsinA and anti-actin antibodies, followed by fluorescently labelled secondary antibodies that were visualised by scanning in an Odyssey CLx (Fig. 3.11). TorsinA antibody staining gave a broad range of non-specific bands, and a major band at the expected size for TorsinA (about 37 kDa) that migrated at the same position as the overexpressed TorsinA (Fig. 3.11, TorsinA lane). This intensity of this immunoreactive band decreased in cells treated with TorsinA siRNA (Fig. 3.11, 10 nM siRNA and 20 nM siRNA lanes) confirming that it does indeed represent endogenous TorsinA. In order to calculate the efficiency of siRNA mediated knock-down, the TorsinA signal intensity was determined and normalised to the actin signal, and expressed relative to the scrambled siRNA treated cells (Fig. 3.11, control lane). The knock-down efficiency of TorsinA was in the range of 80-85% 48h after treatment (Fig. 3.11), with 10 nM siRNA producing a similar level of knock-down to 20 nM. A slightly decreased efficiency was noticed when 80,000 cells were seeded, and longer (72h) siRNA treatment did not increase the efficiency of TorsinA depletion (data not shown). A seeding density of 60,000 cells and 10 nM TorsinA siRNA was selected as the optimal protocol.

A limitation of this quantification of TorsinA knock-down efficiency is that the quantity of proteins loaded in the experiment to optimise siRNA (Fig. 3.11) was outside the linear range for the actin antibody (Fig. 2.3), and thus the quantification of actin from one lane to the other was not entirely reliable.

**Figure 3.11 TorsinA knock-down optimisation after 48h treatment.** Western-blot analysis for TorsinA knock-down. Cells were seeded at a range from 20,000 to 80,000 per well and were treated with 10 nM or 20 nm TorsinA siRNA. Scrambled siRNA was used as a control. Overexpressed TorsinA was used as a positive control. Cells were harvested in SDS-PAGE sample buffer, run on an SDS-PAGE gel then transferred on a membrane. The membrane was incubated with mouse monoclonal anti-actin and rabbit polyclonal anti-TorsinA antibodies followed by fluorescently labelled secondary antibodies. Quantification was normalised to actin then expressed relative to control. Knock-down efficiency was indicated under each lane. TorsinA: about 37 kDa. Actin: about 42 kDa. Arrow: TorsinA. 1k = 1,000.
III. 4.2. GFP-nesprin-2 during the mitotic cycle when TorsinA is reduced

In order to determine whether TorsinA has an effect on the LINC complex during the cell cycle, TorsinA was knocked down. Cells stably expressing GFP-nesprin-2 were transfected with TorsinA siRNA, fixed in paraformaldehyde and stained with antibodies recognising SUN1. All phases of the mitotic cycle were captured on a deltavision microscope, and a maximum intensity Z-projection was performed after deconvolution to have a sharp view of a flattened cell in a 3D space (Fig. 3.12).

Figure 3.12 TorsinA depletion does not have a striking impact on the cell. (A) Representative deltavision fluorescence images of cells stably expressing GFP-nesprin-2 undergoing mitosis. Cells were grown on glass coverslips for 24h, transfected with TorsinA siRNA or scrambled siRNA and treated with doxycycline for 48, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster and anti-SUN1 followed by fluorescently labelled secondary antibodies. Cells undergoing mitosis were identified by the appearance of their DNA and representative images were captured using Metamorph, deconvolved using Huygens Professional then a maximum intensity Z-projection was performed using Fiji ImageJ. Merged pictures included GFP-nesprin-2 in the green channel, SUN1 in the red channel and DAPI in the blue channel. Scale bar: 10 μm. The only difference is that some telophase cells have a larger number of clusters, however this phenotype is only observed in 18% of the cells (counted for 17 cells). (B) Western-blot showing efficient TorsinA knock-down of the cells from the experiments described in A. TorsinA: 37 kDa. Cells were harvested in SDS-Page sample buffer, run on an SDS-PAGE gel then transferred on a membrane. The membrane was incubated with anti-TorsinA antibodies followed by fluorescently labelled secondary antibodies. TorsinA: about 37 kDa.
Except for telophase, there was no noticeable difference between TorsinA knock-down and scrambled siRNA. A limitation of the TorsinA knock-down was the lack of an antibody recognising TorsinA for immunofluorescence microscopy, making it impossible to distinguish whether the observed cells were efficiently depleted for TorsinA. Furthermore, the knock-down may not have been effective enough for the absence of TorsinA to have a noticeable effect during the cell cycle.

III. 5. Overexpressing LAP1-V5-BioID causes an increase in the numbers of GFP-nesprin-2 clusters

As an alternative approach to altering TorsinA function, LAP1 was targeted. LAP1 is a cofactor that is required for TorsinA activity (Zhao et al., 2013) and hence altering LAP1 would be predicted to impact on TorsinA function.

LAP1-V5 with a C-terminal biotin ligase (BioID) was generated as part of a separate set of experiments (Chapter 5). LAP1-V5-BioID (Fig. 3.13) was transfected into cells stably expressing GFP-nesprin-2 to determine whether it has an effect on the distribution of LINC components during the mitotic cycle. Stably transfected cells were stained with antibodies recognising the V5 tag and images of cells in all phases of the mitotic cycle were captured on a deltalvision microscope (Fig. 3.14A). There was no noticeable difference between cells transfected with LAP1-V5-BioID and untransfected cells in most cases. However, in telophase cells a dramatic increase of the number of clusters was observed (Fig. 3.14A, arrows for GFP-nesprin-2 in telophase). Interestingly, LAP1-V5-BioID overlapped with the clusters containing GFP-nesprin-2 (Fig. 3.14A, arrows for LAP1-V5-BioID in telophase, Fig. 3.14B, magnified images). As a control to determine whether presence of the biotin ligase within the NE lumen could be responsible for the phenotype, a construct that targets and maintains the biotin ligase in the ER-NE lumen system was used. This construct, SS-V5-BioID-KDEL possesses an N-terminal signal sequence to direct it to the ER lumen and a KDEL motif to mediate retrieval from the Golgi. Transient expression of this protein had no effect on GFP-nesprin-2 distribution in telophase (Fig. 3.14B).

Figure 3.13 Cartoon illustrating the recombinant LAP1-V5-BioID protein. TMB: transmembrane domain; AF: arginine finger; N-ter: N-terminus; C-ter: C-terminus; INM: inner nuclear membrane. V5 tag is included with the biotin ligase.
Figure 3.14 LAP1-V5-BioID increases the number of GFP-nesprin-2 telophase clusters. (A) Representative deltavision fluorescence images of cells stably expressing GFP-nesprin-2 transfected with LAP1-V5-BioID undergoing mitosis. Cells were grown on glass coverslips for 24h, transfected with LAP1-V5-BioID or untransfected and induced with doxycycline for 24h. fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster and anti-V5 followed by fluorescently labelled secondary antibodies. Cells undergoing mitosis were identified by the appearance of their DNA. Representative images of cells were captured using Metamorph, deconvolved using Huygens Professional then a maximum intensity Z-projection was performed using Fiji ImageJ. Scale bar: 10 μm. Arrows: clusters of GFP-nesprin-2 and clusters of LAP1-V5-BioID. (B) Representative deltavision fluorescence images of cells stably expressing GFP-nesprin-2 transfected with SS-V5-BioID-KDEL in telophase obtained in the same conditions as the experiment in A, alongside the magnified telophase images of LAP1-V5-BioID from A.
Figure 3.15 LAP1-V5-BioID increases the number of GFP-nesprin-2 clusters in early G1. (A) Representative snapshot widefield fluorescence images of cells stably expressing GFP-nesprin-2 transfected with LAP1-V5-BioID in early G1. Cells were grown on glass coverslips for 24h, transfected with LAP1-V5-BioID or untransfected and induced with doxycycline for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster and anti-V5 followed by fluorescently labelled secondary antibodies. Cells in early G1 were identified by the appearance of their DNA, dimmer and larger than in telophase although smaller than late G1. Representative images of cells were captured using Metamorph then processed using Fiji ImageJ. Scale bar: 10 μm. (B) Count of the number of visible clusters from the experiments described in A. n = 13 cells over 3 independent experiments. Error bars show mean ± SEM. **, P < 0.01 using a t-test with Welch's correction. Transfecting LAP1-V5-BioID significantly increases the number of clusters in early G1 cells.

In order to provide a more quantitative measurement of the effect of LAP1-V5-BioID on GFP-nesprin-2 clusters in telophase, cells were grown and transfected as above except that images of cells in early G1 were captured on a snapshot widefield microscope (Fig. 3.15A). The distribution of GFP-nesprin-2 in these images was categorised on the basis of how many GFP-nesprin-2 clusters were present. The number of clusters was counted, and the cells were separated in two categories according to whether they contained more than 5 clusters or less (Fig. 3.15B). This analysis revealed that 92.7% of the cells transfected with LAP1-V5-BioID had more than 5 clusters, whereas none of the untransfected cells had more than 5 clusters. This difference between transfected and untransfected was highly significant (P < 0.0051).

These results suggest that overexpression of LAP1-V5-BioID may cause nesprin-2 and potentially other LINC components to remain stuck in the ER following mitosis and NE reassembly. A question raised is
whether the LAP1-V5-BioID construct is functional. The biotin ligase and the V5 tag are located at the C-terminus of the protein (Fig. 3.13). The N-terminus of this transmembrane protein extends to the nucleoplasm, however the luminal C-terminal domain of LAP1 is necessary for the interaction with TorsinA in the perinuclear space (Zhao et al., 2013). Consequently, the biotin ligase and the V5 could have hindered the function of LAP1 and LAP1-V5-BioID could have had a dominant negative effect.

**III. 6. Discussion**

The initial goal of the project was to determine whether natural occurrences of the LINC complex undergoing assembly or disassembly could be observed. During mitosis, when the cells enter prophase, INM proteins are phosphorylated and lose their interactions with the lamin and the chromatin (reviewed in Güttinger et al., 2009; Ungricht et al., 2017). SUN proteins are phosphorylated as well, and they lose their nucleoplasmic interactions, which could contribute to the disassembly of the nuclear lamina (Patel et al., 2014). Furthermore, functional LINC complexes with a SUN-KASH interaction are required for the removal of membranes from the chromatin (Turgay et al., 2014), indicating that the LINC complex is a key contributor to mitotic progression. Little is known about the fate of the LINC complex during the rest of mitosis, such as whether it remains assembled through the rest of the cycle, or whether it is involved in the resealing of the NE. The nuclear envelope is constructed by the re-organization of the mitotic ER on the chromatin (reviewed in Schooley et al., 2012). Dephosphorylation of INM proteins quickly follows, regulating the recruitment of more NE proteins (review in Schooley et al., 2012). NPCs are fully constituted, allowing the passage of lamins to complete the assembly of the nuclear lamina (reviewed in Ungritch et al., 2017). Following the end of telophase and cytokinesis, the daughter cells should have fully reformed NE (review in Schooley et al., 2012). SUN2 localisation is dependent on the lamins (Hasan et al., 2007; Ostlund et al., 2009) while SUN1 is dependent on the NPCs to localise to the NE (Liu et al., 2007). During telophase, SUN2 was recruited to the newly formed NE earlier than SUN1 (Fig. 3.4), suggesting that SUN2 proteins could have been dephosphorylated and regained their nucleoplasmic interactions prior to SUN1, or that SUN2 was recruited to the assembling lamins prior to SUN1 being recruited by the NPCs. The NPCs may have been incomplete at that point, however Nup153 seemed to have been fully recruited to the NE while SUN1 had not been fully incorporated yet (Fig. 3.1, telophase).

Notwithstanding the difference in timing to get back to the NE, SUN1 and SUN2 also differed by the existence of clusters enriched in SUN1 and nesprin-2 from which SUN2 was absent (Fig. 3.4), as well as nesprin-2 not being recruited to the NE at the same rate as SUN2 considering that SUN2 was fully recruited to the newly formed NE while nesprin-2 was not (Fig. 3.4). These observations suggest that at least a population of nesprin-2 and SUN2 proteins may not be physically associated at this point in cell division. Furthermore, GFP-nesprin-2 and SUN2 were associated with the lagging chromosome while SUN1 was not (Fig. 3.8), indicating that SUN1 and nesprin-2 proteins also do not always interact throughout mitosis. A previous study (Patel et al., 2014) suggested that the LINC complex remains intact during mitosis through interaction of the SUN domain with the KASH domain, however this study looked biochemically at cells arrested in prometaphase with nocodazole. The implication
would be that the LINC complex starts mitosis assembled, then at some point following NEBD there is a possibility that LINC components disassemble and reassemble in a dynamic process. These observations that nesprin-2 was found alternatively without SUN2 or without SUN1 is also interesting because SUN1 or SUN2 knockout mice are viable (Horn et al., 2013), however a SUN1 SUN2 double knockout is lethal (Lei et al., 2009) which was suggesting that the nesprins were not selective as to which SUN protein they bind to. However, if the nesprins are preferentially interacting with one member of the LINC complex rather than the other, there could be a mechanism to regulate this preference. A hypothesis could be that phosphorylation impacts the SUN-KASH interaction, then differential dephosphorylation rates determines which SUN protein the nesprins are able to interact with. A question remains whether LINC complexes are involved in the reformation of the NE at the end of mitosis, possibly for the assembly of the lamins network or the formation of the NPCs. Furthermore, differential dephosphorylation rates could be required to complete these functions in the correct order.

Clusters containing SUN1 and nesprin-2 persisted until early G1 (Fig. 3.10) and disappeared in interphase (Fig. 3.6). The implication is that either the proteins in the clusters were later incorporated to the NE, or they were degraded. If the NE membrane was sealed, then any new ER membrane connecting to the NE would be continuous with the ONM and not the INM (Fig. 1.1). Consequently, SUN1 proteins would freely translocate to the ONM but not to the INM as the NPC barrier is usually believed to restrict free diffusion for domains larger than 60 kDa (Swanson and McNeil, 1987) and SUN1 proteins are about 98 kDa. This view has been challenged and the barrier can be less restrictive during NE reformation (Shimozomo et al., 2009), however the clusters in early G1 are past that stage and towards late mitosis the cells should already have a fully reformed NE (review in Schooley et al., 2012). To accommodate with size constraints dictated by the lateral channels of NPCs, the nucleoplasmic domain of an inner nuclear membrane protein influences targeting (Soullam and Worman, 1995). SUN proteins have several mechanisms to target them to the INM: a predicted INM-SM (inner nuclear membrane-sorting motif), a SUN-NELS (SUN-nuclear envelope localisation signal), and a cNLS (classic nuclear localisation signal) (reviewed by Tapley and Starr, 2013). While a newly synthesised SUN protein will be directed to the INM, SUN proteins in LINC complexes interact with the nesprins with a 3:3 stoichiometry (Sosa et al., 2012) and the nesprins are transmembrane proteins of the membrane opposite SUN1. SUN1 proteins in the clusters are likely interacting with the nesprins, thus if SUN1 was to attempt translocation from the ER to the ONM to the INM while tethering nesprin-2 (Fig. 3.16), that would be physically impossible as the nesprins are bound to a membrane and not transportable through the perinuclear space as SUN1 migrates. In conclusion, LINC complexes that get trapped in the ER after NE resealing are likely to get degraded except if there is a mechanism to disassemble the complexes in the ER. This theory that LINC complexes mislocalised in the ER get degraded will be followed in Chapter IV. Conversely, there exist small nesprin-1 and nesprin-2 KASH variants likely capable of NE entry and localisation to the INM (Mislow et al., 2002; Wheeler et al., 2007) which interact with the N-terminal domain of SUN proteins (Haque et al., 2010), therefore they could theoretically be transported to the NPC alongside SUN1 without remaining ‘trapped’ in clusters.

The second aim of this study was to examine whether perturbing the AAA+ ATPase TorsinA or its cofactor LAP1 has an effect on the assembly or disassembly of the LINC components during telophase.
LINC complexes are formed with a 3:3 stoichiometry between SUN domain proteins and KASH domain proteins, and two facing cysteines could be implicated in a disulphide bridge that would require a protein.

Figure 3.16 Model for SUN1 and nesprin-2 proteins trapped in the ER during early G1. Cartoon illustrating SUN1 and nesprin-2 proteins in the ER and NE during early G1. While there are LINC complexes formed at the NE, SUN1 and nesprin-2 are still detected in the ER. Hypothetical LINC complexes that are assembled by their C-terminus in the ER cannot physically switch from one membrane to the next (rectangles). ER: endoplasmic reticulum; NE: nuclear envelope; INM: inner nuclear membrane; ONM: outer nuclear membrane.

with redox capabilities to link or unlink (Sosa et al., 2012), while TorsinA harbors a redox sensor motif (Zhu et al., 2010). Considering that LINC components may have been disassociated at some point between early mitosis and late mitosis (Fig. 3.4; Fig. 3.8) and that LINC complexes trapped in the ER after NE resealing (Fig. 3.10; Fig. 3.15) may require disassembly, the effect of TorsinA was investigated during late mitosis. Knocking down TorsinA may have a subtle effect in increasing the number of clusters. A caveat to these experiments is that the level of knock-down was not sufficient to trigger an effect for TorsinA, combined to the lack of antibodies to select cells depleted for TorsinA. However, overexpressing a recombinant LAP1 severely increased the number of clusters remaining in the ER up until early G1. A hypothesis could be that contrary to facilitating disassembly of the LINC complex, LAP1 strengthened the interaction by creating a disulphide bridge between the cystines of the SUN and the KASH domain (Sosa et al., 2012), thus trapping the LINC complexes in the ER as the complexes would not be able to pass the NPCs of the sealed NE (Fig. 3.16). A limitation to this interpretation is the
uncertainty whether the LAP1 recombinant was functional, or acted as a dominant negative. The biotin ligase fused to the lumenal domain of LAP1 could have hindered the functional interaction with TorsinA, as the lumenal domain is required for the interaction between TorsinA and LAP1 (Zhao et al., 2013).

TorsinA was overexpressed on its own and there was no effect on the number of clusters (data not shown), although it may be because more TorsinA on its own without an activator should not mechanically increase the rate of its ATPase activity. A similar experiment with an untagged LAP1 coupled with an antibody recognising LAP1 would validate whether LAP1 increases the number of SUN1/nesprin-2 clusters. Nonetheless, this result suggests that the combination of TorsinA/LAP1 may modulate the assembly of the LINC complex, a hypothesis that will be followed in Chapter IV.
IV. LINC COMPLEX DISASSEMBLY AND DEGRADATION

IV. 1. Introduction

The previous chapter suggested that the LINC complex is dynamic and is likely to undergo disassembly and reassembly during mitosis. TorsinA/LAP1 may have an effect on this process. During telophase, the LINC components SUN1 and nesprin-2 are recruited to the NE later than SUN2, and partially remain in the ER forming clusters visible by microscopy. The fate of LINC components that fail to return to the NE is unknown; they could conceivably undergo disassembly and return to the NE through NPCs or could be permanently excluded from the NE and removed from the ER for example by proteolytic degradation. The first aim of this chapter was to determine the fate of LINC components which fail to return to the NE at the end of mitosis.

IV. 2. Nesprin-2 is subjected to proteasomal degradation

SUN1 and nesprin-2 form clusters in the ER during telophase, and these clusters persist in early G1 before disappearing as the cell proceeds into interphase. One possible fate of these ER localised proteins is degradation by the proteasome. This possibility was tested by inhibiting the proteasome and observing the levels of nesprin-2 in the ER.

Cells stably expressing GFP-nesprin-2 were treated with the proteasome inhibitor PSII for 7h or untreated and images of these were captured on a snapshot widefield microscope (Fig. 4.1A). Analysis of cell population was limited to interphase as proteasome inhibitors stop cell cycle progression (Lu et al., 2006). Without treatment, GFP-nesprin-2 was solely present in the ‘ring shaped’ NE. In the presence of proteasome inhibitor, GFP-nesprin-2 appeared both in the NE and what seemed to be the ER network. This could mean either that GFP-nesprin-2 had been retargeted from the NE to the ER and the overall levels of GFP-nesprin-2 had not changed, or that a surplus of GFP-nesprin-2 accumulated in the ER due to not being degraded. In order to verify whether the total level of GFP-nesprin-2 increased upon proteasome inhibition, a quantitative approach was necessary. Intensity of the fluorescence of cells treated with PSII or untreated was measured and compared (Fig. 4.1B). The average fluorescence intensity per cell significantly increased to levels of approximately 138% from 573681 A.U. to 790457 A.U. when the proteasome was inhibited (P < 0.05). Cell extracts were run on a gel and scanned with a in a ChemiDoc Imaging System (Bio-Rad) to visualise GFP fluorescence. A band was revealed at 80 kDa, consistent with GFP-nesprin-2 (Fig. 4.2A). The GFP fluorescence was then normalised to the total protein measured by Coomassie blue stain (Fig. 4.2B). The level of GFP-nesprin-2 increased to levels of approximately 163% in lysates of cells treated with proteasome inhibitor (P = 0.14). These results demonstrate that GFP-nesprin-2 is subjected to proteasomal degradation.
Figure 4.1 Proteasome inhibition causes a mislocalisation and accumulation of GFP-nesprin-2.
Cells stably expressing GFP-nesprin-2 were grown on glass coverslips for 24h, treated with doxycycline for 24h, treated with PSII for 7h or untreated, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI and GFP-booster. (A) Representative images were captured on a snapshot widefield microscope using Metamorph then processed using Fiji ImageJ in order to have a large number of cells to quantify. Scale bar: 10 μm. In the presence of proteasome inhibitor, GFP-nesprin-2 was present in the ER. (B) Average fluorescence intensity per cell from the experiments described in A. n ≥ 59 cells over 3 independent experiments. Error bars show mean ± SEM. *, P < 0.05 using a t-test with Welch’s correction. A.U., arbitrary units.
Figure 4.2 GFP-nesprin-2 is subjected to proteasomal degradation. Cells stably expressing GFP-nesprin-2 were grown on a 12-wells plate for 24h, treated with doxycycline for 24h, harvested with SDS-PAGE sample buffer and run on an SDS-PAGE gel. (A) GFP scan of the gel. GFP-nesprin-2: 80 kDa. (B) Fluorescence intensity from the GFP signal in the experiment described in A was normalised to the Coomassie blue stained total proteins and expressed as a percentage of the fluorescence intensity in untreated cells. n = 3 independent experiments. Error bars show mean ± SEM. ns, not significant using a t-test with Welch’s correction.

GFP-nesprin-2 is expressed 2.6-fold higher levels than the endogenous nesprin-2 (Fig. 3.5C) and has a GFP tag that may affect how the recombinant behaves, and thus this conclusion needs to be verified with endogenous nesprin-2 as well. HeLa-M cells were grown on glass coverslips, treated with the proteasome inhibitor PSII for 7h or untreated, fixed in paraformaldehyde and stained with antibodies recognising nesprin-2. Representative images of the cells were captured on a snapshot widefield microscope (Fig. 4.3A). Without treatment, nesprin-2 was enriched at the nucleus and faint outside of it. In the presence of proteasome inhibitor, nesprin-2 was enriched in the ‘ring shaped’ NE and increased slightly in the rest of the cell when compared with untreated cells. Accumulation of nesprin-2 outside the NE of cells treated with PSII was less visible than in GFP-nesprin-2 cells treated with PSII (Fig. 4.1A), probably due to the lower levels of nesprin-2 present in the cell (Fig. 3.5C) and the antibody stain of anti-nesprin-2 not being ideal. The intensity of the fluorescence of cells treated with PSII or untreated was measured and compared (Fig. 4.3B). The average fluorescence intensity per cell increased to levels of approximately 181% from 175132 A.U. to 316120 A.U. when the proteasome was inhibited. The difference has a p-value of 0.09 which is not statistically significant but still highly suggestive at the P < 0.10 level.

Taken together, these results indicate that nesprin-2 undergoes proteasome mediated degradation. The observation that GFP-nesprin-2 accumulates in what seems to be the ER following inhibition of proteasomal degradation indicates that this may be the site of degradation, which would suggest that nesprin-2 remaining in the ER is extracted from the membrane and degraded by the proteasome.
Figure 4.3 Proteasome inhibition may cause an accumulation of nesprin-2. HeLa-M cells were grown on glass coverslips for 48h, treated with PSII for 7h or untreated, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI and anti-nesprin-2. (A) Representative images were captured on a snapshot widefield microscope using Metamorph then processed using Fiji ImageJ in order to have a large number of cells to quantify. Scale bar: 10 μm. (B) Average fluorescence intensity per cell from the experiments described in A. n ≥ 54 over 3 independent experiments. Error bars show mean ± SEM. ns, not significant using a t-test with Welch’s correction. A.U., arbitrary units. Without proteasomal degradation, the average level of nesprin-2 per cell increased.
However, nesprin-2 could also be degraded at the NE considering that if degradation is inhibited, the excess nesprin-2 that builds up in the NE could spill out into the ER due to saturation of the SUN sites.

**IV. 3. Displacement of GFP-nesprin-2 from the NE**

The results above show that nesprin-2 is a substrate for proteasomal degradation. Targeting nesprin-2 for degradation could conceivably occur either because the protein is not tethered to a SUN domain and the ‘free’ conformation is recognised by degradation machinery such as ERAD, and/or nesprin-2 is in stable LINC complexes that are trapped in the ER following NE resealing, and those complexes unable to pass through the NPC are targeted for degradation.

In order to test this idea, variants of LINC components were used to compete with the KASH or SUN domains, and SUN1 was knocked down to remove tethering sites from the NE.

**Figure 4.4 Model for a KASH recombinant protein GFP-KASH4 saturating the SUN domains at the NE.**

(A) Cartoon illustrating the recombinant KASH4 protein used in this study and its estimated molecular weight. KASH: KASH domain; TM: transmembrane domain; GFP: green fluorescent protein; N-ter: N-terminus; C-ter: C-terminus. The transmembrane domain locates the protein to the ER and NE, and the GFP is located on the side of the cytoplasm. (B) Cartoon illustrating SUN and Nesprin proteins in the ER and NE. A recombinant KASH protein GFP-KASH4 saturating most SUN domains would disrupt the native LINC complexes and cause many nesprins to relocalise in the ER. ER: Endoplasmic Reticulum. NE: Nuclear Envelope.
IV. 3.1. KASH4 recombinant protein does not affect GFP-nesprin-2

A recombinant GFP-KASH4 only featuring the characteristic KASH4 domain of mouse nesprin-4 was used (Fig. 4.4A). The transmembrane domain of KASH4 allows it to insert into the ER membrane and localise the C-terminal KASH domain in the ER/NE lumen where it can interact with SUN domain proteins with the GFP tag exposed to the cytoplasm. Thus, overexpression of GFP-KASH4 is expected to saturate the SUN domains in the INM thereby displacing endogenous nesprins from LINC complexes and mislocalising them to the ER (Fig. 4.4B). It would then be possible to test whether the relocated nesprin-2 was degraded by the proteasome.

Inducible GFP-KASH4 was transiently transfected into HeLa cells and a western blot revealed a band at approximately 37 kDa (Fig. 4.5A) consistent with GFP-KASH4. The recombinant protein was observed on a widefield microscope and appeared successfully enriched in the NE and showed a typical ER distribution (Fig. 4.5B).

Figure 4.5 GFP-KASH4 is a recombinant protein located to the ER and NE. Cells were grown on glass coverslips for 24h, transfected with GFP-KASH4 and treated with doxycycline for 24h or untreated and fixed with methanol. (A) Western-blot showing overexpression of the recombinant protein in the cells described in A. Cells were harvested in SDS-PAGE sample buffer and run on an SDS-PAGE gel then transferred on a membrane. The membrane was incubated with an anti-GFP antibody followed by fluorescently labelled secondary antibodies. GFP-KASH4: 37 kDa. (B) Representative widefield fluorescence images of HeLa cells transfected with GFP-KASH4. Scale bar: 10 μm.

Considering it was not possible to discriminate between GFP-KASH4 and GFP-nesprin-2 by microscopy, the cells stably expressing GFP-nesprin-2 were transfected with GFP-KASH4, harvested and lysates were run on an SDS-PAGE gel followed by scanning to visualise in-gel fluorescence (Fig. 4.6A). Due to the difference in size (80 kDa for GFP-nesprin-2 and 37 kDa for GFP-KASH4), GFP-nesprin-2 could be quantified (Fig. 4.6B). This revealed that the levels of GFP-nesprin-2 when GFP-KASH4 was overexpressed were 96.4% of the untransfected cells (P = 0.9). The lack of an effect of GFP-KASH4 on the levels of GFP-nesprin-2 may be due to the low expression of the GFP-KASH4 recombinant protein, thus failing to compete with GFP-nesprin-2 for the SUN sites.
Figure 4.6 Overexpressing GFP-KASH4 has no effect on the levels of GFP-nesprin-2. (A) Cells stably expressing GFP-nesprin-2 were grown on 12-well plates for 24h, transfected with GFP-KASH4 or untransfected, treated with doxycycline for 24h, harvested with SDS-PAGE sample buffer and run on an SDS-PAGE gel. (A) GFP scan showing the same levels of GFP-nesprin-2 whether the cells have been transfected with GFP-KASH4 or untransfected. Black arrow: GFP-nesprin-2, 80 kDa; white arrow: GFP-KASH4, 37 kDa. (B) Fluorescence intensity of the GFP-nesprin-2 band was quantified and normalised to total protein stained with Coomassie blue, then expressed as a percentage of the levels in untransfected cells. n = 3 independent experiments. Error bars show mean ± SEM. ns, not significant using a t-test with Welch’s correction.

IV. 3.1. SUN1 knock-down decreases GFP-nesprin-2 levels

As an alternative to overexpression of GFP-KASH4, knock-down of SUN1 was used to induce mislocalisation of nesprin-2 to the ER. Instead of having the SUN domains saturated, there would be less SUN sites to tether the nesprins in the NE, and nesprins would be predicted to be localised in the ER.
**Figure 4.7 SUN1 knock-down.** Western-blot analysis for SUN1 knock-down. 60,000 cells per well of cells stably expressing GFP-nesprin-2 in a 12-well dish were treated with 10 nM SUN1 siRNA for 48h. Scrambled siRNA was used as a control. Cells were harvested in SDS-PAGE sample buffer and run on an SDS-PAGE gel then transferred on a membrane. (A) The membrane was incubated with Revert and a mouse monoclonal anti-SUN1 antibody followed by fluorescently labelled secondary antibodies. SUN1: 98 kDa. (B) The membrane was incubated with Revert and a rabbit polyclonal anti-SUN1 antibody followed by fluorescently labelled secondary antibodies. SUN1: 98 kDa. (C) Fluorescence intensity of the mouse anti-SUN1 band was quantified and normalised to the total protein stained with Revert, then expressed as a percentage of the levels in cells treated with scrambled siRNA. n = 3 independent experiments. Error bars show mean ± SEM. *, P < 0.05 using a t-test with Welch’s correction. (D) Fluorescence intensity of the rabbit anti-SUN1 band was quantified and normalised to the total protein stained with Revert, then expressed as a percentage of the levels in cells treated with scrambled siRNA. n ≥ 3 independent experiments. Error bars show mean ± SEM. ns, not significant using a t-test with Welch’s correction.
Cells expressing GFP-nesprin-2 were treated with 10 nM SUN1 siRNA or scrambled siRNA for 48h, and cell lysates were analysed by SDS-PAGE. Blotting was performed with two SUN1 antibodies available, a mouse monoclonal anti-SUN1 (Fig. 4.7A) and rabbit polyclonal anti-SUN1 (Fig. 4.7B). Quantification of the signal intensity revealed an efficient knock-down of SUN1, despite the estimated knock-down being slightly different with the two antibodies. The mouse anti-SUN1 measured a 90% reduction relative to the control (statistically significant with a p-value of 0.04) (Fig. 4.7C), whilst the rabbit anti-SUN1 measured a 71% reduction (not significant with P = 0.028) (Fig. 4.7D).

Cells used to test the SUN1 knock-down (Fig. 4.7) were also quantified for GFP-nesprin-2 (Fig. 4.8A) and the levels of GFP-nesprin-2 when SUN1 was knocked-down were reduced to 48% of the levels of GFP-nesprin-2 in cells treated with scrambled siRNA (Fig. 4.8B), however due to variance between the samples the result is not statistically significant with a p-value of 0.16.

Representative images of cells treated with SUN1 siRNA or scrambled siRNA were captured on a snapshot widefield microscope (Fig. 4.9). Cells transfected with scrambled siRNA had a ‘ring shaped’ NE localisation for SUN1. In SUN1 knock-down cells, SUN1 was more or less lost from the NE and the ‘ring shape’ did not appear as clear as in control cells. Quantification of the fluorescence intensity of SUN1 (Fig. 4.10A) revealed a 62% knock-down (from 692919 A.U. to 262986 A.U., a difference statistically significant with a p-value of 0.0003). GFP-nesprin-2 in cells transfected with scrambled siRNA (Fig. 4.9) also appeared in the ‘ring shaped’ NE, with occasional bleeding outside of the NE. GFP-nesprin-2 in cells treated with SUN1 siRNA also appeared in the ‘ring shape’ NE, however the signal seemed weaker for some of the cells. Conversely, GFP-nesprin-2 seemed slightly enriched in the ER of cells with a SUN1 knock-down. To confirm whether GFP-nesprin-2 levels were reduced in cells treated with SUN1 siRNA, a quantitative measurement was performed (Fig. 4.10B) and it was determined that the levels of GFP-nesprin-2 when SUN1 was knocked-down were reduced to 61% of the levels of GFP-nesprin-2 in cells with scrambled siRNA (from 994904 A.U. to 608295 A.U., a difference statistically highly significant with a p-value of 0.0072).

To confirm whether GFP-nesprin-2 unable to reach the NE was degraded by proteasomal degradation, cells knocked-down for SUN1 were treated with the proteasome inhibitor PSII (Fig. 4.11). Inhibition of proteasomal degradation did not seem to affect the localisation of SUN1, however GFP-nesprin-2 presence in what seems to be the ER dramatically increased in comparison to untreated cells. The average fluorescence intensity per cell was measured and compared (Fig. 4.12) revealing that the levels of GFP-nesprin-2 in cells treated with PSII were increased to 265% the level of untreated cells (from 608295 A.U. to 1613460 A.U., statistically significant with a p-value of 0.002), confirming that GFP-nesprin-2 mislocalised to the ER by SUN1 depletion was degraded by the proteasome. Degradation of GFP-nesprin-2 in the ER could be due to the lack of a SUN partner causing it to be recognised by the ER quality control.
Figure 4.8 SUN1 knock-down may decrease the levels of GFP-nesprin-2. (A) Cells stably expressing GFP-nesprin-2 were grown on 12-well plates for 24h, treated with doxycycline and SUN1 or scrambled siRNA for 48h, harvested with SDS-PAGE sample buffer and run on an SDS-PAGE gel. After transfer, the membrane was blotted with Revert and anti-GFP followed by fluorescently labelled secondary antibodies and scanned. GFP-nesprin-2: 80 kDa. (B) Fluorescence intensity from the GFP band was quantified and normalised to total protein stained with Revert then expressed as a percentage of the levels in cells treated with scrambled siRNA. n = 3 independent experiments. Error bars show mean ± SEM. ns, not significant using a t-test with Welch’s correction.
Figure 4.9 SUN1 knock-down may displace GFP-nesprin-2. Cells stably expressing GFP-nesprin-2 were grown on glass coverslips for 24h, treated with doxycycline and SUN1 or scrambled siRNA for 48h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster and rabbit anti-SUN1 followed by fluorescently labelled secondary antibodies. Representative images were captured on a snapshot widefield microscope using Metamorph then processed using Fiji ImageJ in order to have a large number of cells to quantify. Arrows: nuclei with reduced SUN1. Scale bar: 10 µm.
Figure 4.10 SUN1 knock-down decreases the levels of GFP-nesprin-2. Cells stably expressing GFP-nesprin-2 were grown on glass coverslips for 24h, treated with doxycycline and SUN1 or scrambled siRNA for 48h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster and rabbit anti-SUN1 followed by fluorescently labelled secondary antibodies. (A) Average fluorescence intensity per cell of anti-SUN1 from the experiments described in Fig. 4.9. n ≥ 85 over 3 independent experiments. Error bars show mean ± SEM. ***, P < 0.001 using a t-test with Welch’s correction. (B) Average fluorescence intensity per cell of GFP-nesprin-2 from the experiments described in Fig. 4.9. n ≥ 113 over 3 independent experiments, intensity of cells was measured regardless of SUN1 levels. Error bars show mean ± SEM. **, P < 0.01 using a t-test with Welch’s correction. A.U., arbitrary units.
Figure 4.11 Proteasome inhibition rescues the effect of SUN1 knock-down on GFP-nesprin-2. Cells stably expressing GFP-nesprin-2 were grown on glass coverslips for 24h, treated with doxycycline and SUN1 siRNA for 48h, treated with PSII for 7h or untreated, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster and rabbit anti-SUN1 followed by fluorescently labelled secondary antibodies. Representative images were captured on a snapshot widefield microscope using Metamorph then processed using Fiji ImageJ in order to have a large number of cells to quantify. Scale bar: 10 μm.
Figure 4.12 Proteasome inhibition rescues the effect of SUN1 knock-down on GFP-nesprin-2. Cells stably expressing GFP-nesprin-2 were grown on glass coverslips for 24h, treated with doxycycline and SUN1 siRNA for 48h, treated with PSII for 7h or untreated, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster and rabbit anti-SUN1 followed by fluorescently labelled secondary antibodies. Average fluorescence intensity per cell of GFP-nesprin-2 from the experiments described in Fig. 11. n ≥ 113 over 3 independent experiments. Error bars show mean ± SEM. **, P < 0.01 using a t-test with Welch’s correction. A.U., arbitrary units.

IV. 3.2. ER-localised SUN1 recombinant protein displaces GFP-nesprin-2 to the ER and decreases GFP-nesprin-2 levels

In order to test another method to displace nesprins from the NE, an ER localised soluble variant of the SUN1 protein, termed HA-SUN1-KDEL was used (Fig. 4.13A). This protein contains the SUN domain for interaction with the nesprin-2 KASH domain, but lacks the transmembrane domain and nucleoplasmic domain required for INM localisation and is retained in the ER by a KDEL retrieval motif. Overexpression of this protein is therefore expected to disrupt LINC complexes by competing with endogenous SUN proteins and displace nesprins into the ER (Fig. 4.13A), either targeting them for degradation or protecting the nesprins from ER quality control degradation by interacting with the SUN domain.
Figure 4.13 Model for a soluble SUN1 protein HA-SUN1-KDEL tethering nesprins to the ER. (A) Cartoon illustrating the recombinant SUN1 protein used in this study and its estimated molecular weight. SS: signal sequence; HA: HA tag; CC: coiled-coil domain; SUN: SUN domain; KDEL: KDEL retention signal N-ter: N-terminus; C-ter: C-terminus. The signal sequence targets the protein to the ER, and the KDEL retention signal prevents its export from the Golgi. (B) Cartoon illustrating SUN and Nesprin proteins in the ER and NE. While there are LINC complexes formed at the NE, a soluble ER SUN1 recombinant protein (HA-SUN1-KDEL) would trap many nesprins in the ER. ER: Endoplasmic Reticulum. NE: Nuclear Envelope.

HA-SUN1-KDEL was transiently transfected in HeLa cells and a western blot revealed a band at approximately 57 kDa (Fig. 4.14A) which is consistent with HA-SUN1-KDEL. The recombinant protein was examined on a widefield microscope and showed a typical ER distribution (Fig. 4.14B).

In order to test whether HA-SUN1-KDEL displaced GFP-nespin-2 to the ER, cells stably expressing GFP-nespin-2 were transfected with HA-SUN1-KDEL or left untransfected. Representative images of the cells were captured on a deltavision microscope (Fig. 4.15). In untransfected cells, GFP-nespin-2 was restricted to the ‘ring shaped’ NE. Cells overexpressing HA-SUN1-KDEL lost GFP-nespin-2 from the NE and instead GFP-nespin-2 seemed to localise to what appears to be the ER in regions enriched in HA-SUN1-KDEL. HA-SUN1-KDEL and GFP-nespin-2 in the ER area appeared as ‘beads on a string’ rather than a continuous network of tubules due to the deconvolution interpreting contiguous areas of variable intensity as diffused light from the areas with higher intensity. The overall fluorescence intensity of GFP-nespin-2 in cells transfected with HA-SUN1-KDEL appeared to be weaker than in untransfected cells, which would be consistent with proteasomal degradation of displaced nesprins. However, a
Figure 4.14 HA-SUN1-KDEL is a soluble ER SUN1 recombinant protein. HeLa cells were grown on glass coverslips for 24h, transfected with HA-SUN1-KDEL and treated with doxycycline for 24h or untreated, fixed with methanol and labelled with anti-HA followed by fluorescently labelled secondary antibodies. Scale bar: 10 µm. (A) Western-blot showing overexpression of the recombinant protein. Cells were harvested in SDS-PAGE sample buffer and run on an SDS-PAGE gel then transferred on a membrane. The membrane was incubated with DAPI and an anti-HA antibody followed by fluorescently labelled secondary antibodies. HA-SUN1-KDEL: 57 kDa. (B) Representative widefield fluorescence images of cells treated with doxycycline.

quantitative measurement needed to be performed, and thus representative images of the cells were captured on a snapshot widefield microscope (Fig. 4.16A). Untransfected cells once again had GFP-nesprin-2 enriched in a ‘ring shaped’ NE. GFP-nesprin-2 staining appeared fainter in cells transfected with HA-SUN1-KDEL, especially around the NE as the ‘ring shape’ was partially lost. GFP-nesprin-2 was instead present in what appeared to be the ER where it mostly overlapped with HA-SUN1-KDEL (yellow on the merged picture) HA-SUN1-KDEL extended further to the cell periphery whereas GFP-nesprin-2 seemed more central. Unlike deconvolved pictures (Fig. 4.15), HA-SUN1-KDEL was continuous in the snapshot widefield pictures (Fig. 4.16A) and displayed the characteristic tubular shape of the ER network. GFP-nesprin-2 and the intensity of the fluorescence of cells overexpressing HA-SUN1-KDEL or untransfected was measured and compared (Fig. 4.16B) (for quantification purposes, areas were cropped to leave out untransfected cells from the HA-SUN1-KDEL coverslips and only measure cells overexpressing HA-SUN1-KDEL). The average fluorescence intensity per cell decreased from 57368 A.U. to 362182 A.U. thus a decrease to 63% the initial level even though the p-value of
Figure 4.15 HA-SUN1-KDEL displaces GFP-nesprin-2 from the NE. Cells stably expressing GFP-nesprin-2 were grown on glass coverslips for 24h, transfected with HA-SUN1-KDEL or untransfected, treated with doxycycline for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster and anti-HA followed by fluorescently labelled secondary antibodies. Representative deltainvision fluorescence images were captured using Metamorph, deconvolved using Huygens Professional then maximum intensity projection Z-projection was applied to the deconvolved stack using Fiji ImageJ. Scale bar: 10 μm.

0.12 means that this result is not statistically significant. GFP-nesprin-2 mislocalised in the ER appears to have been degraded faster than GFP-nesprin-2 in untransfected cells.

In order to test whether inhibition of proteasomal degradation would restore GFP-nesprin-2 levels in cells expressing HA-SUN1-KDEL, cells overexpressing HA-SUN1-KDEL were also treated with the proteasome inhibitor PSII (Fig. 4.16A). GFP-nesprin-2 appeared to be concentrated in punctate structures in close proximity to the nucleus rather than being distributed throughout the ER, and did not fully overlap with HA-SUN1-KDEL. The average fluorescence intensity per cell (Fig. 4.16B) was measured at 646997 A.U. in cells treated with PSII, thus 179% the level of untreated cells overexpressing HA-SUN1-KDEL, even though the p-value of 0.12 means that this result is not statistically significant. This data supports that the decrease of GFP-nesprin-2 levels caused by HA-
Figure 4.16 HA-SUN1-KDEL displaces GFP-nesprin-2 to the ER and increases its degradation by the proteasome. Cells stably expressing GFP-nesprin-2 were grown on glass coverslips for 24h, transfected with HA-SUN1-KDEL or untransfected, treated with doxycycline for 24h, treated with PSII for 7h or untreated, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster and anti-HA followed by fluorescently labelled secondary antibodies. (A) Representative images were captured on a snapshot widefield microscope using Metamorph then processed using Fiji ImageJ in order to have a large number of cells to quantify. Scale bar: 10 μm. (B) Average fluorescence intensity per cell from the experiments described in B. n ≥ 50 over 3 independent
experiments. Error bars show mean ± SEM. ns, not significant using a t-test with Welch’s correction. A.U., arbitrary units.

SUN1-KDEL has probably been rescued by blocking proteasomal degradation. These observations suggest that the model of HA-SUN1-KDEL trapping GFP-nesprin-2 in the ER causing it to be degraded by the proteasome inhibitor may be correct.

A second method was employed to quantify the levels of GFP-nesprin-2 based on in-gel fluorescence of GFP. Lysates of cells stably expressing GFP-nesprin-2 transfected with HA-SUN1-KDEL or untransfected, treated with PSII or untreated, were run on a gel and scanned (Fig. 4.17A) which revealed a predominant fluorescent band at 80 kDa consistent with GFP-nesprin-2. The GFP fluorescence was then normalised to the total protein measured by Coomassie blue stain (Fig. 4.17B). The levels of GFP-nesprin-2 decreased to 75% of the control when HA-SUN1-KDEL was overexpressed (P = 0.47), comparable to decrease to 63% recorded by microscopy (Fig. 4.16B). Furthermore, the treatment with PSII rescued the levels of GFP-nesprin-2 fluorescence in cells overexpressing HA-SUN1-KDEL with a level at 145% the level of untreated cells overexpressing HA-SUN1-KDEL, with a p-value of 0.01 reinforcing the statistical significance of this effect.

Figure 4.17 The proteasome inhibitor is responsible for the degradation of GFP-nesprin-2 and HA-SUN1-KDEL favours this degradation. Cells stably expressing GFP-nesprin-2 were grown on a 12-wells plate for 24h, transfected with HA-SUN1-KDEL or untransfected, treated with doxycycline for 24h, harvested with SDS-PAGE sample buffer and run on an SDS-PAGE gel. (A) GFP scan and Coomassie blue stain scan of the gel. GFP-nesprin-2: 80 kDa. (B) Fluorescence intensity from the GFP band was quantified and normalised to the total protein stained with Coomassie blue and expressed as a percentage of the fluorescence intensity in untreated untransfected cells. n = 3 independent experiments. Error bars show mean ± SEM. ns, not significant; *, P < 0.05 using a t-test with Welch’s correction.
These results confirm that HA-SUN1-KDEL traps GFP-nesprin-2 in the ER and facilitates its degradation by the proteasome. GFP-nesprin-2 bound to a SUN domain was degraded at a similar rate than GFP-nesprin-2 in SUN1 depleted cells, which indicates that mislocalisation to the ER is the important factor for degradation of GFP-nesprin-2. This observation has implications for the nesprins remaining in the ER at the end of the cell cycle once the NE has been resealed. If these nesprins were forming LINC complexes with the SUN1 proteins, they would seemingly not be protected from proteasomal degradation and would instead be targeted for degradation due to mislocalisation to the ER.

IV. 4. Displacement of nesprin-2 from the NE

GFP-nesprin-2 is expressed 2.6-fold higher levels than the endogenous nesprin-2 (Fig. 3.5C) and has a GFP tag that may affect how the recombinant behaves. Thus, whether endogenous nesprin-2 displaced to the ER gets degraded by the proteasome similarly to GFP-nesprin-2 needs to be verified.

IV. 4.1. SUN1 knock-down does not affect endogenous nesprin-2 levels

In order to test whether fewer SUN sites to tether nesprin-2 in the NE would reduce the levels of nesprin-2, SUN1 was knocked-down in HeLa cells. HeLa-M cells were treated with SUN1 siRNA or scrambled siRNA and representative images of the cells were captured on a snapshot widefield microscope (Fig. 4.18). Cells transfected with scrambled siRNA had a ‘ring shaped’ NE localisation for SUN1. In SUN1 knock-down cells, SUN1 was more or less lost from the NE and the ‘ring shape’ did not appear as clear as in control cells. Quantification of the fluorescence intensity of SUN1 using the rabbit anti-SUN1 antibody (Fig. 4.19A) revealed a 62% knock-down (from 558497 A.U. to 214283 A.U., a difference statistically highly significant with a p-value of 0.0009). In cells treated with scrambled siRNA (Fig. 4.18), nesprin-2 appeared in the NE and slightly diffuse outside the NE with occasional bright points that could have been artefacts from the anti-nesprin-2 antibody staining. SUN1 knock-down cells appeared to have a crisper staining with nesprin-2 localised in the NE and slightly present in the ER, however there did not seem to be a difference in localisation when compared to cells with scrambled siRNA. Quantification was needed to verify whether the levels of nesprin-2 had changed (Fig. 4.19B), however the levels of nesprin-2 when SUN1 was knocked-down remained close to the nesprin-2 levels with scrambled siRNA (339447 A.U. and 323369 A.U. respectively, statistically not significantly different with a p-value of 0.8). SUN1 depletion was not enough to affect endogenous nesprin-2. A possible reason could be that, at an endogenous level, SUN2 and the remaining SUN1 proteins may be enough to compensate and nesprin-2 had enough SUN sites to be enriched in the NE.
Figure 4.18 SUN1 knock-down had no effect on nesprin-2 localisation. HeLa-M cells were grown on glass coverslips for 24h, treated with SUN1 siRNA or scrambled siRNA for 48h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, anti-nesprin-2 and rabbit anti-SUN1 followed by fluorescently labelled secondary antibodies. (A) Representative images were captured on a snapshot widefield microscope using Metamorph then processed using Fiji ImageJ in order to have a large number of cells to quantify. Scale bar: 10 μm.
Figure 4.19 SUN1 knock-down has no effect on the levels of endogenous nesprin-2. HeLa-M cells were grown on glass coverslips for 24h, treated with SUN1 siRNA or scrambled siRNA for 48h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, anti-vesprin-2 and rabbit anti-SUN1 followed by fluorescently labelled secondary antibodies. (A) Average fluorescence intensity per cell of anti-SUN1 from the experiments described in Fig. 4.18. n ≥ 70 over 3 independent experiments. Error bars show mean ± SEM. ***, P < 0.001 using a t-test with Welch’s correction. A.U., arbitrary units. (B) Average fluorescence intensity per cell of anti-vesprin-2 from the experiments described in Fig. 4.18. n ≥ 60 over 3 independent experiments. Error bars show mean ± SEM. ns, not significant using a t-test with Welch’s correction. A.U., arbitrary units.

IV. 4.2. KASH4 recombinant protein displaces nesprin-2 to the ER and increases nesprin-2 levels

GFP-KASH4 failed to reduce the levels of GFP-vesprin-2 (Fig. 4.6), however endogenous nesprin-2 is expressed at a lower level than GFP-vesprin-2 and the levels of transfected GFP-KASH4 may be more potent at competing with the levels of endogenous nesprin-2.

In order to test whether GFP-KASH4 displaced nesprin-2 to the ER, HeLa-M cells were transfected with GFP-KASH4 or left untransfected. Representative images were captured on a deltavision microscope (Fig. 4.20A). In untransfected cells, nesprin-2 was restricted to the ‘ring shaped’ NE and was not present in the ER. In cells transiently expressing GFP-KASH4, GFP-KASH4 was located in the NE and in what appears to the ER. Nesprin-2 was observed in the NE similarly to the untransfected cells, however some nesprin-2 was located to the ER. This observation confirms the predicted localisation of nesprin-2 if SUN domains were partially blocked by GFP-KASH4 (Fig. 4.4). In order to verify whether the levels of nesprin-2 were affected, representative images were captured on a snapshot widefield microscope (Fig. 4.20B). In untransfected cells, nesprin-2 was present in the NE. In cells transiently transfected with GFP-KASH4, nesprin-2 was present in the NE although the boundaries of the NE were less clear than in untransfected cells, as nesprin-2 was also present outside the NE. The average fluorescence intensity of cells
transfected with GFP-KASH4 or left untransfected was measured and compared (Fig. 4.21). The levels of nesprin-2 increased from 175132 A.U. to 409124 A.U., thus a very significant increase to 234% the levels of untransfected cells (P < 0.01). In conclusion, GFP-KASH4 caused the levels of nesprin-2 to dramatically increase by blocking SUN sites, which is surprising as nesprin-2 was displaced to the ER and this localisation was expected to target the protein for degradation. Nesprin-2 displaced to the ER and not involved in the formation of LINC complexes may not be recognised by ER quality control and degraded like GFP-nesprin-2.

**Figure 4.20 GFP-KASH4 displaces nesprin-2 to the ER.** HeLa-M cells were grown on glass coverslips for 24h, transfected with GFP-KASH4 or untransfected, treated with doxycycline for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster and anti-nesprin-2 followed by fluorescently labelled secondary antibodies. (A) Representative deltavision fluorescence images were captured using Metamorph, deconvolved using Huygens Professional then maximum intensity projection Z-projection was applied to the deconvolved stack using Fiji ImageJ. Scale bar: 10 μm. (B) Representative images were captured on a snapshot widefield microscope using Metamorph then processed using Fiji ImageJ in order to have a large number of cells to quantify. Scale bar: 10 μm.
Figure 4.21 GFP-KASH4 increases the nesprin-2 levels. HeLa-M cells were grown on glass coverslips for 24h, transfected with GFP-KASH4 or untransfected, treated with doxycycline for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, GFP-booster and anti-nesprin-2 followed by fluorescently labelled secondary antibodies. Average fluorescence intensity per cell from the experiments described in Fig. 4.20. n ≥ 46 over 3 independent experiments. Error bars show mean ± SEM. **, P < 0.01 using a t-test with Welch’s correction. A.U., arbitrary units.

IV. 4.3. ER-localised soluble SUN1 recombinant protein displaces nesprin-2 to the ER and increases nesprin-2 levels

Previously, HA-SUN1-KDEL displaced GFP-nesprin-2 from the NE (Fig. 4.13) and reduced its levels (Fig. 4.14; Fig. 4.15). In order to determine whether nesprin-2 behaved differently from GFP-nesprin-2 when displaced to the ER by GFP-KASH4 (Fig. 4.20; Fig. 4.21) because nesprin-2 lacked a SUN partner, HeLa-M cells were transfected with HA-SUN1-KDEL or left untransfected. Representative images were captured on a snapshot widefield microscope (Fig. 4.22A). In untransfected cells, nesprin-2 was restricted to the ‘ring shaped’ NE and only faintly present outside the NE. In cells transiently expressing HA-SUN1-KDEL, HA-SUN1-KDEL was located in the NE and in what appears to the ER. Nesprin-2 was observed in the NE similarly to the untransfected cells, however the clear ‘ring shape’ was lost and in addition nesprin-2 was located to the ER. This observation is compatible with the predicted localisation of nesprin-2 if trapped in the ER by a soluble SUN domain (Fig. 4.11). In order to verify whether the levels of nesprin-2 were affected, the average fluorescence intensity of cells transfected with HA-SUN1-KDEL or left untransfected was measured and compared (Fig. 4.22B). The levels of nesprin-2 increased from 175132 A.U. to 278874 A.U., thus a significant increase to 160% the levels of untransfected cells (P < 0.05). In conclusion, HA-SUN1-KDEL caused the levels of nesprin-2 to dramatically increase when nesprin-2 was displaced to the ER and bound to SUN domains.

Taken together with the previous results, this means that the nesprins displaced to the ER by GFP-KASH4 and free from a SUN1 domain were not protected from degradation by not being recognised by ER quality control. The presence or absence of a SUN domain did not change the observation that nesprin-2 displaced to the ER increased in levels (Fig. 4.20; Fig. 4.21; Fig. 4.22) while GFP-nesprin-2 displaced to the ER decreased in levels (Fig. 4.8; Fig. 4.9; Fig. 4.14; Fig. 4.15). Furthermore, the
Figure 4.22 HA-SUN1-KDEL displaces nesprin-2 to the ER and increases its levels. HeLa-M cells were grown on glass coverslips for 24h, transfected with HA-SUN1-KDEL or untransfected, treated with doxycycline for 24h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, anti-nesprin-2 and anti-HA followed by fluorescently labelled secondary antibodies. (A) Representative images were captured on a snapshot widefield microscope using Metamorph then processed using Fiji ImageJ in order to have a large number of cells to quantify. Scale bar: 10 μm. (C) Average fluorescence intensity per cell from the experiments described in A. n ≥ 62 over 3 independent experiments. Error bars show mean ± SEM. *, P < 0.05 using a t-test with Welch’s correction. A.U., arbitrary units.
proteasome prevents accumulation of both endogenous and exogenous proteins in the ER (Fig. 4.2; Fig. 4.3). These results suggest an additional layer of regulation for nesprin-2 expression. GFP-Nesprin-2 is chemically induced at a stable pace regardless of the fate of the protein, and may more accurately show what happens to the protein in the absence of a cellular context trying to compensate. In the case of endogenous nesprin-2, the expression is regulated by the cell. The presence of nesprin-2 in the ER or its absence from the NE and failure to carry the normal function may trigger an up-regulation of the expression, which could overcome the degradation rate of the protein mislocalised to the ER. Alternatively, the cell could down-regulate the expression of nesprins if the proteins were functional in the NE, and their absence from the NE could induce the cell to stop down-regulating the production of nesprins and thus their expression would overcome the degradation rate of the protein.

IV. 5. Effect of TorsinA and LAP1 knock-down on the LINC complex

The overall aim of this PhD was to examine the relationship of TorsinA to the LINC complex. AAA+ ATPases are usually molecular machines that drive conformational rearrangements of protein complexes, and thus the assembly or disassembly of the LINC components could be a potential target of the AAA+ ATPase TorsinA. Having observed an effect of disrupting the LINC complex on nesprin-2, the impact of TorsinA was next investigated. A good method to see whether the interaction between LINC components is affected by TorsinA would be to deplete the cells of the protein with siRNA. In addition, LAP1 is an activator of TorsinA (Zhao et al., 2013; Sosa et al., 2014) and thus depleting the cells of LAP1 provides an alternative means of disrupting TorsinA function. The effect of a LAP1 depletion may be predicted to be the same as a TorsinA deletion.

IV. 5.1. Optimisation of LAP1 knock-down

LAP1 antibody capable of detecting the endogenous protein could not be obtained. Therefore, in order to test the efficiency of siRNA-mediated LAP1 knock-down, cells stably expressing LAP1-V5-BioID were used and the ability of three different siRNAs targeting the LAP1 coding sequence were tested to reduce levels of LAP1-V5-BioID. Cells expressing LAP1-V5-BioID were treated with doxycycline and scrambled siRNA or 10 nM LAP1 siRNA for 48h, harvested and subjected to western blotting with anti-V5 antibodies (Fig. 4.23). LAP1 siRNA 1 was the only siRNA that effectively depleted LAP1, giving 50% knock-down of LAP1-V5-BioID compared to cells treated with scrambled siRNA. The lack of knock-down with the other two siRNAs is unexpected as the bands seem fainter than with scrambled siRNA. LAP1 siRNA 1 was thus deemed best candidate.
Figure 4.23 LAP1 knock-down. Western-blot analysis for LAP1 knock-down. 60,000 cells per well of cells stably expressing LAP1-V5-BioID in a 12-wells dish were treated with 10 nM LAP1 siRNA 1, 2 and 3 for 48h. Scrambled siRNA was used as a control. Cells were harvested in SDS-PAGE sample buffer, analysed by SDS-PAGE then transferred onto nitrocellulose. The membrane was incubated with Revert protein stain, then with a mouse monoclonal anti-V5 antibody followed by fluorescently labelled secondary antibodies. LAP1-V5-BioID: 100 kDa. Fluorescence intensity from the V5 bands was quantified and normalised to total protein stained with Revert then expressed as a percentage of the levels in cells treated with scrambled siRNA.

IV. 5.2. Disrupting TorsinA decreases the levels of GFP-nesprin-2

In order to test whether interfering with TorsinA function disrupts the interaction between LINC components and thus promotes the degradation of nesprin-2 in the ER, cells stably expressing GFP-nesprin-2 were treated with scrambled, LAP1 or TorsinA siRNA, and representative images were captured on a snapshot widefield microscope (Fig. 4.24A). In cells treated with scrambled siRNA, GFP-nesprin-2 was mostly restricted to the ‘ring shaped’ NE and could be observed outside the NE for some cells. The nuclei were big and round. In cells treated with LAP1 siRNA and TorsinA siRNA, some nuclei were smaller and appeared to have fold lines. GFP-nesprin-2 was mainly present in the ‘ring shaped’ NE similarly to cells treated with siRNA, and GFP-nesprin-2 also appeared outside the NE of some cells.

Although there was no clear change in the localisation of GFP-nesprin-2 in the presence of the various siRNA, the quantification (Fig. 4.24B) showed that TorsinA siRNA decreased the levels of GFP-nesprin-2 to 75% of the control cells, from 994904 A.U. to 742056 A.U., statistically significant with a p-value of 0.04. When looking at LAP1 siRNA, there was also a decrease of the GFP-nesprin-2 levels to 75% of the control, from 994904 A.U. to 744552 A.U. Due to the variance the result is not statistically significant, but with a p-value of 0.099 it remains highly suggestive at the P < 0.10 level.
As a conclusion, TorsinA and maybe LAP1 decreased the levels of GFP-nesprin-2. There are several possibilities that could explain this result. Lack of TorsinA activity could lead to increased degradation of GFP-nesprin-2, or lack of TorsinA could disrupt the formation of stable LINC complexes resulting in GFP-nesprin-2 being less effectively recruited to the NE and thus degraded in the ER according to the previous observations (Fig. 4.8; Fig. 4.9; Fig. 4.14; Fig. 4.15). However, there was no clear loss of GFP-nesprin-2 observed in the NE nor an appearance in the ER, hence this effect was subtle.

**Figure 4.24** TorsinA knock-down decreases the levels of GFP-nesprin-2. Cells stably expressing GFP-nesprin-2 were grown on glass coverslips for 24h, treated with doxycycline and TorsinA or LAP1 or scrambled siRNA for 48h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with GFP-booster. (A) Representative images were captured on a snapshot widefield microscope using Metamorph then processed using Fiji ImageJ in order to have a large number of cells to quantify. Scale bar: 10 μm. (B) Average fluorescence intensity per cell of GFP-nesprin-2 from the experiments described in A. n ≥ 90 over 3 independent experiments. Error bars show mean ± SEM. *, P < 0.005; ns, not significant using a t-test with Welch’s correction.
IV. 5.3. TorsinA knock-down increases the levels of endogenous LINC components

If the absence of TorsinA and maybe LAP1 increases the degradation or mislocalisation of endogenous nesprin-2, the anticipated effect would be an increase in the levels of nesprin-2 (Fig. 4.20; Fig. 4.21; Fig. 4.22). HeLa-M cells were treated with scrambled siRNA, LAP1 siRNA or TorsinA siRNA (estimated at 68% efficiency, Fig. 4.25) and representative images were captured on a snapshot widefield microscope (Fig. 4.26). In cells treated with scrambled, LAP1 and TorsinA siRNA, SUN1 was in the 'ringed shaped' NE. In cells treated with scrambled siRNA, endogenous nesprin-2 was in the NE and slightly present outside the NE. In cells treated with LAP1 siRNA, nesprin-2 was mostly in the NE. In cells treated with TorsinA siRNA, nesprin-2 did not seem to be clearly localised to the 'ring shaped' NE and instead appeared to be more spread throughout the cell outside the NE.

In order to determine whether the levels of endogenous nesprin-2 were affected by the siRNA treatments, the average fluorescence intensity of nesprin-2 staining per cell was quantified (Fig. 4.27A). The levels of nesprin-2 staining in cells depleted of LAP1 were 339721 A.U., which was close to the 323369 A.U. for scrambled siRNA treated cells (P = 0.9). Strikingly, however, the levels of nesprin-2 in the absence of TorsinA increased to 141% the level of control cells at 454575 A.U., and the difference is statistically significant with a p-value of 0.03.

The average fluorescence intensity per cell of SUN1 staining was also measured (Fig. 4.28B), and once again the levels between scrambled siRNA and LAP1 siRNA were close (558497 A.U. and 539456 A.U. respectively, with P = 0.9). However, TorsinA siRNA increased the levels of SUN1 to 140% the levels in control cells at 783437 A.U. This difference is not statistically significant, however the p-value of 0.05 is highly suggestive at the P < 0.10 level.

These results demonstrate that interfering with TorsinA affected the localisation of endogenous nesprin-2 by displacing it to the ER (Fig. 4.26) and that TorsinA increased both the levels of nesprin-2 (Fig. 4.27A) and SUN1 (Fig. 4.28B). If lack of TorsinA function was responsible for increased degradation of GFP-nesprin-2 (Fig. 4.24), then it would be surprising that lack of TorsinA function was responsible for decreased degradation of endogenous nesprin-2. The reverse effect of TorsinA depletion on GFP-nesprin-2 and nesprin-2 is consistent with the previous observations of LINC complex disruption throughout this study, suggesting that lack of TorsinA could disrupt the formation of stable LINC complexes resulting in nesprins being less effectively recruited to the NE and thus degraded in the ER. In addition, the increase in SUN1 levels appears to be consistent with the idea that perturbing the function of the LINC complex involves an additional layer of regulation to produce more LINC component proteins.
Figure 4.25 TorsinA knock-down. HeLa-M cells were grown on glass coverslips for 24h, treated with TorsinA or scrambled siRNA for 48h, harvested with SDS-PAGE sample buffer and run on an SDS-PAGE gel then transferred on a membrane. (A) The membrane was incubated with Revert total protein stain, then with an anti-TorsinA antibody followed by fluorescently labelled secondary antibodies. TorsinA: 37 kDa. (B) Fluorescence intensity from the TorsinA band was quantified and normalised to the total protein stain with Revert and expressed as a percentage of the fluorescence intensity in cells treated with scrambled siRNA. n = 3 independent experiments. Error bars show mean ± SEM. *, P < 0.05 using a t-test with Welch’s correction.
Figure 4.26 TorsinA knock-down displaces endogenous nesprin-2 to the ER. HeLa-M cells were grown on glass coverslips for 24h, treated with TorsinA, LAP1 or scrambled siRNA for 48h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, anti-nesprin-2 and rabbit anti-SUN1 followed by fluorescently labelled secondary antibodies. Representative images were captured on a snapshot widefield microscope using Metamorph then processed using Fiji ImageJ in order to have a large number of cells to quantify. Scale bar: 10 μm.
TorsinA knock-down increases the levels of endogenous nesprin-2 and SUN1. HeLa-M cells were grown on glass coverslips for 24h, treated with TorsinA, LAP1 or scrambled siRNA for 48h, fixed with 3% paraformaldehyde, permeabilised with 0.2% triton and labelled with DAPI, anti-nesprin-2 and rabbit anti-SUN1 followed by fluorescently labelled secondary antibodies. (A) Average fluorescence intensity per cell of anti-nesprin-2 from the experiments described in Fig. 4.26. n ≥ 67 over 3 independent experiments. Error bars show mean ± SEM. *, P < 0.05; ns, not significant using a t-test with Welch’s correction. A.U., arbitrary units. (B) Average fluorescence intensity per cell of anti-SUN1 from the experiments described in Fig. 4.26. n ≥ 67 over 3 independent experiments. Error bars show mean ± SEM. ns, not significant using a t-test with Welch’s correction. A.U., arbitrary units.

IV. 5.4. TorsinA knock-down increases the mobility of GFP-nesprin-2

In order to investigate whether loss of TorsinA affects the interaction between SUN1 and nesprin-2, the mobility of GFP-nesprin-2 was examined. Fluorescence recovery after photobleaching (FRAP) is a technique that allows to measure the mobility of a protein. Fluorophores like GFP can irreversibly lose their ability to emit light over time, and this process can be accelerated by illuminating the fluorophore with a strong laser pulse at the corresponding excitation wavelength to photobleach it. Photobleaching a small area prevents the fluorophore from that area to emit light, and thus light gradually appearing in this area is emitted by new fluorophores moving into that area. The rate of fluorescence recovery informs on the mobility of a protein equipped with a fluorophore.

Cells stably expressing GFP-nesprin-2 were grown on glass coverslips and observed in a spinning disk confocal microscope fitted for live cell imaging. Several cells were photobleached in a small area of the nuclear envelope (Fig. 4.28A), and the half-time of recovery of GFP-nesprin-2 was measured (Fig. 4.28B). The half-time was 7.6 minutes for control cells and 5.3 minutes for cells depleted for TorsinA, thus the recovery was 43% faster with loss of TorsinA and the difference is statistically significant with a p-value of 0.02.

Depletion of TorsinA increased the mobility of GFP-nesprin-2. Indirect effects could be responsible for this observation, such as lipid composition of the membranes affect by TorsinA (Grillet et al., 2016).
Figure 4.28 TorsinA knock-down decreases the mobility of GFP-nesprin-2. (A) Cells stably expressing GFP-nesprin-2 were grown on glass dishes for 24h, treated with doxycycline and TorsinA siRNA or scrambled siRNA for 48h and observed under a spinning disk confocal microscope equipped for live cell imaging. Laser exposure was used to photobleach areas of the NE and the fluorescence intensity of these areas was measured over time. (A) Representative images were captured using Slide Book then processed using Fiji ImageJ. White rectangles indicate the photobleached areas. Scale bar: 10 μm. (B) Average fluorescence intensities of the photobleached areas from the experiments described in A were extracted, normal photo-bleaching was compensated for, intensities were normalised to the initial value after photobleaching and a non-linear regression formula was performed to determine the half-time. Half-time values were compared for cells with scrambled siRNA and cells with TorsinA siRNA. n ≥ 14 over 2 independent experiments. Error bars show mean ± SEM. *, P < 0.005 using a t-test with Welch’s correction.

Direct effects may be more likely as TorsinA can associate with the nesprins (Nery et al., 2008). Such direct effects may be a change in the conformation of GFP-nesprin-2 that impacted its mobility, a disassembly of the LINC complex or an impact on the strength of the interaction between SUN and KASH domains.

IV. 6. Discussion

Association between SUN domains and KASH domains of SUN proteins and nesprins (Sosa et al., 2012) is the basis for the formation of LINC complexes and their function. Together they transmit forces through the nucleus (Razafsky et al., 2014) and permit deformation of the nucleus (Anno et al., 2012), such as during cell migration (Lombardi et al., 2011). Another example of LINC complexes being required for a mechanical function is in TAN lines. TAN lines are SUN2 and nesprin-2G arrays connecting actin cables to the nucleus for rearward nuclear movement during centrosome reorientation in migrating fibroblasts (Luxton et al., 2011). Recently, TorsinA and LAP1 have been shown to be required for TAN line assembly (Saunders et al., 2017), suggesting that TorsinA is implicated in the dynamics of the LINC complex. Previously, TorsinA was shown to interact with the KASH domain (Nery et al., 2008) and displace LINC components in the NE (Vander Heyden et al., 2009). Elucidating how
TorsinA affects the LINC complex could help understand EOTD and some nuclear envelopathies to facilitate treatment of these diseases.

Damaged, mislocalised and misassembled proteins are usually recognised by quality control and sent to the proteasome for degradation (reviewed in Tomko and Hochstrasser, 2013). The proteasome was demonstrated to prevent accumulation in the ER of GFP-nesprin-2 (Fig. 4.1) as well as the endogenous protein nesprin-2 (Fig. 4.3). Thus, nesprins can be recognised by quality control and sent for degradation to keep the levels under control.

In order to determine whether nesprins displaced to the ER were degraded by the proteasome, several methods to disrupt the LINC complex were used. These methods disrupted the LINC complex by taking advantage of the interaction between the SUN and KASH domains that tethers the nesprins to the NE (Ostlund et al., 2009). LINC complex function had previously been studied by disrupting the complex (Stewart-Hutchinson et al., 2008). Soluble SUN1 proteins and recombinant KASH proteins were observed to displace nesprins to the ER. This aforementioned study demonstrated the motif length required to match SUN and KASH domains, but did not measure the levels of LINC components nor the fate of misplaced proteins. These unanswered questions were explored in the present study. SUN1 depletion displaced some of GFP-nesprin-2 from the NE to the ER (Fig. 4.9) and decreased the levels of GFP-nesprin-2. A soluble ER-localised SUN1 recombinant protein (Fig. 4.13) displaced GFP-nesprin-2 to the ER (Fig. 4.15) and reduced the levels of GFP-nesprin-2 (Fig. 4.16; Fig. 4.17). GFP-nespin-2 reduced levels were reversed by inhibiting the proteasome (Fig. 4.12; Fig. 4.16; Fig. 4.17), suggesting that nesprins misplaced to the ER were degraded by the proteasome whether they are ‘free’ or bound to a SUN domain. This model could indicate that the fate of LINC complexes present in ER clusters at the end of the cell cycle (Fig. 3.4; Fig. 3.10) was to be targeted for proteasomal degradation, in the event that these complexes were not able to disassemble and translocate to the NE.

SUN1 depletion did not affect the levels of endogenous nesprin-2 (Fig. 4.19), indicating that SUN2 may have been able to compensate which is consistent with previous studies showing that SUN1 or SUN2 knockout mice are viable (Horn et al., 2013) and their functions are at least partially redundant. When endogenous nesprin-2 was displaced to the ER by a soluble ER-localised SUN1 recombinant protein, an increase in nesprin-2 levels was observed (Fig. 4.22). This effect was consistent with a saturation of SUN domain sites with a recombinant KASH4 protein (Fig. 4.4). The recombinant KASH4 protein displaced endogenous nesprin-2 to the ER (Fig. 4.20) and nesprin-2 levels increased (Fig. 4.21). On one hand, when disrupting the LINC complex GFP-nesprin-2 demonstrated a direct effect of protein degradation by the proteasome. On the other hand, disrupting the LINC complex increased levels of endogenous nesprin-2, which indicates that in the cellular context an additional layer of regulation of nesprin-2 expression is involved. Several studies have shown that SUN1 interacts with the chromatin (Chi et al., 2007; Ding et al., 2007), which could suggest a direct control of the LINC complex on its own expression leading to a down-regulation of its production if the LINC complex is performing as expected. Conversely, another idea is that the lack of correct LINC complex function or mislocalisation of LINC components could trigger an up-regulation of the expression of nesprins at a sufficient rate to overcome ER degradation by the proteasome. RT-qPCR could provide insight on the regulation of LINC complex components gene expression.
According to these findings, a model for LINC complex disruption can be proposed. When the nesprins cannot get enriched to the NE due to lack of SUN sites or due to being tethered to the ER, then the nesprins are displaced into the ER and are targeted for degradation. In the case of GFP-nespin-2, this can be seen as a decrease in protein levels, an effect that is reversed in the presence of proteasome inhibitor. Conversely, in the situation of endogenous nespin-2, there is an increase of these levels as the cell up-regulates the protein to compensate for the disruption. If a protein such as TorsinA or LAP1 disrupts the LINC complex by impacting the interaction between the SUN and Nesprins, similar effects would very likely be observed.

Figure 4.29 Model for a disruption of the LINC complex. Cartoon illustrating SUN and Nesprin proteins in the ER and NE. Disrupting the LINC complex and relocating the nesprins to the ER causes their degradation and an up-regulation of LINC components. ER: Endoplasmic Reticulum. NE: Nuclear Envelope.

Therefore, the model of perturbing the location of nesprins to affect its levels was used to observe the impact of TorsinA function on the LINC complex. Both TorsinA and LAP1 knock-down decreased the levels of GFP-nespin-2 (Fig. 4.24), indicating that interfering with TorsinA was responsible for an increased degradation of GFP-nespin-2. Several studies proposed that TorsinA may be involved in ERAD. TorsinA binds to and promotes the degradation of misfolded ε-sarcoglycan (Esapa et al., 2007). In addition, increasing levels of TorsinA facilitated degradation of misfolded proteins, and TorsinAΔE is impaired in this function resulting in an increased sensitivity to stress in the cells (Nery et al., 2011). However, if the implication is that TorsinA was involved in the degradation of GFP-nespin-2 through the ERAD pathway, then TorsinA depletion would have protected GFP-nespin-2 rather than reducing the levels of GFP-nespin-2.
Interfering with TorsinA increased the mobility of GFP-nesprin-2 (Fig. 4.28) from 7.6 minutes half-time recovery rate to 5.3 minutes half-time recovery rate. Ostlund et al. estimated GFP-mini-nesprin-2G half-time recovery rate at 2.5 minutes (Ostlund et al., 2009), however GFP-mini-nesprin-2G was transiently transfected. Preliminary studies on transiently transfected GFP-nesprin-2 showed a half-time recovery rate of 2 minutes (data not shown), which is consistent with their data.

TorsinA knock-down displaced endogenous nesprin-2 to the ER and increased the levels of nesprin-2 (Fig. 4.26; Fig. 4.27) which is consistent with the model for LINC complex disruption (Fig. 4.29). An idea is that if depletion of TorsinA disrupted the interaction between SUN and KASH domains, then function of TorsinA was to maintain or strengthen this interaction. However, there are other possible explanations for these results. A recent study in Drosophila demonstrated that TorsinA has a role in lipid metabolism (Grillet et al., 2016). The implication is that the lipidic composition of the NE may have been changed due to TorsinA depletion and in turn impacted the LINC complex. Consistent with the idea that TorsinA was required to strengthen the interaction between SUN and KASH, Saunders proposed that TorsinA could act as a molecular chaperone to structurally remodel LINC complexes containing nesprin-2G (Saunders et al., 2017). Furthermore, KASH and SUN contain cysteines (Sosa et al., 2012) and the formation of a disulphide bridge could be crucial for the transmission of forces through the LINC complex (Jahed et al., 2015). TorsinA harbours a redox sensor motif (Zhu et al., 2010), and redox activity was necessary for the effect of TorsinA on TAN lines assembly and persistence (Saunders et al., 2017).

SUN1 levels increased with nesprin-2 levels when TorsinA was depleted (Fig. 4.27), suggesting that nesprins and SUN proteins may be regulated together by the same mechanism. It would be interesting to see if SUN1 levels also increase when the LINC complex is disrupted with a soluble ER-localised SUN1 protein or a KASH recombinant protein. Furthermore, levels of SUN2 could also be monitored to see whether all LINC components are regulated together.

The absence of effect of LAP1 towards the levels of endogenous LINC components (Fig. 4.27) may be due to the stoichiometry of the proteins in presence and their function; GFP-KASH4 proteins were not enough to outcompete GFP-nesprin-2 proteins even though their levels successfully competed with endogenous nesprin-2 (Fig. 4.2), and SUN1 depletion was likely compensated by SUN2 proteins at an endogenous level (Fig. 4.19) while SUN2 alone was not enough to provide domains to tether the all of the overexpressed GFP-nesprin-2 proteins and protect them from degradation (Fig. 4.10; Fig. 4.12). LAP1 depletion had a perceptible effect on GFP-nesprin-2 proteins (Fig. 4.24) but at an endogenous level it is possible that LULL1 was enough to compensate for the absence of LAP1, although the function of LULL1 is still unclear. TorsinA being the common link for the AAA+ ATPase activity of these two proteins, TorsinA depletion was the bottleneck that had an effect both at the level of GFP-nesprin-2 and at an endogenous level.

In conclusion, the model proposed is that TorsinA (and by association LAP1 and LULL1) is responsible for strengthening the interaction between SUN and the nesprins. This mechanism may be regulated by the formation of a disulphide bond between cysteines in the KASH and SUN domains. This may explain why LAP1-V5-BioID increased the number of GFP-nesprin-2 clusters in late mitosis (Fig. 3.14; Fig. 3.15); the interaction between LINC components may have been strengthened by disulphide bonds,
thus the complexes may have lost their capacity to disassemble and were not able to translate back to the NE. Overexpressing LULL1 would in theory yield similar results of increasing late mitosis clusters of LINC components. On another note, live cell imaging with fluorescently tagged LINC components such as GFP-nesprin-2 and RFP-SUN1 could help determine whether LINC components in late mitosis clusters are degraded or incorporated back in the NE.
V. **INTERACTOME NETWORK WITH PROXIMITY LABELLING**

V. 1. **Introduction**

The AAA+ ATPase TorsinA and by extension its activators LAP1 and LULL1 have been proposed to regulate the LINC complex, however little is known about what triggers these proteins to perform their function, or whether they have other substrates in addition to the LINC complex.

In order to determine potential interactors of proteins, a method was developed by Brian Burke and Kyle Roux: proximity-dependent biotin identification, also named BioID (Roux et al., 2012). This method uses a biotin ligase such as BirA to biotinylate primary amines (mostly lysines) of proteins in the direct vicinity of the enzyme. The BirA or a shorter version the BioID can be added to the sequence of a protein of interest, the bait, to direct the enzyme to the usual environment of the bait and biotinylate the surrounding proteins upon addition of biotin to the cell culture medium. Streptavidin is able to bind biotin, and thus streptavidin beads allow the capture of biotinylated proteins and their subsequent identification by mass spectrometry to uncover the potential interactome of the bait.

In this study, LAP1, LULL1 and TorsinA and its mutants were used as bait to examine their interactome and find potential substrates for these proteins.

V. 2. **Generation of cells stably expressing the baits**

LAP1 and LULL1 are TorsinA activators (Zhao et al., 2013) and thus using them as baits for proximity is very likely to yield interactors and substrates relevant to TorsinA. Considering that these baits are located to the ER and NE compartments, they will naturally randomly encounter other proteins in this location despite having a function unrelated to TorsinA. A potential control to evaluate which proteins are likely to be false-negatives would be a bait randomly biotinylating other proteins it encounters in the ER and NE compartments, and thus SS-V5-BioID-KDEL was conceived (Fig. 5.1A). An N-terminal signal sequence (SS) targets the recombinant protein to the ER, and a KDEL retrieval signal mediates retrieval from the Golgi, maintaining an ER localisation.

As the AAA ATPase domains of TorsinA, LAP1 and LULL1 interact in the lumen of the ER/NE (Sosa et al., 2014), the substrates for their AAA ATPase activity are most likely to be found in that same compartment. Thus, the biotin ligase was chosen to be fused next to their luminal domain. Placing the biotin ligase at the N-terminus of TorsinA (Fig. 5.1B) may disrupt the way TorsinA interacts with the membranes (Callan et al., 2007) and therefore impact the dynamics of the protein. Placing the biotin ligase in N-terminus of LAP1 (Fig. 5.1C) would position the biotin ligase in the nucleoplasm (Goodchild et al., 2005). Placing the biotin ligase in N-terminus of LULL1 (Fig. 5.1D) would position the biotin ligase in the cytoplasm (Goodchild et al., 2005). While alternate locations for the biotin ligase may be informative in and on themselves, it was decided for better comparison between the baits to place the biotin ligase in C-terminus of these proteins, as it is where these proteins supposedly interact with their substrate in the perinuclear space/ER lumen.
When this study was started, in addition to the original BirA protein (Burke et al., 2012) the Roux lab had developed a second-generation biotin ligase referred to as BioID (Kim et al., 2016). The BioID encodes a minimal biotin ligase domain and being smaller may be less disruptive to the function of these proteins in case the added protein hinders the interaction sites. ER lumen is oxidising and has a potential for glycosylation which could affect activity (Margittai et al., 2015); since no previous reports have utilised proximity labelling inside the ER/NE lumen environment, it was decided to generate TorsinA baits with both of these biotin ligases (Fig. 5.1B). TorsinA mutants were also used as baits, as their characteristic mutations (Table 1.2) may change how TorsinA interacts with a substrate. TorsinAKA has a mutation in the Walker A motif and cannot bind ATP, and supposedly cannot bind to a substrate either (Babst et al., 1998; Matveeva et al., 1997). TorsinAEQ has a mutation in the Walker B motif and cannot hydrolyse ATP, and supposedly cannot separate from the substrate thus acting as a 'substrate trap' version of the AAA ATPase (Weibezahn et al., 2003; Dalal et al., 2004; Babst et al., 1998). TorsinAΔE is responsible for EOTD (Ozelius et al., 1997) and accumulates at the NE (Gerace et al., 2004). Finally, LAP1 was fused to BioID (Fig. 5.1C) while LULL1 was fused to BirA (Fig. 5.1D).

Figure 5.1 BioID and BirA baits. Cartoon illustrating the different recombinant proteins used in this study and their expected localisation. (A) SS-V5-BioID-KDEL. (B) TorsinA, TorsinAKA, TorsinAEQ and TorsinAΔE associated with either V5-BirA or V5-BioID. (C) LAP1-V5-BioID. (D) LULL1-V5-BirA. SS: Signal sequence; KDEL: KDEL retention signal; ER: endoplasmic reticulum; C-ter: C-terminus; N-ter: N-terminus; BirA: biotin ligase; BioID: BirA second generation.

Stably transfected cell lines were made in HeLa-M cells using the pTRIPZ lentiviral vector and selected using puromycin. Stably transfected cells were induced with doxycycline and lysates were analysed by western blotting with anti-V5 (Fig. 5.2) to determine whether they had incorporated their recombinant protein and whether it was expressed at the right molecular weight. TorsinA-V5-BioID and mutants
displayed bands at about the expected molecular weight of 65 kDa. TorsinA-V5-BirA and mutants had bands at a slightly higher molecular weight, about the expected 70 kDa. SS-V5-BioID-KDEL displayed a band around the expected molecular weight of 25 kDa. Finally, LAP1-V5-BioID and LULL1-V5-BirA had

![Western blot analysis](image)

**Figure 5.2 Stable expression of the BioID and BirA baits.** Western-blot analysis for expression ratios of the different constructs. Cells stably expressing the different recombinant proteins were treated with doxycycline for 24h. Cells were harvested in SDS-PAGE sample buffer and run on an SDS-PAGE gel then transferred on a membrane. The membrane was incubated with Revert and a mouse monoclonal anti-V5 antibody followed by fluorescently labelled secondary antibodies. Estimated molecular weights: TorsinA-V5-BioID and mutants: 65 kDa; LAP1-V5-BioID: 100 kDa; SS-V5-BioID-KDEL: 25 kDa; TorsinA-V5-BirA and mutants: 70 kDa; LULL1-V5-BirA: 100 kDa.

bands at the expected molecular weight of 100 kDa. Fluorescence intensity of the bands was quantified and normalised to the Revert total proteins. TorsinAEQ-V5-BirA was the least expressed of the baits, so the expression ratio for the other constructs were compared to this one. The bands of proteins with BioID appeared as doublets. Expression ratios showed that TorsinAEQ-V5-BirA is the least expressed of the baits, followed by TorsinA-V5-BioID and TorsinAΔE-V5-BioID. On the other end of the spectrum, TorsinA-KA-V5-BioID and SS-V5-BioID-KDEL were the most expressed.

The next step was to establish whether the recombinant proteins were localised in the right compartment and whether the distribution was similar to the known distribution of these proteins (Gerace et al., 2004; Naismith et al., 2004; Goodchild et al., 2005). Cells were grown on coverslips, fixed in methanol and stained with antibodies recognising V5. Representative images were captured on a widefield...
microscope (Fig. 5.3A), TorsinA-V5-BioID was present in what appears to be the ER and NE. TorsinAKA-V5-BioID was displayed in the NE but enriched in what appears to be the ER. TorsinAEQ-V5-BioID was enriched in the NE and slightly appeared in the ER. TorsinAΔE-V5-BioID was clearly in the NE while barely in the ER. LAP1-V5-BioID was only present in the NE. SS-V5-BioID-KDEL was localised in the NE but enriched in what appeared to be the ER.

A

|------------------|-------------------|------------------|-------------------|---------------|-----------------|

B

<table>
<thead>
<tr>
<th>TorsinA-V5-BirA</th>
<th>TorsinAKA-V5-BirA</th>
<th>TorsinAEQ-V5-BirA</th>
<th>TorsinAΔE-V5-BirA</th>
<th>LULL1-V5-BirA</th>
</tr>
</thead>
</table>

Figure 5.3 Cellular localisation of the BioID and BirA baits. Cells stably expressing the different constructs were grown on glass coverslips, treated with doxycycline for 24h, fixed methanol, and labelled with DAPI and anti-V5 followed by fluorescently labelled secondary antibodies. Representative fluorescence images were captured on a widefield microscope using Metamorph and processed with ImageJ. Scale bar: 10 μm. (A) Cells stably expressing BioID baits. (B) Cells stably expressing BirA baits.

For the BirA baits (Fig. 5.3AB), TorsinA-V5-BirA was enriched in the NE and in what appears to be the ER. TorsinAKA-V5-BirA was located in the NE but was mostly present in what appears to be the ER.
TorsinAEQ-V5-BirA was mainly enriched in the NE and only slightly localised in the ER. TorsinAΔE-V5-BirA was also found in the NE while barely appearing in the ER. Finally, LULL1-V5-BirA was localised in the NE but enriched in what appears to be the ER. All the localisations were consistent with previous observations of these proteins without the biotin ligase (Gerace et al., 2004; Naismith et al., 2004; Goodchild et al., 2005). The fact that TorsinAEQ mutants were still enriched in the NE despite the BioID or BirA sequences present in C-terminus suggests that the biotin ligases did not disrupt the function of these proteins, as their localisation was proposed to reflect their function (Gerace et al., 2004).

V. 3. Characterisation of the BioID biotin ligase

The western-blot analysis of the baits (Fig. 5.2) showed doublets for all the constructs with the BioID biotin ligase. This could potentially represent proteins that have not been translocated into the ER and thus retain the N-terminal sequence (Callan et al., 2007). Even though recombinant proteins with BioID did not seem to display a cytoplasmic localisation (Fig. 5.3), non-translocated and truncated proteins may still be present in the cytoplasm. Further investigations were required in order to fully characterise the localisation of this biotin ligase.

First, a protease protection assay was performed to test whether TorsinA-V5-BioID was present in the cytoplasm and accessible to proteolytic degradation or was protected within the lumenal environment. Cells stably expressing TorsinA-V5-BioID were permeabilised using two methods. Triton X-100 solubilises all the membranes, exposing all luminal proteins to the proteinase K added to the cells. At low concentrations, digitonin selectively permeabilises cholesterol rich membranes such as the plasma membrane, leaving internal membranes such as ER membrane, ONM and INM intact (Jamur et al., 2010). Hence, if a protein is located in the cytoplasm, it is vulnerable to the action of proteinase K in cells permeabilised with either digitonin or triton X-100, whereas if a protein is in the ER/NE lumen it is protected from the proteinase K in digitonin-treated cells. Kinesin-1 heavy chain (KIF5B) was used as a control; it is a cytoplasmic protein which should get degraded under both permeabilisation methods and confirm the effectiveness of proteinase K. Indeed, KIF5B was degraded upon addition of proteinase K when the membrane had been permeabilized with triton or digitonin (Fig. 5.4). Conversely, TorsinA-V5-BioID was degraded under triton permeabilisation but not under digitonin permeabilisation (Fig. 5.4). In conclusion, TorsinA-V5-BioID is correctly located to the ER/NE system and not in the cytoplasm.

Next, the localisation of SS-V5-BioID-KDEL was characterised by permeabilising the membranes with digitonin and triton X-100 followed by microscopy. If the recombinant protein was not properly translocated into the ER and had been expressed in the cytoplasm, the V5 tag would be accessible after triton X-100 permeabilisation and digitonin permeabilisation. If the recombinant protein was correctly localised in the ER/NE, the V5 tag would only be detected after triton X-100 permeabilisation. Protein disulphide isomerase (PDI), an ER resident protein, was used as a control luminal protein which should
Figure 5.4 TorsinA-V5-BiolD is not expressed in the cytoplasm. Western-blot analysis for a protease protection assay on TorsinA-V5-BiolD. Cells stably expressing TorsinA-V5-BiolD were treated with doxycycline for 24h, permeabilised with 0.2% triton or 0.02% digitonin, treated with up to 20 µL proteinase K, up to 20, TCA precipitated, resuspended in SDS-Page sample buffer, run on an SDS-gel, transferred on a membrane and incubated with antibodies recognising TorsinA and KIF5B followed by fluorescently labelled secondary antibodies. Estimated molecular weights: TorsinA-V5-BiolD: 65 kDa; KIF5B: 110 kDa. Red stars: bands for KIF5B.

Figure 5.5 SS-V5-BiolD-KDEL is localised to the ER and NE. Cells stably expressing SS-V5-BiolD-KDEL were subjected to different treatments before representative images were captured on a widefield microscope. Scale bar: 10 µm. (A) Treated with doxycycline for 24h, permeabilised with 0.2% triton or 0.02% digitonin and labelled with DAPI, anti-PDI and anti-V5 followed by fluorescently labelled secondary antibodies. (B) Treated with doxycycline for 24h, permeabilised with 0.2% triton or 0.02% digitonin and labelled with DAPI, anti-PDI and anti-tubulin.

only be observed in cells following triton x-100 permeabilisation. Cells expressing SS-V5-BiolD-KDEL were permeabilised using triton or digitonin then representative images were captured with a widefield microscope (Fig. 5.5A). The antibodies recognising V5 and PDI did not detect their antigens when the cells were permeabilised with digitonin. However, when the cells were permeabilised with triton X-100 both antibodies recognising PDI and antibodies recognising V5 tag were clearly visible in what appears
to be the ER as well as in the NE. To prove that this was not due to the digitonin not working efficiently and not permeabilising the cell membrane, tubulin was also used as a control (Fig. 5.5B). The cells permeabilised with digitonin showed clear tubulin staining but not V5 staining.

In conclusion, the majority of TorsinA-V5-BioID and SS-V5-BioID-KDEL are located in the ER/NE, suggesting that the double band is not caused by non-translocated or mislocalised proteins.

Another possibility for the existence of the band doublets would be the presence of glycosylation. TorsinA is known to have two N-linked glycosylation sites (Breakefield et al., 2001) but it would not explain why TorsinA-V5-BioID has a double band while TorsinA-V5-BirA does not. NetNGlyc was used to predict glycosylation sites. The algorithm detected the two known sites in the sequence of TorsinA (Fig. 5.6A). Notably, whilst no glycosylation sites were predicted on the sequence of BirA (Fig. 5.6B), a single consensus site for N-glycosylation was identified in the sequence of BioID (Fig. 5.6C).

![Figure 5.6 Predicted N-glycosylations in the sequence of the recombinant baits.](image)

To examine whether this site was in fact glycosylated, lysates from the cells stably expressing TorsinA-V5-BirA and TorsinA-V5-BioID were digested with PNGase F, an enzyme that cleaves N-linked glycans (Fig. 5.7). This resulted in a downward shift in the mobility of endogenous TorsinA (Fig. 5.7, lower panel). TorsinA-V5-BirA also had a band shift (Fig. 5.7, red arrows), very likely to be the band shift of the TorsinA fragment in the recombinant protein. TorsinA-V5-BioID had the two bands collapse into a single lower band (Fig. 5.7, blue arrows), confirming that the two different bands were caused by glycosylated forms.
In conclusion, the double bands seen with recombinant proteins that include the BioID biotin ligase is due to glycosylation forms and not due to a mislocalisation to the ER.

**Figure 5.7 The bait recombinant proteins are glycosylated.** Western-blot analysis for N-linked glycosylation on TorsinA-V5-BirA and TorsinA-V5-BioID. Cells stably expressing the different recombinant proteins were treated with doxycycline for 24h, harvested in SDS-Page sample buffer, digested with PNGase F, TCA precipitated, resuspended in SDS-Page sample buffer, run on an SDS-gel, transferred on a membrane and incubated with antibodies recognising TorsinA followed by fluorescently labelled secondary antibodies. Estimated molecular weights: TorsinA-V5-BioID: 65 kDa; TorsinA-V5-BirA: 70 kDa. Black arrows: band shift of TorsinA. Red arrows: band shift of TorsinA-V5-BirA. Blue arrows: band shift of TorsinA-V5-BioID.

### V. 4. Pull-down and biotinylation optimisation

The action of the biotin ligase itself could be tested with streptavidin attached to a fluorophore that would bind biotinylated proteins. Stable SS-V5-BioID-KDEL cells were induced with doxycycline and treated with biotin or left untreated, fixed and permeabilised with triton. Then, the cells were stained with streptavidin and antibodies recognising PDI, to use as an ER control. Representative images were captured on a widefield microscope (Fig. 5.8). In the absence of doxycycline and biotin, there was no streptavidin staining. After doxycycline and biotin treatment, there was a streptavidin staining that overlapped with the ER staining of antibodies recognising PDI. After doxycycline induction in the absence of biotin, the biotin ligase had no biotin to conjugate onto the proteins. Without doxycycline but in the presence of biotin, there was a faint streptavidin staining that could potentially be caused by endogenous biotinylation or a leaky expression of SS-V5-BioID-KDEL combined with saturating amounts of biotin. All other baits were tested in the same manner and yielded the same result of biotinylation restricted to the ER/NE (*data not shown*).

The pull-downs were performed according to a protocol obtained via personal communication from Alexandre Chojnowski (IMB, Singapore), based on the standard protocol developed by Brian Burke and Kyle Roux (Burke *et al.*, 2012). Cells were induced by doxycycline for 24h, supplemented with biotin for 24h. Then, the cells were lysed in a buffer containing 0.4% SDS to ensure efficient lysis of the NE, and also to disrupt protein-protein interactions that could lead to isolation of non-biotinylated proteins. Cell lysates were passed through a syringe to shear the DNA in order to make the samples less viscous,
then biotinylated proteins were captured by incubating the samples with streptavidin beads. Beads were washed with a buffer containing 1% NP-40 and then a Tris buffer, resuspended in SDS-PAGE sample buffer then run on a gel. Initial pull-downs of biotinylated protein gave the impression of capturing a relatively low amount protein. To optimise the capture protocol, there were two possibilities. One was to increase the concentration of SDS to better break the cells and separate the proteins, and the other possibility was to change the method to shear the DNA. Shearing with a syringe was the established method in the protocol, however residual lysate might get stuck in the syringe. Moreover, this was not a time-efficient method for large-scale experiments. Thus, sonication could be used as an alternative method to reduce viscosity of the samples by shearing DNA.

Figure 5.8 SS-V5-BioID-KDEL biotinylates proteins in the presence of biotin. Cells stably expressing SS-V5-BioID-KDEL were untreated or treated with doxycycline and/or biotin for 24h, permeabilised with 0.2% triton and labelled with DAPI, anti-PDI and streptavidin. Representative images were captured on a widefield microscope. Scale bar: 10 μm.

Cells stably expressing SS-V5-BioID-KDEL were induced for 24h in the presence of biotin, lysed with buffer containing 0.4% or 1% SDS and sonicated or sheared with a syringe before the capture on streptavidin beads and a western-blot analysis with streptavidin and anti-V5 antibodies (Fig. 4.9). On one hand, a higher concentration of SDS did not capture more proteins. SDS is an ionic detergent, and thus will denature proteins. Thus, a high concentration may have interfered with the interaction of streptavidin with biotin. On the other hand, sonication slightly improved the amount of proteins captured and was therefore chosen as the method of choice for lysis of the cells before pull-down.
In order to compare biotinylation in several baits to background biotinylation in the parental cell lines, cells stably expressing SS-V5-BioID-KDEL, TorsinA-V5-BirA, TorsinA-V5-BioID and HeLa-M cells were induced with doxycycline for 24h, incubated with biotin for 24h, lysed in a buffer containing 0.4% SDS and sonicated. After capture of biotinylated proteins with streptavidin beads, a western-blot analysis was performed using anti-V5 antibodies (Fig. 5.10). The parental cell line HeLa-M showed a background biotinylation that reflects natural biotinylation in cells (Dakshinamurti et al., 1981). Relative to parental cells, an increase in the number of thick bands appeared in the SS-V5-BioID-KDEL sample. Likewise, TorsinA-V5-BirA sample displayed many thick bands. TorsinA-V5-BioID also had extra bands that were not present in the HeLa-M sample, however a lower number of thick bands were visible in this sample than in samples of the other baits. The BirA biotin ligase on TorsinA seemed to biotinylate more proteins than the BioID biotin ligase on TorsinA. The difference could be due to the fact that TorsinA-V5-BirA is expressed at about twice the levels of TorsinA-V5-BioID (Fig. 5.2), or it could be due to the difference in size that gives BirA a longer reach to biotinylate farther proteins, as BioID is about 28 kDa and BirA is about 38 kDa (Kim et al., 2016).

![Western-blot analysis for SS-V5-BioID pull-downs under different conditions.](image)

**Figure 5.9 Lower SDS ratio and sonication yield more proteins.** Western-blot analysis for SS-V5-BioID pull-downs under different conditions. Cells stably expressing TorsinA-V5-BioID were treated with doxycycline for 24h and biotin for 24h, separated in three tubes after trypsinisation, lysed in a buffer containing 0.4% or 1% SDS, sonicated or sheared in a syringe, incubated with Dynabeads for 2h, resuspended in SDS-Page sample buffer, run on an SDS-gel, transferred on a membrane and incubated with streptavidin bound to a fluorophore and antibodies recognising V5 followed by fluorescently labelled secondary antibodies. Estimated molecular weight: SS-V5-BioID-KDEL: 25 kDa.

Processing a first batch of samples on mass spectrometry revealed a lot of background, especially cytoplasmic background that should in theory not be caused by the baits. Notwithstanding the natural biotinylation background, the sepharose in the beads used for the capture may aggregate proteins regardless of the streptavidin-biotin affinity and this is a well-known issue in experiments with mass spectrometry analysis following a pull-down.
In order to try to determine the identity of the biotinylated proteins, samples were analysed by mass spectrometry. Cells stably expressing TorsinA-V5-BioID were induced with doxycycline for 24h, incubated with biotin for 24h, lysed in a buffer containing 0.4% SDS and sonicated. After capture of biotinylated proteins with streptavidin beads, the beads were digested with trypsin and Lys-C, then the samples were desalted, precipitated and sent to a MS/MS spectrometer. Detected peptides were identified with Mascot. Analysis of a trial set of samples revealed a lot of cytoplasmic proteins that should not be biotinylated by a bait located in the ER/NE lumen. It was possible that the sepharose in the beads used for the capture may have bound non-specifically to non-biotinylated proteins. This is a well-known issue in experiments with mass spectrometry analysis following a pull-down (Vermeulen et al., 2008).

**Figure 5.10 Baits and background biotinylation.** Western-blot analysis for the biotinylation of parental and cells stably expressing the baits. Cells stably expressing SS-V5-BioID-KDEL, TorsinA-V5-BioID, TorsinA-V5-BirA and the parental cell line were treated with doxycycline for 24h and biotin for 24h, lysed in a buffer containing 0.4% SDS, sonicated, incubated with Dynabeads for 2h, resuspended in SDS-PAGE sample buffer, run on an SDS-gel, transferred on a membrane and incubated with streptavidin and antibodies recognising V5 followed by fluorescently labelled secondary antibodies. Estimated molecular weights: SS-V5-BioID-KDEL: 25 kDa; TorsinA-V5-BioID: 65 kDa; TorsinA-V5-BirA: 70 kDa.

In order to reduce background binding to the streptavidin beads, the pull-down was conducted with the addition of plain sepharose beads. While biotinylated proteins have an affinity for the streptavidin beads, part of the contaminant proteins might bind to the additional sepharose beads. The Dynabeads streptavidin beads being magnetic, proteins bound non-specifically to the sepharose beads would in theory be separated after washing the beads on a magnet while the Dynabeads would be retained. Cells stably expressing TorsinA-V5-BioID were induced with doxycycline for 24h, incubated with biotin for 24h, lysed in a buffer containing 0.4% SDS and sonicated. Proteins were captured with 100 µl
Dynabeads, 50 µl Dynabeads or 100 µl Dynabeads supplemented with 100 µl plain sepharose beads. After capture, the beads were separated on a magnet, digested with trypsin and Lys-C, then the samples were desalted, precipitated and sent to a MS/MS spectrometer. Detected peptides were identified with Mascot and Uniprot database was used to classify the hits according to their cellular compartments (Fig. 5.11A). 284 cytoplasmic proteins were detected with the inclusion of sepharose beads, while there were less than 200 cytoplasmic proteins when the samples were captured without additional sepharose beads. On the contrary, it dramatically increased isolation of proteins located in compartments that would preclude their biotinylation by the NE/ER localised biotin ligase. Furthermore, proteins from the ER and the NE, where the biotin ligase was located were relatively

![Graph A](image1)

![Graph B](image2)

**Figure 5.11 Optimising pull-down of biotinylated proteins.** Hit analysis of the proteins pulled-down with different beads. Cells stably expressing TorsinA-V5-BioID were treated with doxycycline for 24h and biotin for 24h, lysed in a buffer containing 0.4% SDS, sonicated, incubated with 50 µL or 100 µL Dynabeads or 100 µL Sepharose beads for 2h, digested, desalted and processed by mass spectrometry. The proteins corresponding to the resulting hits were found by Proteome Discoverer and Mascot, and their cellular localisation was found in Uniprot.

Unaffected with approximately 20 proteins per sample in the endoplasmic reticulum, and approximately 10 proteins per sample in the nuclear membrane. In comparison to 50 µl streptavidin
beads pull-down, 100 µl streptavidin beads captured more proteins in all compartments. Hits unique for each method were then compared (Fig. 5.11B). There were 18 unique hits in the ER for the samples with additional sepharose beads while the other samples had 3 unique hits in the ER. This result showed that addition of sepharose beads added a lot of unique proteins that were not detected in the other samples, hinting that those were background proteins not captured by streptavidin. Using less streptavidin beads did not provide a tangible difference in the number of unique hits, therefore 100 µl streptavidin beads and no sepharose was kept as the optimised pull-down protocol.

V. 5. Analysis of the pull-downs

The parental cell line HeLa-M as well as cells stably expressing TorsinA-V5-BioID, TorsinAΔE-V5-BioID, TorsinAEQ-V5-BioID, TorsinAKA-V5-BioID, LAP1-V5-BioID, SS-V5-BioID-KDEL, LULL1-V5-BirA, TorsinA-V5-BirA, TorsinAΔE-V5-BirA, TorsinAEQ-V5-BirA and TorsinAKA-V5-BirA were incubated with biotin then lysed in a buffer containing 0.4% SDS and sonicated. Three independent biological repeats of the experiments were performed. Lysed samples were incubated with 100 µl streptavidin beads to capture the biotinylated proteins, digested with trypsin and Lys-C then processed through mass spectrometry in an MS/MS procedure.

Raw data were then imported in the software Progenesis. The data for each sample was visually displayed as a 2D “ion map” with m/z in the x axis and retention time in the y axis (Fig. 5.12), with darker areas representing the abundance (number) of peptide ions in the MS/MS signal as if this was an electrophoresis gel. Specific peptides have a similar “fingerprint” among samples, and some of these “fingerprints” could be seen across all samples if they were part of the common background. These “fingerprints” were used to manually align the “ion map” of all samples and reduce chromatography shifts that occurred when the samples were run through the machine. Once all samples were aligned, Progenesis compared the similar patterns in all the samples and automatically designated one of the samples to normalise the abundance of each ion in all samples with an appropriate coefficient. Normalisation of the abundance between samples corrected for experimental variation when running samples, such as differences in sample loading. Once proteins were identified by Mascot, the abundance of each protein was established by the sum of its unique peptide ion abundances.

Mascot was used to identify peptides by searching for their profile in its database. The data was then exported to Excel. Uniprot and DAVID were used to consolidate the identifiers of each peptide in a custom Excel file designed by Alexandre Chojnowski (IMB, Singapore). The Excel file first cleaned up the database from redundant entries (same protein identified several times under different entry names) and peptides that had not been identified to a known protein profile. The cellular localisation of each protein was obtained from Uniprot database and added to the Excel file. Furthermore, the Excel file also allowed triplicates to be grouped together and calculate a mean for the abundance of each protein of each bait. Abundance ratios for each protein of each bait were expressed as a percentage of abundance relative to the bait displaying the highest abundance of each specific protein.
Figure 5.12 Ion map from a MS/MS sample. Screen capture of the Progenesis software during analysis of a biotinylation pull-down.

The spectral count for each protein was scored against known background contaminants from CRAPome (Mellacheruvu et al., 2013) and proteins isolated in pull-downs using the parental cell line in order to score protein hits. Background contaminants were given a lower score, and proteins abundant in bait pull-downs were given a higher score. STRING was used to cluster the proteins into networks according to suggested interactions from the literature and interaction databases. Finally, Cytoscape was used to visualise the data (Fig. 5.12A). Each bait (Fig. 5.12B) was colour-coded to display a histogram for the abundance ratio of each protein (Fig. 5.12C). As a reminder, the abundance of each protein is the sum of its peptide ion abundances detected in the pull-down of a bait. Abundance ratios for each bait are protein abundances expressed as a percentage relative to the protein that had the highest abundance in one of the pull-downs. For example, if the highest abundance of tubulin was 10 peptide ions with one bait, then if tubulin had 5 peptide ions with another bait its relative abundance would be 50%. Finally, the score determined the background colour of the rectangle displaying each protein. Proteins that had quantitatively more peptide ions than other proteins had a higher score, and proteins that were detected in the CRAPome contaminant database or in pull-downs of the parental cell line had a lower score. The score was displayed from bright blue (lowest score) to bright red (highest score).
Figure 5.12 Cytoscape representation. (A) Raw representation in Cytoscape of the 1656 unfiltered hits of the MS/MS experiment combined from all pull-downs. (B) Cartoon illustrating the different recombinant proteins used in this study and the colour used for the abundance ratio charts in Cytoscape. 1 = TorsinA-V5-BioID; 2 = TorsinAΔE-V5-BioID; 3 = TorsinAEQ-V5-BioID; 4 = TorsinAKA-V5-BioID; 5 = TorsinA-V5-BirA; 6 = TorsinAΔE-V5-BirA; 7 = TorsinAEQ-V5-BirA; 8 = TorsinAKA-V5-BirA; 9 = LAP1-V5-BioID; 10 = LULL1-V5-BirA; 11 = SS-V5-BioID-KDEL. BirA: biotin ligase; BioID: BirA second generation. (C) Graphical representation in Cytoscape of the abundance ratio charts for all baits described in B. Rectangle: zoomed-in view of a group of proteins. The names represent the gene symbol for each protein, and the background colours from blue to red represent the scoring. (D) Clustered display of the 53 remaining hits by STRING score of C after filtering by cellular localisation to remove hits that were not annotated for "endoplasmic", "nucleus envelope", "nuclear pore complex" and had a final score inferior to 0.5.
The 1656 combined hits detected in all pull-downs were filtered for their cellular localisation. Anything that was not annotated for "endoplasmic", "nucleus envelope", "nuclear pore complex" was removed, and 181 hits remained. Then the score for the CRAPome background contaminants and parental cell line pull-downs was taken into account, and any hit with a low score (<0.5) was removed, 89 hits remained. Then hits with a higher abundance ratio in SS-V5-BioID-KDEL were removed, 52 hits (3% of the total) remained and the STRING score was used to cluster the proteins in groups (Fig. 5.12D). The proteins from these clusters and the individual proteins with a high score were then individually analysed according to their abundance ratios for each bait and information about them found in the literature.

**Figure 5.13 Cytoscape representation.** Zoom-in on Fig. 4.12D with the cluster containing TOR1A, TOR1AIP1, TOR1AIP2 and FBXO2.

**Table 5.1 Hits and their localisation.** Description of the hits in Fig. Z1.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TOR1AIP2</td>
<td>Torsin-1A-interacting protein 2 (Lumenal domain-like LAP1) (LULL1)</td>
<td>Nucleus inner membrane.</td>
</tr>
<tr>
<td>FBXO2</td>
<td>F-box only protein 2</td>
<td>Cytoplasm, microsome membrane.</td>
</tr>
</tbody>
</table>

TorsinA, LAP1, LULL1 and F-box only protein 2 were part of a cluster (Fig. 5.13; Table 5.1). TorsinA was more abundant with the TorsinA BioID pull-downs than with the TorsinA BirA pull-downs, possibly due to the way the biotin ligases are physically positioned relative to the carrier due to the different size of the two enzymes (BirA, 38 kDa, is larger than BioID, 28 kDa – Kim et al., 2016). A higher ratio for the dystonia mutant protein makes sense as TorsinAΔE is known to form higher order heterooligomers with TorsinA (Goodchild et al., 2004). LAP1 (Fig. 5.13; Table 5.1) was only detected in LAP1-V5-BioID pull-downs which is surprising as TorsinA and LAP1 are involved in heterohexamers (Sosa et al., 2014).
Maybe the conformation was not right for the biotin ligase on the other baits to touch this protein. This result suggests that this experiment may not be successful. LULL1 (Fig. 5.13; Table 5.1) was mainly detected by itself, and not at a high ratio for any of the other baits. Again, the conformation was maybe not right for a good interaction, or the activity of TorsinA independently of LAP1 and LULL1 may be enough for the protein to perform as the cells were growing in the flasks. F-box only protein 2 (Fig. 5.13; Table 5.1), also known as F-box/G-domain protein 1 (FBG1), is a ubiquitin ligase involved in ERAD known to degrade neuronal glycoproteins in the cytosol (Nelson et al., 2006). It has a non-essential role on the degradation of TorsinA and TorsinAΔE (Gordon et al., 2012). Considering that TorsinA is degraded through macroautophagy while TorsinAΔE is targeted to the ubiquitin-proteasome pathway, the high ratio for the TorsinAΔE-V5-BioID may hint that FBXO2 directly interacted with the mutant protein to target it for degradation. However, since it is a cytosolic protein with no transmembrane domain, it should only have interacted with proteins in the cytoplasm.

Figure 5.14 Cytoscape representation. Zoom-in on Fig. 4.12D with the cluster containing SUN1, LMNB1, NUP88 and NUTF2.

<table>
<thead>
<tr>
<th>NUP88</th>
<th>Nuclear pore complex protein Nup88</th>
<th>Nucleus, nucleus envelope, nuclear pore complex.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUN1</td>
<td>SUN domain-containing protein 1</td>
<td>Nucleus inner membrane.</td>
</tr>
<tr>
<td>LMNB1</td>
<td>Lamin-B1</td>
<td>Nucleus inner membrane; Lipid-anchor; Nucleoplasmic side.</td>
</tr>
</tbody>
</table>

Table 5.2 Hits and their localisation. Description of the hits in Fig. Z12.

Lamin-B1, SUN1, Nup88 and NTF-2 were part of a cluster (Fig. 5.14; Table 5.2). Lamin-B1 was a surprising hit as it forms higher order polymers in the nucleoplasm (Burke et al., 2013). LAP1 has been
shown to interact with Lamin-B1 during interphase in the nucleoplasmic side of the nuclear envelope, but also in vesicles after nuclear envelope breakdown (Maison et al., 1997). Furthermore, knock-down of LAP1 has been shown to decrease the levels of Lamin-B1 (Santos et al., 2015). LULL1 and TorsinA pull-downs detected Lamin-B1, thus these proteins may also interact with Lamin-B1 together with LAP1 and it would be worth studying further. However, it is surprising that the biotin ligase detected a protein on the other side of the membrane. SUN1 (Fig. 5.14; Table 5.2) was detected predominantly in LAP1-V5-BioID pull-down. On one hand, it could be because both are inner nuclear membrane transmembrane proteins and thus they are very likely to be in close proximity. On the other hand, it could hint at a direct interaction and LAP1 could mediate the AAA+ ATPase activity of TorsinA on the LINC complex. Nuclear pore complex protein Nup88 (Fig. 5.14; Table 5.2) acts as a binding site for Lamin-A (Lussi et al., 2011) as part of the nuclear pore complex. Nup88 was detected by the TorsinA baits (especially TorsinAΔE-V5-BioID) as well as in SS-V5-BioID-KDEL pull-downs. NUP88 could act as a recruiter for TorsinA or proteins related to TorsinA. Nuclear transport factor 2 (Fig. 5.14; Table 5.2) mediates the import of some proteins from the cytoplasm to the nucleus, and in doing so recruits them to the nuclear pore complex (Paschal et al., 1995). NTF-2 was only a hit for the highly overexpressed TorsinAKA-V5-BirA which is expected not to be functional (Gerace et al., 2004).

![Figure 5.15 Cytoscape representation.](image)

**Figure 5.15 Cytoscape representation.** Zoom-in on Fig. 4.12D with the cluster containing YES1, PTRF, EGFR and BECN1.

<table>
<thead>
<tr>
<th>YES1</th>
<th>Tyrosine-protein kinase</th>
<th>Cell membrane, nucleus, cytoplasm, Golgi apparatus, membrane.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTRF</td>
<td>Polymerase I and transcript release factor (Cavin-1)</td>
<td>Membrane, caveola, Cell membrane, Microsome, Endoplasmic reticulum, Cytoplasm, cytosol.</td>
</tr>
<tr>
<td>EGFR</td>
<td>Receptor protein-tyrosine kinase</td>
<td>Membrane, endoplasmic reticulum, Golgi, nucleus membrane, endosome, nucleus.</td>
</tr>
<tr>
<td>BECN1</td>
<td>Beclin-1</td>
<td>Cytoplasm, Golgi membrane, endoplasmic reticulum membrane, mitochondrial membrane, endosome.</td>
</tr>
</tbody>
</table>

**Table 5.3 Hits and their localisation.** Description of the hits in Fig. Z13.
EGFR, PTRF, BECN1 and YES1 were part of another cluster (Fig. 5.15; Table 5.3). EGFR, epidermal growth factor receptor, is implicated in downstream signalling to regulate growth, survival, proliferation and differentiation (Oda et al., 2005). EGFR mediates the phosphorylation of Beclin-1 (Fig. 5.15; Table 5.3) (Wei et al., 2013), PTRF (Fig. 5.15; Table 5.3) (Guha et al., 2008) and Yes (Fig. 5.15; Table 5.3) (Sutton et al., 2013). PTRF, also known as Cavin-1, plays an important role in caveolae formation and organisation (Hill et al., 2008). There does not seem to be an obvious link to the proteins studied, it was detected largely by the bait LULL1-V5-BirA but also in SS-V5-BioID-KDEL pull-downs. The tyrosine-protein kinase YES1 may be involved in cytokinesis (Jung et al., 2011), and so is Beclin-1 (Thoresen et al., 2010; Sagona et al., 2010). EGFR was detected by most of the baits but also heavily detected in SS-V5-BioID-KDEL pull-downs, it could have simply been pulled down while binding with biotinylated proteins. However, PTRF and YES1 had high abundance ratios for the LULL1 bait, which could hint at a potential role of LULL1 during cytokinesis that may be worth investigating.

Figure 5.16 Cytoscape representation. Zoom-in on Fig. 4.12D with the clusters containing SEC63, SSR1, SRPRB, SEC23A and SEC31A.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEC63</td>
<td>Translocation protein SEC63 homolog</td>
</tr>
<tr>
<td>SSR1</td>
<td>Translocon-associated protein subunit alpha</td>
</tr>
<tr>
<td>SRPRB</td>
<td>Signal recognition particle receptor subunit beta</td>
</tr>
<tr>
<td>SEC23A</td>
<td>Protein transport protein Sec23A</td>
</tr>
<tr>
<td>SEC31A</td>
<td>Protein transport protein Sec31A</td>
</tr>
<tr>
<td>SEC63</td>
<td>Endoplasmic reticulum membrane; Multi-pass membrane protein.</td>
</tr>
<tr>
<td>SSR1</td>
<td>Endoplasmic reticulum membrane.</td>
</tr>
<tr>
<td>SRPRB</td>
<td>Endoplasmic reticulum.</td>
</tr>
<tr>
<td>SEC23A</td>
<td>Smooth endoplasmic reticulum membrane, Golgi.</td>
</tr>
<tr>
<td>SEC31A</td>
<td>Cytoplasm, endoplasmic reticulum membrane.</td>
</tr>
</tbody>
</table>

Table 5.4 Hits and their localisation. Description of the hits in Fig. Z14.

The proteins translocation protein SEC63 homolog, translocon-associated protein, signal recognition particle receptor, transport protein Sec23A and transport protein Sec31A were part of two interconnected clusters (Fig. 5.16; Table 5.4) The proteins in these clusters are involved in the translocation pathway (reviewed by Mori and Ito, 2001). They drive the transmembrane movement of
newly synthesized proteins and integration of some membrane proteins. These proteins may simply have been biotinylated while the baits were being translated to the endoplasmic reticulum.

Ras-related protein Rab-2A and syntenin were part of a cluster (Fig. 5.17; Table 5.5). Ras-related protein Rab-2A is required for protein transport from the endoplasmic reticulum to the Golgi complex or ER-associated degradation (Sugawara et al., 2014). Considering that it was detected by all the baits and was almost present at a similar abundance ratio in the parental cell line, it may be a false positive. Syntenin (Fig. 5.17; Table 5.5) is implicated in a diverse array of functions, including trafficking of transmembrane protein and ubiquitination (Philley et al., 2016). Interestingly, it was mostly detected by the TorsinAEQ-V5-BioID bait and then in second position by the TorsinAEQ-V5-BirA bait, which are the ‘substrate trap’ mutants (Naismith et al., 2004), making the syntenin a very good candidate for further studies.

![Cytoscape representation](image)

**Figure 5.17 Cytoscape representation.** Zoom-in on Fig. 4.12D with the clusters containing SDCBP and RAB2A.

<table>
<thead>
<tr>
<th>SDCBP</th>
<th>Syndecan binding protein (Syntenin), isoform CRA_a (Syntenin-1)</th>
<th>Cytoplasm, nucleus, endoplasmic reticulum membrane, membrane, cell junction, melanosome, secreted.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAB2A</td>
<td>Ras-related protein Rab-2A</td>
<td>Endoplasmic reticulum membrane, Golgi membrane.</td>
</tr>
</tbody>
</table>

**Table 5.5 Hits and their localisation.** Description of the hits in Fig. Z15.
**Figure 5.18 Cytoscape representation.** Zoom-in on Fig. 4.12D with the clusters containing PDIA5, FKBP2 and POGLUT.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>Localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDIA5</td>
<td>Protein disulphide-isomerase A5 (Protein disulphide isomerase-related protein)</td>
<td>Endoplasmic reticulum lumen.</td>
</tr>
<tr>
<td>FKBP2</td>
<td>Peptidyl-prolyl cis-trans isomerase FKBP2 (Rotamase)</td>
<td>Endoplasmic reticulum membrane; Peripheral membrane protein.</td>
</tr>
<tr>
<td>POGLUT1</td>
<td>Protein O-glucosyltransferase 1</td>
<td>Endoplasmic reticulum lumen.</td>
</tr>
</tbody>
</table>

**Table 5.6 Hits and their localisation.** Description of the hits in Fig. Z16.

Protein disulphide-isomerase 5, protein O-glucosyltransferase 1 and peptidyl-prolyl cis-trans isomerase FKBP2 were part of a cluster (Fig. 5.18; Table 5.6). Protein disulphide-isomerase 5 catalyses the rearrangement of disulphide bonds in proteins (Hayao et al., 1995). Little is known about this protein; however, it is interesting that it was detected by the TorsinA-V5-BirA bait, the TorsinA-V5ΔE-BirA bait and the highest ratio was for the LULL1-V5-BirA pull-down. This would be a protein of interest for further study, especially considering that we postulated that the effect of TorsinA on the LINC complex may be on the disulphide bridge between the SUN domain and the KASH domain. Peptidyl-prolyl cis-trans isomerase FKBP2 (Rotamase) (Fig. 5.18; Table 5.6) is implicated in the formation of collagen (Ishikawa et al., 2017). Protein O-glucosyltransferase 1 (Fig. 5.18; Table 5.6) is implicated in the regulation of NOTCH (Basmanav et al., 2014). Both these proteins have been detected by most baits, and also in the SS-V5-BioID-KDEL pull-down. They are also present in the pull-down from the parental cell line. Furthermore, a closer look at their relationship in the STRING database does not yield any meaningful link between these proteins or with PDIA5. They are very likely to be false positives.
Figure 5.19 Cytoscape representation. Zoom-in on Fig. 4.12D with the clusters containing TMX3, SEL1L and USP19.

<table>
<thead>
<tr>
<th>TMX3</th>
<th>Protein disulphide-isomerase TMX3</th>
<th>Endoplasmic reticulum membrane; Single-pass membrane protein.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEL1L</td>
<td>Protein sel-1 homolog 1</td>
<td>Endoplasmic reticulum membrane.</td>
</tr>
<tr>
<td>USP19</td>
<td>Ubiquitin carboxyl-terminal hydrolase 19</td>
<td>Endoplasmic reticulum.</td>
</tr>
</tbody>
</table>

Table 5.7 Hits and their localisation. Description of the hits in Fig. Z17.

Figure 5.20 Cytoscape representation. Zoom-in on Fig. 4.12D with the clusters containing USP10, ERP44 and TXNDC5.

<table>
<thead>
<tr>
<th>USP10</th>
<th>Ubiquitin carboxyl-terminal hydrolase 10</th>
<th>Cytoplasm, endoplasmic reticulum membrane (Hypothesis).</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERP44</td>
<td>Endoplasmic reticulum resident protein 44</td>
<td>Endoplasmic reticulum lumen.</td>
</tr>
<tr>
<td>TXNDC5</td>
<td>Thioredoxin domain-containing protein 5</td>
<td>Endoplasmic reticulum lumen.</td>
</tr>
</tbody>
</table>

Table 5.8 Hits and their localisation. Description of the hits in Fig. Z18.

Protein disulphide-isomerase TMX3, protein sel-1 homolog 1 and ubiquitin carboxyl-terminal hydrolase 19 were part of a cluster (Fig. 5.19; Table 5.7). Protein disulphide-isomerase TMX3 is a probable disulphide isomerase, which participates in the folding of proteins containing disulphide bonds (Haugstetter et al., 2005). It was detected by all baits including SS-V5-BioID-KDEL, thus despite the
potential interest in its function, this protein may be a false positive. Protein sel-1 homolog 1 (Fig. 5.19; Table 5.7) plays a role in ERAD (Lilley et al., 2005). Ubiquitin carboxyl-terminal hydrolase 19 (Fig. 5.19; Table 5.7) is a deubiquitinating enzyme that regulates the degradation of various proteins (Hassink et al., 2009). Considering that they were mostly detected by the TorsinAKA-V5-BirA bait that was expressed at much higher levels than the other baits (Fig. 5.2), these proteins could have been involved in the quality control of the overexpressed protein and are not selected as interesting for this study.

Ubiquitin carboxyl-terminal hydrolase 10, endoplasmic reticulum protein 44 and Endoplasmic reticulum protein 46 were part of a cluster (Fig. 5.20; Table 5.8). Ubiquitin carboxyl-terminal hydrolase 10 is a hydrolase that can remove conjugated ubiquitin from target proteins such as p53/TP53, BECN1, SNX3 and CFTR (Wang et al., 2015). This protein has mainly been detected by TorsinAKA-V5-BirA and LAP1-V5-BirA baits. Just like for the previous proteins involved in quality control, it could be because these baits were the most highly expressed (Fig. 5.2), except that it was not much abundant in the SS-BioID-V5-KDEL pull-downs. This protein may potentially be of interest for further studies, however it could be a false positive. Endoplasmic reticulum protein 44 (Fig. 5.20; Table 5.8) may be involved in the control of oxidative protein folding and may favour the maturation of disulphide-linked oligomeric proteins and their quality control (Anelli et al., 2002; Anelli et al., 2003). Endoplasmic reticulum protein 46 (Fig. 5.20; Table 5.8) belongs to the protein disulphide isomerase family (Li et al., 2017). Their functions could be of interest to the present study, however they have been detected in most pull-downs including SS-V5-BioID-KDEL and thus are likely to be false positives.

Figure 5.21 Cytoscape representation. Zoom-in on high score single protein hits from Fig. 4.12D EMD, VAPB and CKAP4.

|-----|--------|-------------------------------------------------------------------------------------------------|

Table 5.9 Hits and their localisation. Description of the hits in Fig. Z19.

Emerin, vesicle-associated membrane protein-associated protein B/C and cytoskeleton-associated protein 4 were not part of a cluster (Fig. 5.21; Table 5.9). Emerin mediates anchoring to the cytoskeleton, and mutations can cause muscular dystrophy (Barateau et al., 2017). It was highly
detected by TorsinAKA-V5-BirA and LULL1-V5-BirA, but also to a less extent by TorsinA-V5-BioID. Interestingly, LAP1 and emerin appeared to function together in mice skeletal muscle (Shin et al., 2014). This protein is an excellent candidate for further exploration. Vesicle-associated membrane protein-associated protein B/C (Fig. 5.21; Table 5.9) participates in the endoplasmic reticulum unfolded protein response (Kanekura et al., 2006). It was mainly detected by the TorsinAKA-V5-BirA bait, and mildly detected by the LULL1-V5-BirA bait, thus by the baits expressed at the highest levels except for SS-BioID-V5-KDEL (Fig. 5.2). There does not seem to be enough reasons to consider this differently than a false-positive. Cytoskeleton-associated protein 4 (Fig. 5.21; Table 5.9) mediates the anchoring of the endoplasmic reticulum to microtubules (Osseni et al., 2016). Despite the interesting and relevant function, this protein was detected by all baits and very highly by the SS-V5-BioID-KDEL bait which may help decide in favour of a false positive.

Transmembrane emp24 domain-containing protein 5, phosphatidylinositol 5-phosphate 4-kinase type-2 beta, charged multivesicular body protein 4b, lanosterol synthase and translin were not part of any cluster (Fig. 5.22; Table 5.10). Transmembrane emp24 domain-containing protein 5 has a potential role in vesicular protein trafficking between Golgi stacks (Koegler et al., 2010). It was mostly detected by the TorsinAEQ-V5-BioID and TorsinAΔE-V5-BirA baits, as well to a lesser extent by the same mutants with a BirA. While the known function does not seem to relate, the detection pattern makes it worth investigating. Translin (Fig. 5.22; Table 5.10) exhibits both single-stranded and double-stranded endoribonuclease activity (Aoki et al., 1995). It was solely detected by LULL1-V5-BirA and is very likely a false positive. Phosphatidylinositol 5-phosphate 4-kinase type-2 beta (Fig. 5.22; Table 5.10) participates in the biosynthesis of phosphatidylinositol 4,5-bisphosphate (Castellino et al., 1997). It was mainly detected by the LULL1-V5-BirA bait, and to some extent by the TorsinA baits which may makes it a protein of interest. Charged multivesicular body protein 4b (Fig. 5.22; Table 5.10) plays a role in the endosomal sorting pathway ESCRT-III (Baietti et al., 2012; Xu et al., 2017). The ESCRT-III complex is involved in nuclear envelope reformation (Olmos et al., 2015). The detection of the protein by the bait LAP1-V5-BioID is of interest, however its abundance ratio is also high for the bait SS-V5-BioID-KDEL. This may be a relationship worth investigating despite the low confidence in this detection. Lanosterol synthase (Fig. 5.22; Table 5.10) is implicated in the reaction that forms the sterol nucleus (Baker et al., 1995). It was mostly detected by the EQ variants of TorsinA that act as ‘substrate traps’ (Naismith et al., 2004), TorsinAEQ-V5-BioID and TorsinAEQ-V5-BirA, as well as LAP1-V5-BirA. Due to these observations, Lanosterol synthase seems like a good candidate for further investigations.
Figure 5.22 Cytoscape representation. Zoom-in on Fig. 4.12D with the single proteins hits TMED5, LSS, TSN, PIP4K2B and CHMP4B.

Table 5.10 Hits and their localisation. Description of the hits in Fig. Z20.

<table>
<thead>
<tr>
<th></th>
<th>Description of the hits in Fig. Z20.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMED5</td>
<td>Transmembrane emp24 domain-containing protein 5</td>
</tr>
<tr>
<td>LSS</td>
<td>Lanosterol synthase</td>
</tr>
<tr>
<td>TSN</td>
<td>Translin</td>
</tr>
<tr>
<td>PIP4K2B</td>
<td>Phosphatidylinositol 5-phosphate 4-kinase type-2 beta</td>
</tr>
<tr>
<td>CHMP4B</td>
<td>Charged multivesicular body protein 4b (Chromatin-modifying protein 4b)</td>
</tr>
</tbody>
</table>

Table 5.10 Hits and their localisation. Description of the hits in Fig. Z20.

V. 6. Discussion

Proximity-dependent biotin identification is a novel method aimed at identifying proteins in the immediate proximity of a bait (Roux et al., 2012). A protein of interest is fused to a recombinant biotin-ligase which biotinylates primary amines of proteins close to the bait. Streptavidin is used for its affinity with biotin to capture biotinylated proteins which are then analysed by mass spectrometry. A bioinformatics analysis subsequently identifies the captured proteins. Normalised spectral counts permit to normalise the abundance of the captured proteins ('hits') relative to each other.

In this study, LAP1, LULL1 and TorsinA and its mutants were used as bait (Fig. 5.1). They were fused either to a first-generation biotin ligase (Roux et al., 2012) or a second-generation biotin ligase (Kim et al., 2016), considering that none had previously been tested in the ER system. Furthermore, the different sizes of the enzymes (28 kDa and 38 kDa, respectively) could potentially detect different proteins as evidenced when flexible linkers of different lengths were used (Kim et al., 2016).

Doublet bands were observed on gel for the second-generation BioID (Fig. 5.2) that could potentially represent proteins that had not been translocated into the ER and thus retained the N-terminal sequence.
(Callan et al., 2007). However, the baits were not located in the cytoplasm (Fig. 5.4; Fig. 5.5) and glycosylated forms were shown to be responsible for the doublet (Fig. 5.6; Fig. 5.7).

An important number of hits (~1500) was detected, however this number likely reflected background biotinylation in cells (Dakshinamurti et al., 1981) in addition to typical mass spectrometry and pull-down contaminants (Mellacheruvu et al., 2013). Optimisation of the protocol by Alexandre Chojnowski (IMB, Singapore) did not reduce the background of the pull-down (Fig. 5.11), the only gain was slightly better yield of captured proteins when switching to sonication rather than using a syringe to shear the DNA (Fig. 5.9). Background contaminants were likely bound to the sepharose or to other captured proteins, but increasing the concentration of the ionic detergent SDS to remove background also prevented legitimate proteins from being captured (Fig. 5.9). The difference in expression of the baits was up to 14.9-fold (Fig. 5.2) which likely meant that the highly expressed baits encountered more proteins as well as cell systems specialised in quality control (Fig. 5.19; Fig. 5.20; Table 5.7; Table 5.8). A better approach of the optimisation would have been to express the baits at similar levels.

Once the protocol was established, TorsinA-V5-BioID, TorsinAΔE-V5-BioID, TorsinAEQ-V5-BioID, TorsinAΔE-V5-BioID, LAP1-V5-BioID, TorsinA-V5-BirA, TorsinAΔE-V5-BirA, TorsinAEQ-V5-BirA, TorsinAKA-V5-BirA and LULL1-V5-BirA were used as baits to biotinylate proteins in close proximity. After pull-down and a MS/MS procedure, the resulting hits in the ER/NE were analysed and filtered to a select few (Table 5.11). Several proteins have been detected with a particularly high abundance ratio in favour of LULL1-V5-BirA. While this could be due to the higher expression level of this bait; it may also be related to the function of LULL1 or to proteins that direct LULL1 to TorsinA to trigger their activity. The proteins of interest are Lamin-B1 (Fig. 5.14; Table 5.2) that is a known partner for LAP1, PTRF (Fig. 5.15; Table 5.3) and YES-1 (Fig. 5.15; Table 5.3) that have a role in cytokinesis, protein disulphide-isomerase 5 (Fig. 5.18; Table 5.6) that rearranges disulphide bridges and finally emerin (Fig. 5.21; Table 5.9) that is able to bind the SUN proteins (Haque et al., 2010) and also seems to work with LAP1 (Shin et al., 2014). Phosphatidylinositol 5-phosphate 4-kinase type-2 beta (Fig. 5.22; Table 5.10) was detected by LULL1-V5-BirA, and the TorsinA baits, which could implicate these proteins in a signalling pathway. LAP1-V5-BioID detected charged multivesicular body protein 4b (Fig. 5.22; Table 5.10), linked to the ESCRT-III complex that is involved in nuclear envelope reformation. With the detection of proteins linked to cytokinesis, there is a lot of potential for TorsinA and its interactors to be somehow related to the manipulation of the nuclear envelope. Several proteins were detected by the mutants that are believed to act as ‘substrate traps’ (Naismith et al., 2004). Those proteins are transmembrane emp24 domain-containing protein (Fig. 5.22; Table 5.10) involved in vesicular protein trafficking between Golgi stacks and syntenin-1 (Fig. 5.17; Table 5.5) implicated in trafficking of transmembrane protein. This may suggest a new role for TorsinA related to membrane trafficking. The detection of lanosterol synthase (Fig. 5.22; Table 5.10), implicated in the reaction that forms the sterol nucleus, further implicates TorsinA and its interactors in the manipulation of the nuclear envelope. Nup88 (Fig. 5.14; Table 5.2) was also detected by the TorsinA baits, perhaps to recruit the proteins for a role close to the NPC.
### Table 5.11 Identified proteins selected as the ten best interaction candidates.

Selected hits after proximity-dependent biotin identification of TorsinA, LAP1 and LULL1 followed by manual filtering.

<table>
<thead>
<tr>
<th>Candidate proteins</th>
<th>Bait</th>
<th>Potential involvement</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emerin</td>
<td>LULL1</td>
<td>Anchoring of the nuclear envelope to the cytoskeleton</td>
<td>Barateau et al., 2017</td>
</tr>
<tr>
<td>PTRF</td>
<td>LULL1</td>
<td>Cytokinesis</td>
<td>Guha et al., 2008</td>
</tr>
<tr>
<td>YES-1</td>
<td>LULL1</td>
<td>Cytokinesis</td>
<td>Sutton et al., 2013</td>
</tr>
<tr>
<td>Nup88</td>
<td>LULL1, TorsinA</td>
<td>Nuclear pore complex, binding site for Lamin-A</td>
<td>Lussi et al., 2011</td>
</tr>
<tr>
<td>Lamin-B1</td>
<td>LULL1, TorsinA</td>
<td>Structure and function of the lamina</td>
<td>Burke et al., 2013</td>
</tr>
<tr>
<td>Protein disulphide-isomerase 5</td>
<td>LULL1, TorsinAΔE</td>
<td>Rearrangement of disulphide bridges</td>
<td>Hayao et al., 1995</td>
</tr>
<tr>
<td>Lanosterol synthase</td>
<td>LAP1, 'substrate trap' TorsinA</td>
<td>Formation of the sterol nucleus</td>
<td>Baker et al., 1995</td>
</tr>
<tr>
<td>Phosphatidylinositol 5-phosphate 4-kinase type-2 beta</td>
<td>LAP1, 'substrate trap' TorsinA</td>
<td>Nuclear envelope reformation</td>
<td>Castellino et al., 1997</td>
</tr>
<tr>
<td>Syntenin-1</td>
<td>TorsinA</td>
<td>Trafficking of transmembrane protein</td>
<td>Philley et al., 2016</td>
</tr>
<tr>
<td>Transmembrane emp24 domain-containing protein</td>
<td>-substrate trap' TorsinA</td>
<td>Vesicular protein trafficking between Golgi stacks</td>
<td>Koegler et al., 2010</td>
</tr>
<tr>
<td>Emerin</td>
<td>LULL1</td>
<td>Anchoring of the nuclear envelope to the cytoskeleton</td>
<td>Barateau et al., 2017</td>
</tr>
</tbody>
</table>

The final score calculated by low presence background and high abundance ratio was used to narrow down the candidates to a final score above 1 (red in cytoscape representation, Fig. 5.12). Resulting top candidates were emerin and Lamin-B1 (Table 5.12). LULL1 and TorsinA have been shown to coimmunoprecipitate with Lamin-B1, while LAP1 knock-down decreases Lamin-B1 levels (Santos et al., 2015). Their detection is consistent with their ability to coimmunoprecipitate, thus giving strong credibility this ‘hit’. LAP1 and emerin appeared to function together in mice skeletal muscle (Shin et al., 2014). Consequently, these two hits appear to be ideal candidates for further studies. However, this final selection may be too stringent as it only includes suspected interactors rather than new potential targets.

### Table 5.12 Identified proteins with the best score.

Selected hits with the highest score (>1) after proximity-dependent biotin identification of TorsinA, LAP1 and LULL1 followed by manual filtering.

<table>
<thead>
<tr>
<th>Candidate proteins</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emerin</td>
<td>Anchoring of the nuclear envelope to the cytoskeleton</td>
<td>Barateau et al., 2017</td>
</tr>
<tr>
<td>Lamin-B1</td>
<td>Structure and function of the lamina</td>
<td>Burke et al., 2013</td>
</tr>
</tbody>
</table>
TorsinA was more abundant with the TorsinA BioID pull-downs than with the TorsinA BirA pull-downs (Fig. 5.13; Table 5.1), confirming that different lengths of the enzyme have a different reach (Kim et al., 2016) and BirA may have been too big to biotinylate the neighboring protomer. TorsinA and LAP1 are involved in heterohexamers (Sosa et al., 2014) and TorsinA should have been detected by the LAP1 bait, however LAP1 was only detected in LAP1-V5-BioID pull-downs (Fig. 5.13; Table 5.1). Similarly, LULL1 was not detected at a high ratio by TorsinA (Fig. 5.13; Table 5.1). SUN1 was not abundantly detected by TorsinA (Fig. 5.14; Table 5.2), although SUN1 is involved in the proper localisation of TorsinAΔE to the NE (Jungwirth et al., 2001). Finally, nesprins were not detected although this may be because the lysines are not accessible due to the conformation of the protein. Taken together, these results show that known proteins were not detected as expected and casts doubt on the significance of the absence of detection of other potential interactors.

The analysis of pull-downs from biotin ligase proximity ligation was complicated by a high signal-to-noise ratio that made it difficult to distinguish whether a protein was interacting with the baits, whether a protein was simply in the physical environment of the baits, whether a protein was brought along by binding to other proteins, whether a protein was naturally biotinylated by the cell, or whether a protein was pulled down in the beads despite having no affinity with streptavidin. Indeed, 97% of the detected proteins were not in the same compartment as the baits (Fig. 5.11), which furthermore casts doubt on the validity of the remaining 3%. The parental cell line as well as the SS-V5-BioID-KDEL acted as controls to help identify false positives, however they may also have coloured the detection of genuine hits as false positives.

Using a large number of baits allowed to refine the interpretation of the hits, although a major caveat was the differential expression levels between the baits. Highly expressed baits may have accumulated in the ER and biotinylated proteins that the baits would not have encountered otherwise. There was also no guarantee that these proteins hindered by the biotin ligases were still functional and would meet their standard partners.

A few candidates have been picked that may elicit interest in further studies (Table 5.11), but the assay itself was not enough to validate an interaction or to draw solid conclusions on the potential function of TorsinA. Other assays such as co-immunoprecipitation or PCR duelling will be required to confirm the interaction with newly detected candidates.
VI. GENERAL DISCUSSION AND FUTURE WORK

LINC components act as spacers (Sosa et al., 2012) and maintain the functional and structural integrity of the nuclear envelope (Burke and Roux, 2009). However, during mitosis the NE undergoes rearrangement and the INM proteins are phosphorylated and incorporated into a tubulated ER fused with membranes that previously constituted the NE (Ungricht et al., 2017). This study aimed to answer the question whether SUN and KASH interaction is maintained as the cell undergoes mitosis. An early hypothesis was that the LINC complex remains intact during mitosis (Patel et al., 2014). However, the results of the present study demonstrated that a population of nesprin-2 is present in clusters without SUN2 throughout telophase, and these clusters persist in early G1 after the NE has been resealed. SUN1 overlaps with nesprin-2 in these clusters, and their C-terminus interaction may prevent the assembled LINC complexes from being translocated back to the NE through the NPCs. During a rare phenomenon known as ‘anaphase lag’ (Pampalona et al., 2016), SUN2 and nesprin-2 were observed at the cleavage furrow. However, SUN1 did not overlap with the ‘lagging chromosome’ at the cleavage furrow, which could be because SUN1 was not yet dephosphorylated to regain its ability to interact with chromatin (Patel et al., 2014). Taken together, these results suggest that nesprin-2 and SUN1 may not always interact throughout mitosis and at least a population of these proteins may have undergone disassembly at some point, and that nesprin-2 and SUN2 may not always interact throughout the cell cycle. Interestingly, these findings also suggest that nesprin-2 could potentially discriminate between SUN1 and SUN2 and that nesprin-2 and SUN2 have a different timing to go back to the NE. This timing may depend on the phosphorylation/dephosphorylation rate of the proteins (Patel et al., 2014), or the assembly status of the NPCs (Dultz et al., 2008) as SUN1 seems to associate with and localise to NPCs (Liu et al., 2007). The fate of the clusters also remains unanswered; the hypothesis of this study is that LINC components trapped in ER clusters are targeted for degradation by the proteasome, but this may seem like a wasteful process. Alternatively, LINC complexes in these clusters may have taken a longer time to dissociate and be retargeted to the NE. Live fluorescence with GFP-tagged SUN1 and RFP-tagged SUN2 would give insight on the fate of these clusters and whether they return to the NE with a different timing, or whether they are degraded outside the NE. Furthermore, mutated proteins with their phosphorylation sites removed could possibly affect their timing of NE relocalisation. Most importantly, depleting SUN1 or SUN2 and observing the localisation of nesprin-2 may determine whether they were interacting or not. If SUN1 was depleted and not present in telophases clusters anymore and nesprin-2 was absent from the clusters, this hypothetical observation would mean that an interaction between SUN1 and nesprin-2 was necessary to trap nesprin-2 in telophase clusters. Deleting the C-terminus or N-terminus parts of SUN1 would help determine which parts of the protein are necessary for the binding of nesprin-2. Conversely, if nesprin-2 was present in telophase clusters without SUN1, this would mean that nesprin-2 may not be interacting with SUN1 and only shares a similar timing in returning to the NE.

Another aim of this study was to investigate the role of TorsinA, a protein belonging to the AAA+ ATPase family of proteins. AAA+ ATPases are hexameric proteins typically involved in protein folding and unfolding, disassembly of protein complexes and degradation of proteins by proteolysis (reviewed in Hanson and Whiteheart, 2005). However, TorsinA lacks a conserved arginine near the nucleotide
binding site. LULL1 and LAP1 lack other AAA+ ATPase nucleotide binding motifs, but they form heterohexamers with TorsinA and are suspected to be necessary to the ATPase activity of TorsinA by providing an ‘arginine finger’ to the nucleotide binding pocket of TorsinA (Sosa et al., 2014). The LINC complex was suggested to be a substrate for the activity of TorsinA in the NE (Nery et al., 2008; Jungwirth et al., 2011), possibly targeted for degradation by LULL1-activated TorsinA (Vander Heyden et al., 2009). In order to investigate the effect of TorsinA, a model of LINC disruption was first established with dominant negative LINC components and SUN1 depletion (Fig. 4.29). When the LINC complex was disrupted, the recombinant protein GFP-nesprin-2 mislocalised to the ER was degraded. However, when the LINC complex was disrupted, levels of the endogenous nesprin-2 increased. This may be caused by cellular regulation to compensate for the degradation of nesprin-2 or the lack of LINC complexes in a stable conformation. Lack of TorsinA and to some extent LAP1 achieved similar effects of LINC complex disruption, as well as decreasing the mobility of nesprin-2 in the NE. Taken together, these results suggest that TorsinA and by extension LAP1 strengthen the interaction of the LINC complex. This result is consistent with the increase of telophase clusters containing nesprin-2 when LAP1 was overexpressed, as LAP1 and nesprin-2 overlapped in these clusters and stable LINC complexes may have been unable to translocate back to the NE through the NPCs. Furthermore, this result is also consistent with a study demonstrating that TorsinA and LAP1 are necessary for TAN lines assembly and persistence (Saunders et al., 2017). Considering that redox activity was necessary for the effect of TorsinA (Zhu et al., 2010; Saunders et al., 2017), TorsinA might have formed a disulphide bond between the facing cysteines in the SUN and KASH domains (Sosa et al., 2012). To prove this hypothesis, mutating the cysteine residues in the redox motif of TorsinA would decrease the number of nesprin-2 and SUN1 clusters in telophase even when LAP1 is overexpressed. Similarly, TorsinA with mutated cysteins or LAP1 without an ‘arginine finger’ would increase the mobility of GFP-nesprin-2 with FRAP assays as the SUN-KASH interaction could be less stable without a disulphide bridge, and inversely overexpressed wild-type LAP1 would decrease the mobility of GFP-nesprin-2. Furthermore, the impact of TorsinA mutants on the strength of the KASH-SUN interaction could be studied with cross-linking assays or coimmunoprecipitation. Conversely, unlike LAP1 deletion, LULL1 depletion did not affect TAN lines formation (Saunders et al., 2017) nor affected the NE or mortality in mice (Goodchild et al., 2005), which suggests a different role for LULL1 than LAP1. Overexpression of LULL1 may have a different effect on SUN1/nesprin-2 telophase clusters, and depletion of LULL1 may have a different effect than TorsinA/LAP1 depletion on the levels of nesprin-2 or the mobility of nesprin-2 in the NE. Furthermore, the disruption model established in this study was almost exclusively based on the levels of nesprin-2, but an increase of the levels of SUN1 suggests that SUN1 and SUN2 should also be monitored when the interaction between LINC components is disrupted by the lack of an interacting partner or when the function of TorsinA and its cofactors is perturbed. Besides demonstrating whether these proteins are degraded by the proteasome, ubiquitination assays could inform whether the proteins are targeted for degradation when the LINC complex is disrupted. Furthermore, RT-qPCR could provide insight on the regulation of LINC components gene expression when the complex is disrupted.

Proximity-dependent biotin identification (BioID) (Roux et al., 2012) was used to identify potential interactors of TorsinA, LAP1 and LULL1 by biotinylation of proteins in the vicinity of the recombinant baits fused with a biotin ligase. The signal-to-noise ratio was estimated at approximatively 3%, and known
interactors such as TorsinA, LAP1 and LULL1 were barely detected by each other. This could be due to the availability of lysines in the conformation of the adjacent proteins as well as the length and reach of the biotin ligase fused to the bait proteins (Kim et al., 2016). The presence of background and absence of several known interactors casts doubt on the relevance of any ‘hits’ identified during the filtering and selection of potential candidates. Conversely, proteins selected as best candidates after merging user-based and mathematical filtering are emerin and Lamin-B1, which have been previously shown to be potential interactors: LAP1 and emerin seem to function together in mice skeletal muscle (Shin et al., 2014), LAP1 depletion decreases Lamin-B1 levels, and LULL1 and TorsinA coimmunoprecipitate with Lamin-B1 (Santos et al., 2015). Considering that these top hits were legitimate interactor candidates, the other proteins identified in the final selection may be worth investigation further (Table 5.11). Coimmunoprecipitation or cross-linking could validate these proteins as interactor candidates. To study them further, their function could be monitored alongside the function of TorsinA, LAP1 and LULL1 when one of these proteins is depleted.

In conclusion, this study described novel findings on the behaviour of the LINC complex during mitosis, suggesting a differential timing between SUN1 and SUN2 to the NE in formation during telophase, and the existence of SUN1/nesprin-2 telophase clusters which persist until early G1. The fate of these clusters requires further examination, although a model for LINC components disruption suggests that nesprin-2 trapped in the ER is targeted for degradation by the proteasome. The role of TorsinA seems to strengthen the SUN-KASH interaction as nesprin-2 is more mobile and is degraded in the absence of TorsinA, which further establishes TorsinA as a key player in the regulation and stabilisation of the LINC complex. However, more studies will be necessary to validate this hypothesis, and new research should put emphasis on the disulphide bridge between the SUN and KASH domains as well as the redox sensor motif of TorsinA. Finally, BioID allowed to strengthen the potential role of Lamin-B1 and emerin as TorsinA/LAP1/LULL1 interactors, and some new proteins were selected as candidates to validate their interaction with TorsinA/LAP1/LULL1.
REFERENCES


Burke, B. (2012). It takes KASH to hitch to the SUN. Cell 149, 961-963.


