

# A three-dimensional view of the interface between nuclear envelope and chromatin

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# ABBREVIATIONS

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Aa	Amino Acid
AD-EDMD	Autosomal Dominant-Emery Dreifuss Muscular Dystrophy
APS	Atypical progeria syndromes
AR-EDMD	Autosomal Recessive-Emery Dreifuss Muscular Dystrophy
ATP	Adenosine triphosphate
BAF	Barrier-to-autointegration factor
CEA	French Alternative Energies and Atomic Energy Commission
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CMC	Cardiomyopathy with conduction defects
CMT	Charcot-Marie-Tooth disease
CTSL	Cathepsin L
DSBs	Double strand breaks
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid
DTT	Dithiothréitol
<i>E. Coli</i>	<i>Escherichia coli</i>
EDMD	Emery-Dreifuss muscular dystrophy
EDTA	Acide éthylène diamine tétra-acétique
EM	Electron Microscopy
ER	Endoplasmic Reticulum
F-actin	Filamentous actin
FBTSA	Fluorescence-based Thermal Shift Assay
FLIP	Fluorescence loss in photobleaching
FMP	Leibniz-institut für molekulare pharmakologie

FPLD	Dunnigan-type familial partial lipodystrophy
FRET	Fluorescence resonance energy transfer
G-actin	Globular actin
GCL	Germ cell less
GF	Gel Filtration
GFP	Green Fluorescent Protein
GST	Glutathione-S-transferase
HDAC3	Histone deacetylase 3
HGPS	Hutchinson-Gilford progeria syndrome
HhH motif	Helix-hairpin-Helix motif
HSQC	Heteronuclear single quantum coherence spectroscopy
Icmt	Isoprenylcysteine carboxyl methyltransferase
IgFold domain	Immunoglobulin-like domain
INM	Inner Nuclear Membrane
IPTG	Isopropyl $\beta$ -D-1-thiogalactopyranoside
ITC	Isothermal Titration Calorimetry
KASH domain	Klarsicht, ANC-1 and SYNE/Nesprin-1 and 2 Homology domain
LADs	Lamina-associated domains
LAS	Lamina-associated sequences
LB	Lysogeny Broth
LBR	Lamin-B receptor
LBSR	Structural Biology and Radiobiology Laboratory
LEBS	Enzymology and Structural Biochemistry Laboratory
LEM domain	LAP2-Emerin-Man1 domain
LGMD	Limb-girdle muscular dystrophy type 1B

LINC complex	Linker of Nucleoskeleton and Cytoskeleton complex
Lmo7	Lim-domain-only-7
MAD	Mandibuloacral dysplasia
MSC motif	MAN1/Src1p/C-terminal motif
NARF	Nuclear Prelamin A Recognition Factor
NE	Nuclear Envelope
NGPS	Nestor Guillermo Progeria Syndrome
NHEJ	Nonhomologous end-joining
Ni-NTA	Nickel-nitrilotriacetic acid
NLS	Nuclear Localization Signal
NMR	Nuclear Magnetic Resonance
NPC	Nuclear Pore Complex
NRF2	Nuclear Factor (erythroid-derived 2)-like 2
OD	Optical Density
O-GlcNA	O-linked-Nacetylglucosamine
ONM	Outer Nuclear Membrane
PCR	Polymerase chain reaction
PDB	Protein data bank
PEG	Polyethylenglycol
PIC	Pre-integration complex
PKA	Protein Kinase A
PLA	Proximity Ligation Assay
PMSF	Phenylmethyl Sulfonyl Fluoride
PS	Progeroid syndromes
Ran-GTP	RAs-related nuclear protein-Guanosine Triphosphate

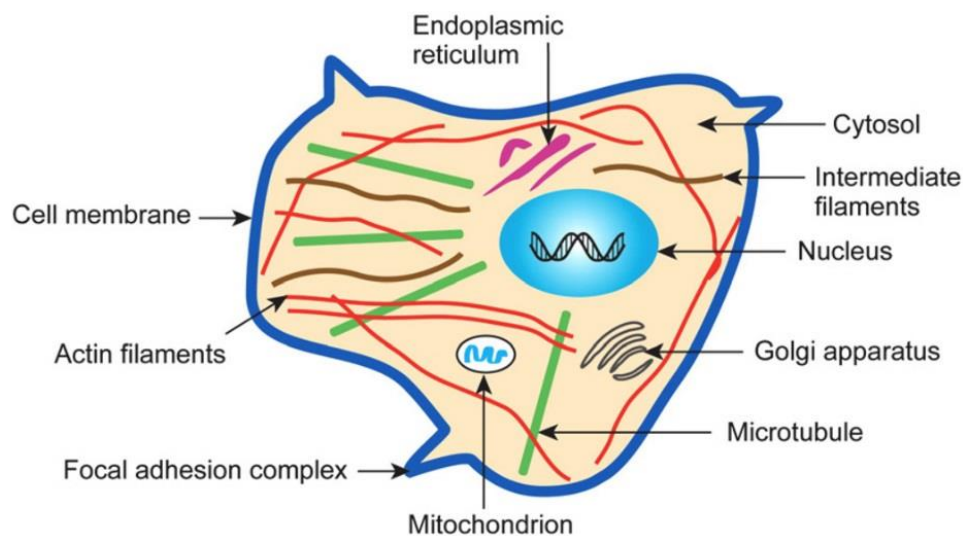


Rce1	Ras Converting CAAX Endopeptidase 1
RD	Restrictive dermopathy
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
<i>S.cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	Sodium dodecyl sulfate
SDS-PAGE	Polyacrylamide gel electrophoresis with SDS
Src	Tyrosine kinase Src
SUN domain	Sad1 and UNC-84 domain
TAN lines	Transmembrane actin-associated nuclear lines
TEV	Tobacco etch virus protease
ThT	Thioflavin
TM	Transmembrane domain
Trp	Translocated promoter region protein
UHM motif	U2AF homology motif
WT	Wild-Type
XL-EDMD	X-linked-Emery Dreifuss Muscular Dystrophy
53BP1	P53-binding protein 1

# INTRODUCTION

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The defining feature of eukaryotic cells is that they have membrane-bound organelles such as mitochondria and the Golgi apparatus (figure 1). The bigger organelle specific to eukaryotic cells is the nucleus, with a diameter of 10 to 20  $\mu\text{m}$ . It has two major functions: it stores cell hereditary material (DNA), and it coordinates cell activities, which include growth, intermediary metabolism, protein synthesis, and cell division. Eukaryotes usually have a single nucleus, but a few cell types, such as mammalian red blood cells, have no nucleus, and a few others, as some muscle cells, have many nuclei<sup>1,2</sup>.



**Figure 1 : Scheme of a eukaryotic cell with its different components<sup>3</sup>.**

A nuclear envelope separates the cytoplasm from the nucleoplasm (figure 2). This envelope contains the outer nuclear membrane, the inner nuclear membrane and a lot of proteins that can be gathered into three groups. The first group contains the nuclear pore complexes, which are large protein complexes crossing the nuclear envelope. They mediate the selective exchange of components, including RNA, ribosomal proteins, signaling molecules and lipids between the cytoplasm and the nucleus. The second group is composed of integral membrane proteins, as for example the LEM domain proteins Emerin, MAN1 and LAP2 (for Lamina-Associated Protein 2)<sup>4</sup>. The last group corresponds to the nuclear lamina, which is a meshwork of lamin filaments underlying the nuclear envelope (figure 2).

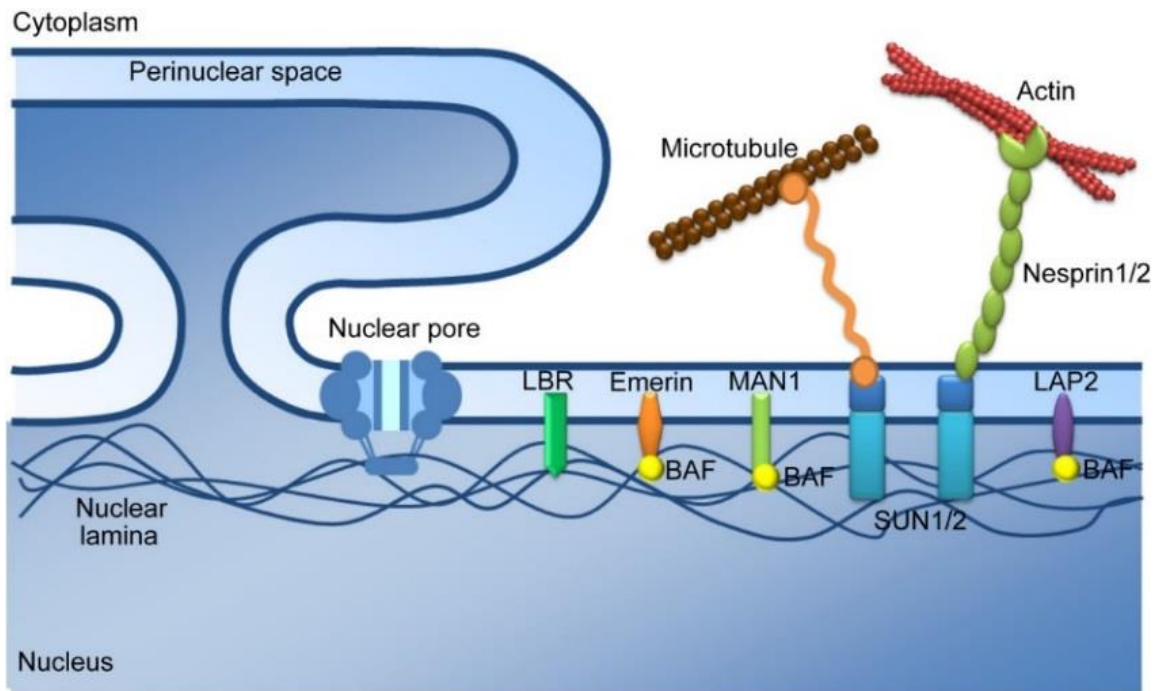


Figure 2 : Architecture of the nuclear envelope<sup>5</sup>.

The nuclear envelope (NE) is the focus of an increasing number of studies because mutations in genes encoding NE proteins cause a panel of human diseases called nuclear envelopopathies. A large number of NE-associated diseases are caused by inherited mutations in the gene *LMNA* coding for A-type lamins<sup>6</sup>. These laminopathies include Emery-Dreifuss muscular dystrophy, dilated cardiomyopathy with variable muscular dystrophy, Dunnigan-type familial partial lipodystrophy, Charcot-Marie-Tooth type 2 disease, mandibuloacral dysplasia, and the Hutchinson-Gilford progeria syndrome. Research on laminopathies and more generally on nuclear envelopopathies has provided novel clues about nuclear envelope function.

# I. NUCLEAR ENVELOPE: STRUCTURE

Each component of the nuclear envelope (outer and inner nuclear membranes, nuclear pore complexes, membrane proteins and nuclear lamina) forms distinct domains but all are interconnected. The architecture of these domains is poorly characterized and their structural study remains nowadays challenging.

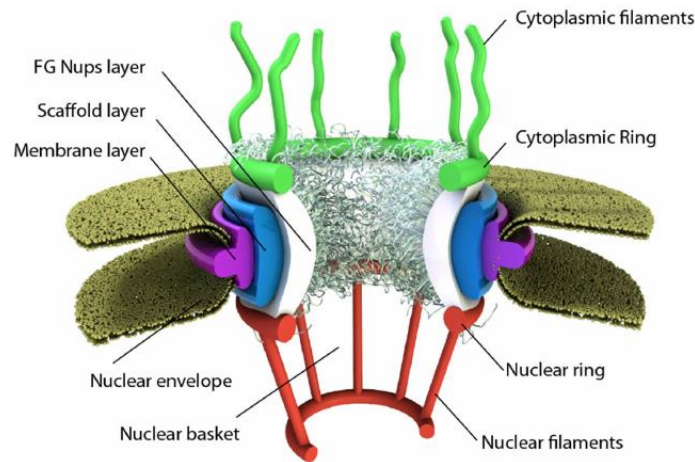
## 1. THE NUCLEAR PORE COMPLEX

### *a. Structure*

The vertebrate nuclear pore complex (NPC), which is one of the largest macromolecular complexes in the cell with an estimated molecular mass of 120 kDa, is embedded into the outer and the inner nuclear membranes and mediates macromolecular transport across the NE<sup>7</sup>. In mammalian cells, the number of nuclear pore complexes varies between 4000 and 6000 and they appear like a ring of a 100 nm of diameter<sup>8</sup>.

Several 3D structures of these nuclear pore complexes were recently reported. For example, the groups of O. Medalia at University of Zürich and M. Beck at EMBL Heidelberg solved the 3D structure of a human nuclear pore complex, using cryo-electron tomography<sup>9</sup> and molecular modeling combined with cross-linking mass spectrometry and cryo-electron tomography, respectively<sup>10</sup>. The group of A. Hoelz at Caltech obtained the 3D structure of the inner ring complex using X-ray crystallography and cryo-electron tomography<sup>11</sup>.

The nuclear pore exhibits an octagonal cylindrical structure. More precisely, it is composed of two rings, cytoplasmic and nucleoplasmic, with 8 spokes to connect both rings, one central region, 16 filaments (8 cytoplasmic and 8 nucleoplasmic) and one terminal ring ([figure 3](#)). The 8 nucleoplasmic filaments adopt a basket arrangement (the nuclear basket) stabilized by the presence of the terminal ring.



**Figure 3 : Model of nuclear pore complex substructures.**

**The central channel exhibits eight-fold rotational symmetry and has eight cytoplasmic filaments as well as eight nuclear filaments protruding into the cytoplasm and nucleoplasm respectively. The nuclear filaments are bound via a ring, resulting in a basket structure<sup>11</sup>.**

This protein complex comprises approximately 50 to 100 proteins termed nucleoporins. All nucleoporins share basic structural components and can be classified in three classes<sup>12</sup>. The first group contains transmembrane  $\alpha$ -helix proteins, which mostly constitute the outside of NPC central region, whereas the second group, which contains  $\beta$ -propeller and  $\alpha$ -solenoid folds, constitutes the inside of the NPC. The last group is composed of the FG-nucleoporins. The FG-nucleoporins contain a structured domain that serves as an NPC anchor point and a largely unstructured domain composed of phenylalanine and glycine repeat sequences that forms some kind of entropic barrier to diffusion. This group may contribute to the formation of the NPC inner central framework and peripheral structures.

### *b. Nuclear import/export in cells*

Small molecules (less than 40kDa), ions and metabolites are able to diffuse freely inside the nucleus but it is not the case of proteins, various types of RNA and ribonucleoproteins (RNPs), which are actively translocated through the NPCs. These molecules can be thought of as cargoes<sup>13,14</sup>.

Concerning the import, the first step is the recognition of a specific sequence on the cargoes, the nuclear localization signal (NLS), which can be complex but is predominantly a stretch of basic residues. This NLS is recognized by importin- $\alpha$ , which will, after binding to the NLS, become an adapter for importin- $\beta$  and then, the complex is translocated through the NPC into the nucleus, where importins are dissociated from the complex by Ran-GTP.

Ran (RAS-related Nuclear protein) belongs to the family of small proteins G with a GTPase activity and is able to hydrolyze GTP in GDP when activated by a Ran GTPase activating protein<sup>13</sup>.

Afterwards, importins can diffuse to the cytoplasm, where the GTP molecules are hydrolyzed into GDP, which leads to the release of  $\alpha$  and  $\beta$  importins then available for a new protein-NLS import cycle (figure 4).

The export complex is formed inside the nucleus based on the recognition of a nuclear export signal (typically a leucine rich sequence) by exportins, in the presence of Ran-GTP (figure 4). After passing through the nuclear pores, exportins release the cargo molecule due to activation of the Ran GTPase activity.

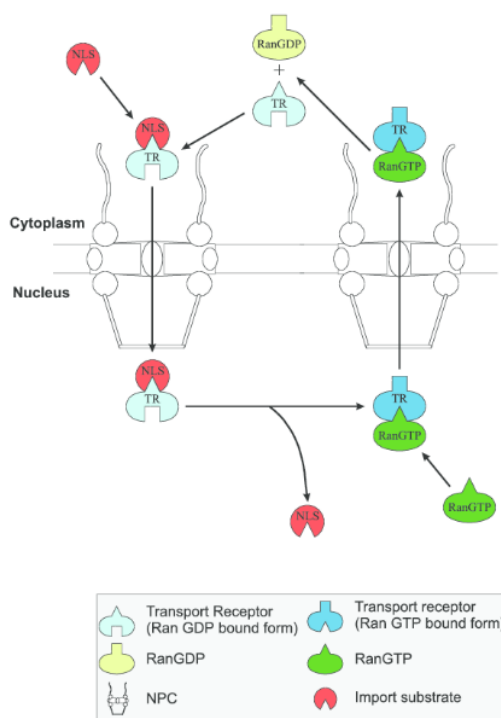


Figure 4 : Import and export through the NPC<sup>13</sup>.

### *c. Nuclear lamina acts as anchor for NPCs*

An interaction between NPCs and the lamina was first proposed after the observation of filamentous structures associated with the baskets of NPCs in amphibian oocytes<sup>15,16</sup>. Afterwards, it was shown that NPCs are motionless after assembly, and, from the study of nuclear membrane disintegration using several detergents, it was proposed that lamina could be able to cradle the nuclear pores<sup>17</sup>.

Recently, using a BioID approach, A-type lamin C (LaC) was found to be a specific partner of NPCs, more than A-type lamin A (LaA) or B-type lamin (LaB)<sup>18</sup>. Indeed, LaC interacts prominently compared to LaA or LaB with the Trp protein (Translocated promoter region protein), a component of the nuclear basket, and with protein Nup214, a FG-Nup of the NPCs. In more details, it was shown that interaction between NPCs and lamins is regulated by the status of the lamin post-IgFold tail and that A-type lamins may contribute directly to NPCs distribution.

## **2. THE NUCLEAR LAMINA**

### *a. Expression and localization*

The nuclear lamina, a filamentous protein network underlying the inner nuclear membrane, has emerged as a critical player from basic cell biology to human health<sup>19</sup>. Lamin genes are found in all metazoa examined to date, but are absent in plants and unicellular organisms<sup>20</sup>. Three lamin genes are present in mammalian genomes<sup>21</sup>. On one hand, *LMNB1* and *LMNB2* genes encode lamin B1 and B2 (B-type lamins), proteins expressed at all stages of development and essential for cell survival<sup>22,23</sup>. On the other hand, the *LMNA* gene encodes prelamin A and lamin C (A-type lamins), which are expressed later in organism development. In addition to these 4 major isoforms, 3 minor isoforms are also produced: AΔ10, C2 and B3<sup>24,25</sup>.



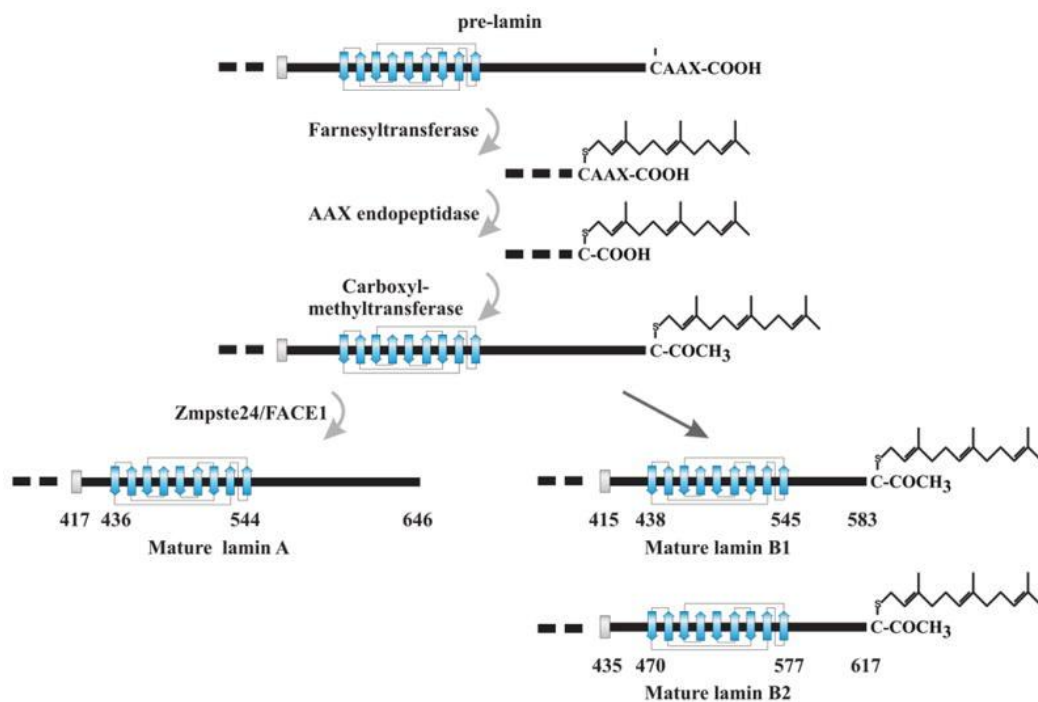
A-type and B-type lamins show different behaviors and spatial distributions. To observe these different properties, the most evident is to focus on lamin behavior during mitosis. At the onset of prophase, nuclear lamin is phosphorylated in order to be disassembled. A-type lamins are first phosphorylated by Cdk1 and become soluble whereas B-type lamins are phosphorylated by protein kinase C and stay associated to the nuclear envelope (likely due to the presence of the farnesyl moiety) as it is dispersed into the endoplasmic reticulum<sup>26–28</sup>. At this stage, both lamins are distributed throughout the cytoplasm. Afterwards, A and B-type lamins also show different temporal orders of re-assembly at the nuclear envelope. After lamin dephosphorylation by PP1A, B-type lamins bound to nuclear membranes accumulate around chromosomes<sup>29,30</sup>. Although A-type lamins are thought to bind chromatin at a later stage, after the major components of the NE including NPC are assembled<sup>30</sup>, live-cell imaging analyses allowed the visualization in early anaphase of a small pool of A-type lamins at specific chromosome regions, together with BAF, emerin and LAP2alpha, from where it extends at later stages<sup>29,31</sup>.

### *b. Maturation*

Like for all proteins, the *LMNA* and *LMNB1/2* transcription step takes place inside the nucleus. Then, the produced mRNA is exported to the cytoplasm where it is translated into lamin C, lamin B1, lamin B2 or prelamin A, the precursor form of lamin A, these 3 last lamins all containing a prenylation motif, the CaaX box (figure 5)<sup>28,32</sup>. Lamin C lacks a –CAAX sequence and its carboxyl terminus is not further modified after synthesis, whereas other lamins are modified in order to become mature. First maturation steps are common to lamins A, B1 and B2.

Initiation of the -CaaX sequential processing consists of the addition of a farnesyl group onto the cysteine residue by a farnesyltransferase. This modification leads to the removal of the three terminal amino acids by an endopeptidase. The cleavage of the –aaX motif is done by different enzymes according to the lamin type. Concerning prelamin A, the Zinc metalloprotease ZMPSTE24 is used for this step whereas for lamins B1 and B2, it is a different protease, Rce1 (Ras-converting enzyme 1).

A final common step for all farnesylated lamins is the carboxymethylation of the terminal cysteine by the isoprenylcysteine carboxyl methyltransferase (Icmt). After this step, lamin B1 and lamin B2 have reached their mature states. This is not the case of prelamin A, which possesses a second cleavage site for ZMPSTE24. This last step leads to the removal of 15 amino acids from the C-terminus of lamin A, including the farnesylated/carboxymethylated cysteine residue. The presence of a farnesyl residue attached to B-type lamins suggests that these lamins are more closely associated to the inner nuclear membrane than A-type lamins<sup>33</sup>.



**Figure 5 : The maturation process of lamins A, B1 and B2<sup>28</sup>. The common C-terminal CAAX motif is composed of a cysteine (C), followed by two aliphatic amino acids (aa) and a random amino acid (X). Its farnesylation is the first step of the lamin maturation process.**

The prelamin A maturation is a complex process, and some genetic mutations driving to failures to generate mature lamin A are responsible for diseases. In particular, it was observed that a mutation modifying the second cleavage site for ZMPSTE24 (Leu647Arg)<sup>34</sup> or mutations modifying the enzyme ZMPSTE24 cause progeroid syndromes in humans<sup>35,36</sup>.

A *de novo* dominant splice-donor site mutation within exon 11 (G608G) was observed in several patients<sup>37</sup>. It results in the synthesis of a mutant prelamin A, called progerin (figure 6). This protein is characterized by a deletion of 50 amino acids near the C-terminal tail of prelamin A, but its –CaaX motif is intact. The deletion eliminates the site for the second endoproteolytic cleavage by ZMPSTE24.

The mutated protein can undergo farnesylation, endoproteolytic release of the –aaX, and carboxyl methylation but stays, at the end of the maturation process, in a farnesylated form. It remains associated with the nuclear membrane, eliciting nuclear blebbings and other nuclear abnormalities in cells, including disrupted lamin-heterochromatin interactions and alterations in gene transcription. All these defects cause a known syndrome, the Hutchinson–Gilford progeria syndrome (HGPS), characterized by the rapid appearance of aging at the beginning of childhood<sup>37,38</sup>. Affected children look normal at birth and in early infancy, but then grow more slowly than other children and show different abnormalities as stiffness of joints, cardiovascular disease, atherosclerosis and loss of body fat and hair.

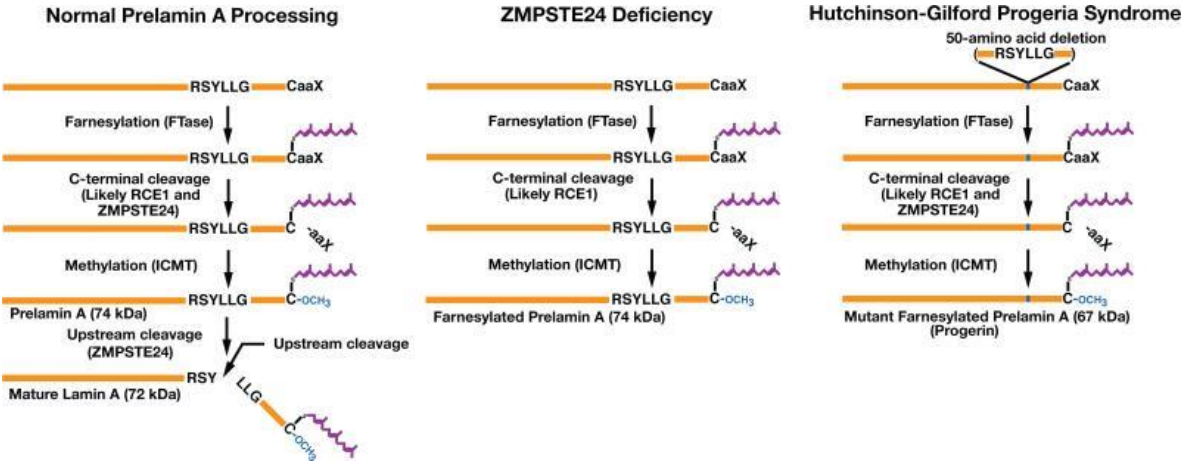


Figure 6 : Biogenesis of lamin A in healthy humans as well as in patients with either a ZMPSTE24 deficiency or the silent mutation causing HGPS<sup>39</sup>.

### c. Structure

The structure of lamins is composed of three units that are common among intermediate filament proteins. These are a long central-helical rod domain of 40 kDa, an N-terminal (head) domain and a C-terminal (tail) domain (figure 7a)<sup>32,40</sup>.

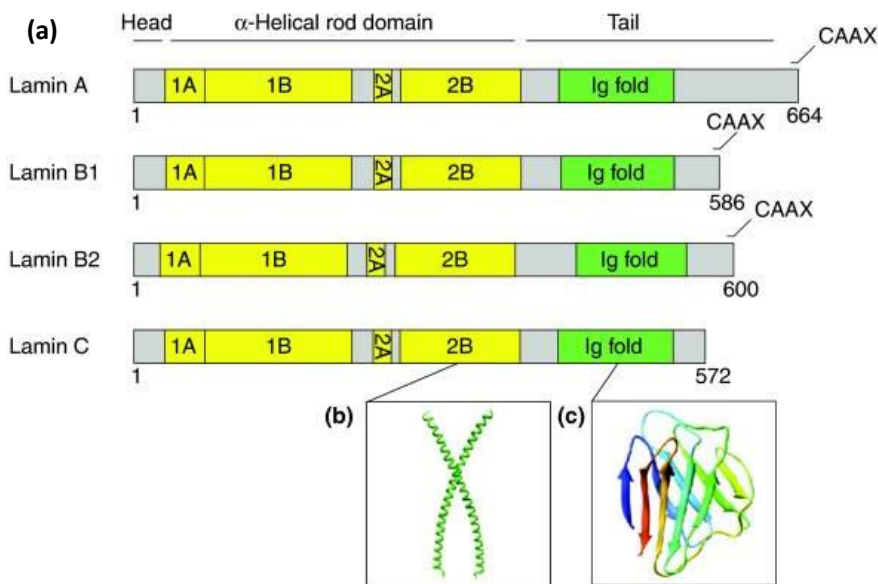


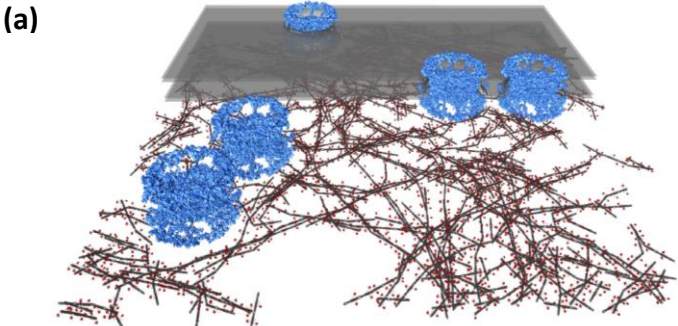
Figure 7: Structural organization of nuclear lamins.

(a) Architecture of major lamin variants. (b) X-ray structure of the human lamin A/C coiled-coil 2 fragment (PDB code: 1X8Y)<sup>41</sup>. (c) NMR structure of the human lamin A/C IgFold domain (PDB code: 1IVT)<sup>42</sup>.

The central domain contains  $\alpha$ -helical segments, called 1A, 1B, 2A and 2B, showing the heptad repeat periodicity of coiled-coil proteins, and connected by short linkers. A 3D structure of the coiled-coil 2B fragment corresponding to human lamin A was solved by crystallography<sup>41</sup> (figure 7, b). The amino-terminal head domain is variable in size but generally shorter than 40 residues long. It is predicted as unstructured. The carboxy-terminal tail domain contains a NLS and a globular immunoglobulin-like domain (IgFold). In B-type lamins, this globular domain is followed by a CAAX box, whereas this box is absent in lamin C and is cleaved during maturation in lamin A. The 3D structure of the human A-type lamin Igfold was solved by NMR<sup>42</sup> and crystallography<sup>43</sup> (figure 7, c). It shows a compact  $\beta$ -sandwich formed by two  $\beta$ -sheets and nine  $\beta$ -strands connected by short loops. This Igfold is reported to mediate diverse protein-protein and protein-ligand interactions. However no 3D structure of a complex involving this Igfold was reported until now.

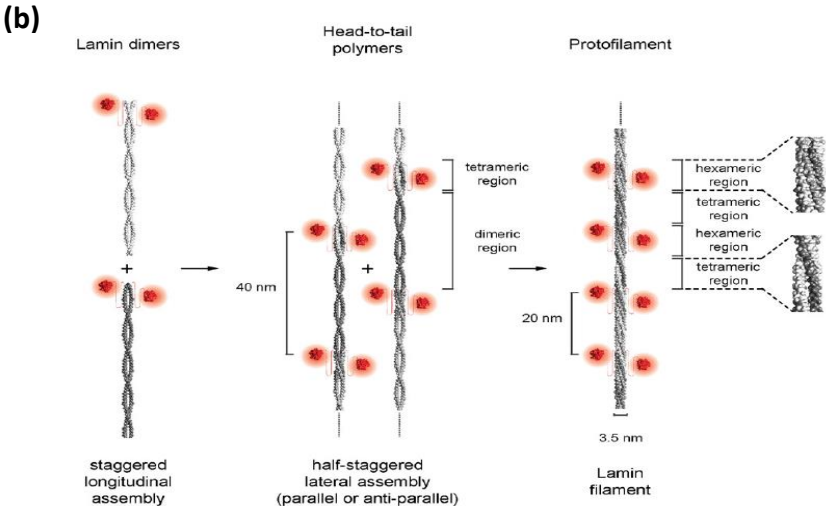
Then, first high resolution pictures of lamin filaments were recently obtained using cryo-electron tomography by the groups of W. Baumeister at Martinsried<sup>44</sup> and the group of O. Medalia at University of Zürich<sup>45</sup>. The first group obtained images of vitrified interphase HeLa cells, and identified entangled, 4-nm-diameter filaments in a volume of 30 nm in thickness underlying the NE, which they assigned to lamins.

The second group obtained images of vimentin-null mouse embryonic fibroblasts, which were treated as following: the plasma membrane and cytoplasmic content were removed by a short exposure to a mild detergent, and most of the chromatin was removed by treating the nuclei with nuclease. The lamin filaments were identified using gold-labelled antibodies against lamins. Thus this group reported the presence of ~3.5 nm thick lamin filaments forming a complex meshwork within a 14±2 nm thick layer at the nuclear periphery. Statistical analysis of these filaments indicated a length of 380±122 nm. In figure 9, I have shown 2 views of lamins presented in the paper of Medalia and coworkers, showing the filament network at the nuclear periphery (figure 8a) and a model of the lamin oligomer consistent with the cryoelectron tomography data (figure 8b).



**Figure 8 : Lamin models as deduced from the analysis of cryo-electron tomography images.**

(a) The lamin network (rod domain in dark blue and Igfold in red), NPCs (in blue) and the lipid bilayers (in light grey) were modeled into a region of a cryo-tomogram containing the lamin meshwork. (b) Averaging and classification of lamin filament images obtained *in situ* confirmed that lamin filaments are composed of at least two half-staggered head-to-tail polymers. Igfold domains are displayed in red<sup>46</sup>.





Both A and B-type lamins are able to oligomerize at the nuclear envelope. As it is well described in figure 8b, lamins form dimers through their  $\alpha$ -helical rod domain, oriented in a parallel manner, and these dimers form the basic building block of higher-order lamin assemblies. Indeed, each dimer can further associate with another dimer through head to tail interactions, to form a polymer. At least two polymers finally associate laterally in a staggered conformation to form a filament showing a diameter of about 3-4 nm<sup>45</sup>.

A- and B-type lamin meshworks are similar in appearance, but are mostly separated from each other, with some points of colocalization due to interactions between lamins<sup>46</sup>. Recently, A and B-type lamins were visualized directly in cells using super resolution microscopy, in order to confirm and extend these previous data (figure 9)<sup>18</sup>. It was observed that lamins are assembled into structurally and functionally separated meshworks or microdomains, with no preferential alignment.

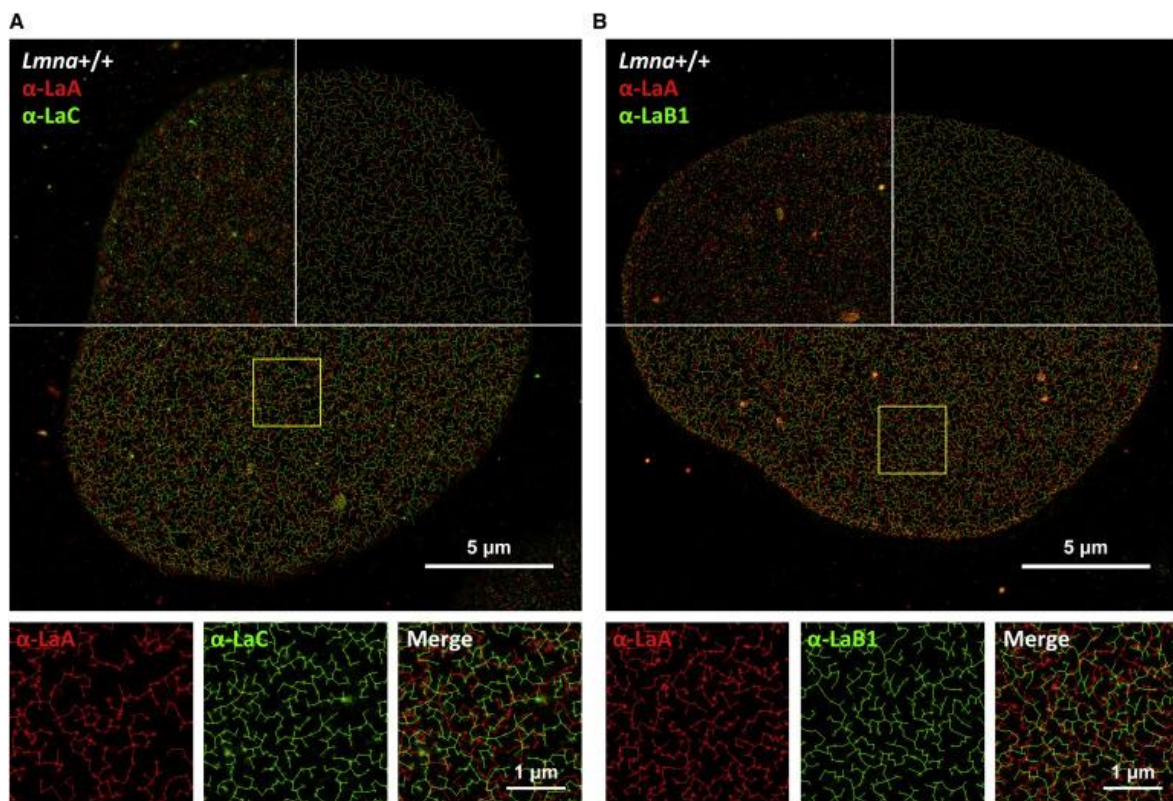


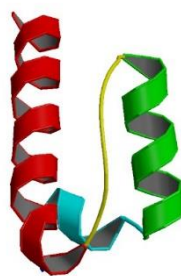
Figure 9 : Endogenous lamins observed using super resolution microscopy (d-STORM analysis), based on conventional indirect immunofluorescence labeling employing primary antibodies against lamins A, C, and B1<sup>18</sup>.

### 3. THE INNER NUCLEAR MEMBRANE PROTEINS

The outer nuclear membrane (ONM) is continuous with the endoplasmic reticulum and has ribosomes bound to its cytoplasmic surface. However, the ONM contains specific proteins, which are not found in the endoplasmic reticulum membrane<sup>47</sup>. The inner nuclear membrane adheres to the nuclear lamina matrix and proteomic studies revealed that this membrane is enriched in at least 50 integral membrane proteins<sup>48</sup>. I will focus below on the most studied of these inner nuclear membrane proteins.

#### *a. LEM domain proteins*

Among the inner nuclear membrane proteins, a prominent family shares the ability to directly bind to the nuclear lamina and to tether the nuclear envelope to chromatin, through interactions with a conserved chromatin-associated protein named barrier-to-autointegration factor (BAF). This family is formed by the LAP2-emerin-MAN1 (LEM) domain proteins, which all exhibit a LEM domain, whose 3D structure is known<sup>49-52</sup> (figure 10). This domain is composed of approximately 40 amino acids, which form a three residue N-terminal helical turn (in blue) and two parallel  $\alpha$ -helices (in red and green) connected by a long extended loop (in yellow).



**Figure 10** : Emerin LEM domain 3D structure, as solved by NMR (*PDB 2ODC*)<sup>52</sup>.

In mammals, LEM domain proteins can be categorized into three groups (figure 11)<sup>53</sup>. Group I is composed of proteins with an N-terminal LEM domain (or a LEM-like domain, i.e. a domain structurally homologous to the LEM domain)<sup>50,51,54</sup>, followed by a large and disordered region. Some of these proteins possess a single transmembrane domain at the C-terminus that anchors the protein to the inner nuclear membrane, whereas some are soluble nuclear proteins. In this group, we find proteins emerlin and LAP2. The 3D structure of emerlin LEM domain in complex with BAF was solved on the basis of NMR data<sup>52</sup>. This LEM domain binds to BAF with an affinity of 200-600 nM<sup>52,55</sup>. LAP2 can be expressed as six isoforms. Two of these isoforms, LAP2 $\alpha$  and LAP2 $\zeta$ , lack a transmembrane domain and localize in the nucleoplasm. All these isoforms possess an N-terminal LEM-like domain (residues 5-48) followed by a LEM domain (residues 109-153)<sup>50,52</sup>. Two-hybrid assays revealed that the LAP2 N-terminal region common to all isoforms was sufficient to bind BAF<sup>56</sup>. More precisely, region 67-195, but not region 1-67 nor region 138-373, was capable of BAF binding. As the LEM domain comprises residues 109 to 153, the linker between the LEM-like and the LEM domain together with the LEM domain seem sufficient for BAF binding. Cai et al.<sup>51</sup> further confirmed using NMR that the LEM domain is the main LAP2 binding site for BAF. However they observed that the complex between LAP2 LEM and BAF has a short lifetime of about 1.5-2 ms. Finally, Huang et al.<sup>57</sup> in the same team also confirmed later using NMR that the LEM domain of MAN1, another protein belonging to group I, binds to BAF.

Group II is composed of proteins with an N-terminal LEM domain, followed by an unfolded nucleoplasmic region, two transmembrane domains and a carboxyl-terminal winged-helix MSC (for MAN1/Src1p/C-terminal motif) domain, that directly binds to DNA. Among proteins of this group, there are LEMD2 and MAN1, which possesses an additional C-terminal UHM (U2AF homology motif) domain<sup>58</sup>. The MSC domain of LEMD2 was recently shown to directly interact with the ESCRT-II/ESCRT-III hybrid protein Cmp7p (CHMP7), in order to recruit additional ESCRT-III proteins to holes in the nuclear membrane and thus contribute to seal the membrane holes<sup>59</sup>. The UHM domain of MAN1 is essential for binding to SMAD2/3, which are important actors of the TGF- $\beta$  pathway<sup>60</sup>. Finally, group III is composed of proteins that carry internal LEM domains and multiple ankyrin repeats. Representatives of this group include LEM3/Ankle1 and LEM4/Ankle2.



LEM3 shuttles between the cytoplasm and the nucleoplasm<sup>61</sup> whereas LEM4 has a transmembrane domain and localizes either at the endoplasmic reticulum in human cells, or at the inner nuclear envelope in worms<sup>62</sup>. LEM4 is essential for BAF dephosphorylation at mitotic exit. It acts in part by inhibiting BAF's mitotic kinase VRK-1 and by recruiting PP2A. It is required for PP2A to dephosphorylate BAF.

The composition of the LEM domain protein family differs between organisms. In metazoa, at least one member from each group is present, whereas in yeast only group II proteins were identified<sup>53</sup>.

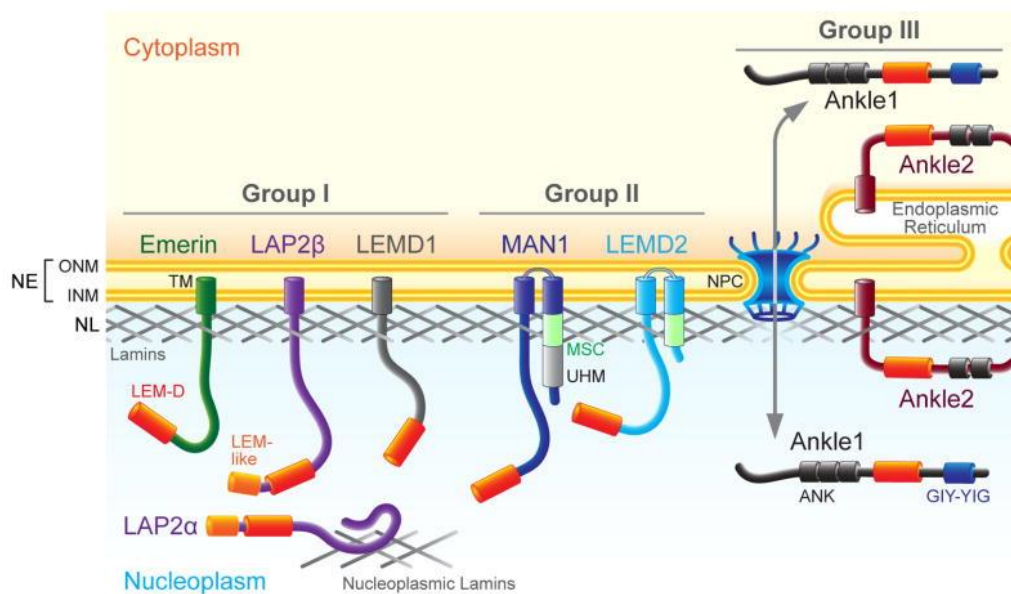


Figure 11 : Human LEM domain protein family<sup>53</sup>.

I inserted in the following page of this thesis, the link for the accepted version of a review on LEM proteins written for Methods in Enzymology and to which I contributed.

# Purification and structural analysis of LEM-domain proteins

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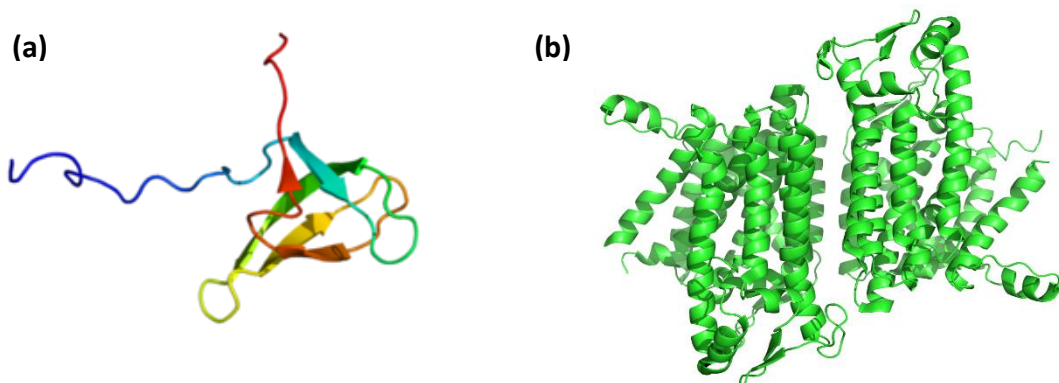
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## b. LBR

LBR, for Lamin-B receptor, was first described in a publication from the Blobel laboratory, in 1988<sup>63,64</sup>. It is an inner nuclear membrane protein with two functions: its N-terminal nucleoplasmic region, which contains a TUDOR domain (figure 12a), interacts with both chromatin and the nuclear lamina, while its C-terminal multi-pass transmembrane domain is a C14 sterol reductase (figure 12b). Mutations in LBR are associated with rare human diseases, Pelger-Huët anomaly and Greenberg skeletal dysplasia, which cause harmless anomalies of blood cells to fatal developmental defects<sup>66-68</sup>. The severity of these diseases depends on the nature of the specific mutation, and whether one or both copies of the gene are affected. All these diseases are characterized by a defect in cholesterol synthesis due to loss of LBR-associated sterol C14 reductase activity<sup>68</sup>. Several disease-associated LBR point mutations reduce sterol C14 reductase activity by decreasing the affinity of LBR for the reducing agent NADPH. Other mutations lead to LBR truncations.

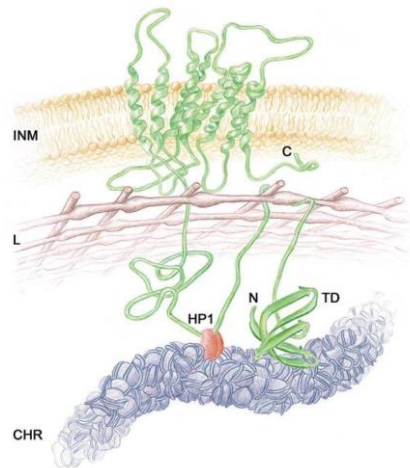


**Figure 12 : Architecture of LBR.**

**(a) 3D structures of the TUDOR domain of LBR (PDB code: 2L8D)<sup>70</sup> (b) and the integral membrane region of a LBR homolog (PDB code: 4QUV)<sup>71</sup>.**

LBR was initially characterized because of its ability to bind to the nuclear lamin B, and more precisely, its capacity to mediate interaction between chromatin and lamin B. However, it was shown that the TUDOR domain of LBR is not sufficient for binding to lamins<sup>69</sup>. It is able to bind to free histone H3. It also recognizes H4 dimethylated on lysine 20 and is essential for chromatin compaction<sup>70</sup> (figure 13). A specific motif in the N-terminal nucleoplasmic region of LBR directly binds to the chromo-shadow domain of the chromatin-associated protein HP1 $\alpha$  which interacts with histones<sup>71,72</sup> (figure 13).

It was reported that LBR and lamin A/C are responsible for two different mechanisms that tether heterochromatin to the NE<sup>73</sup>. These two mechanisms are sequentially used during cellular differentiation and development. The absence of both LBR and lamin A/C leads to loss of peripheral heterochromatin and an inverted architecture with heterochromatin localizing to the nuclear interior.



**Figure 13 : Cartoon representation of LBR embedded within the nuclear envelope<sup>74</sup>.**

### *c. The LINC complex*

Nuclear positioning is essential for many cellular processes, as cell division, migration, differentiation and polarization. In eukaryotes, this process requires the activity of actin, microtubules and microtubule-dependent motors. However, several studies revealed that another complex is fundamental to provide a structural support to the nucleus: the Linker of Nucleoskeleton and Cytoskeleton complex (LINC complex).

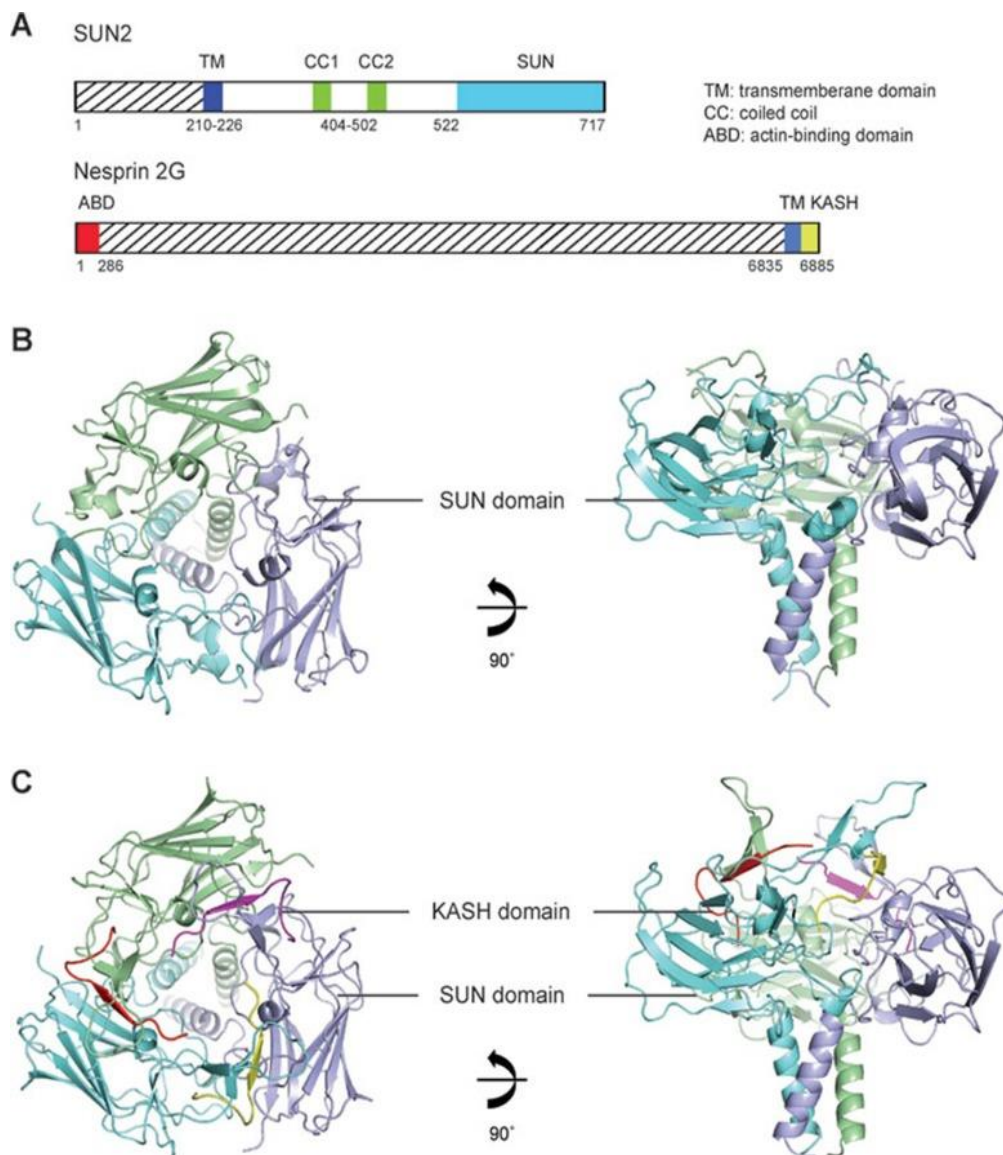
The LINC complex is conserved from yeast to human. It is a protein complex associated with the inner and outer membranes of the nucleus. It is composed of ONM-resident KASH-domain proteins (Klarsicht, ANC-1 and SYNE/Nesprin-1 and 2 Homology) and INM-resident SUN-domain proteins (Sad1 and UNC-84)<sup>75,76</sup>. Defects in these LINC complex proteins are associated with a broad spectrum of muscle pathologies (e.g., Emery-Dreifuss muscular dystrophy and dilated cardiomyopathy) and neuronal disorders<sup>77-79</sup>.

KASH-domain proteins are membrane proteins that share several common features<sup>76</sup>. They are composed of large cytoplasmic domains extending into the cytoplasm and interacting with actin filaments, microtubules, intermediate filaments or centrosomes. They possess a single transmembrane helix at their N-terminus, followed by a short fragment of around 30 residues, containing a PPPX motif (X is a variable residue) and located in the perinuclear space. The transmembrane helix together with the luminal tail form the KASH domain, a characteristic signature of the family<sup>80</sup>. Six KASH proteins are known in humans. Four of them, called Nesprins (1-4), contain spectrin repeats in their cytoplasmic domains. Nesprin 1 and 2, in mammals, are actin-binding proteins and are characterized by the fact that they are very large, with extensive fibers that span the cytoplasm<sup>81-83</sup>. Nesprin-3, a shorter molecule, binds to actin too, but through binding to a giant protein, plectin<sup>82</sup>. The last known Nesprin, Nesprin-4, is found in lower abundance, only in a few cell types, and is a microtubule-binding protein<sup>83</sup>.

Sad1/UNC-84 or SUN proteins were originally described in fission yeast (Sad1) and *Caenorhabditis elegans* (UNC-84)<sup>84,85</sup>. Afterwards, five mammalian family members (SUN1-5) have been identified<sup>86</sup>. Whereas SUN3, SUN4 (SPAG4), and SUN5 seem to be specifically expressed in testicles, SUN1 and SUN2 are ubiquitously expressed and can bind to all four Nesprins<sup>87,88</sup>. Sun proteins contain a variable nucleoplasmic region at their N-terminus, followed by a single transmembrane domain that spans the inner nuclear membrane and a region, localized into the perinuclear space, which comprises two coiled-coil repeats (CC1 and CC2) that are shown to be essential for trimerization and are also believed to act as rigid spacers to delineate the distance between the inner and the outer nuclear membranes<sup>80,89</sup>. Finally, the most highly conserved region between family members is the C-terminal SUN-domain (figure 14, a)<sup>90</sup>.

The 3D structure of a part of the LINC complex has recently been solved by X-ray crystallography<sup>90,91</sup> (figure 14, b). This structure contains the SUN domain of SUN-2, which folds into a compact  $\beta$ -sandwich. It exhibits at its N-terminus a helical extension (residues 525–540) involved in the formation of a coiled-coil, followed by 20 residues (residue 567 to 587) that are assembled in order to form a flexible domain, called the KASH-lid<sup>92</sup>. Residues 593–601 form a well-defined loop, which surrounds and coordinates a bound cation, and is structured by an intrachain disulfide bond formed between conserved cysteines at positions 601 and 705.

It was shown that this SUN2 fragment forms a stable trimer and binds to the KASH domain of nesprin<sup>90,91</sup>. The SUN homotrimer serves as a platform to interact with three KASH peptides. The peptides are individually bound in three deep grooves, which are symmetrically distributed close to the trimeric interface of the SUN domain homotrimer (figure 14, c). It is interesting to observe that addition of KASH peptide does not induce large structural changes in the SUN domain. Indeed, when the structure of apo-SUN-2 is superimposed with KASH-SUN-2 structure, only the region around the KASH-lid shows significant changes.



**Figure 14 : Structure of free and complexed SUN2<sup>90</sup>.**

**(A)** Schemes of the domain organization for human SUN2 and Nesprin 2G. **(B)** The SUN domain homotrimer side and top views represented in cartoon (PDB: 3UNP). The three protomers of the SUN homotrimer are colored in cyan, light blue and pale green. **(C)** Top and side views of the SUN-KASH complex. The three KASH domain peptides are colored in yellow, magenta and red.

Unexpectedly, when the two coiled-coil regions that are located before the SUN domain were studied separately, it was showed that, while the first coiled-coil is indeed trimeric, the second coiled-coil alone is monomeric<sup>93</sup>. The 3D structure of the coiled-coil 2 and SUN domain revealed that this coiled coil can fold into a three helix bundle and lock the SUN domain in a monomeric and inactive conformation (figure 15). Thus, structural changes within coiled-coil 2 dictate the oligomeric state of SUN2 and thus regulate the formation of the LINC complex.

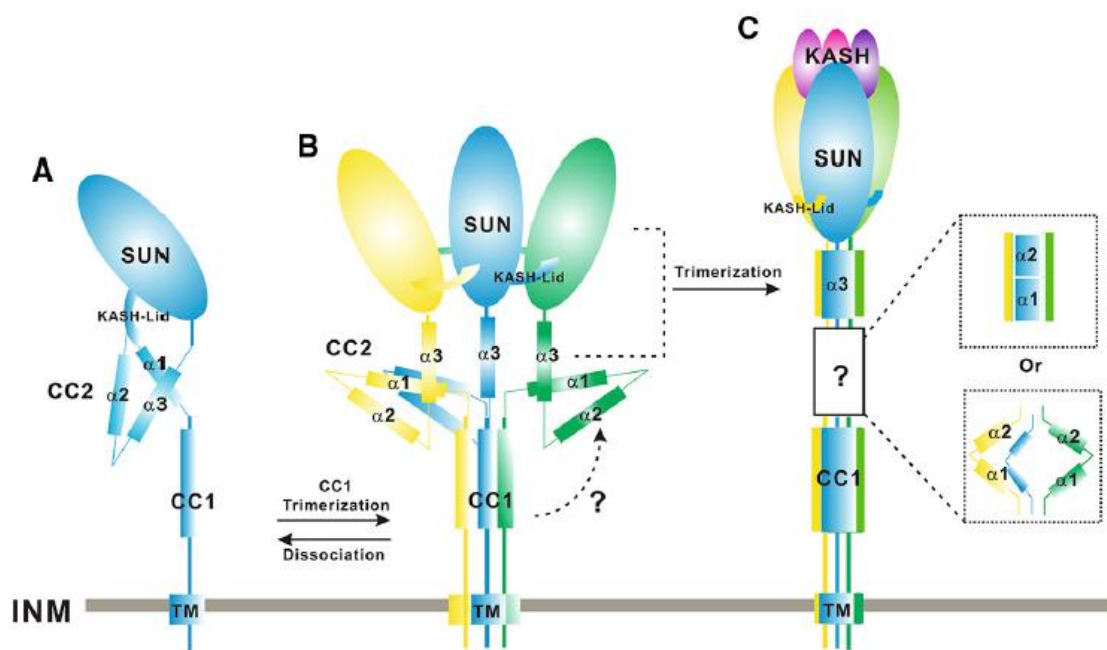


Figure 15 : Structural insights into SUN-KASH complexes across the nuclear envelope<sup>93</sup>.

In the monomeric form of the SUN2 protein, coiled-coil 2 forms a three-helix bundle that interacts with the SUN domain and lock it in an inactive state. SUN2 trimerization mediated by coiled-coil 1 probably induces a conformational change within coiled-coil 2. This change releases helix 3 of coiled-coil 2 that is then able to trimerize, thus triggering the formation of three KASH binding sites on the SUN2 trimer.



## II. NUCLEAR ENVELOPE: FUNCTIONS

### 1. STRUCTURAL AND MECHANICAL PROPERTIES OF THE NUCLEUS

The nuclear envelope provides sturdiness to the nucleus and this function may be more or less important depending on the tissue. Indeed, stiffness and strength of a tissue are related to the physical stress sensed in that tissue. For example, brain or fat tissues bear little stress, while adult bone tissue sustains high stress, which promotes bone growth and stiffening to match the stress<sup>94</sup>.

In this part, I will first describe the implication of the nuclear lamina, the LINC complex, nuclear actin and emerin in the mechanical properties of the nucleus and in the cellular response to a mechanical stress. Then, I will present the implication of another protein, BAF, in nuclear shape maintenance through regulation of nuclear envelope and chromatin interaction.

#### *a. Lamin composition determines the nucleus stiffness*

Lamins contribute to nuclear stiffness<sup>95</sup>. In lamin-depleted *Xenopus* egg extracts, assembled nuclei are highly fragile<sup>96</sup>. Cells from mouse *LMNA*<sup>-/-</sup> were found to be mechanically weak<sup>97</sup>. Later, direct mechanical measurements on *Xenopus* oocyte nuclei confirmed that lamina acts as a stiff<sup>95</sup>. In addition, mass spectrometry analyses showed that elasticity of bulk tissue is strongly correlated to the composition of the nuclear lamina (figure 16). Indeed, the ratio of A-type lamin to B-type lamin is increasing with the stiffness of the tissue. This observation could explain the fact that only vertebrates possess A-types of lamins<sup>94</sup>.

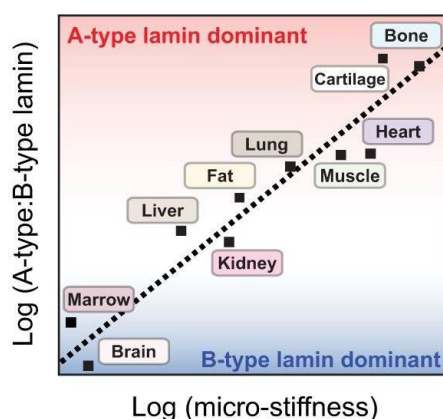
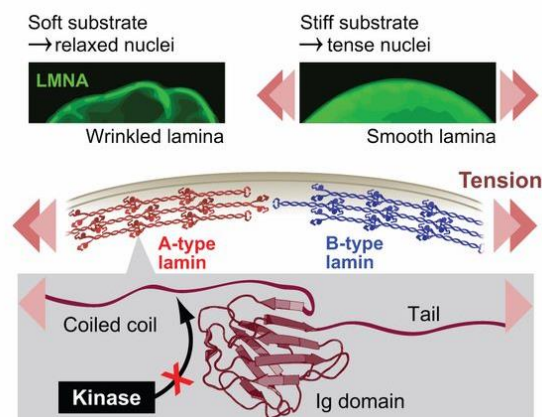


Figure 16 : Relationship between lamin composition and tissue rigidity.

A logarithmic scale representation of the ratio of A-type lamin (in red) to B-type lamin (in blue) in tissues according to their rigidity<sup>96</sup>.



Moreover, lamins have been shown to be regulated during mechanical stress. Lamin implication in nucleus mechanical properties was first studied in nuclei with different lamina compositions, by micropipette aspiration experiments<sup>95,98</sup>. It was shown that B-type lamins contribute to the elastic response whereas A-type lamins contribute to the viscosity. Afterwards, lamins behavior during mechanical stress was studied in mesenchymal stem cells (MSCs) that were cultivated in a medium mimicking the extra cellular matrix of either brain or bone (figure 17). This experiment showed that in the nuclear envelope of cells grown on soft substrates, lamins are wrinkled and relaxed, whereas, on stiff substrates, lamins appear smooth. On stiff substrates, the rate of phosphorylation at four sites decreases. Phosphorylation of intermediate filament proteins modulates their solubility, conformation and organization. Lamins are known to be highly phosphorylated during mitosis, in order to be disassembled. We can do the hypothesis that decreasing phosphorylation leads to a diminution of A-type lamin solubility and an increase of lamin strengthening<sup>94</sup>. More than dephosphorylation, a partial unfolding of the Igfold domain could be triggered by a mechanical stress. In contrary, on soft substrates, A-type lamins are more extensively phosphorylated, more soluble and could be eventually degraded.



**Figure 17 : Impact of mechanical stress on the Igfold domain structure<sup>94</sup>.**

**Nuclei of MSCs were cultured on soft and stiff substrates. Cysteine-shotgun MS was used to identify cysteines that are exposed by stress. Cys522 of A-type lamin Igfold was identified as a stress-sensitive site.**

Finally, migration of the cells in complex environments requires cellular deformability, which is limited by the cell nucleus. Indeed, lamins form a rigid shell underneath the inner nuclear membrane. To overcome this limitation, some migrating cells express a low level of lamins, whereas others secrete proteases to enlarge pores in the extracellular matrix<sup>99</sup>. It was recently shown that dendritic cells, when migrating through narrow channels, answer to nuclear rigidity by rapidly assembling a branched actin network around the nucleus in an Arp2/3 and Wave2 dependent manner<sup>100</sup>. This triggers local ruptures of the lamin A/C shell. If lamin A/C expression is suppressed, then this mechanism is no longer necessary. Formins, which are actin nucleating proteins generating unbranched actin filaments, also contribute to the formation of perinuclear actin filaments. In particular, FMN2 associates with and generates a perinuclear actin system that controls nuclear shape and positioning in cells migrating on 2D surfaces<sup>101</sup>. This actin-based cage protects the nucleus and its contents from damage when cells migrate through tiny spaces. It may prevent nuclear envelope rupture or facilitate nuclear envelope repair by either ESCRT III or lamin-dependent<sup>102</sup> or –independent pathways.

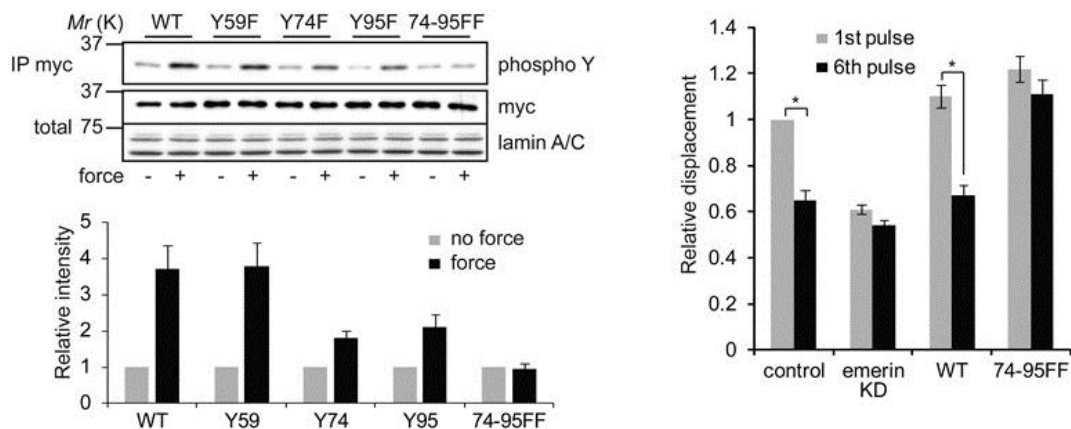
*b. Interaction between the LINC complex and actin contributes to nucleus positioning*

Nuclear envelope proteins together with actin control the position of the nucleus in the cell, in order to regulate cell division, differentiation and again cell migration<sup>103</sup>. In particular, actin is an essential actor of the rearward movement of the nucleus to orient the centrosome and polarize cells for directed migration. Actin-dependent nuclear movement in fibroblasts and myoblasts involves a LINC complex composed of nesprin-2G and SUN2. The association of these proteins with dorsal actin cables results in their assembly into linear arrays termed transmembrane actin-associated nuclear (TAN) lines<sup>104</sup>. Interaction between actin and the outer nuclear envelope involves the CH domains of nesprin-2G. The formin family member FHOD1 provides a second site of interaction between the actin cable and nesprin-2G<sup>105</sup>. The KASH domain of nesprin-2G interacts with the SUN domain of SUN2 in the perinuclear space. Finally TAN lines are positioned because of association of SUN2 with A-type lamins<sup>106</sup>. Emerin is also found in TAN lines and may contribute to their anchoring.

### c. Emerin mediates nuclear response to a mechanical stress

Emerin, a protein which belongs to the group I of LEM-domain proteins, is implicated in the nucleus response to a mechanical stress, in particular through phosphorylation events, like lamins. Analysis of the role of nuclear envelope proteins in the nucleus response to a mechanical stress was done through application of a force on isolated nuclei via the LINC complex component Nesprin-1<sup>107</sup>. Magnetic tweezers were used to stimulate magnetic beads that were coated with anti Nesprin-1 antibodies. Successive pulses with a constant force were applied on the nucleus and bead displacement diminution was observed which means that application of a constant force leads to an increase of the nucleus resistance.

Because induction of protein phosphorylation is one of the first events that appears when mechanical force is applied to cells, tyrosine phosphorylation of nuclear proteins from isolated nuclei subjected to force was observed. Several nuclear proteins were phosphorylated after mechanical stress, but one of these proteins was more strongly phosphorylated on its tyrosines: it is the emerin protein. More precisely, emerin phosphorylation on two tyrosines (Y74 and Y95) by the kinase Src was essential for the response of the nucleus to a mechanical stress (figure 18). When these tyrosine were mutated, the nucleus could not answer anymore to force application. However the molecular consequences of these tyrosine phosphorylations are still unknown.



**Figure 18 : Emerin is phosphorylated by Src on its tyrosines 74 and 95 in response to a mechanical stress<sup>107</sup>.**

#### d. BAF mediates interaction between nuclear envelope and chromatin

The small protein barrier-to-autointegration-factor (BAF) was identified in a yeast two-hybrid screen designed to find partners of the inner nuclear membrane protein LAP2<sup>56</sup>. BAF is a highly conserved 89-amino acid protein that is localized in the cytoplasm and the nucleoplasm of metazoan cells<sup>108,109</sup>. It forms a dimer in solution. The 3D structure of this dimer was solved by NMR<sup>110</sup> (PDB: 2EZK) and by X-ray crystallography<sup>111</sup> (PDB: 2BZF) (figure 19).

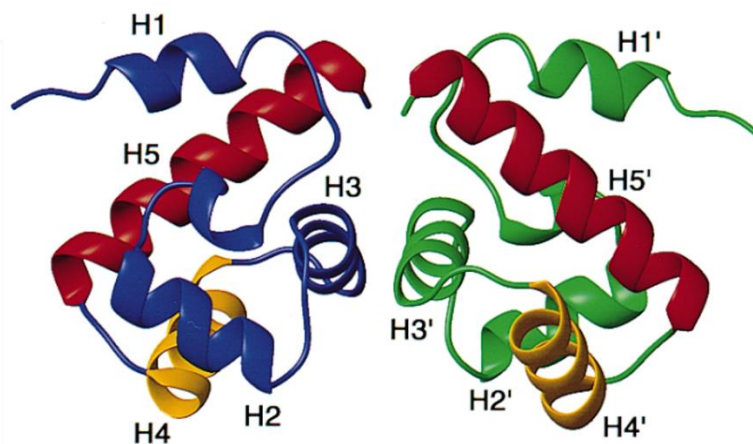


Figure 19 : Solution structure of BAF dimer, as solved by NMR (PDB: 2EZK)<sup>110</sup>.

Ribbon diagram of the BAF dimer, with first and second helices of the DNA recognition helix-turn-helix motif in orange and red, respectively and other helices in blue for one subunit and green for the other.

Each monomer of BAF is mainly composed of five helices (H1-5). The presence of one copy and one pseudo copy of a nonspecific DNA-binding motif (helix-hairpin-helix, HhH) was observed for each monomer. This motif is characterized by two helices connected by a short turn and is formed by helices H2 and H3 and helices H4 and H5. The 3D structure of the BAF dimer in interaction with a 7-bp-DNA was then solved by X-ray crystallography, at a resolution of 2.87 Å<sup>111</sup>. This structure shows that the interaction is mediated by both HhH motifs (figure 20) and the N-terminal of the helix 1 (in yellow in figure 20). In the presence of a short DNA, each BAF monomer is able to bind to one DNA molecule, whereas it was shown before that BAF is able to form higher-order complexes in presence of longer DNA molecules<sup>112</sup>.

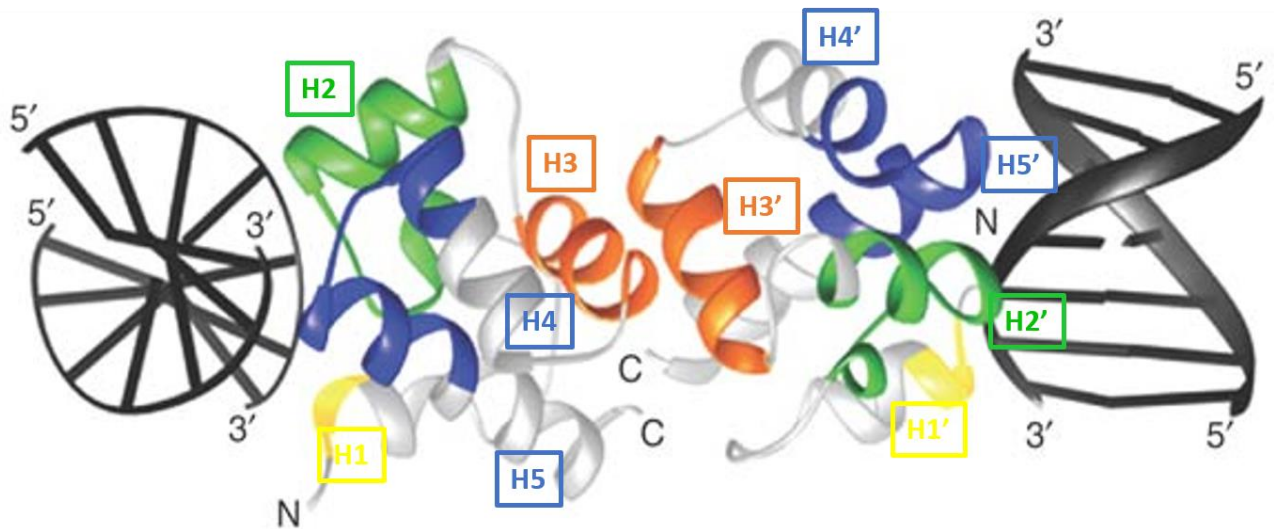


Figure 20 : Structure of the BAF dimer in interaction with a 7-bp-DNA, solved by X-ray crystallography (PDB: 2BZF)<sup>111</sup>.

The HhH and pseudo HhH motifs are formed by helices H2 (in green) and helices H4 and H5 (in blue), respectively, with the connecting helix H3 (in orange). In yellow is represented the interacting region of  $\alpha 1$ ; black, DNA; N and C, protein termini.

A first link between BAF function and chromatin was found several years ago by a group who did an assay to identify and purify the host factor conferring protection to the retroviral DNA against auto-integration<sup>113</sup>. More precisely, BAF was found as an essential actor for the biogenesis of MoMLV and HIV-1 retroviruses. For example, during biogenesis of MoMLV, BAF is recruited to cytoplasmic pre-integration complexes (PICs), in order to block autointegration of the viral DNA, whereas in absence of BAF, self-destruction of viral DNA is observed. BAF seems to protect DNA against integration by bridging DNA.

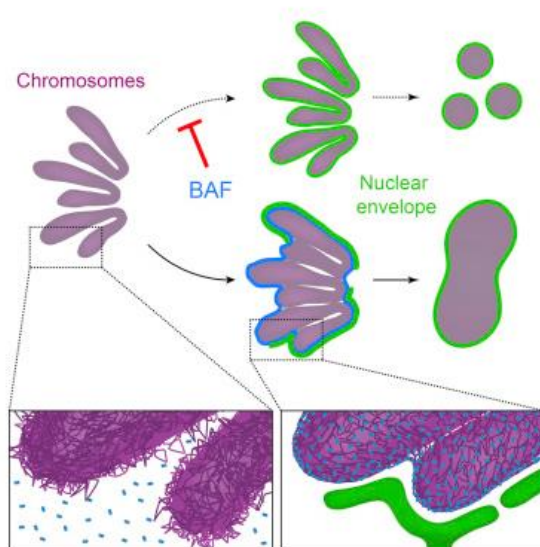
The most important role of BAF is its capacity to interact with chromatin in order to regulate nuclear assembly and organization. Indeed, *in vivo* studies have demonstrated that depletion of BAF from *C. elegans* embryos<sup>112,114</sup> or *D. melanogaster* larvae<sup>115</sup> induce strong effects on chromosome condensation and segregation, notably the retention of mitotic chromatin modification during late anaphase and telophase. In addition, a direct effect of BAF on nuclear envelope assembly and function was observed. Indeed, depletion of BAF using short RNAi resulted in nuclear shape and size defects<sup>116</sup> and moreover, this depletion compromised severely chromatin segregation. Furthermore, protein kinase VRK-1 phosphorylates BAF *in vivo* to regulate its localization and function<sup>116</sup>.

Phosphorylation of BAF not only abrogates its DNA binding activity by introducing negative charges on the DNA interacting surface, but reduces the binding to LEM domain proteins and leads to mislocalization from the newly formed NE<sup>117</sup>. Co-expression of VRK1 and GFP-BAF greatly diminishes the association of BAF with the nuclear chromatin/matrix and leads to its dispersal throughout the cell.

On the opposite, depletion of VRK-1 by RNAi for 13h causes strong accumulation of BAF on chromatin throughout mitosis, which clearly indicates that VRK-1 activity is required for BAF to dissociate from mitotic chromatin. In addition, an increase in chromatin association of BAF in anaphase was observed in absence of VRK-1 in embryo cells. Finally, more than dissociation from mitotic chromatin, depletion of VRK1 and the consequent reduction in BAF phosphorylation caused abnormalities in the nuclear envelope of interphase cells. One explanation could be that an increased fraction of unphosphorylated BAF could extend the duration of interactions between BAF and its DNA or protein partners which could be responsible for the distorted structure of the nuclear envelope<sup>118</sup>.

Analysis of BAF localization during the cell cycle in HEK293 cells confirmed that during interphase, total BAF and phosphorylated BAF are present in both the cytoplasm and the nucleus<sup>119</sup>. In early telophase, phosphorylated BAF accumulates at the central region of chromosomes, and in late telophase, it already dispersed to the cytoplasm, while total BAF translocates to the periphery of chromosomes. Thus, unphosphorylated BAF is mainly localized at the periphery of chromosomes through telophase, where the new NE is formed. Emerin localizes to the central region of chromosomes in a BAF-dependent manner<sup>31</sup>. BAF phosphorylation controls the recruitment of emerin to the central region of telophase chromosomes, which is essential for new NE assembly.

In addition to its role in nuclear envelope association around chromosomes, BAF was recently found as a spindle factor for shaping a single nucleus instead of an ensemble of individualized mitotic chromosomes<sup>120</sup>. Indeed, a micronucleation phenotype was caused by a siRNA targeting BAF. Moreover, BAF mediated DNA cross-bridging in order to form a network at the chromatin surface and this increased the mechanical resistance of chromatin. Finally, they showed that this chromatin compaction could be reversed by addition of recombinant VRK1. In conclusion, as it is represented on the [figure 21](#), BAF-mediated DNA cross-bridging allows formation of a compact and mechanically stiff chromatin surface that shapes a single nucleus and this BAF-DNA interaction is disrupted by BAF phosphorylation by VRK1 at the early stages of mitosis<sup>120</sup>.



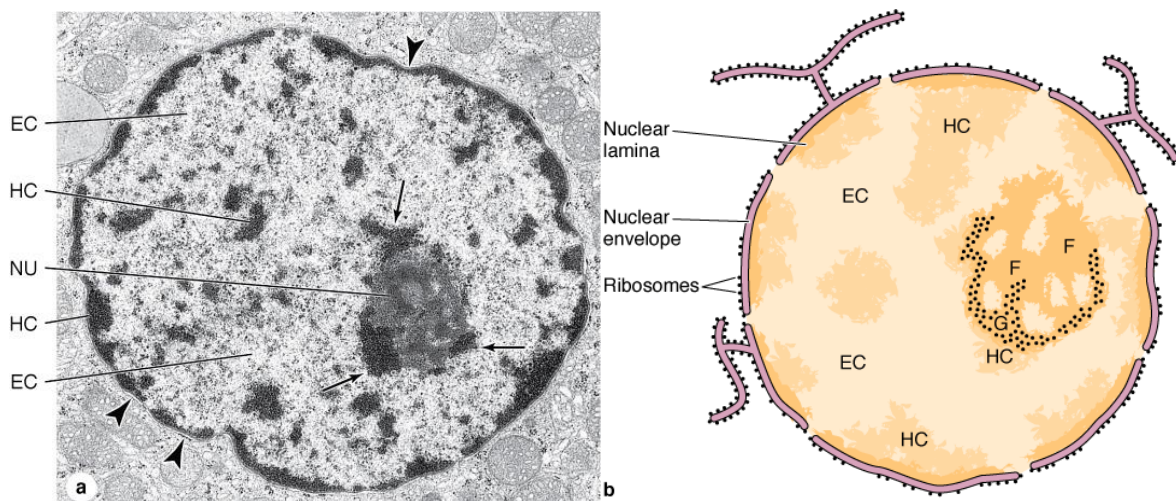
**Figure 21 : Model of the BAF-DNA network forming at the surface of anaphase chromosomes prior to nuclear envelope reassembly.**

**BAF, in blue, increases chromatin (in purple) surface stiffness and forces formation of a single nucleus. Nuclear envelope is labelled in green<sup>122</sup>.**



## 2. GENE EXPRESSION

Since several years, different studies focused on the organization of chromatin at the nuclear periphery (heterochromatin) (figure 22). Its nature and its role differ from those of chromatin located in the nuclear interior (euchromatin)<sup>121</sup>. Indeed, chromatin at the nuclear periphery is compact and transcriptionally silent. Inversely, internal chromatin is unpacked and transcriptionally active. The association of a promoter with the inner nuclear membrane (INM) is neither necessary nor sufficient for repression. So how does subnuclear localization influence gene expression? Recent work argues that the common denominator between genome organization and gene expression is the modification of histones and in some cases of histone variants<sup>122</sup>.



**Figure 22 : Organization of the chromatin at the nuclear periphery<sup>123</sup>.**

**(a) Structure of euchromatin (EC), heterochromatin (HC) and nucleolus (NU) observed by electron microscopy (b) Schematic representation of the nucleus and of the nucleolus (with the pars granulosa (G) and pars fibrosa (F)).**

The nuclear envelope is a tethering point for chromatin<sup>124,125</sup>. Several nuclear envelope components are important for this function, as the mammalian nuclear pore complexes, composed of elements that can be separated in two groups, some which could activate gene expression whereas others could have a silencing function<sup>126,127</sup> and the lamina, which is close to compact heterochromatin.



### *a. Nuclear pore complexes and transcription activation*

NPCs have been implicated in chromatin organization and gene regulation<sup>128,129</sup>. Studies in yeast revealed that Nups can associate with promoters of active genes<sup>130</sup> and that the expression of inducible genes is increased by interactions with nuclear pores<sup>131</sup>. For example, *in yeast*, the nucleoporin Nup84 of pore complexes and its subcomplex components are able to activate transcription when they are fused to a DNA-binding domain<sup>132</sup>. In the absence of Nup84, transcriptional activation by most nucleoporins is impaired.

However, a genome-wide study of Nup-chromatin association in human cells revealed a correlation between the binding sites of Nups and regions enriched in repressive histone modifications<sup>133</sup>, which exhibited characteristics of sequences known to associate with the nuclear periphery<sup>134</sup>. In higher eukaryotes, some nucleoporins have two separate pools: one activates genes in the nucleoplasm while the other in the peripheral NPC structures has a silencing function<sup>126,127</sup>.

### *b. Lamins and gene silencing*

Nuclear periphery has been implicated in gene expression regulation. Molecular mapping of chromatin regions associated to the lamina identified large genomic regions called lamina-associated domains (LADs)<sup>135</sup>. Such LADs are proposed to help organize chromosomes inside the nucleus and have been associated with gene repression. Furthermore, localization of these LADs close to the nuclear lamina is facilitated by the presence of lamina-associated sequences (LAS), which contain the GAGA motif<sup>136</sup>. This repeated motif directs lamina association and is bound by the transcriptional repressor cKrox, in a complex with HDAC3 and Lap2 $\beta$ . Knockdown of cKrox or HDAC3 results in dissociation of LASs/LADs from the nuclear lamina.

In both mammals and nematodes, chromatin associated with LADs is densely packed and enriched for repressive histone modifications, most notably H3K9me2/me3<sup>122,134,137</sup>. In nematodes, elimination of two histone methyltransferases, MET-2 and SET-25, compromised INM binding<sup>138</sup>.

In metazoans, LEM-domain proteins are found in regions enriched in repressive epigenetic marks and which contain all features that define LADs<sup>53</sup>. This suggests that LEM-domain proteins may contribute to LAD establishment or maintenance. In addition, proteins which promote the formation of repressed chromatin may contribute to LAD maintenance. Two LEM-domain proteins, emerin<sup>139</sup> and LAP2 $\beta$ <sup>140</sup> are able to bind the histone deacetylase HDAC3, which allows the deacetylation of histone H4, in order to inhibit transcription activity. This confirms that LEM-domain proteins can maintain LADs by recruiting partners that contribute to repressed chromatin states.

### *c. BAF and gene expression*

BAF's roles in transcriptional regulation are major open questions, and might involve interactions with a variety of players including transcription factors, chromatin modifiers and nuclear lamina components. A first example is BAF association with Sox2 in embryonic stem cells that could contribute to the formation or regulation of Sox2 complexes to maintain pluripotency<sup>141</sup>. Another example is found in the photoreceptor development and function field. Indeed, BAF was identified in a yeast two-hybrid screen that was designed to identify partners of the homeodomain transcription factor Crx, implicated in regulating the expression of photoreceptor and pineal genes<sup>142</sup>. BAF is able to repress the Crx-dependent reporter activity *in vivo* by directly interacting with Crx<sup>142</sup>. This study also shows that Crx is not the only transcription factor that can bind to BAF, but four other paired-like homeodomain proteins (Chx10, Pax-6, Otx1, and Otx2) also have the ability to interact with BAF *in vitro*.

### 3. GENOMIC INSTABILITY AND DNA DAMAGE RESPONSE

Genomic instability is defined as a tendency of the genome to acquire mutations and epimutations as well as alterations in gene or chromosome dosage. The origin of this phenomenon is none other than failures in processes important for genome stability and replication<sup>143</sup>. DNA damage is the result of constant attack of the genome by endogenous and exogenous agents. It can result from side products of our normal metabolic activities as free radicals and reactive oxygen (ROS) or reactive nitrogen species; it can also result from environmental factors such as UV radiation, X-rays and chemical compounds. Furthermore, DNA lesions might be due to deficiencies in DNA replication or loss of telomere function. The most deleterious kinds of lesions, which are DNA double-strand breaks, can be repaired by nonhomologous end-joining (NHEJ) or homologous recombination (HR).

Several lines of evidence have linked laminopathies with increased genomic instability<sup>143</sup>. In particular, expression of several A-type lamin mutants has been associated with impairment in the ability of cells to properly repair DNA damage and maintain telomere homeostasis. Defects in DNA repair were first reported in the premature aging laminopathy HGPS and the *Zmpste24*<sup>-/-</sup> mouse model of progeria<sup>144</sup>. Later studies indicated that accumulation of unreparable DNA damage in laminopathies such as HGPS and Restrictive Dermopathy is in part due to elevated levels of ROS and greater sensitivity to oxidative stress<sup>145</sup>. A whole variety of molecular mechanisms have been proposed to contribute to the defects in DNA repair and the increased genomic instability in progeria.

From the observation that progeria cells exhibit delayed recruitment of 53BP1 and RAD51 to  $\gamma$ -H2AX-labeled DNA repair foci after irradiation, the role of lamins in HR and NHEJ pathways was studied. The role of lamins in telomere maintenance was also examined.

### *a. A-type lamins and 53BP1*

P53-binding protein 1 (53BP1) is an important regulator of the cellular response to DNA double strand breaks (DSBs) that can bind to chromatin close to DSBs in order to carry out several functions<sup>146</sup>.

In NHEJ processes, 53BP1 was shown to be important because it participates in the repair of short-range DNA DSBs by inhibiting end-resection and facilitating the recruitment of the NHEJ DNA repair machinery.

A-type lamins are known to stabilize this protein and one of the mechanisms behind genomic instability in A-type lamins-deficient cells is the loss of the DNA repair factor 53BP1. This was observed in several studies and notably through experiments showing that depletion in A-type lamins by lentiviral transduction with shRNAs leads to decreased levels of the 53BP1 protein but not of its transcript levels<sup>143</sup>.

A search for mechanisms by which A-type lamins affect DNA DSB repair revealed a role for cathepsin L (CTSL) in regulating 53BP1 protein stability<sup>143</sup>. CTSL is a cysteine protease ubiquitously expressed in tissues that was recently shown to be able to localize in the nucleus instead of its classical localization in the lysosomes. It was found that loss of A-type lamins induces transcriptional upregulation of CTSL or increased stability of its transcripts. Moreover, increase in CTSL is directly responsible for the decrease in 53BP1 protein levels so that A-type lamins seems to stabilize 53BP1 by inducing transcriptional downregulation of CTSL.

In addition, in progeria cells, loss of components of the NuRD complex and decreased levels of the histone acetyltransferase Mof were observed and associated with impaired recruitment of DNA repair factors and increased basal DNA damage<sup>147,148</sup>. This decrease in Mof levels correlates with lower global levels of H4K16 acetylation, a histone mark associated with chromatin compaction, and decreased recruitment of DNA repair factors such as 53BP1. The importance of this H4K16 acetylation is demonstrated by the restoration of 53BP1 recruitment upon ectopic expression of Mof.

### *b. A-type lamins defects and alteration in telomere biology*

There is evidence in the literature that A-type lamins associate with telomeres, putatively via telomere-binding proteins. This association has been shown to be important for the proper distribution of telomeres within the 3D nuclear space. A study compared nuclear distribution of telomeres between wild-type mouse embryonic fibroblasts and fibroblasts devoid of A-type lamins, and showed a clear difference in telomere distribution between the two genotypes<sup>149</sup>.

This confirmed that A-type lamins participate in the correct distribution of telomeres throughout the entire nuclear volume and that defects in these lamins lead to a nuclear decompartmentalization of telomeres.

A second important feature is the maintenance of a heterochromatic structure for telomere length homeostasis and interestingly, A-type lamins participate in the maintenance of histone marks found at telomeric heterochromatin. Indeed, in mice fibroblasts expressing a lamin A mutant associated with HGPS (HGPS), a down-regulation of H3K9me3 and H3K27me3 and an up-regulation of H4K20me3 were observed<sup>150–152</sup>. These changes found in HGPS cells are different from changes observed in mouse fibroblasts expressing no lamin A.

Indeed, in this second case, no change in H3K9me3 levels was observed whereas a significant decrease in H4K20me3 levels was noted. Altogether, these results confirm implication of A-type lamins in the maintenance of histone marks<sup>149</sup>.

Then, A-type lamin mutants exhibited a defective ability to process dysfunctional telomeres through the NHEJ pathway<sup>149</sup>. First, loss of 53BP1 function was recently associated with defective processing of dysfunctional telomeres by the NHEJ repair pathway<sup>153</sup>. Then, chromosome fusion percentages were evaluated in mouse fibroblasts, expressing or not A-type lamins. Clearly, in presence of a dominant-negative mutant of the telomeric binding protein TRF2, which induces telomere dysfunction and chromosome end-to-end fusions, the number of fusions increased in mouse fibroblasts expressing no A-type lamins<sup>149</sup>. Because of these two important observations, we can speculate that A-type lamins provide a platform for the association of 53BP1 to dysfunctional telomeres.

Finally, another study confirmed the implication of lamin A/C in dysfunctional telomeres processing. First, co-immunoprecipitation of endogenous TRF2 together with lamin A/C in IMR90s revealed an interaction between both proteins<sup>154</sup>. Then, the specificity of this interaction was confirmed by pull-down assays using endogenous lamin A/C and GFP-TRF2 (functional TRF2), GFP-TRF2 $\Delta$ B $\Delta$ M (non-functional TRF2) or wild-type GFP-TRF1, in IMR90s. Indeed, only interaction between lamin A/C and functional TRF2 (GFP-TRF2) was observed in these conditions. In addition, pull-down assays revealed that lamin A mutant that causes HGPS (GFP-progerin) could not interact with endogenous TRF2.

TRF2 has been shown to bind interstitial telomeric sequences<sup>155</sup>, thus stabilizing the telomeric t-loop; the impact of a decrease in lamin A/C levels or the *LMNA* mutation that causes HGPS (progerin) was observed. Both led to a reduction of interstitial t-loop formation and to telomere loss.

#### *c. Nuclear envelope and mobility of DNA breaks*

A recent study also implicated components of the nuclear envelope, such as the LINC complex and associated microtubule motor proteins (Kinesins), in the mobility of dysfunctional telomeres as well as of DNA DSBs within the nucleus. In addition, 53BP1/LINC/microtubule-dependent movement is important for NHEJ-based repair of dysfunctional telomeres and DSBs<sup>156</sup>. All together, these studies indicate that damaged DNA/chromatin exhibits increased mobility within the nucleus, and that this mobility is critical for its proper repair. The DNA repair factor 53BP1, LINC-microtubule connections, and the lamin network, all seem to play a role in these processes. Characterizing how these connections are established and regulated will be important for a mechanistic understanding of how nuclear architecture impacts genome stability.

#### *d. A-type lamins and replicative stress*

Lamin A/C is present at sites of early replication in normal human fibroblasts<sup>152,157</sup>. Lamin A/C-depleted cells are unable to restart most replication forks after treatment with HU compared to cells with lamin A/C, indicating that lamin A/C is required for the resolution of stalled replication forks<sup>158</sup>. This suggests that lamin A/C provides a platform for the resolution of stalled replication fork intermediates.

### III. EMERIN, A NUCLEAR ENVELOPE PROTEIN

#### 1. EMERIN STRUCTURE

Emerin is a type II integral INM protein of 254 amino acids (aa). Its gene was discovered in 1994 because it is mutated in patients with X-linked EDMD<sup>159</sup>. Emerin shows ubiquitous localization with the highest expression in skeletal and cardiac muscles and is mainly present at the inner nuclear membrane<sup>160,161</sup>. Emerin possesses a single transmembrane (TM) domain (aa 223-243) for anchoring at the inner nuclear membrane and in addition, is composed of a small C-terminal domain in the luminal space (aa 244-254) and a large hydrophilic N-terminal region localized in the nucleoplasm (aa 1-222). This N-terminal region exhibits a small globular LEM domain and a large region predicted as unstructured. Structure of the LEM domain was solved by NMR<sup>49</sup> and as all LEM-domain proteins (as presented in the introduction), it is composed of one three-residue N-terminal  $\alpha$ -helix and two large parallel  $\alpha$ -helices separated by a loop with conserved hydrophobic residues<sup>51</sup>.

Most mutations that cause EDMD or other myopathies yield to premature stop codons, and truncated emerin is rapidly degraded in cells<sup>162</sup>. However a small number of mutations are missense or short in-frame deletions. Seven of these mutations were reported in patient cells, and an eighth mutation was recently identified in the group of Dr G. Bonne (Institut de Myologie, Paris). During my PhD, I focused my work on these eight mutations. Five of them affect residues of the predicted unstructured nucleoplasmic region (S54F,  $\Delta$ 95-99, Q133H, P183T, and P183H) and cause a classical EDMD phenotype<sup>163</sup>. Two missense mutations P22L and T43I (Bonne, personal communication) and the short in-frame deletion mutation  $\Delta$ K37<sup>164</sup> affect the LEM domain and are associated with isolated cardiac defects.

Recent studies provided biochemical and cellular evidences that emerlin can interact with itself<sup>165</sup>. First, yeast 2-hybrid experiments revealed that region 1-225 of emerlin binds to itself, indicating that C-terminus truncated emerlin can form homodimers and/or multimers<sup>166</sup>. Second, *in vitro* GST-pulldown experiments using recombinant emerlin fragment 1-221 and co-immunoprecipitation of full-length emerlin from HEK293T cell extracts also strongly suggested that emerlin could oligomerize both *in vitro* and in cells<sup>165</sup>. A model was proposed to explain emerlin intermolecular association data (figure 23)<sup>165</sup>.

The R region (aa 187 to 220) could be involved in homotypic interactions (R to R) and heterotypic interactions between itself and the S region (aa 159 to 162), whereas the LEM domain (aa 1 to 47) could interact with the AR region (aa 170 to 220). Consequently, this model proposes two emerlin “backbone” configurations depending on LEM domain positions. A down backbone configuration (figure 23. A), which corresponds to BAF- or chromatin-associated positions of the LEM domain, and an up backbone configuration (figure 23. B), which allows emerlin self-association.

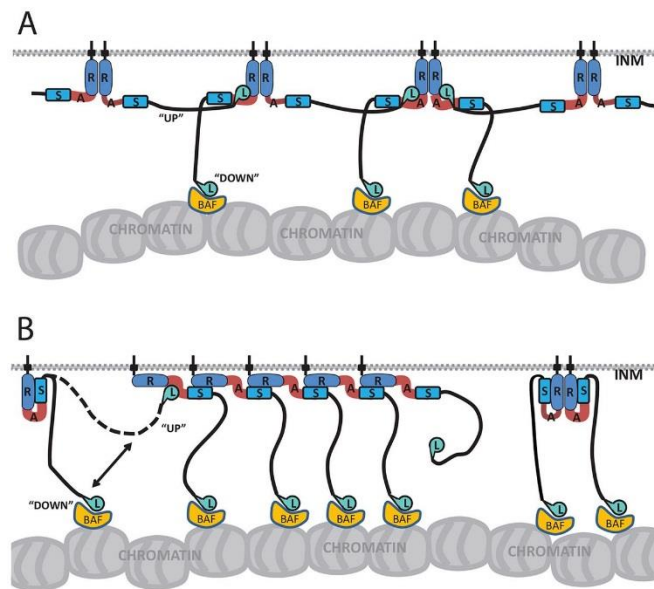


Figure 23 : Model for emerlin intermolecular association<sup>165</sup>.

(A) Down backbone configuration and (B) up backbone configuration.



## 2. EMERIN INTERACTING PROTEINS

Emerin interacts with several proteins essential for nuclear structure and chromatin organization. Best described emerin binding partners are A-type lamins and BAF. In addition, human emerin directly binds to at least 14 other proteins *in vitro* (figure 24)<sup>167</sup>.

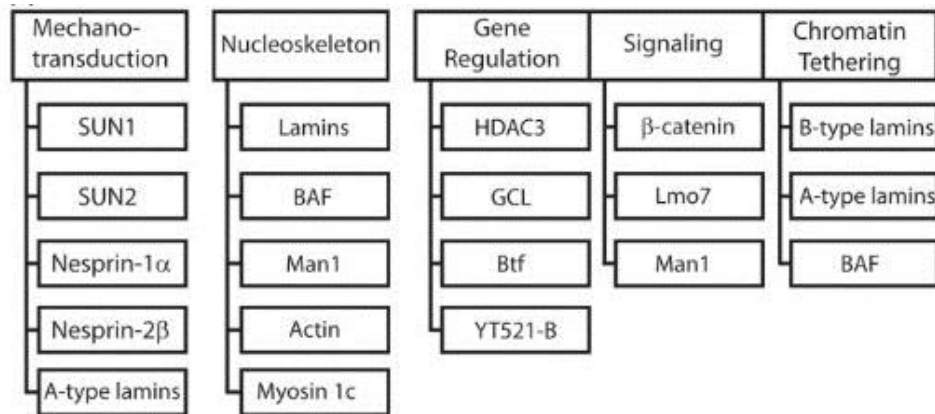


Figure 24 : Direct binding partners of emerin<sup>167</sup>.

### a. Emerin forms a ternary complex with BAF and A-type lamins

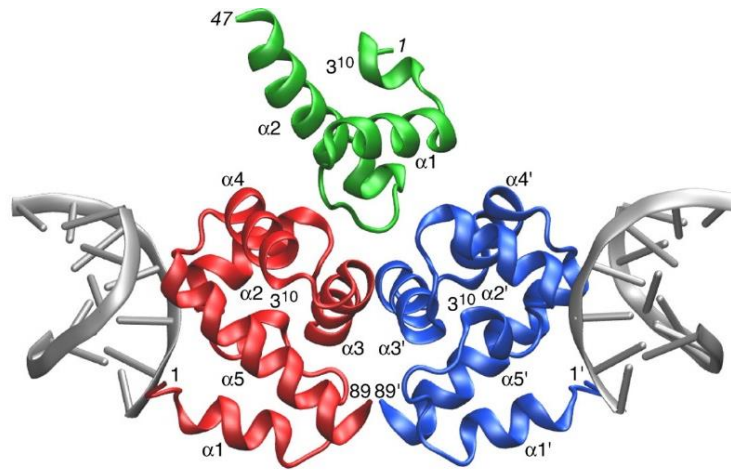
All LEM-domain proteins localized at the INM are supposed to interact with A-type lamins but interestingly, no consensus lamin-binding motif has been identified in LEM-domain proteins.

Several studies report the existence of an interaction between emerin and lamins and confirm that this interaction provides the structural backbone for proper nuclear localization and retention of emerin. A first observation of this interaction was done using immunofluorescence in Green Monkey Kidney cells: co-localization of emerin with A and B-type lamins was revealed in interphase cells<sup>162</sup>. Afterwards, this interaction was confirmed by several co-immunoprecipitation experiments<sup>168,169</sup>. The fact that the emerin and A-type lamins directly interact was demonstrated using Biomolecular Interaction Analysis (BIA)<sup>170</sup> and yeast two hybrid assays<sup>166</sup>. Finally, structural associations between emerin and lamins were investigated in four human cell lines. Absence of lamins A/C led to a mis-localization of endogenous and exogenous emerin in the endoplasmic reticulum. In addition, in cell lines which express lamin C, but not lamin A, both emerin and lamin C were mis-localized<sup>169</sup>.

Because of these results, a hypothesis could be that lamin A mediates association of lamin C with the lamina and then, lamin C stabilizes association of emerin with the inner nuclear membrane.

Concerning molecular details of this interaction, only few data were reported. The region of lamin A responsible for emerin binding was delimited using yeast two-hybrid experiments<sup>166</sup>. It consists of the lamin A tail domain, and more precisely, the region between amino acids 384 to 566. On the other hand, the region of emerin responsible for its interaction with A and B-type of lamin tails was deduced from GST-pulldown experiments. Two regions of emerin (residues 55-132 and 159-178) were found as necessary for lamin A and B1 tails binding and two regions of emerin (residues 1-132 and 159-220) were found to be sufficient for these bindings<sup>165</sup>.

Emerin interacts with BAF through its LEM domain. The 3D structure of the complex between emerin LEM domain and BAF was solved using NMR and molecular modeling<sup>52</sup> (figure 25). On one hand, the LEM domain of emerin and more in details, its helix  $\alpha 1$ , its subsequent loop, and the N-terminal end of its helix  $\alpha 2$  (in green) can bind directly to the dimer of BAF. On the other hand, BAF dimer interacts with the LEM domain through the C-terminal end of helix  $\alpha 2$ , the hairpin turn and the helix  $\alpha 3$  of its first monomer (in red) and through the hairpin turn between  $\alpha 2$  and  $\alpha 3$ , the C-terminal end of  $\alpha 3$ , and the central portion of  $\alpha 4$  of its second subunit (in blue). In addition, superimposition of the two known 3D structures of BAF dimer in interaction with either DNA or the LEM domain clearly revealed that BAF binding sites for both molecules do not overlap (figure 25). Thus, BAF can probably simultaneously bind to emerin and DNA.



**Figure 25 : The BAF<sub>2</sub> (in blue and red) - Em<sup>LEM</sup> (in green) interface (PDB: 2ODG) does not overlap the BAF<sub>2</sub>-DNA<sub>2</sub> (in grey) interface (PDB: 2BZF), as deduced from the superimposition of the 3D structures of BAF dimer bound to either emerlin or DNA<sup>52</sup>.**

A direct interaction between emerlin and BAF at the nuclear envelope of living cells was demonstrated by Fluorescence Resonance Energy Transfer (FRET)<sup>171</sup>. But interestingly, in the same study, Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Loss In Photobleaching (FLIP) experiments showed that, whereas BAF was highly mobile at the nuclear envelope, emerlin was much less mobile. Thus, a “touch and go” model was proposed according to which BAF interacts frequently but transiently with emerlin in interphase cells.

It was proposed that emerlin interacts with A-type lamins in order to provide structural support to the nucleus and with BAF in order to regulate gene expression. Recently, studies showed that the three proteins are able to interact together to form a ternary complex. First, autoradiography experiments using 150 nM <sup>35</sup>S-lamin A, 250 nM <sup>35</sup>S-BAF, or both proteins, which were incubated with immobilized emerlin, showed that A-type lamins and BAF together form stable complexes with emerlin<sup>55</sup>. Moreover, K. Wilson and co-workers published that binding of <sup>35</sup>S-emerlin to prelamin A tail-containing strips is enhanced fourfold by wild-type BAF<sup>172</sup>. Then, it was shown, *in vitro* and in HeLa cells, that phosphorylation of BAF on serine 4 leads to mis-localization of emerlin, which failed to be at the inner nuclear envelope. Binding of prelamin A tail to BAF was also significantly reduced in the case of BAF mutant S4E. In contrast, BAF S4E had no effect on emerlin binding to lamin A<sup>172</sup>.

## *b. Structural binding partners*

In addition to lamins, emerin interacts with other proteins that provide structural support to the nucleus. First, emerin was reported to directly interact with actin. Cytoplasmic actin was well studied during several years but afterwards actin was also found in the nucleus<sup>173</sup>. It was described as a component of chromatin remodeling complexes. It was also suggested as involved in nuclear architecture. Globular actin (G-actin) was shown to co-immunoprecipitate with emerin in myoblast extracts<sup>168</sup>. Another study demonstrated that emerin can form a complex with lamin A and actin, but only in differentiated muscle cells<sup>174</sup>. This binding was strongly increased in the presence of an active serine-threonine phosphatase. Thus dephosphorylation is required to allow emerin-actin binding. Afterwards, it was demonstrated that emerin can also bind and stabilize filamentous actin (F-actin) by capping its pointed end<sup>175</sup>. Recently, emerin polypeptides bearing various mutations have been tested *in vitro* for binding to different emerin partners and interaction with actin was confirmed.

It was shown that the LEM domain as well as the central region of emerin are implicated in this binding<sup>167</sup>. Finally, emerin is able to bind nuclear myosin I, in cells and directly *in vitro*<sup>176</sup>.

Other important emerin partners are SUN-domain and Nesprin proteins. This group of proteins clearly plays a structural role in the nucleus because they form the LINC complex (first part of this introduction). Nesprin-1 $\alpha$  binds to emerin *in vitro*, as shown by blot overlay<sup>177</sup>. Interestingly, this protein was found as binding also lamin A, suggesting that it might enhance a crosslink between emerin and A-type lamins at the nuclear envelope. More recently, another Nesprin isoform, Nesprin-2 was found to bind both emerin and lamin A/C tail<sup>178</sup>. Moreover, it was shown that absence of Nesprin-2 in cells leads to mis-localization of emerin. Finally, concerning SUN-domain proteins, it was demonstrated, by immunoprecipitation, that emerin binds to the nucleoplasmic domains of SUN1 (aa 223–302) and SUN2 (aa 1-174). Although a weak emerin interaction was also observed with the proximal end (residues 1–208) of SUN1<sup>179</sup>.

### c. Gene regulatory binding partners

Supporting a role for emerin in gene expression, several gene regulatory proteins have been reported to interact with emerin. First, emerin associates directly with the histone deacetylase 3 (HDAC3)<sup>139</sup> and is able to enhance its enzymatic activity by 2.5 fold *in vitro*.

Second, co-immunoprecipitation assays from HeLa cell nuclear extracts, and a microtiter well binding assay, demonstrated that three different regions of emerin (residues 34-83, 175-196 and 207-217) interact with the protein germ cell less (GCL)<sup>55</sup>. GCL is a conserved protein that binds to the transcription factor E2F, which activates genes required for entry into S-phase and it was shown that emerin interacts with GCL in order to co-repress E2F. Interestingly, competition assays demonstrated that BAF and GCL compete for binding to emerin *in vitro*<sup>55</sup>.

Adding to the list of gene regulatory binding emerin partners, a yeast-two-hybrid screening of a human heart cDNA library identified a nuclear splicing associated factor called YT521-B<sup>180</sup>. More in details, it was demonstrated that the C-terminus of YT521-B is sufficient to bind emerin *in vitro* and influence splice-site selection *in vivo*. On the other hand, it was shown that this protein binds emerin on the two regions flanking the lamin A binding domain, partially overlapping the BAF and lamin A binding regions.

In a similar yeast-two-hybrid assay, the pro-apoptotic transcription repressor called Btf was identified as an emerin interacting protein<sup>181</sup>. On one hand, this interaction was shown to require emerin residue 45-83 and 175-217 and on the other hand, it does require residues 377–646 of Btf. It is important to note that Btf can relocalize from the nuclear interior to the nuclear envelope during apoptosis induction, which suggests that emerin could be required to signal apoptosis within the nucleus.

The latest indication of emerin implication in gene regulation involves  $\beta$ -catenin and Lim-domain-only-7 (Lmo7), two signaling transcription factors that shuttle between the cell surface and the nucleus. The first one,  $\beta$ -catenin, is known to mediate Wnt signaling, and absence of emerin in cells seems to increase Wnt signaling, which suggests that emerin normally attenuates this pathway<sup>182</sup>. The second one, Lmo7, is a transcription factor that activates many genes including the emerin gene<sup>176</sup>. This suggests that Lmo7 binding to emerin protein can feedback-regulate emerin gene expression.

### 3. EMERIN POST-TRANSLATIONAL MODIFICATIONS

Emerin plays several functions at the nuclear envelope through binding to a lot of different partners. These interactions are differentially regulated by post-translational modifications. Major modifications in cells are phosphorylations and for example, emerin can be phosphorylated on several sites, like serine and threonine residues including S49, S66, T67, S120, S163 and S175<sup>183-186</sup>. Then, five residues in human emerin were identified as undergoing cell cycle-dependent phosphorylation using a *Xenopus* egg mitotic cytosol model system<sup>184</sup>. In addition, emerin is hyperphosphorylated in four different forms in metaphase and early S-phase cells<sup>163</sup>. Finally, during infection by Herpes simplex virus type 1, emerin is phosphorylated by several kinases<sup>187,188</sup>.

#### a. Emerin phosphorylation

Human emerin possesses at least 42 published sites of phosphorylation *in vivo* (25 serines, 4 threonines and 13 tyrosines)<sup>167,189</sup> (figure 26). It was reported that emerin is a major target of phosphorylation during mitosis and interphase, but the proteins and pathways that mediate phosphorylation on these sites, and the functional consequences of these modifications, remain poorly understood.

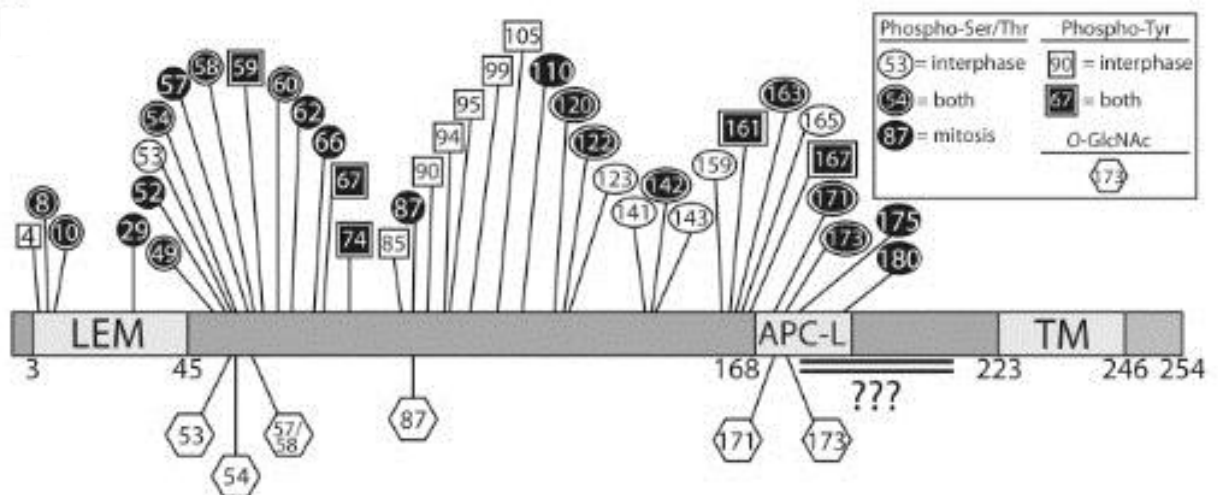
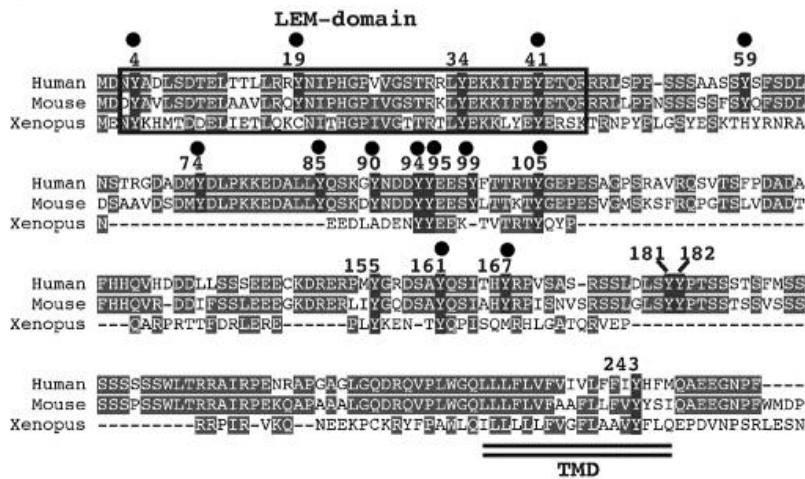


Figure 26 : Emerin phosphorylation sites identified by mass spectrometry in cells<sup>167</sup>.

Hexagons, O-GlcNAc sites; circles, phospho-Ser/Thr; squares, phospho-Tyr; white, asynchronous cultures; black, mitotic cultures and conditions; black with outline, sites identified in both asynchronous and mitotic cells. The double-underlined region has at least two O-GlcNAc sites and potentially other modifications that are uncharacterized due to the large size of the corresponding tryptic peptide and poor recovery by mass spectrometry.

## 1) Tyrosine phosphorylation

Several independent proteomic studies were achieved in order to identify tyrosine phosphorylation sites on emerin and 13 sites were found (figure 27). These sites are indicated with black dots on figure 27. All these sites found in human emerin are conserved in mouse and only six are conserved in *Xenopus*. In cells, tyrosine phosphorylation of emerin was validated by immunoprecipitation assays<sup>189</sup>.



**Figure 27 : Tyrosine phosphorylation sites of the emerin and amino acid sequences of emerin from human, mouse and *Xenopus laevis*<sup>190</sup>.**

At least two non-receptor Tyrosine kinases target emerin directly: Src and Abl. LC-MS/MS analysis revealed three major tyrosines that are phosphorylated by Src: Y59, Y74 and Y95<sup>190</sup>. Concerning Abl, one site was recovered and identified as Y167.

Tyrosine phosphorylation might regulate emerin interactions with specific partners and in particular, it was found as important for emerin binding to BAF<sup>184,190</sup>. First, BAF interacts with the LEM domain of emerin, yet this domain has four tyrosines in human, with at least three that are phosphorylated by unknown kinases *in vivo*. In addition, five tyrosines were found as impacting the interaction between BAF and emerin: Y4, Y19, Y34 and Y41 and interestingly, Y161, which is not localized closed to the LEM domain. Substitutions that removed a single hydroxyl moiety decreased (Y19F, Y34F, Y161F) emerin binding to BAF *in vitro* and *in cells*<sup>190</sup>. Concerning Y4F substitution, it was shown to reduce emerin 1-176 binding to BAF *in vitro*, but conferred enhanced binding to BAF when expressed in cells as full-length GFP-emerin<sup>190</sup>.



## 2) Serine and threonine phosphorylation

Concerning emerlin phosphorylation on serines and threonines, several known modifiers were suggested as protein kinase A (PKA), GSK3 $\beta$ , PKC $\delta$  and ERK2/MAPK.

For example, ERK2 was shown to directly interact with emerlin *in vivo* during lentiviral infection<sup>191</sup> and this led to a hyperphosphorylation of emerlin that caused an altered distribution of emerlin at the inner nuclear envelope and a mislocalization of emerlin to the outer nuclear membrane<sup>188</sup>.

### b. Emerlin O-glycosylation

Emerlin is highly modified through serine, threonine and tyrosine phosphorylations but it also possesses all features characteristic of O-linked-Nacetylglucosamine-modified proteins by, which are the presence of large disordered regions containing a lot of serine and threonine residues. O-linked-Nacetylglucosamine is a single sugar modification of serine or threonine residues that is abundant, reversible and dynamic, and this modification can compete or augment phosphorylation to regulate signaling, transcription, mitosis, and stress responses.

Immunoprecipitation assay with HeLa cells confirmed that emerlin is O-GlcNAcylated *in vivo*<sup>167</sup>. Afterwards, different emerlin polypeptides were O-GlcNAcylated *in vitro*, in order to identify which emerlin residues are modified. At least two regions, comprising residues 1–70 and 170–220, were targeted by the OGT enzyme *in vitro*. More in details, eight emerlin residues were O-GlcNAcylated *in vitro* and five residues were identified: Ser53, Ser54, Ser87, Ser171 and Ser173. In cells, only serine 173 was identified as significantly O-GlcNAcylated.

Thereafter, interaction between O-GlcNAcylated emerlin and BAF was studied. Interestingly, it was shown that only modification on Ser173 could impact BAF binding to emerlin and more in details, phosphorylation instead of O-GlcNAcylated at Ser173 reduced significantly BAF binding. This result could be explained by different mechanisms. First, a phosphorylation versus an O-GlcNAcylation at Ser173 could change emerlin conformation. Another hypothesis is that modifications on Ser173 could impact binding to a third partner, which could impact binding between emerlin and BAF afterwards.



## IV. THE NUCLEAR ENVELOPE AND DISEASES

Many human diseases, called envelopathies, are associated with NE protein defects. A large proportion of them are associated with mutations in lamin A/C and emerin.

### 1. EMERY-DREIFUSS MUSCULAR DYSTROPHY (EDMD)

EDMD is a group of genetic and degenerative diseases that primarily affects voluntary muscles. Its name comes from two physicians, Alan Emery and Fritz Dreifuss, who first described this disorder among a large Virginian family in 1966<sup>192</sup>. EDMD is characterized by contractures of major tendons, slowly progressive skeletal muscle wasting and weakness, and dilated cardiomyopathy with potentially lethal ventricular conduction system defects that can cause sudden cardiac arrest.

Mutations in *EMD* (encoding emerin) and *FHL1* (encoding FHL1) cause X-linked EDMD (XL-EDMD), whereas mutations in *LMNA* (encoding lamin A and C) cause an autosomal dominant (AD-EDMD) or autosomal recessive EDMD (AR-EDMD). For all forms of EDMD the diagnosis is based on clinical findings and family history. The diagnosis of X-linked EDMD also relies on failure to detect emerin or FHL1 protein in various tissues and molecular genetic testing of *EMD* or *FHL1*. The diagnosis of AD-EDMD and AR-EDMD relies on molecular genetic testing of *LMNA*<sup>193</sup>.

#### a. X-Linked EDMD

The first gene responsible for the X-linked form was identified in 1994<sup>159</sup>. It corresponds to the *STA* (or *EMD*) gene and mutations in this gene were found in several patients. Moreover, this gene was found as coding for the emerin protein.

DNA extraction from peripheral blood lymphocytes was done to detect mutations on the emerin gene which cause X-linked EDMD. Almost 100 mutations were found in this *STA* gene and these are approximately composed of 39.5% of small deletions, 31% of non-sense mutations, 15.5% of mutations in splice sites, 4% of large deletion, 8.5% of missense mutations and 1.5% of mutations in the promoter.

Although, the majority of mutations results in complete loss of emerin, some mutations have been identified as reducing expression level and protein amount<sup>194</sup>.

Interestingly, despite the different mutations in EDMD producing varying effects on emerin expression, the clinical phenotype of all the patients is similar.

Several studies focused on the emerin mutants that are still present in cells. Five emerin variants were observed at the nuclear membrane: S54F,  $\Delta$ 95-99, Q133H and P183T/H. Interestingly, all the corresponding mutations are detected in the predicted unfolded part of emerin. The behavior of four of these mutants (S54F,  $\Delta$ 95-99 and P183H/T) was studied by transfection of GFP-mutants in C2C12 myoblasts<sup>168</sup>. All mutants displayed reduced targeting and retention at the nuclear envelope in comparison to wild-type emerin and this was particularly significant in the case of mutant  $\Delta$ 95-99. The observed immunofluorescence intensity decrease might correspond to a decrease in expression, in stability or to a wrong localization.

Concerning mutation Q133H, it was shown to reduce the amount of emerin compared to control<sup>195</sup> and study of the corresponding GFP-mutant in COS-7 cells showed a correct localization.

Furthermore, emerin polypeptides bearing four of these mutations (S54F,  $\Delta$ 95-99, Q133H and P183H) were tested *in vitro* for binding to different partners: BAF, lamin A, GCL, Btf, YT521-b, Lmo7, HDAC3, F-actin and MAN1<sup>196</sup> (figure 28). On one hand, all tested mutants are still able to bind BAF. On the other hand, interaction between all emerin mutants and HDAC3 is disrupted. Interestingly, mutant  $\Delta$ 95-99 is the most functionally affected. Indeed, this mutant cannot bind anymore, in addition to HDAC3, A-type lamins, GCL, Btf, actin and MAN1. Like mutant  $\Delta$ 95-99, mutant Q133H cannot bind actin and MAN1 whereas mutant S54F cannot bind Btf. Finally, mutant P183H is the only one that cannot bind to Lmo7.

Recently a new in frame *EMD* deletion was found:  $\Delta$ K37. This mutation affects the LEM domain and is associated with isolated cardiac defects in patients<sup>164</sup>.

	S54F	Δ95-99	Q133H	P183H
BAF	+	+	nt	+
Lamin A	+		nt	+
GCL	+		+	+
Btf			+	+
YT521-B	nt	nt	+	+
Lmo7	+	+	+	
HDAC3				
Actin	+			+
MAN1	+			+

Figure 28 : X-linked EDMD mutation impacts on emerin binding to its partners<sup>196</sup>.

Scoring: normal binding (+), weakened binding (± and gray), and undetectable binding (black box). nt, not tested.

### b. Autosomal EDMD

Although most cases of EDMD are X-linked, a rare autosomal dominant form (AD-EDMD) was also reported. The gene responsible for AD-EDMD was identified in 1999. It is localized on chromosome 1q11-q23 and corresponds to the *LMNA* gene, which encodes A-type lamins<sup>197</sup>.

Unlike X-linked EDMD, the majority of mutations which cause AD-EDMD are missense mutations and lead to the production of an equimolar mixture between normal and mutated lamins<sup>198,199</sup>.

Three mechanisms are possible to cause autosomal dominance. The first one is a mechanism in which the reduced level of functional lamin A/C is not sufficient for normal lamina function. The second proposed mechanism is that lamin A/C mutants play a deleterious function. The last one is that, knowing that lamins form dimers in order to form filaments, a functional multimer exists, which cannot tolerate a defective subunit.

## 2. OTHER LAMINOPATHIES

More than EDMD, mutations in the *LMNA* gene have been shown to cause a large spectrum of diseases and the term of laminopathies has been adopted to describe them<sup>200–203</sup>. So far, at least eight different laminopathies have been associated to mutations in *LMNA*, including dilated cardiomyopathy with conduction defects (CMC), Limb-girdle muscular dystrophy type 1B (LGMD), Dunnigan-type familial partial lipodystrophy (FPLD), Charcot-Marie-Tooth disease (CMT), mandibuloacral dysplasia (MD), restrictive dermopathy (RD) and Hutchinson-Gilford progeria syndrome (HGPS). A representative set of mutations causing these different pathologies, including AD-EDMD, is represented on figure 29<sup>199</sup>.

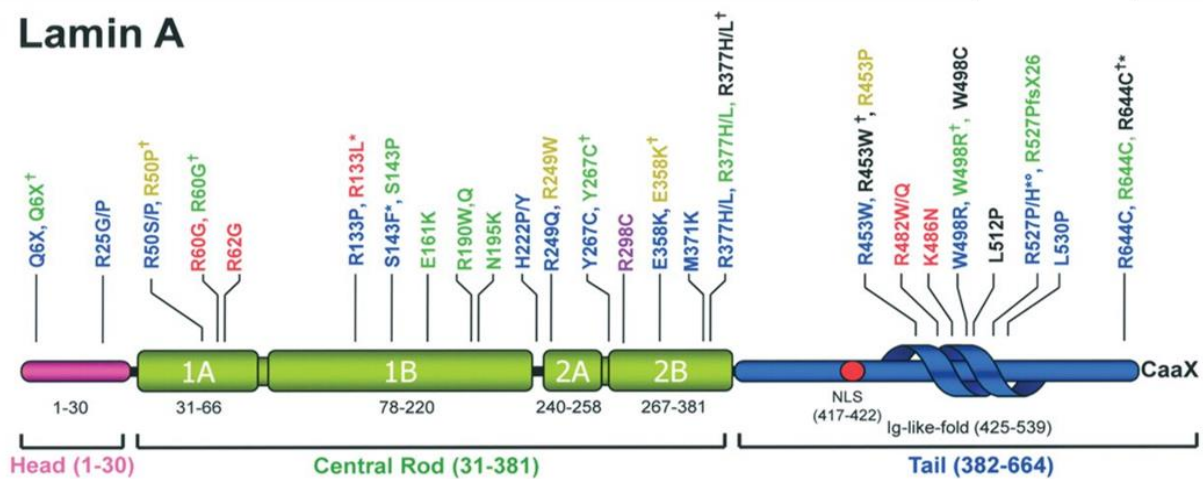


Figure 29 : Distribution of lamin A mutations and their related laminopathies, observed after clinical diagnosis<sup>204,205</sup> with A-EDMD in blue, DCM in green, LGMD1B in black, CMD in gold, FPLD2 in red and CMT2B in purple.

'†' indicates that the same amino acid change causes different laminopathies. \*R133L also causes WRN, \*S143F additionally results in HGPS, \*R527H causes MAD too and \*R644C also gives rise to a range of other disorders. °Patients carrying R527C develop either a severe form of MAD and/or progeria<sup>199</sup>.

### *a. Cardiomyopathy with conduction defects (DCM)*

Cardiomyopathies are characterized by cardiomyocyte dysfunction and tissue-wide remodeling of the myocardium leading to functional decline. *LMNA* was found as implicated in this kind of disease in 1999<sup>206</sup>, by Fatkin et al., and accounts for approximately 6-8% of CMD cases in humans. The number of mutations causing CMD increased and at least eight mutations are known<sup>198</sup> (figure 29).

This kind of disease can occur as an isolated phenotype or more frequently in combination with a skeletal muscle dystrophy such as EDMD or Limb girdle muscular dystrophy<sup>207,208</sup>.

### *b. Limb-girdle muscular dystrophy type 1B (LGMD-1B)*

LGMD-1B, like other muscular dystrophies, is primarily a disorder of voluntary muscles. Indeed, it affects mainly the proximal limb-girdle musculature<sup>209</sup>. This group of diseases comprises 15 different types inherited as both autosomal dominant and recessive forms.

As it is represented on figure 29, LGMD1B mutations (black on figure 29) tend to cluster in both the Ig-like fold and coil 2.

### *c. Dunnigan-type familial partial lipodystrophy (FPLD)*

Lipodystrophies are a group of diseases characterized by the absence or reduction of subcutaneous adipose tissue. Dunnigan-type FPLD is a rare autosomal dominant disease characterized by loss of subcutaneous adipose tissue from extremities and trunk and accumulation of fat in the head and neck areas<sup>210</sup>.

A first mutation was identified, R482Q<sup>211</sup> and afterwards, five other mutations were found. As it is shown on figure 29, at least half of the known mutations that cause this pathology are localized in the lamin tail region<sup>147</sup>.

### *d. Charcot-Marie-Tooth disease (CMT)*

Charcot-Marie-Tooth diseases form a heterogeneous group of hereditary motor and sensory neuropathies. This group can be classified in two subgroups: the first one is composed of demyelinating type diseases (type 1) and the second one of axonal type diseases (type 2)<sup>38</sup>.

Mutations in the *LMNA* gene are associated with axonal CMT. The first mutation was discovered in three consanguineous Algerian families. Genome analysis identified a missense mutation causing an R298C substitution.

#### *e. Progeroid syndromes (PS)*

Mutations in lamins cause muscle diseases, adipose tissue diseases and neuropathies, as shown in Figure 29. They also cause accelerated aging syndromes also called progeroid syndromes, as presented in the following paragraphs.

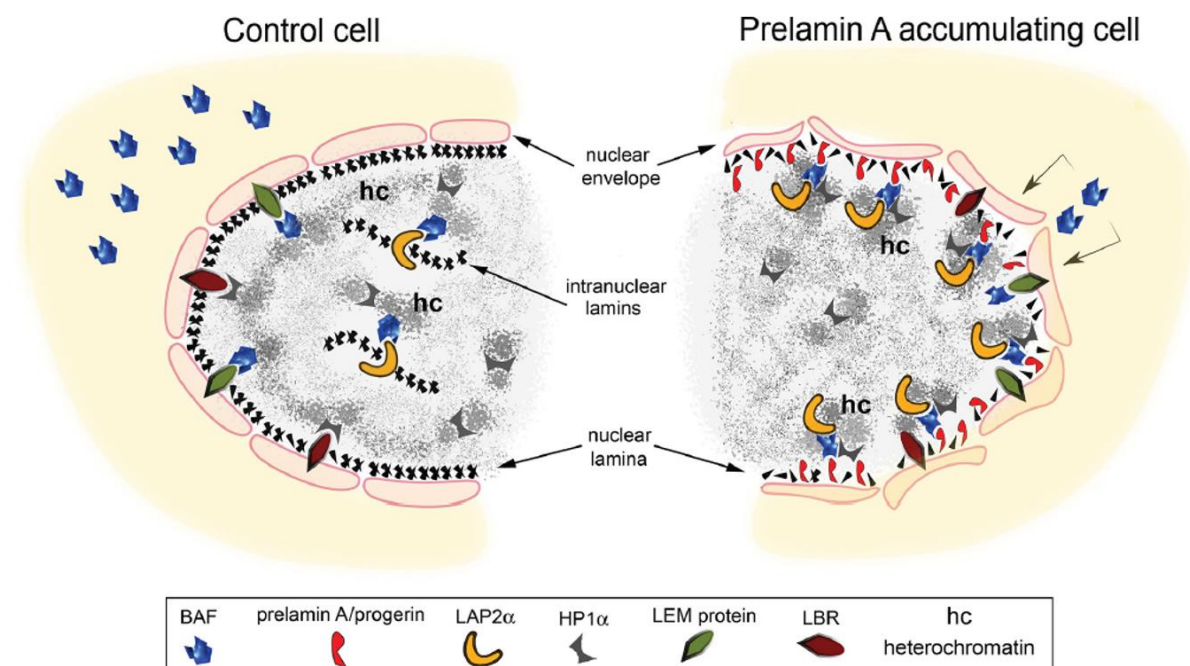
##### 1) Hutchinson-Gilford progeria syndrome (HGPS)

Hutchinson-Gilford progeria (HGPS) is a rare genetic disorder that causes an average age of death at 13.4 years due to coronary artery disease<sup>37</sup>. It is characterized by abnormalities in skin, bone, fat tissue, hair and blood vessels. The most common HGPS mutation is located at codon 608 and like I already explained in the first part of this introduction, it is a silent mutation G608G. However, this mutation induces a cryptic splicing donor site that generates a 150-nucleotide deletion in the mRNA sequence. In consequence, the resulting prelamin A bears a 50-amino acid in-frame deletion and lacks the proteolytic cleavage site for ZMPSTE24, which is essential for C-terminal farnesyl group release<sup>37,212</sup>. The incompletely processed prelamin A is commonly called progerin. In cells, progerin accumulation correlates with nuclei that exhibit altered size and shape with nuclear envelope interruptions. In addition, the presence of progerin induces a loss of heterochromatin and H3K9 trimethylation, whereas H4K20 trimethylation level increases<sup>151,152,213,214</sup>. Progerin also impairs the response to oxidative stress through, for example, NRF2 (Nuclear Factor (erythroid-derived 2)-like 2) transcription factor sequestration, which leads to an accumulation of ROS (reactive oxygen species) and an impairment of the DNA damage response.

Because of its farnesylation, progerin enhances some interactions that might depend on its farnesyl moiety. For example, it was shown that farnesylated prelamin A interacts with NARF (Nuclear Prelamin A Recognition Factor)<sup>215</sup> and SUN1 proteins<sup>216</sup> whereas interaction with HP1 $\alpha$  is disrupted<sup>217</sup>.

Because it was demonstrated *in vivo* that BAF could interact with different prelamin A forms<sup>218</sup>, and because accumulation of BAF in the nucleus of HGPS cells was observed, it was proposed that some chromatin defects could be triggered by the progerin-BAF interaction. To confirm this hypothesis, a recent study demonstrated first that progerin could bind BAF in HEK293 cells. Indeed, after expression of FLAG-tagged progerin in HEK293 cells, recruitment of endogenous BAF at the nuclear rim was observed<sup>219</sup>. In addition, co-immunoprecipitation studies confirmed that progerin is able to bind BAF WT.

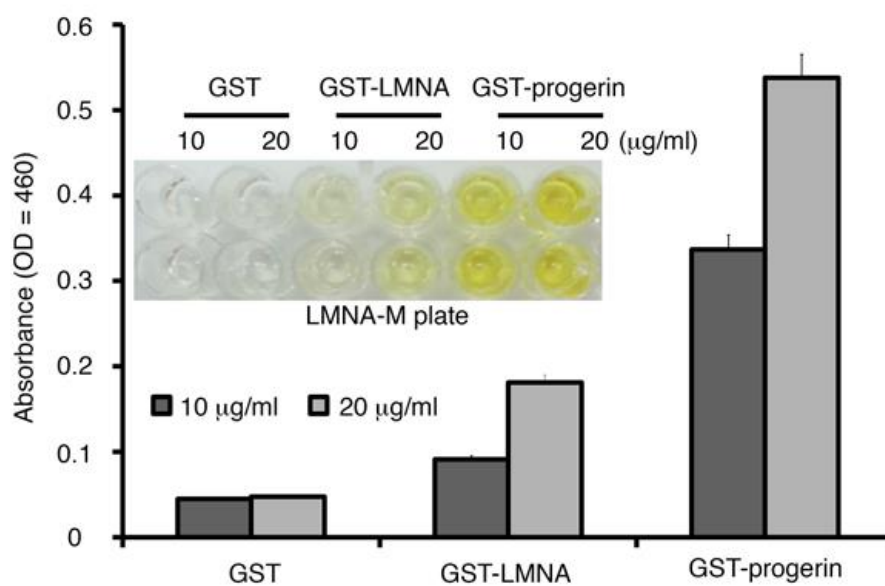
Interestingly, in this same study, it was shown that BAF is necessary to mediate both prelamin A and progerin effects on nuclear distribution of LAP2- $\alpha$  and HP1 $\alpha$ , which are proteins involved in chromatin-related processes (figure 30). These observations could explain how the progerin-BAF interaction plays a role on chromatin organization: accumulation of progerin induces BAF translocation from the cytoplasm to the nuclear lamina where both proteins interact and this could help progerin to interact with DNA organizing proteins to modify chromatin organization through BAF.



**Figure 30 : Speculative cartoon of the mechanism involving prelamin A-BAF interaction in chromatin organization changes associated to prelamin A accumulation<sup>219</sup>.**

Another protein was found as interacting with progerin and could contribute to the development of the senescence phenotype of HGPS and aged cells. Indeed, different techniques as proteomic studies, exploiting RNA interference technology to target the mutated pre-spliced or mature LMNA mRNAs<sup>220</sup> or two-hybrid approaches using a cDNA library to search for progerin-interactive partner proteins<sup>221</sup>, have identified lamins A/C as progerin-binding partners<sup>222</sup>. More in details, a direct interaction was found between progerin and A-type lamins whereas no interaction was detected with B-type lamins.

Interestingly, an ELISA assay in which His-tagged lamin A middle region (His-LMNA-M; from residues 301 to 564) was fixed in a 96-well plate and incubated with GST-fused recombinant lamin A C terminal region (GST-LMNA) or with progerin C-terminal region (GST-progerin) clearly revealed that progerin–lamin A binding affinity is more than 2-fold stronger than that of lamin A–lamin A binding (figure 31).



**Figure 31 : ELISA results showing His-LMNA-M/ GST-LMNA and His-LMNA-M/GST-progerin interactions<sup>222</sup>.**

This result is in agreement with the HGPS cell phenotype in which A-type lamins form an irregular fiber arrangement<sup>222</sup>. Indeed, stronger affinity of progerin for lamin A could induce a tighter but irregular bundle formation compared to intermediate filaments formed by interaction between several lamin A/C proteins.



Another hypothesis is that interaction between progerin and lamin A may induce nuclear deformation, because it was shown that progerin alone did not induce nuclear deformation in lamin A/C-deficient cells whereas co-transfection with lamin A/C could induce it<sup>222</sup>.

All these results are consistently leading to the hypothesis that the strong affinity of progerin for lamin A/C could contribute to nuclear lamina alteration in HGPS cells.

## 2) Atypical progeroid syndromes (APS)

Besides HGPS, several atypical progeria syndromes (APS) caused by *LMNA* gene mutations have been described, such as atypical Restrictive Dermopathy (the most severe), Werner syndrome and mandibuloacral dysplasia. Some mutations causing atypical progeroid syndromes were identified. First, heterozygous mutations localized in lamin A/C C-terminus like T623S<sup>223</sup> were found. Secondly, some mutations were identified in the coiled-coils 1A and 1B of lamin A/C, which are required for the polymerization of the nuclear lamins into higher order structures. Indeed, concerning coiled-coil 1A, the heterozygous mutants A57P<sup>224</sup> and both E55K and E55G<sup>225</sup> were observed. Similarly, several heterozygous *LMNA* mutations in the coiled-coil 1B were identified as R133L<sup>226</sup>, L140R<sup>224</sup>, S143F<sup>227</sup> and E145K<sup>37</sup>. Finally, a large number of mutations were found in the globular domain of lamin A/C, the Igfold: heterozygous mutations as R527C<sup>228</sup> and T528M associated to M540T<sup>229</sup> or homozygous mutations like R435C<sup>230</sup>, R471C<sup>203</sup>, R527C<sup>231</sup>, R527H<sup>232–235</sup>, A529T<sup>236</sup>, A529V<sup>237</sup> and K542N<sup>238</sup>. All these mutations are reported on figure 32.

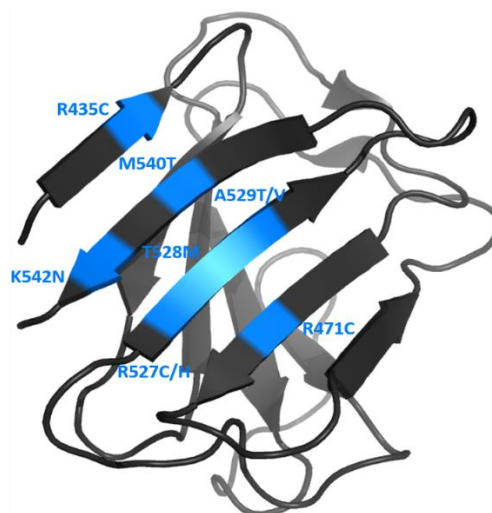


Figure 32 : Localization of Igfold residues that are mutated in APS (PDB: 1IFR<sup>43</sup>).

#### a) Restrictive dermopathy (RD)

Restrictive dermopathy is the most severe of these progeroid syndromes and was first described by Witt et al. in 1986<sup>239</sup>. It is a rare lethal genetic disorder that causes congenital tautness of the skin with characteristics of facial features (small mouth, small pinched nose, and micrognathia), bone mineralization defects including thin, dysplastic clavicles, and pulmonary hypoplasia<sup>240</sup>.

A first group screened *LMNA* coding sequence and exon-intron boundaries in nine children affected by RD and did the same for the endoprotease *ZMPSTE24* of seven patients. As I already mentioned, this enzyme is essential for post-translational processing of prelamin A and allows a correct maturation of lamin A to be inserted into nuclear lamina. In one case, they identified the most common heterozygous mutation responsible of HGPS (G608G) and in another case, they identified a novel splicing mutation specifically affecting lamin A. Both mutations were leading to the production and accumulation of truncated prelamin A. Concerning *ZMPSTE24*, they found a unique heterozygous insertion leading to the creation of a premature termination codon<sup>240</sup>.

Then, the same group did another analysis on ten patients affected by RD and found three novel null mutations in *ZMPSTE24* and in all cases, a complete absence of both *ZMPSTE24* and mature lamin A was observed<sup>36</sup>.

Another study analyzed genomic DNA extracted from peripheral blood lymphocytes of a 2-year girl affected by RD. This analyze revealed the presence of a homozygous mutation, R435C<sup>230</sup>. Thereafter, presence of this mutation R435C was recovered in other patients<sup>241</sup>.

#### b) Werner syndrome

The Werner syndrome is characterized by scleroderma-like skin changes, short stature, thinning of the hair, diabetes mellitus, soft tissue calcification and premature atherosclerosis. It was first described as a progeroid syndrome caused by mutations in the *WRN* gene, which codes for a DNA helicase but afterwards, it was shown that some features of this disorder are also present in laminopathies caused by mutations in *LMNA*. In addition, it was found that individuals with atypical Werner syndrome with mutations in *LMNA* had a more severe phenotype than those affected by the disorder due to mutated *WRN*.

Three *LMNA* missense mutations, localized in lamin A/C coiled-coil domain 1, were identified: A57P, R133L and L140R. Immunofluorescence studies on patient fibroblasts were done and revealed that these mutants cause abnormal nuclear shape and apparition of chromatin in the cytoplasm<sup>224</sup>.

#### c) Mandibuloacral dysplasia (MAD)

Mandibuloacral dysplasia is a rare autosomal recessive disorder characterized by postnatal growth retardation, skeletal malformations, craniofacial anomalies, joint contractures and types A and B patterns of lipodystrophy. Owing to its slowly progressive course, the syndrome was observed in adults, and pediatric case reports are scarce<sup>232</sup>.

A homozygous mutation, R471C, was identified in a 7-year girl and it was report that this mutation causes a severe early MAD combined with progeroid features<sup>203</sup>.

Another homozygous mutation, R527C, was found as causing severe MAD during infancy<sup>231</sup>. Then, this mutation was identified in the *LMNA* gene of two children who were suffering from atypical HGPS<sup>242</sup>.

Furthermore, one study analyzed five consanguineous Italian families, containing patients affected by typical MAD and after sequencing of the *LMNA* gene, identified a homozygous missense mutation, R527H<sup>232</sup>. Patient cells showed nuclei with abnormal lamin A/C distribution and abnormal nuclear shape. Several studies supported the implication of this mutation in MAD<sup>233–235</sup>.

Finally, two homozygous missense *LMNA* mutations A529V<sup>237</sup> and A529T<sup>236</sup> were identified. A529V was found in a male and a female patients with MAD<sup>237</sup> and mutation analysis of a 56-year-old Japanese woman with MAD and type A lipodystrophy revealed the second one, A529T<sup>236</sup>.

### 3) Nestor Guillermo Progeria Syndrome (NGPS)

Recently, two unrelated patients who exhibited Hutchinson-Gilford Progeria syndrome-like phenotypes were described<sup>243</sup>. These patients did not present all symptoms of HGPS syndromes. For example, they did not show signs of ischemia or atherosclerosis and these patients were much older than the average life span of progeroid patients.

The new syndrome was called Nestor Guillermo Progeria Syndrome (NGPS) and after genome sequencing, a homozygous mutation in the *BAF* gene was discovered. This mutation, A12T, does not disrupt the dimerization of the protein *in vitro* but affects BAF protein level<sup>219,244</sup>. Indeed, BAF was detectable in NGPS nuclei but hardly visible in the cytoplasm. Finally, this mutant also affects nuclear morphology.

Unlike in other progeroid syndromes, in NGPS cells, prelamin A processing is normal. But a recent study demonstrated that A12T mutation affects the ability of prelamin A to modify chromatin organization<sup>219</sup>. To prove this, an electron microscopy study was performed on HEK293 cells expressing FLAG-tagged proteins in combination with BAF. Normal chromatin organization was observed in cells expressing WT A-type lamins (LA-WT) in combination with WT BAF, whereas when WT A-type lamins were co-expressed with BAF A12T, chromatin architecture was affected (figure 33). Indeed, peripheral heterochromatin did not adhere anymore to the nuclear lamina. Interestingly, co-expression of prelamin A (LA-C661M) with WT BAF induced a specific recruitment of the heterochromatin with impressive beads-on-a-string appearance whereas in presence of BAF A12T, this beads-on-a-string appearance was not visible anymore, but only some heterochromatin clustering was observed.

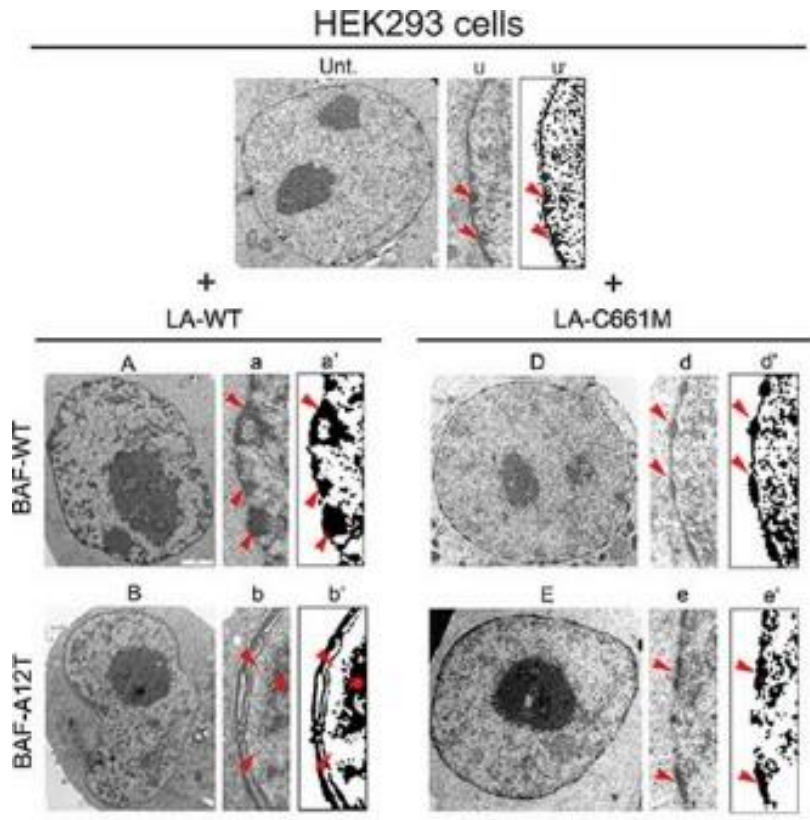


Figure 33 : Electron microscopy evaluation of the impact of prelamin A and BAF mutations on chromatin organization<sup>219</sup>.

This observation led to new hypotheses concerning the role of BAF interaction with A-type lamins. Indeed, the authors showed that in NGPS, BAF mutation compromises prelamin A-BAF interaction but also impairs prelamin A-mediated H3K9m3 intranuclear recruitment; it could perturb the epigenetic function of BAF, affecting its ability to interact with histone H3 or involved in histone H3 modification. In HGPS, the persistence of BAF-progerin binding could impair different BAF interactions or slow down the dynamics of BAF protein complexes.

## V. HYPOTHESIS AND THESIS OBJECTIVES

The nuclear envelope is composed of a double lipid bilayer, corresponding to the inner and outer nuclear membranes, and a large number of proteins. It contributes to the shape and the position of the nucleus, to the cell structure and mobility, to the organization of the genome and to the regulation of signaling pathways. Mutations in genes coding for nuclear envelope proteins, like mutations in the lamin genes, cause a large number of human diseases, called envelopopathies. Structural information that describes the 3D arrangement of these proteins at the nuclear envelope is lacking, and this hampers the description of their normal and pathogenic functional mechanisms.

The LBSR (Structural Biology Laboratory and Radiobiology) works since several years on this structural characterization of protein complexes that are mutated in human diseases. The aim of my thesis was to characterize the interaction between lamin A/C and emerin, in order to obtain molecular details about the complex *in vitro*, and to understand the impact of disease-causing mutations on this interaction *in vitro* and in cells.

To achieve this goal, we decided to first describe the structure of emerin. During the beginning of my thesis, Isaline Herrada, a PhD student in the lab, showed that emerin fragment 1 to 187 oligomerizes *in vitro*. Simultaneously, the group of K. Wilson in Baltimore published that emerin could interact with itself through several regions *in vitro* and in cells<sup>165</sup>. Moreover Guilluy et al.<sup>107</sup> published that emerin is phosphorylated by Src during a mechanical stress on tyrosines 74 and 95, and this is a critical event to trigger the mechanical response of the nucleus<sup>107</sup>. Therefore we decided to study the impact of oligomerization and phosphorylation on emerin binding to lamin A/C.

In this manuscript I will report:

- (1) the structural characterization of emerin (partly performed in collaboration with Dr Isaline Herrada).**
- (2) the structural description of two different complexes involving emerin and lamin.**
- (3) the impact of disease-causing mutations on these interactions, as observed *in vitro* but also in cells (collab. with Dr Brigitte Buendia, Univ. Paris Diderot).**
- (4) the role of phosphorylation by Src on emerin structure and binding properties.**

# RESULTS

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# I. STRUCTURE OF THE INNER NUCLEAR ENVELOPE PROTEIN EMERIN

The function of emerin at the nuclear envelope depends on its oligomerization state, 3D arrangement and modifications. At the beginning of my PhD, I worked together with another PhD student, Dr Isaline Herrada, in order to describe the oligomerization states of emerin fragment 1 to 187, also called EmN. This work was triggered by two observations:

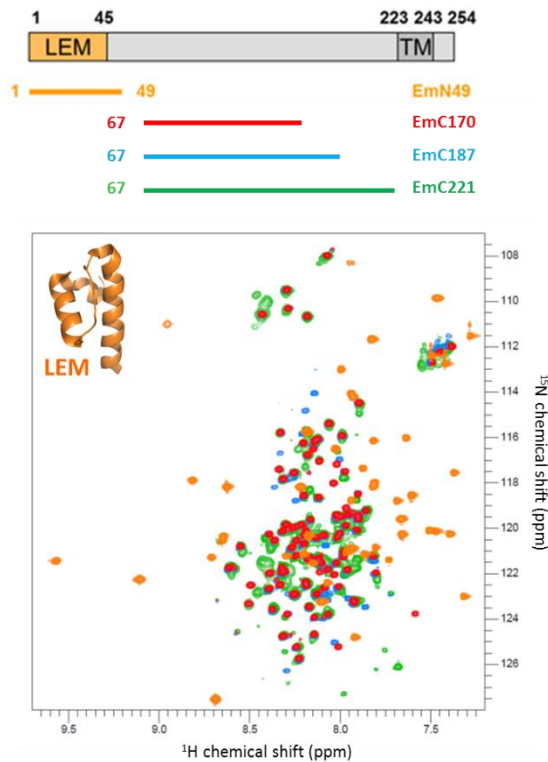
- Emerin nucleoplasmic region binds to lamin A tail. Indeed, emerin nucleoplasmic region (residues 1 to 225) binds to lamin A/C (residues 1 to 566) as well as the tail region of lamin A (residues 385 to 664) as observed by yeast 2-hybrid experiments<sup>166</sup> and this interaction is impaired by mutations in emerin region 70 to 178 as seen by blot overlay assays<sup>245</sup>. However we did not observe any binding between monomeric EmN and lamin A tail by NMR.
- Emerin oligomerizes. Indeed, emerin nucleoplasmic region (residues 1 to 225) interacts with itself as observed by yeast 2-hybrid experiments<sup>166</sup>. Later during my PhD it was also reported that emerin self-associates *in vitro* and in cells<sup>165</sup>.

Our hypothesis was that oligomerization of emerin could regulate lamin A tail binding. I have here inserted the 2 papers in which we describe the oligomerization of EmN from a biophysical point of view. Then I describe additional data that I obtained on the phosphorylation of EmN by a set of kinases involved in cell cycle regulation and mechanotransduction.

## 1. STRUCTURAL CHARACTERIZATION OF EMERIN NUCLEOPLASMIC REGION

The structure of emerin nucleoplasmic region (residues 1 to 221) was analyzed by solution NMR (figure 34). The LEM domain presents a <sup>1</sup>H-<sup>15</sup>N spectrum typical of a  $\alpha/\beta$  fold<sup>49</sup>. The narrow distribution of the peaks present on the <sup>1</sup>H-<sup>15</sup>N spectra of fragments 67-170, 67-187 and 67-221 suggested that these fragments are unstructured. Assignment of the NMR signals of fragment 67-170 confirmed that this fragment is intrinsically disordered (see in [Appendix 1: Samson et al., \*Biomol NMR Assign\* 2016](#)). Thus emerin nucleoplasmic region is composed of a small globular LEM domain and a large disordered fragment.





**Figure 34 : Architecture of emerlin and superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  spectra of fragments 1-49, 67-170, 67-187 and 67-221 of emerlin, recorded in 20mM phosphate buffer pH 6.5, 30mM NaCl, at 700 MHz and 303K.**

In the papers inserted below, I focus on the description of the oligomeric states of emerlin. The whole nucleoplasmic region of emerlin, from residue 1 to residue 221, was systematically aggregating during the dialysis against a urea-free buffer. Therefore I studied oligomerization of both EmN (from residue 1 to residue 187) and EmC221 (from residue 67 to residue 221). I first characterized EmN oligomers because EmN contains the region proposed to bind to lamin A/C.

The first paper reports that EmN can self-assemble into long curvilinear filaments. These oligomers were observed by negative-staining Transmission Electron Microscopy (EM) thanks to the facilities of Dr Maïté Paternostre in our laboratory in Saclay and Dr Dmytro Puchkov at the FMP Berlin. Emerlin-emerlin proximities in cells were consistently detected by *in situ* Proximity Ligation Assays (collab. with Dr Brigitte Buendia, Uni Paris Diderot). Defects in self-assembly and cellular distribution were observed for several emerlin variants causing Emery-Dreifuss Muscular Dystrophy. In particular, for variant del95-99, both a lack of self-assembly *in vitro* and a decrease in emerlin-emerlin and emerlin-lamin A/C proximities in cells were observed. These first results were consistent with our first hypothesis that emerlin self-assembly is essential for emerlin function and in particular for lamin recognition.

# **Muscular dystrophy mutations impair the nuclear envelope emerin self-assembly properties**

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Keywords: nuclear envelope, lamin, protein filaments, nuclear structure, protein oligomerization, electron microscopy

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<https://doi.org/10.1021/acscchembio.5b00648>

The second paper aims at describing the structural organization of EmN oligomers. I showed that emerlin fragment from residue 1 to residue 132 is necessary and sufficient for self-assembly. The LEM domain and the unstructured region of this fragment both contribute to the structural core of the filaments, as shown by solid-state NMR (collab. with Prof Adam Lange, FMP Berlin) and mass spectrometry (collab. with Dr Jean Armengaud, CEA Marcoule). However the poor quality of the solid-state NMR spectra, probably due to conformational heterogeneity present in the EmN filaments, impaired a more detailed description of the 3D structure of emerlin oligomers. Destabilization of the LEM domain favors filament formation. This is in particular observed in the case of emerlin variant delK37, which causes a muscular dystrophy with cardiac defects. Our results predicted that, if emerlin self-assembly regulates lamin binding, variant delK37 should still bind to lamin A/C. This was confirmed using NMR.

Finally another PhD student in the lab, Florian Celli, showed that EmC221 also self-assembles. This oligomerization process is so efficient that it was very difficult to obtain a sample of monomeric EmC221 for solution-state NMR. I will come back to the EmC221 oligomers later when discussing about the emerlin – lamin interaction.

## **Emerin self-assembly mechanism: role of the LEM domain**

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Running title: Nuclear envelope emerin self-assembled structure.

Keywords: Nuclear envelope. Nucleoskeleton. Lamin. Oligomerization. Intrinsically disordered region. Folding.

Conflicts of interest: The authors declare no competing interest.

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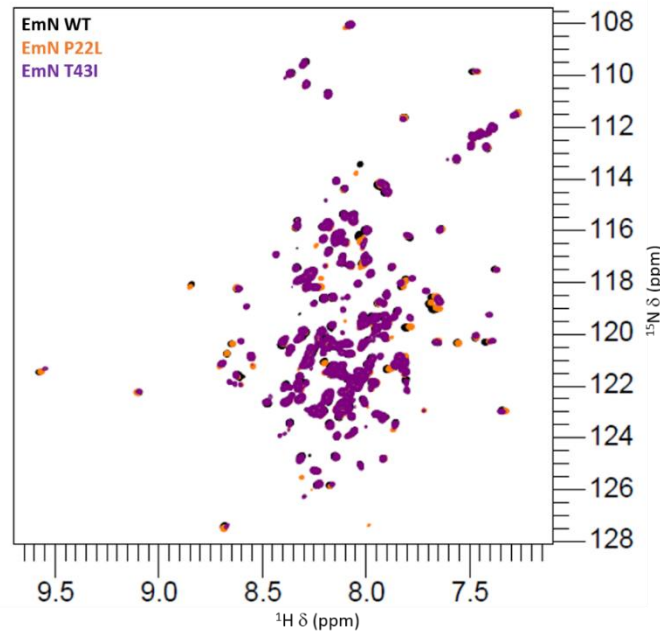
<https://doi.org/10.1111/febs.13983>

## 2. IMPACT OF EMERIN LEM DOMAIN MUTATIONS ON EMERIN SELF-ASSEMBLY

In the first article, we studied the impact of five mutants (S54F,  $\Delta$ 95-99, Q133H and P183T/H) that show a defect in emerin unstructured region and cause Emery-Dreifuss Muscular Dystrophy, on EmN self-assembly, and found that mutant  $\Delta$ 95-99 cannot form filaments anymore. In the second paper I presented, we worked with another mutant showing a defect in emerin LEM domain and known to cause cardiac defects, mutant  $\Delta$ K37. This mutant is particularly interesting because it exhibits a destabilized LEM domain and the EmN mutant  $\Delta$ K37 is able to form filaments faster than EmN WT.

In parallel, we worked with two other mutants showing a defect in emerin LEM domain and associated to the same symptoms as  $\Delta$ K37: mutants P22L and T43I (G. Bonne, personal communication). I here report my results on the 3 mutants of the LEM domain, which will be published in another article.

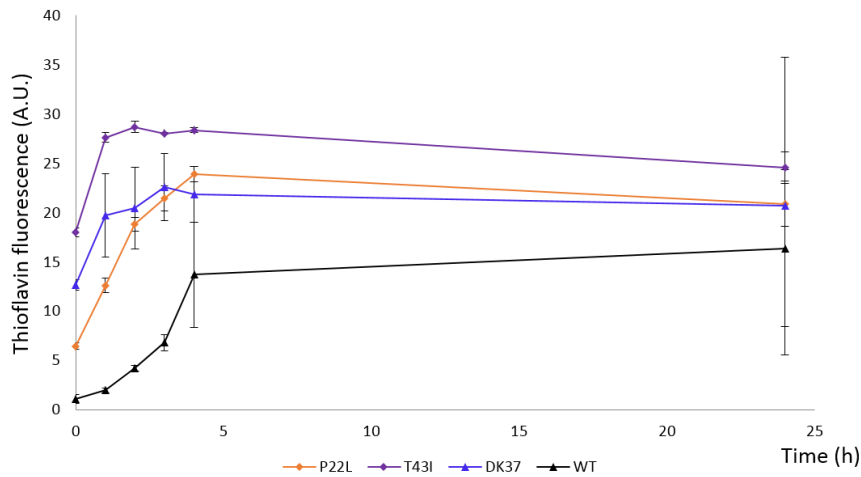
First, together with a PhD student in the lab, Nada Essawy, I studied the structure of EmN mutants (P22L and T43I) by NMR. After recording  $^{15}\text{N}$ - $^1\text{H}$  HSQC spectrum of each mutant (figure 35), we observed no chemical shift differences compared to the spectrum that we obtained for EmN WT, except for some peaks, corresponding to amino acids close to the mutated residues. We concluded that both mutations do not impact the structure of EmN monomers.



**Figure 35 : Structure comparison between EmN monomer WT and mutants.**

**Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on an EmN WT sample at  $100\mu\text{M}$  (in black), an EmN P22L sample at  $100\mu\text{M}$  (in orange) and an EmN T43I sample at  $100\mu\text{M}$  (in purple), in 20mM Phosphate pH6.5, 30mM NaCl, 5mM DTT, at 303K and 750MHz (FMP Berlin).**

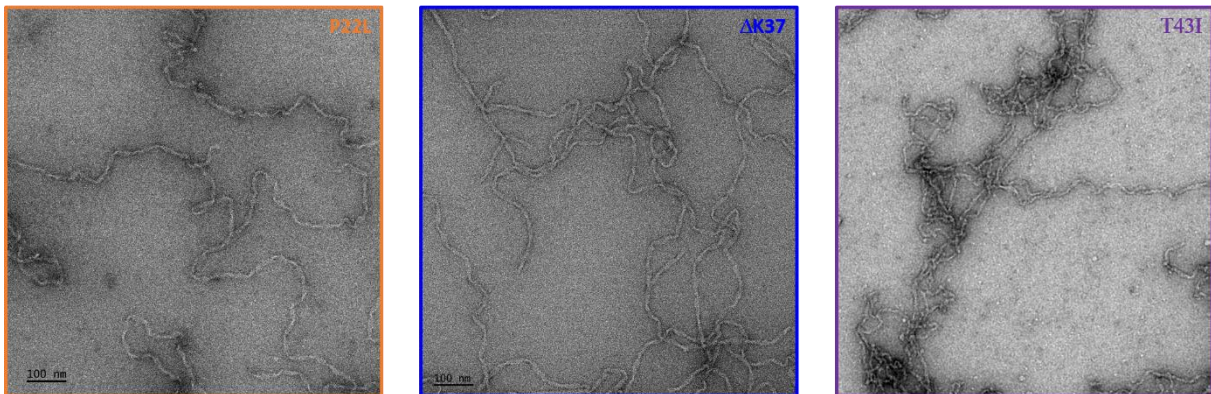
Second, I studied the self-assembly kinetics of the three mutated EmN fragments using thioflavin T fluorescence. As it is described in the second paper I presented, we used this technique because thioflavin T is a benzothiazole dye that increases in fluorescence upon binding to amyloid-like oligomers<sup>246,247</sup>. It gives a fluorescence signal at 480nm after excitation at 440nm, and this signal is significantly enhanced when thioflavin T is bound to these amyloid-like structures, especially the “cross- $\beta$ ” structures, characterized by an extended  $\beta$ -conformation with the strands running perpendicular to the long axis of the assembly of multiple  $\beta$ -sheets<sup>248</sup>. We first verified that thioflavin T fluorescence is not enhanced in the presence of EmN monomers, and we showed that it nicely increases during EmN self-assembly<sup>4</sup>. In the case of the three mutants, I concentrated them up to  $300\mu\text{M}$  and incubated each protein sample at 310K, during one day. I took a sample for fluorescence measurement at 480nm every hour during 4h and then the next day. For each measurement, I diluted my protein sample to  $40\mu\text{M}$  in the kinetic buffer (20mM Tris-HCl pH8, 30mM NaCl) and added  $10\mu\text{M}$  of thioflavin T (figure 36).



**Figure 36 : Self-assembly kinetics of EmN T43I, P22L and  $\Delta$ K37 mutants, compared to EmN WT, as followed by thioflavin T fluorescence.**

The fluorescence intensities at 480nm of EmN WT, T43I, P22L and  $\Delta$ K37 are represented in black, purple, orange and blue, respectively. Experiments were performed in 20mM Tris-HCl pH8, 30mM NaCl, at 310K.

My results show that the three EmN mutants self-assemble faster than EmN WT. This was never observed for any of the five mutants showing a defect in the disordered part of EmN<sup>4</sup>. To confirm that these three mutants could form filaments, we used negative staining electron microscopy. In more details, we concentrated the three proteins up to 600 $\mu$ M and observed the samples after one day at room temperature by EM (figure 37). We confirmed that these three EmN mutants are able to form filaments, which look like EmN WT filaments.



**Figure 37 : Negative-staining EM pictures of the three EmN mutants.**

Pictures were obtained in the presence of 2% uranyl acetate, using a Tecnai Spirit transmission electron microscope (FEI) equipped with a LaB6 filament (Dr Ana-Andreea Arteni, I2BC, Gif-sur-Yvette). Filaments were obtained after incubation at 600 $\mu$ M, during one day, at room temperature. P22L,  $\Delta$ K37 and T43I images are framed in orange, blue and purple, respectively.

### 3. DESCRIPTION OF THE PHOSPHORYLATION OF EMN BY FUNCTIONALLY RELEVANT KINASES

Emerin is heavily phosphorylated in cells. Forty-two phosphorylation sites were reported on emerin *in vivo* (25 serine, 4 threonine and 11 tyrosine)<sup>196</sup>. However, responsible kinases, phosphorylation kinetics and functional consequences on emerin oligomerization and interaction with partners remain poorly understood. For example, emerin phosphorylation by the Src kinase during a mechanical stress was observed on isolated nuclei<sup>107</sup>. However, the consequences of this phosphorylation process on emerin oligomerization and lamin binding is not known. As, after a mechanical stress, lamin A/C was shown to be recruited to the LINC complex in order to reinforce nuclear stiffness<sup>107</sup>, and because it was recently shown that application of a strain could increase emerin levels at the outer nuclear membrane without altering total emerin protein level<sup>249</sup>, I did the hypothesis that emerin phosphorylation by Src could disrupt emerin binding to the nuclear lamina.

Here, first, I present the results I obtained by NMR and mass spectrometry, concerning emerin phosphorylation by Src *in vitro*. I tried to characterize which emerin residues are phosphorylated by Src kinase. Then, because Src phosphorylates emerin on residues 74 and 95, and because we found that this region is important for filament formation, I choose to study impact of Src phosphorylation on emerin oligomerization. Finally, I observed emerin phosphorylation by two other kinases predicted as able to phosphorylate emerin (CK1 and CK2) and in cell extracts.



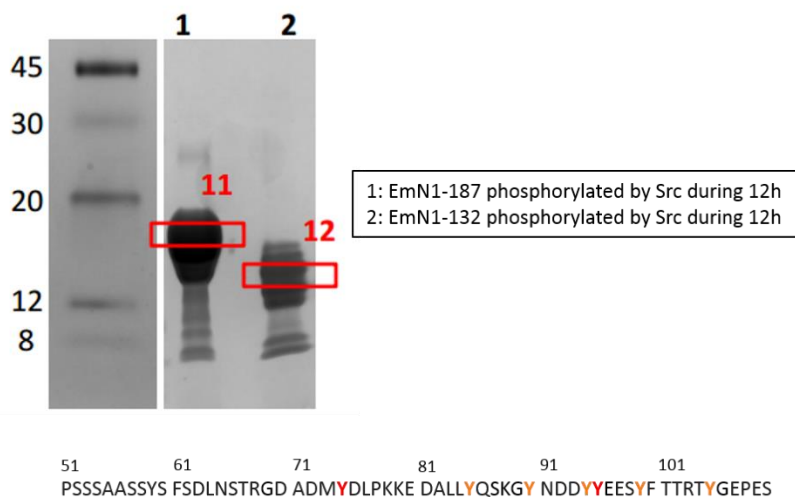
## a. Characterization of emerin phosphorylation *in vitro*

### 1) Emerin phosphorylation by Src kinase, *in vitro*

The Src kinase phosphorylates emerin tyrosines 74 and 95 during application of a mechanical force on the nucleus; mutation of these two residues impair nuclear response to a mechanical stress<sup>107</sup>. I first identified which emerin residues are phosphorylated *in vitro* by Src, through two different techniques which are NMR and mass spectrometry.

I also did some preliminary studies about the impact of Src phosphorylation on emerin oligomerization, following emerin self-assembly by fluorescence and electron microscopy. The final aim was to observe impact of Src phosphorylation on emerin binding to lamin A/C.

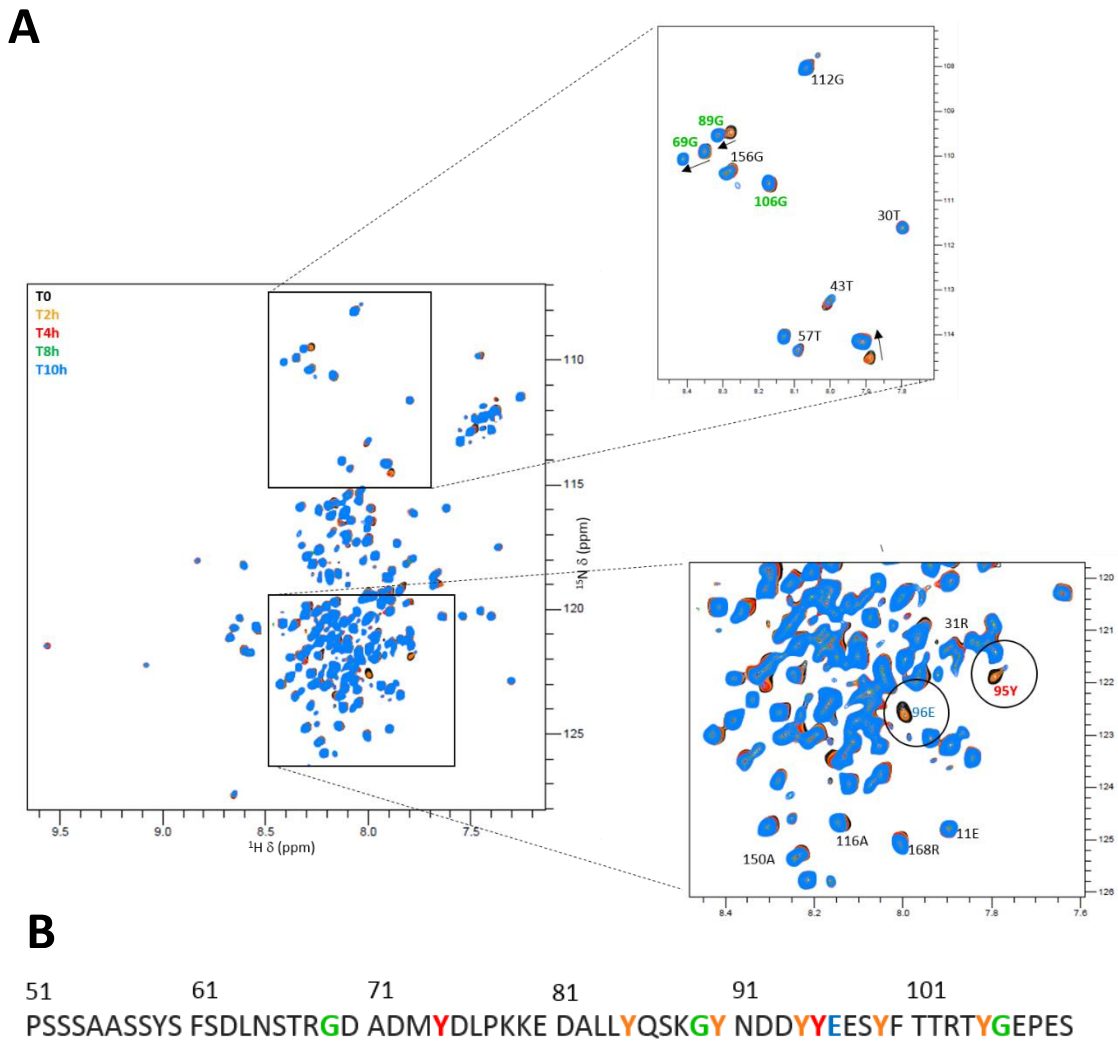
First, I studied emerin modifications in the presence of Src kinase by mass spectrometry (figure 38). I worked with two emerin constructs: Emerin 1-187 (EmN) and 1-132 (EmN132). I mixed the emerin fragment with 10µl of Src kinase and 2mM ATP and incubated them during 12h at 303K. Then I observed the phosphorylated sample by SDS-PAGE and sent the band corresponding to the phosphorylated emerin fragment to the group of Dr Jean Armengaud (CEA Marcoule), who analyzed it by MS/MS mass spectrometry with an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled to an UltiMate 3000 nRSLC system (Dionex, ThermoFisher). This experiment revealed that only Tyr74 and Tyr95 are phosphorylated by Src at more than 20% in our conditions. Tyr161 was phosphorylated at about 15% and Tyr85, Tyr94, Tyr99 and Tyr105 were phosphorylated at about 5%. Tyrosine 74 and 95 are labelled in red in the emerin sequence from figure 38 and interestingly, it corresponds to both tyrosines found as phosphorylated during a mechanical stress. Other tyrosines are labelled in orange in the emerin sequence.



**Figure 38 : SDS-PAGE gel of EmN and EmN132 phosphorylated by Src.**

Samples labelled with number 11 and 12 were then analyzed by mass spectroscopy in order to identify which residues are modified by the kinase. Tyrosines found as phosphorylated are labelled on the emerlin sequence (more than 20% phosphorylated: at red, less than 15% phosphorylated: orange).

To confirm the result obtained by mass spectrometry, I studied EmN phosphorylation kinetics by NMR. I recorded a  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum on  $^{15}\text{N}$  labelled EmN in the presence of 5mM  $\text{MgCl}_2$  and 2mM ATP first, as a reference, and then I added 10 $\mu\text{l}$  of Src kinase in my NMR sample and recorded a spectrum every 2 hours (figure 39), using single tube. Several chemical shift modifications were observed during this kinetics. I had previously assigned the unphosphorylated EmN spectrum<sup>250</sup>. It was thus easy to follow the disappearance of the signal from the unphosphorylated tyrosine 95 (labelled on the figure 39), which is one of the tyrosines reported as modified by Src in the literature<sup>107</sup> (and shown as phosphorylated by Src in our mass spectrometry experiments). Contrariwise, it was difficult to assign all EmN modified residues because of too much spectral overlaps and changes during the kinetics. All these chemical shift differences were due to amino acid modifications and to changes in chemical environment of neighboring residues. The assignment problem is well illustrated though the observation of the peaks corresponding to the glycine residues of the protein (First Zoom on the figure 39). Indeed, many chemical shift differences were observed in this peak area, because of the presence of modified tyrosines (labelled in red and orange on the emerlin sequence) next to several glycines, labelled in green on the emerlin sequence (figure 39, B). The peak corresponding to residue E96 (Second Zoom on the figure 39) also totally shifts from its initial position after Src phosphorylation, probably due to phosphorylation of Y95.

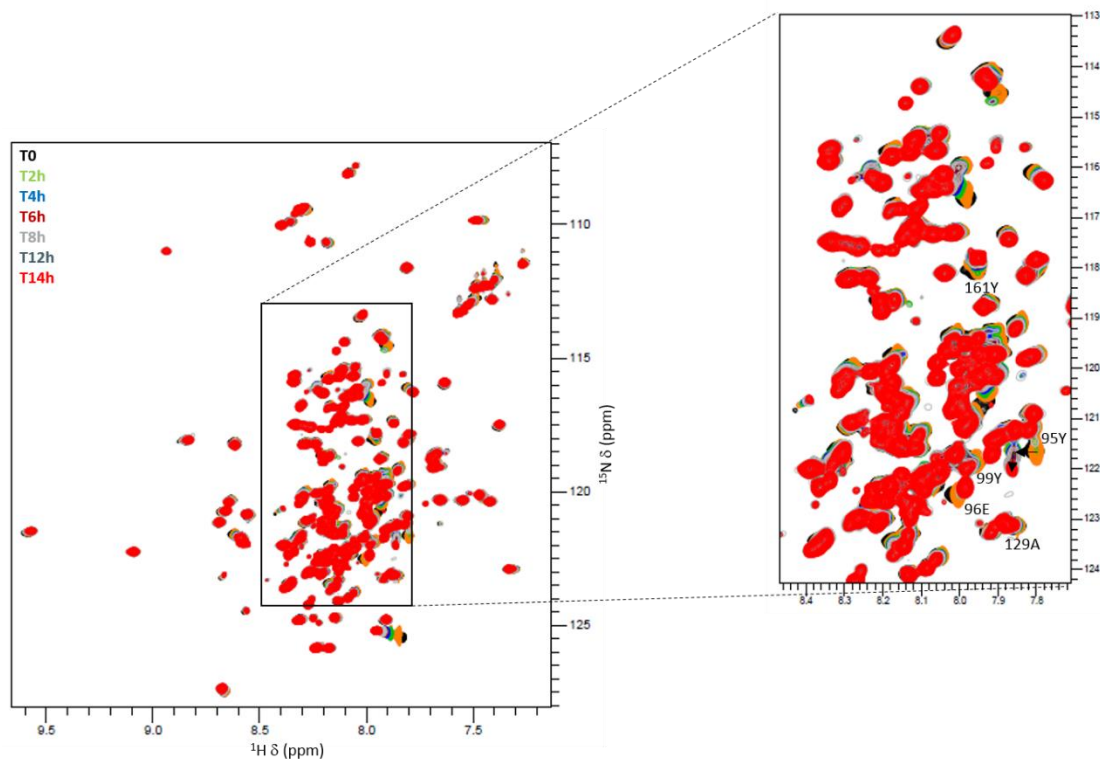


**Figure 39 : EmN phosphorylation kinetics, in the presence of Src.**

(A) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra, recorded every two hours on a EmN sample at  $100\mu\text{M}$  in  $20\text{mM}$  Phosphate pH7,  $30\text{mM}$  NaCl,  $10\text{mM}$   $\beta$ -mercaptoethanol,  $2\text{mM}$  ATP,  $5\text{mM}$   $\text{MgCl}_2$  and  $10\mu\text{l}$  of Src kinase, at  $303\text{K}$  and  $750\text{MHz}$  (FMP Berlin). (B) Emerin sequence with tyrosines identified as more than 20% phosphorylated by mass spectroscopy labelled in red, tyrosines less than 15% phosphorylated labelled in orange, glycines next to modified tyrosines labelled in green and the glutamate next to tyrosine 95 labelled in blue.

To override this problem, I studied EmN132 modifications as I did for EmN (figure 40), by NMR. As in the EmN study, several modifications appeared over time during the kinetics. Then, I produced a double labelled sample of EmN1-132 that I phosphorylated by Src kinase during 12h at  $303\text{K}$  in order to record subsequently 3D NMR experiments to try to assign the phosphorylated sample. Because of too much signal superimpositions due to a lot of changes after phosphorylation and because the protein aggregated in phosphorylation conditions, it was not possible to assign all signals corresponding to amino acids affected by Src phosphorylation.

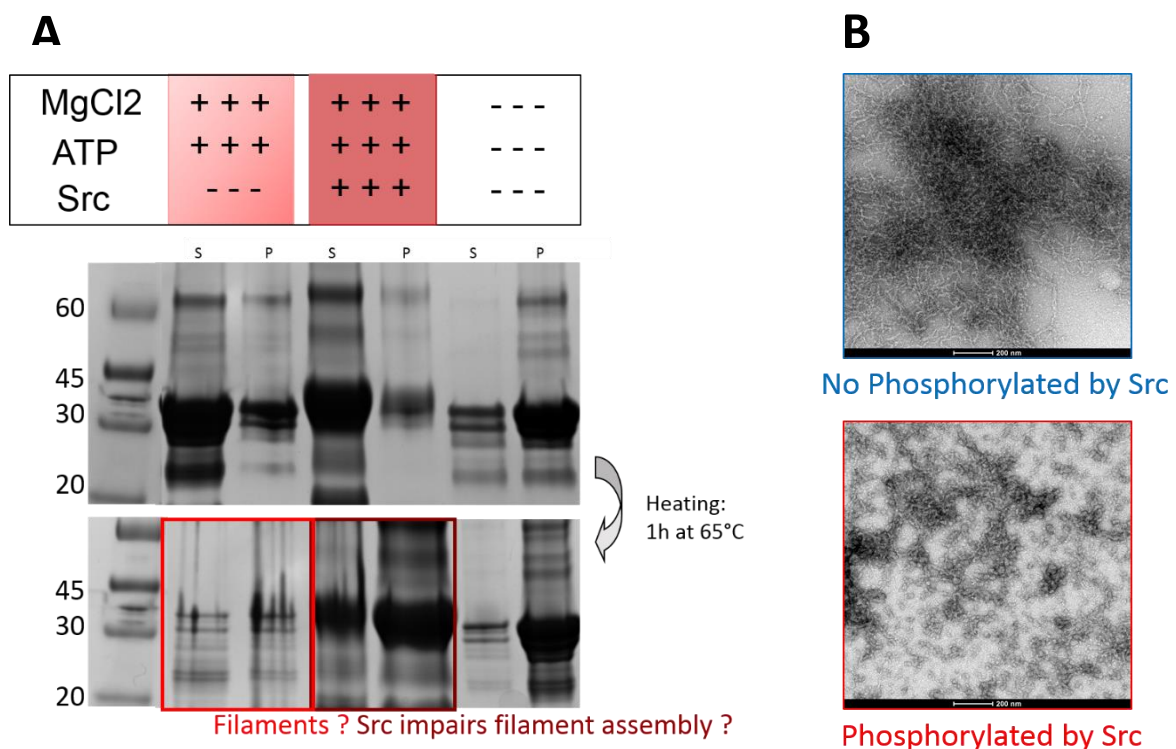
Only both tyrosines found as more than 20% phosphorylated by mass spectroscopy, Tyr74 and Tyr95 were assigned in their phosphorylated states. To continue on this phosphorylation study by NMR and to succeed in protein signal NMR assignment, we need to produce EmN mutants, in which each tyrosine, one per one, is mutated.



**Figure 40 : Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded, every two hours, on an EmN132 sample at  $100\mu\text{M}$  in  $20\text{mM}$  Phosphate pH7,  $30\text{mM}$  NaCl,  $10\text{mM}$   $\beta$ -mercaptoethanol,  $2\text{mM}$  ATP,  $5\text{mM}$   $\text{MgCl}_2$  and  $10\mu\text{l}$  of Src kinase, at  $303\text{K}$  and  $750\text{MHz}$  (FMP Berlin).**

After trying to identify which amino acids are modified by Src, we did preliminary studies on the impact of Src phosphorylation on EmN oligomerization. We studied this by SDS-PAGE, EM and thioflavin T fluorescence. Results obtained using thioflavin T fluorescence experiments are not presented here because they were complicated to reproduce and interpret. One question here in particular is: can thioflavin T bind to phosphorylated filaments, given that it is reported as preferentially interacting with tyrosine rich cross- $\beta$  surfaces (see for example Biancalana and Koide<sup>248</sup>)? I studied EmN oligomerization in the presence of Src using SDS-PAGE, because I observed that EmN filaments could not enter the SDS-PAGE gels in the absence of  $\beta$ -mercaptoethanol and heating.

I first dialyzed the EmN sample in 20mM Tris-HCl pH8, 30mM NaCl and 10mM  $\beta$ -mercaptoethanol, and after concentrating EmN to 600 $\mu$ M, I prepared three different samples: one with EmN, 5mM MgCl<sub>2</sub> and 2mM ATP, one with EmN, MgCl<sub>2</sub>, ATP and 10 $\mu$ l of Src kinase and one containing just the concentrated EmN. Afterwards, I heated each sample during one hour at 338K and I prepared SDS-PAGE samples of the supernatant and the pellet, after 10 minutes of centrifugation at 12000 g, for each condition, before and after heating at 338K (figure 41, A).



**Figure 41 : Src phosphorylation impact on EmN filament assembly, as observed by SDS-PAGE and EM.**

(A) Picture of SDS-PAGE gels obtained with EmN samples, phosphorylated or not by Src, in the presence or absence of MgCl<sub>2</sub> and ATP, before and after heating samples during one hour at 338K. (B) Picture obtained by negative staining EM using 0.5% of uranyl acetate on an EmN sample heated during one hour at 338K, phosphorylated or not during 15h by Src, using a JEOL MET 1400 (120KeV) (Dmytro Puchkov, FMP Berlin).

I observed the disappearance of EmN on SDS-PAGE gels only in one condition, i.e. EmN in the presence of MgCl<sub>2</sub> and ATP after one hour of heating. In the presence of Src, it was only the solubility of the protein sample that was different before and after heating. This experiment suggested that phosphorylation by Src impairs EmN filament assembly.

To confirm this, I observed the impact of Src phosphorylation using electron microscopy (figure 41, B). First, I dialyzed EmN in 20mM phosphate pH7 and 30mM NaCl and I concentrated it until 250 $\mu$ M.

Then, I splitted the EmN sample in two and added 5mM MgCl<sub>2</sub> and 2mM ATP to both of them. I added the Src kinase in one of the two samples and incubated both samples at 303K during 15h. Afterwards, samples were dialyzed against buffer with 20mM Tris-HCl pH8 and 30mM NaCl, concentrated until 600 $\mu$ M and put at 338K during one hour before being placed at room temperature during three days. Finally, both samples were observed by electron microscopy (figure 41, B). We observed the presence of filaments in our non-phosphorylated sample whereas aggregates were observed in the phosphorylated sample. This experiment also suggested that Src phosphorylation inhibits EmN filament formation or decreases the filament self-assembly rate. To confirm this, I have to repeat all these experiments. Secondly, I would like to try another protocol to phosphorylate EmN. Indeed, because we have to be in specific conditions to phosphorylate a protein, depending on the kinase, I would like to phosphorylate EmN before cleavage of its tag, in order to purify again EmN after phosphorylation and to see impact of this phosphorylation on filament assembly without the presence of kinase additives.

## 2) Emerin phosphorylation by CK1 kinase, *in vitro*

Analysis of emerin sequence using KinasePhos2<sup>251</sup> revealed that different serines are predicted as phosphorylated by the CK1 kinase, but two with a better confidence index: Ser120 (SVM score = 0.886429) and Ser123 (SVM score = 0.871596). These two serine are phosphorylated *in vivo*<sup>196</sup>. Therefore I studied by NMR phosphorylation of the EmN132 construct by CK1 (Appendix 2, figure 100). After 12h of phosphorylation, no clear chemical shift differences were observed between the unphosphorylated emerin 1-132 spectrum (in black) and the spectrum obtained after 12h of phosphorylation (in red). I concluded that CK1 could not phosphorylate EmN132 in our conditions.

### 3) Emerin phosphorylation by CK2 kinase, *in vitro*

With the help of the KinasPhos2 software, I found that 6 serines are predicted as phosphorylated by CK2: Ser8, 123, 141, 142, 143 and 175. All these serines are phosphorylated *in vivo*<sup>196</sup>. I studied by NMR phosphorylation of EmN132 by CK2 (Appendix 2, figure 101).

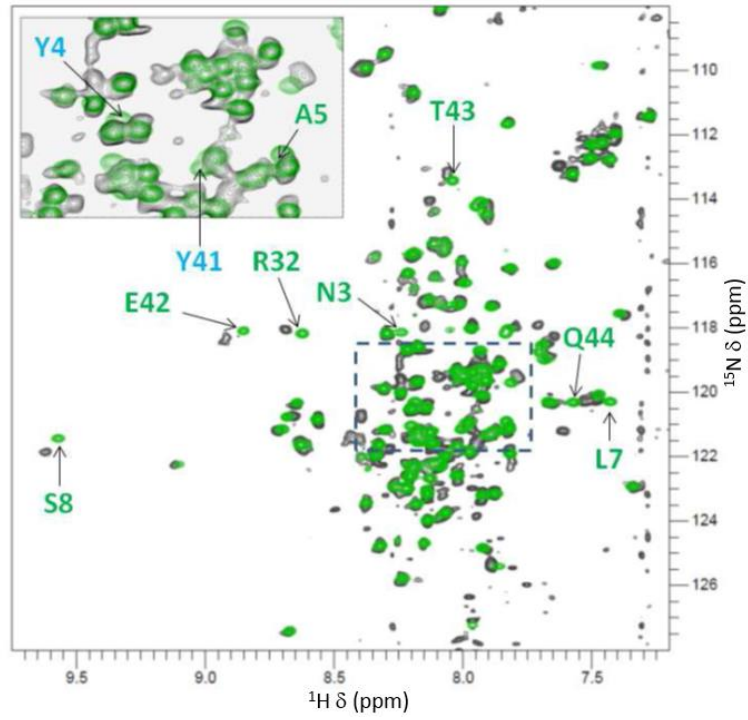
After superimposition of the non-phosphorylated emerin spectrum (in black) and the spectrum of emerin that was phosphorylated during 12h by CK2 (in green), only some minimal chemical shift differences were observed, so I concluded that CK2 does not seem to phosphorylate EmN132 in our conditions.

### 4) Characterization of EmN132 modifications in cell extracts

I did one preliminary study of EmN132 modifications in cell extracts. In more details, I produced our <sup>15</sup>N labelled EmN132 construct with a Histidine Tag at the N-terminus. Then, I concentrated the protein up to 200µM and incubated it in 293T cell extracts (10mg/ml) in the presence of 10mM ATP, 2mM MgCl<sub>2</sub>, 2mM DTT and protease and phosphatase inhibitors. Finally, the protein was purified again by affinity chromatography and concentrated in 20mM Phosphate buffer pH6.5 and 30mM NaCl. NMR <sup>1</sup>H-<sup>15</sup>N HSQC spectra were recorded on the EmN132 sample before (figure 42, in green) and after (figure 42, in black) addition of cell extracts.

Different changes were observed between the two conditions. For example, it was easy to assign peaks corresponding to Tyr4 and Tyr41 (labelled in blue) as well as surrounding residues (labelled in green) and to observe that peaks corresponding to these residues were shifted, suggesting that these LEM domain tyrosines (or their neighboring residues) were phosphorylated in cell extracts.





**Figure 42 : Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a non-phosphorylated EmN132 sample (in black) or an EmN132 sample phosphorylated by 293T cell extracts (in green) at 100 $\mu\text{M}$  in 20mM Phosphate pH6.5, 30mM NaCl, 10mM  $\beta$ -mercaptoethanol, 2mM ATP and 5mM  $\text{MgCl}_2$ , at 303K and 700MHz (CEA Saclay).**



## II. Emerin-lamin interactions

Lamins are known to be essential for nuclear functions. On the one hand, as cytoplasmic intermediate filaments, nuclear lamins play structural functions by forming a nucleoskeletal scaffold to provide physical stability to the nucleus, to define nuclear shape and to position nuclear pore complexes<sup>15,16</sup>. On the other hand, lamins are found as interacting with chromatin-associated proteins, which means that lamins are implicated in higher order chromatin organization<sup>252</sup>. Lamins are proposed to be involved in epigenetic control mechanisms and may also take part in DNA replication.

Since several years, a lot of studies have been initiated in order to understand how lamins interact with their partners at the molecular level. However, when I arrived at the lab, nothing was known at this level. In particular, an interaction between A-type lamins and emerin was confirmed by several techniques but the molecular details of this functionally critical interaction were not known. Co-immunoprecipitation assays demonstrated that an interaction, direct or indirect, exists between lamin A and emerin<sup>166</sup>. Then, surface plasmon experiments showed that this interaction is direct<sup>170</sup>. In addition, yeast two hybrid<sup>166</sup> and blot overlays<sup>245</sup> assays confirm these results. Finally, only binding regions on both sides were characterized: the C-terminal tail of lamins, and more particular region 384-664 which contains the globular Igfold domain, was reported to bind to emerin and mutations in emerin region between residues 70 to 164 were reported to impair the interaction between A-type lamins and emerin.

Our collaborators, the team of Dr Brigitte Buendia from Diderot University in Paris, observed, using the technique of Proximity Ligation Assay (PLA) that GFP-emerin and endogenous A-type lamins were close, i.e. separated by less than 30-40 nm, in HeLa cells ([figure 43](#)).

In my laboratory, the study of a direct interaction between EmN and the lamin A tail from amino acid 389 to 646 had been performed *in vitro* by the previous PhD student, Isaline Herrada. She did not observe a direct interaction between these two protein fragments by NMR and ITC.

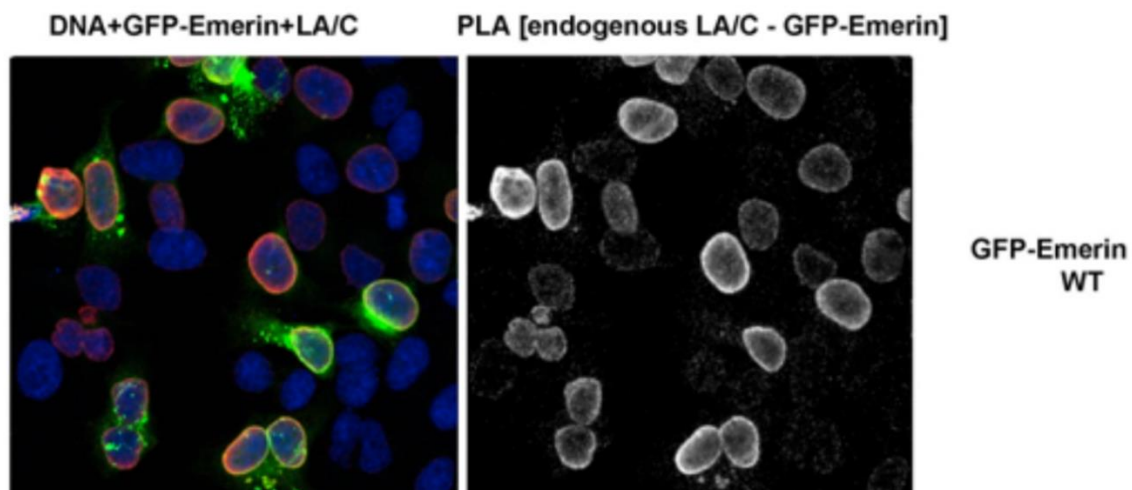


Figure 43 : Localization of emerin-lamin complexes in cells.

HeLa cells overexpressing WT GFP-emerin were fixed, labeled with anti-GFP and anti-LA/C antibodies and processed for PLA before analysis using a confocal microscope. The PLA signals are displayed either merged with DNA staining and GFP fluorescence (on the left) or alone (on the right).

At the beginning of my thesis, I tried again to observe a direct interaction between EmN and the tail of the lamin A/C. To test this interaction, different constructs of the lamin A/C tail were available in our laboratory. I worked with a construct obtained thanks to a collaboration with the laboratory of Prof. Howard J. Worman from Columbia University. It was a GST-tagged construct, with a thrombin cleavage site and coding only for the globular domain of the lamin A/C, from amino acid 411 to 566. We called this construct Igfold. Production and purification of this protein fragment were efficient (the final yield was typically 10 mg of purified protein per liter of bacterial culture) and it was important to add some reducing agents in the sample because of the presence of a solvent-exposed cysteine (Cys522).

First, I recorded the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of the Igfold alone, which was  $^{15}\text{N}$  labeled during bacterial expression, and then the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of the  $^{15}\text{N}$  labeled Igfold in presence of one equivalent of non-labeled EmN monomer (figure 44). After superimposition of both spectra, no difference was detected. We concluded that the Igfold does not directly interact with EmN, in our conditions.

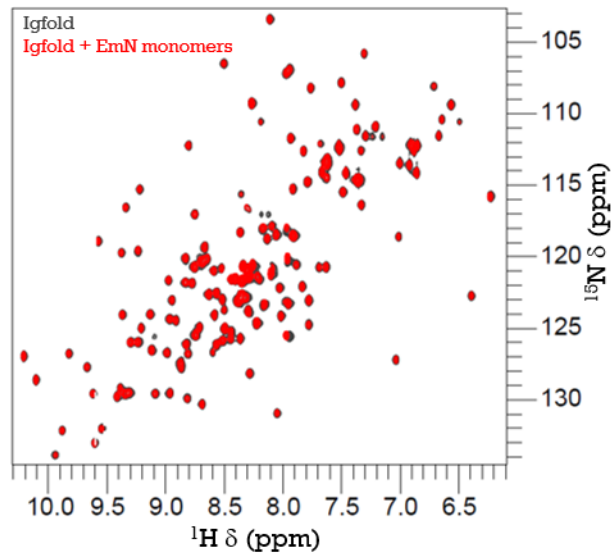


Figure 44 : Study of the EmN monomer/Igfold interaction, by NMR.

Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on an Igfold sample at  $100\mu\text{M}$  alone (in grey) or in presence of  $100\mu\text{M}$  of EmN monomer (in red), in  $20\text{mM}$  Tris-HCl pH8,  $30\text{mM}$  NaCl, at  $303\text{K}$  and  $700\text{MHz}$  (CEA Saclay).

## 1. A FIRST MODEL OF THE INTERACTION BETWEEN THE NUCLEOSKELETON AND THE CHROMATIN

Because we did not observe a direct interaction between emerin and the lamin A/C Igfold, we thought that this interaction could be mediated by a third partner. As both proteins were shown as interacting with the BAF protein independently, we decided to look at the formation of a ternary complex between emerin, BAF and the globular domain of the A-type lamin (Igfold). The formation of this complex was already suggested by several studies<sup>55,253,254</sup>.

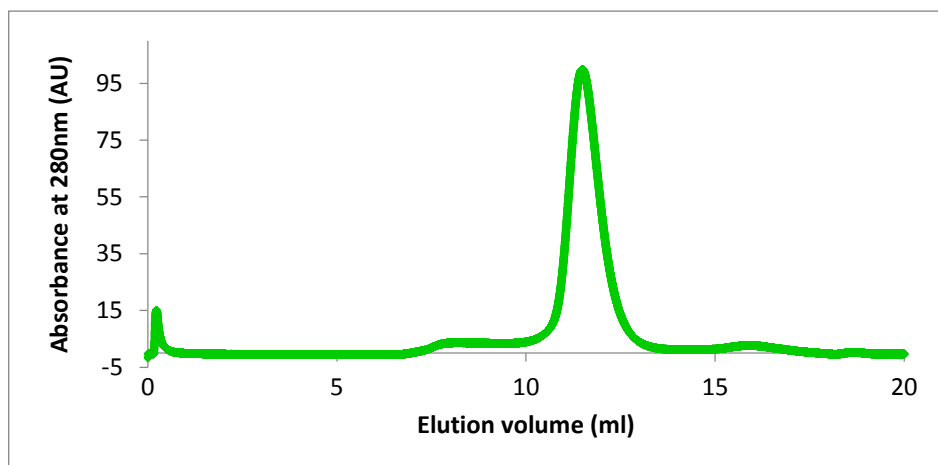
### a. Interaction between emerin and BAF

Interaction between emerin and BAF was already well characterized and the 3D structure between the LEM domain of emerin and BAF was solved (Cf. Introduction). In more details, it was shown that the LEM domain of emerin was able to bind a dimer of BAF.

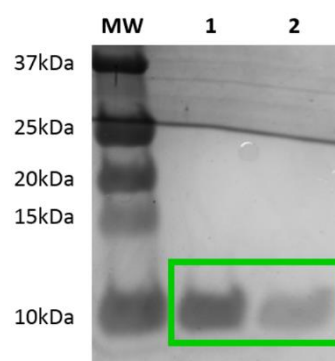
First, I tried to reproduce this interaction *in vitro*, by NMR, ITC and size exclusion chromatography. I worked with two emerin fragments: EmN and the LEM domain alone (fragment EmN49 comprising residues 1 to 49). Contrary to EmN, EmN49 was soluble in bacteria and during purification.

Concerning the BAF protein, because of a lot of oligomerization problems during purification and because BAF protein possesses 4 cysteines, which are not completely conserved between species, we chose to work at the end with a new BAF construct, with all cysteines being replaced by alanines. We called this construct BAF<sub>CtoA</sub>. As for EmN, BAF<sub>CtoA</sub> production was efficient but the protein was produced in inclusion bodies, requiring a purification in urea. After a first purification step, using a nickel column, the protein was renatured by dialysis. Then, BAF<sub>CtoA</sub> was injected onto a gel filtration column in order to observe its oligomerization states (figure 45, A). BAF is a protein of 10kDa and just after purification, BAF<sub>CtoA</sub> formed a dimer (one peak was observed at 11.8ml, which corresponds to a protein of 25kDa, on a Gel Filtration Superdex 75 10/300 GL), but we observed that the number of BAF<sub>CtoA</sub> oligomers increased with time. The dimeric protein was observed on a SDS-PAGE gel and corresponded to a protein of 10kDa (figure 45, B).

**A**



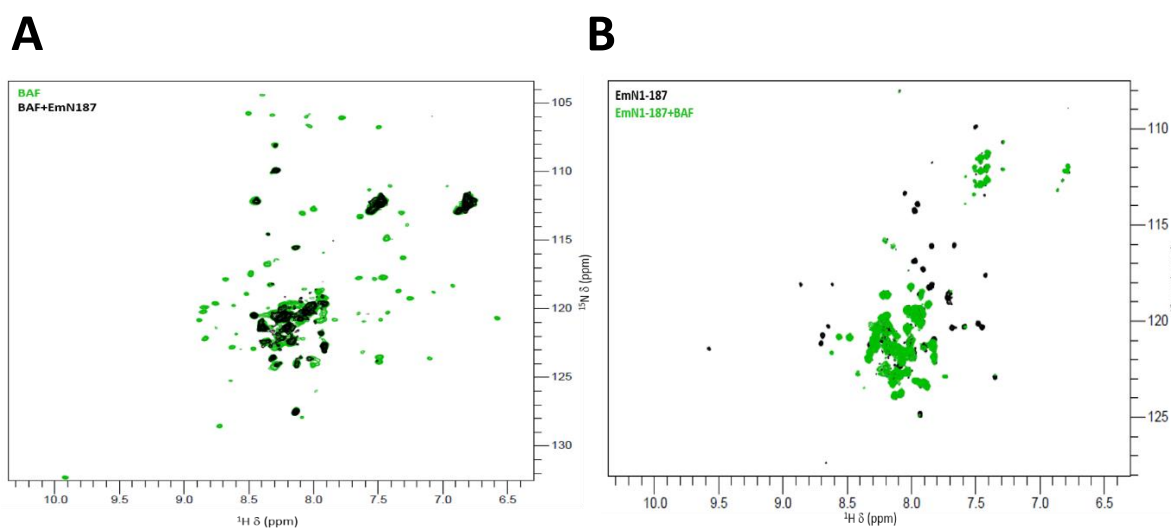
**B**



**Figure 45 : BAF<sub>CtoA</sub> purification.**

(A) Size exclusion chromatography using a GF Superdex 75 10/300 GL, equilibrated with 20mM Tris-HCl pH8 and 100mM NaCl. (B) SDS-PAGE gel obtained after size exclusion chromatography.

After protein production, I first tried to observe an interaction between the BAF<sub>CtoA</sub> dimer and EmN by NMR. To do so, I labeled each protein (BAF<sub>CtoA</sub> and EmN) with <sup>15</sup>N separately and recorded a <sup>1</sup>H-<sup>15</sup>N HSQC spectrum of each protein alone and with its non-labeled partner (figure 46). I observed a decrease of the signal intensity due to addition of the partner in both cases, which was characteristic of an interaction between both proteins. I thus confirmed that BAF<sub>CtoA</sub> and EmN interact *in vitro* and therefore, I obtained a first proof that mutation of BAF cysteines into alanines and refolding by dialysis for both proteins (BAF<sub>CtoA</sub> and EmN) did not impair the interaction. So, I estimated that using these purification protocols, I produced functional proteins.

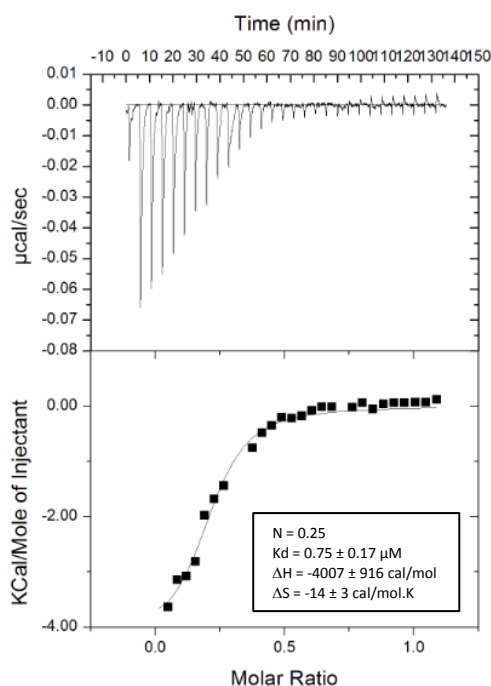


**Figure 46 : Study of BAF<sub>CtoA</sub> /EmN monomer interaction, by NMR.**

**(A) Superimposition of 2D NMR <sup>1</sup>H-<sup>15</sup>N spectra recorded on a BAF<sub>CtoA</sub> sample at 100μM alone (in green) or in presence of 100μM of EmN (in black), in 20mM Phosphate pH6.5, 150mM NaCl, at 293K and 700MHz (CEA Saclay). (B) Superimposition of 2D NMR <sup>1</sup>H-<sup>15</sup>N spectra recorded on a EmN sample at 100μM alone (in black) or in presence of 100μM of BAF<sub>CtoA</sub> (in green), in 20mM Tris-HCl pH7.5, 100mM NaCl, at 303K and 600MHz (CEA Saclay).**

To confirm the affinity and stoichiometry of the interaction reported by the group of Dr Marius Clore, I studied this interaction by ITC (figure 47). Like this other group, I found a dissociation constant of around 0.5μM, a favorable enthalpic contribution to the binding free energy of about 4 kcal/mol and a defavorable entropic energy of about 10 cal/mol.K<sup>52</sup>. This result confirmed that mutation of BAF cysteines into alanines did not change its affinity for emerlin.

However, surprisingly, my stoichiometry was different from 2 as found by Clore and co-workers. Indeed, I found that one emerin could interact with 3 to 4 BAF, depending on the experiment. But this can be explained by the fact that BAF forms oligomers, so part of my BAF protein was not able to interact, which distorted the stoichiometry.



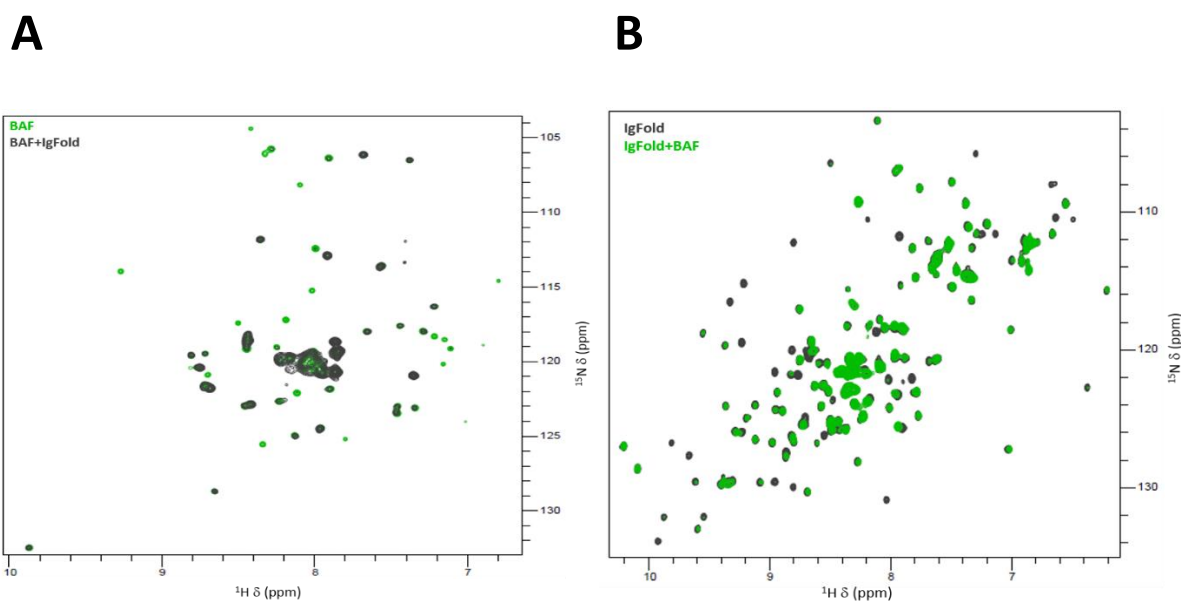
**Figure 47 : Characterization of the EmN/BAF interaction by ITC.**

Successive injections of 10µl of EmN concentrated at 200µM in a BAF sample concentrated at 40µM. Both proteins were dialyzed against the same buffer (20mM Tris-HCl pH8, 100mM NaCl, 10mM β-mercaptoethanol, protease inhibitors Roche). The experiment was done three times, at 288K, on a VP-ITC and we found in average a  $K_d$  of 0.75µM. All ITC values are listed in appendix 2 (Table 5).

### *b. Interaction between the globular domain of lamin A/C and BAF*

Interaction between BAF and the globular domain of lamin A/C (Igfold) was suggested by different studies but no direct interaction was previously described. I tried to observe this interaction by NMR, ITC and size exclusion chromatography. I worked with our lamin fragment called Igfold (from residues 411 to 566) and the same BAF<sub>CtoA</sub> already produced to analyze its interaction with EmN.

First, I studied this interaction by NMR. I produced the <sup>15</sup>N labeled Igfold and added non-labeled BAF<sub>CtoA</sub> and I produced the <sup>15</sup>N labeled BAF<sub>CtoA</sub> and added non-labeled Igfold domain (figure 48). I observed a decrease of intensity after addition of the partner in both cases.



**Figure 48 : Study of BAF<sub>CtoA</sub> /Igfold interaction by NMR.**

**(A)** Superimposition of 2D NMR <sup>1</sup>H-<sup>15</sup>N spectra recorded on a BAF<sub>CtoA</sub> sample at 115μM alone (in green) or in presence of 115μM of Igfold (in grey), in 20mM Phosphate pH6.5, 150mM NaCl, at 293K and 600MHz (CEA Saclay). **(B)** Superimposition of 2D NMR <sup>1</sup>H-<sup>15</sup>N spectra recorded on a Igfold sample at 150μM alone (in grey) or in presence of 150μM of BAF<sub>CtoA</sub> (in green), in 20mM Phosphate pH7, 100mM NaCl, at 303K and 600MHz (CEA Saclay).

To identify which part of the Igfold was implicated in this interaction, I produced a <sup>15</sup>N/<sup>13</sup>C labeled Igfold sample and assign its NMR signals (on the basis of the published Igfold assignment)<sup>42</sup>. Therefore, I recorded and analyzed 3D HNCO, HNCACO, CBCACONH and HNCACB spectra. Afterwards, I quantified intensity differences for each <sup>1</sup>H-<sup>15</sup>N HSQC peak corresponding to the Igfold 411-566 amino acids, before and after addition of one BAF equivalent. In more details, I did the following calculation for each peak:

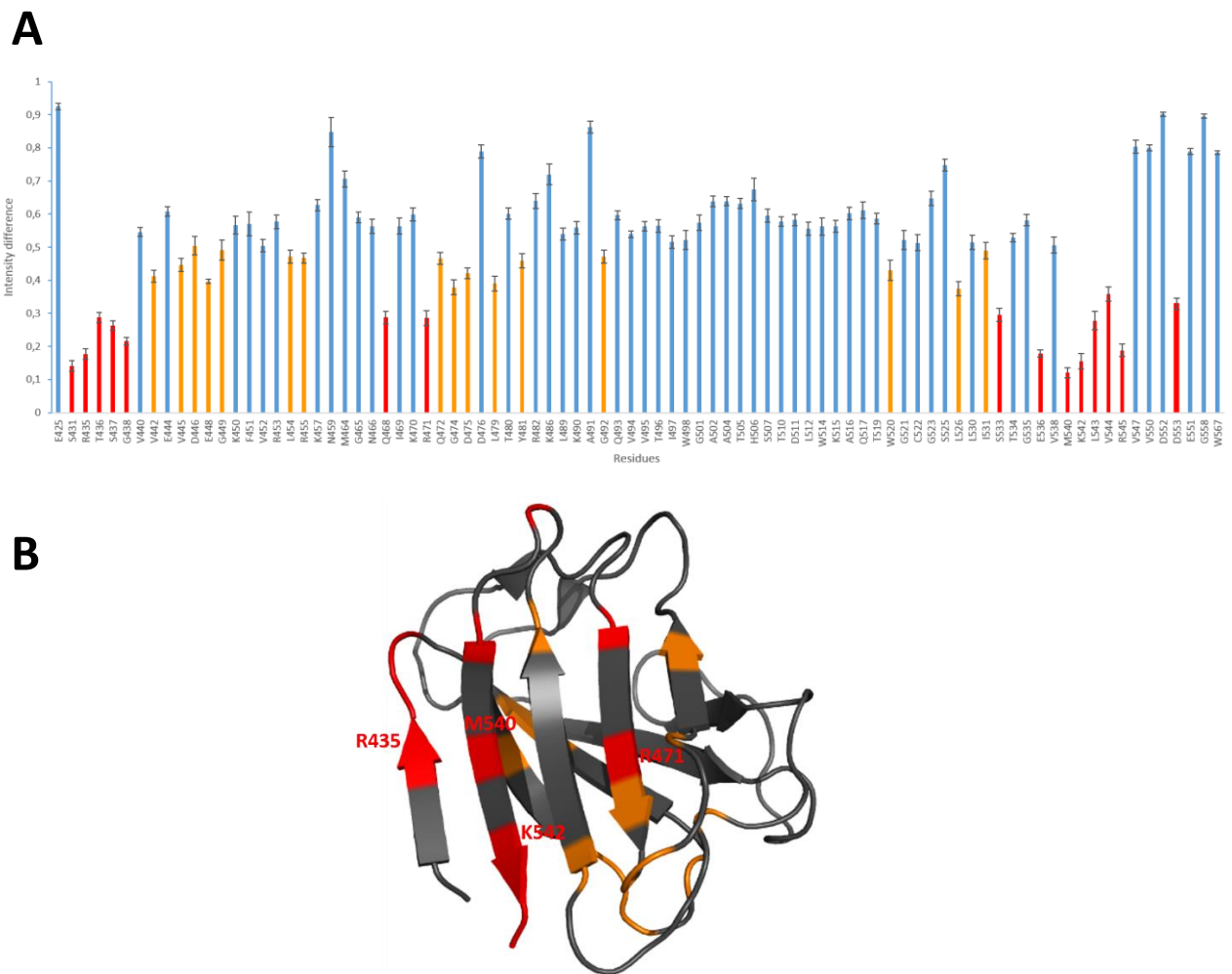
$$\text{Intensity Ratio} = \frac{\text{Peak Intensity on the Igfold spectrum after addition of one BAF equivalent}}{\text{Peak Intensity on the Igfold alone spectrum}}$$

In addition, I calculated an average of the error to substrate it for each peak. To do this, I measured the average intensity of 10 dots belonging to the background noise (BNA). I did the following calculations for each peak:

$$\text{Maximum ratio value} = \frac{\text{Peak intensity on the Igfold spectrum after addition of one BAF equivalent} - \text{BNA}}{\text{Peak intensity on the Igfold alone spectrum} + \text{BNA}}$$

$$\text{Minimum ratio value} = \frac{\text{Peak intensity on the Igfold spectrum after addition of one BAF equivalent} + \text{BNA}}{\text{Peak intensity on the Igfold alone spectrum} - \text{BNA}}$$

With these calculations, I obtained the ratio of intensity between peaks corresponding to the same amino acid in the free and bound protein states and I identified peaks showing more than 50% and 70% of intensity decrease (in orange and red, respectively). Finally, I localized the residues corresponding to these peaks on the known Igfold 3D structure obtained by X-ray crystallography (PDB: 1IFR) using the same color labelling (figure 49, B).



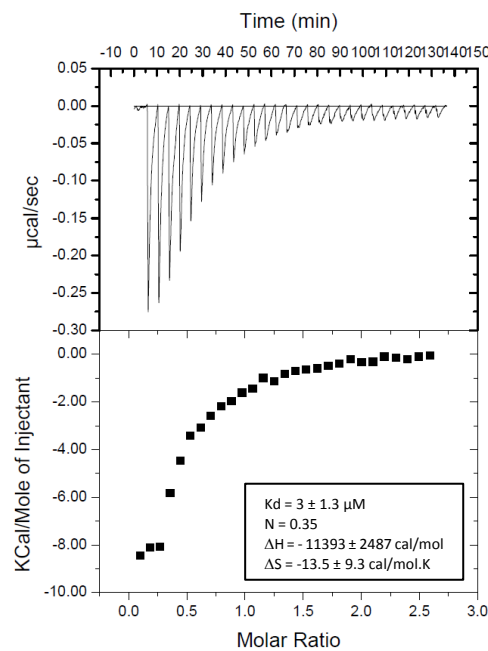
**Figure 49 : Intensity ratio measurement after addition of one BAF<sub>CtoA</sub> equivalent onto the <sup>15</sup>N labeled Igfold.**

**(A)** Ratio as a function of the sequence. Bars corresponding to peaks losing more than 50% and 70% of intensity after BAF<sub>CtoA</sub> addition are labeled in orange and red, respectively. **(B)** Three-dimensional-structure of the Igfold domain (PDB: 1IFR) with residues colored as a function of (A). Residue labelled correspond to residues that are mutated in progeroid syndromes.



Interestingly,  $^1\text{H}$ - $^{15}\text{N}$  HSQC peaks which were the most impacted by  $\text{BAF}_{\text{CtoA}}$  addition (in red) corresponded to several residues that are mutated in progeroid syndromes: R435, M540, K542 and R471. Thus, our NMR data demonstrate that BAF can directly interact with the globular domain of lamin A/C *in vitro* and suggest that mutations of the Igfold that cause progeroid syndrome impair this interaction.

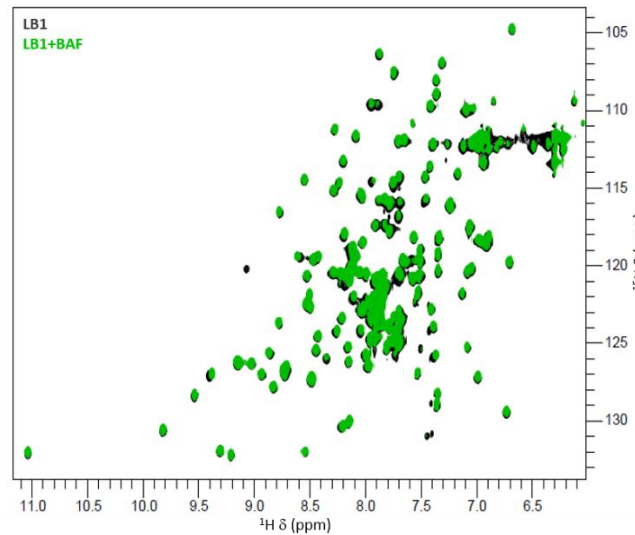
I used ITC to measure the affinity and stoichiometry of the Igfold /  $\text{BAF}_{\text{CtoA}}$  interaction (figure 50). I found that the Igfold of lamin A/C can interact with  $\text{BAF}_{\text{CtoA}}$  with a micro molar affinity, ( $K_d = 3.2\mu\text{M} \pm 1.2\mu\text{M}$  at 288K, as calculated from 5 independent experiments). Concerning the stoichiometry, I found a number of sites between 0.21 and 0.43 (0.32 in average) as a function of the experiment. This again suggests that the Igfold binds to a  $\text{BAF}_{\text{CtoA}}$  dimer and that all the purified  $\text{BAF}_{\text{CtoA}}$  protein is not active.



**Figure 50 : Characterization of the Igfold/  $\text{BAF}_{\text{CtoA}}$  interaction by ITC.**

Successive injections of  $10\mu\text{l}$  of Igfold concentrated at  $270\mu\text{M}$  in a  $\text{BAF}_{\text{CtoA}}$  sample concentrated at  $22.5\mu\text{M}$ . Both proteins were dialyzed against the same buffer ( $20\text{mM}$  Tris-HCl pH8,  $150\text{mM}$  NaCl,  $10\text{mM}$   $\beta$ -mercaptoethanol, protease inhibitors Roche). The experiment was done five times, at 288K on a VP-ITC and we found in average a  $K_d$  of  $3\mu\text{M}$ . All ITC values are listed in appendix 2 (Table 5).

Because B-type lamins also possess an Igfold domain, I asked if the C-terminal domain of lamin B1 could also interact with BAF. Thus, I produced the lamin B1 fragment from amino acid 409 to 586 of lamin B1 (LB1) and I tested its interaction with BAF<sub>CtoA</sub> by NMR. LB1 was <sup>15</sup>N labeled (figure 51, black) and I added non-labeled BAF<sub>CtoA</sub> (figure 51, green).



**Figure 51 : Study of BAF<sub>CtoA</sub> /LB1 interaction by NMR.**

**Superimposition of 2D NMR <sup>1</sup>H-<sup>15</sup>N spectra recorded on a LB1 sample at 200μM alone (in grey) or in presence of 200μM of BAF<sub>CtoA</sub> (in green), in 20mM Phosphate pH7, 150mM NaCl, at 293K and 600MHz (CEA Saclay).**

No intensity change or no chemical shift difference was observed after BAF<sub>CtoA</sub> addition, so we concluded that LB1 cannot interact with BAF<sub>CtoA</sub>, *in vitro*, in our conditions (which are the same as the conditions in which BAF<sub>CtoA</sub> can interact with lamin A/C Igfold). This result is consistent with a study that showed that in cells, in presence of BAF RNAi, A-type lamins and emerin signals at the nuclear envelope were significantly weaker whereas the nuclear envelope localization of lamin B1 was unchanged<sup>253</sup>.

c. Existence of a ternary complex bridging nuclear membrane, nucleoskeleton and chromatin

The existence of a ternary complex involving lamin A/C, BAF and emerin was postulated in cells<sup>55</sup>. I tried to observe the existence of this complex *in vitro*. To do this, I used size exclusion chromatography (figure 52) and I tested if the three protein fragments that I produced, Igfold, EmN and BAF<sub>CtoA<sub>7</sub></sub>, could co-elute. In parallel, I performed this experiment with LB1.

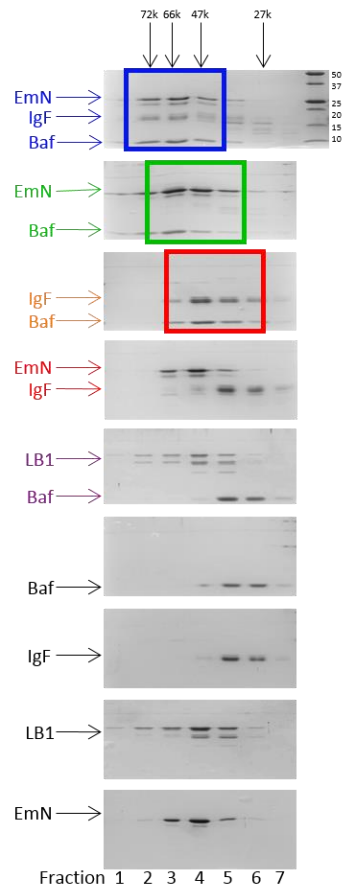


Figure 52 : Identification of a ternary complex between emerin, BAF and lamin A/C by size exclusion chromatography.

Each gel corresponds to the result of one size exclusion chromatography. Proteins were injected in a volume of 500µl and the GF column Superdex 75 10/300 GL was equilibrated in a buffer containing 20mM Tris-HCl pH8 and 30mM NaCl. Bands revealing an interaction are boxed with different colors.

Different conclusions were drawn from this study. First, we confirmed that interaction between lamin Igfold and BAF<sub>CtoA</sub> is specific to lamin A/C, because an interaction was observed between lamin A/C Igfold and BAF<sub>CtoA</sub> (boxed in red), whereas no interaction was observed between LB1 and BAF<sub>CtoA</sub>. Furthermore, no direct interaction was observed between the Igfold of lamin A/C and emerin monomers. This result confirmed what we had already observed by NMR. Then, interaction between BAF<sub>CtoA</sub> and EmN was confirmed once again (boxed in green) and the most important was the observation of a ternary complex between EmN/BAF<sub>CtoA</sub>/Igfold (boxed in blue). Indeed, after injection of the three proteins pooled together at a concentration of 150µM, a predominant pic was observed that corresponded to a complex of approximately 60kDa, which is in accordance with the weight of a complex between a dimer of BAF<sub>CtoA</sub> (20kDa) in interaction with one EmN (25kDa) and one Igfold (15kDa).

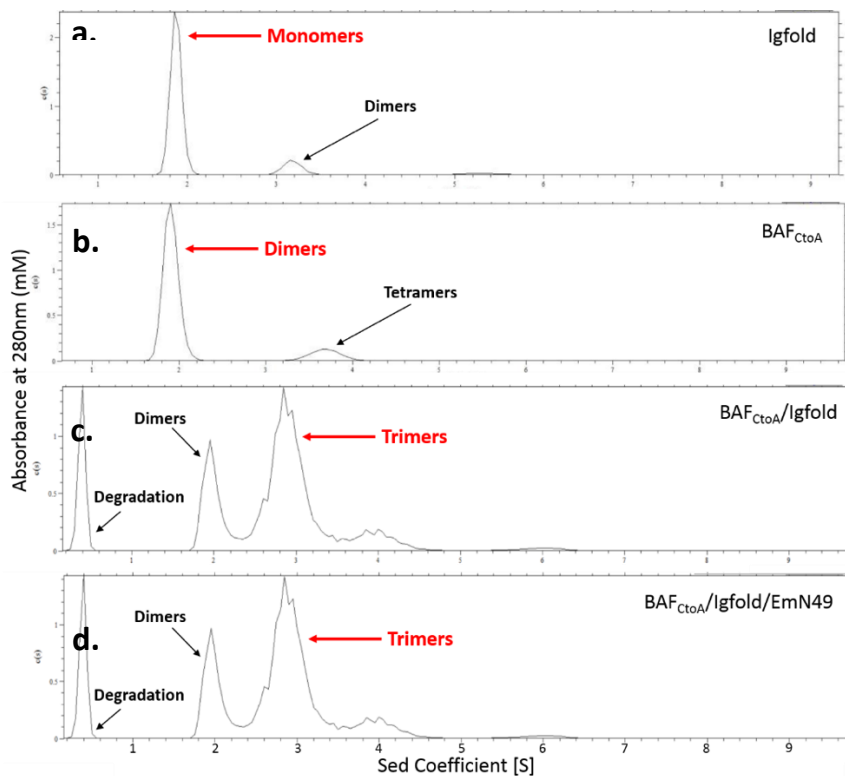
After observation of a ternary complex formation between BAF<sub>CtoA</sub>, EmN and Igfold by gel filtration, we wanted to confirm that this ternary complex exists in solution and that this complex was composed of two BAF, one Igfold and one EmN, as it was supposed by ITC results. For this reason, we analyzed our complexes by analytical ultracentrifugation. In addition, because BAF was shown to interact with EmN49 and not with another part of emerin, we decided to look at the presence of EmN49 only and not EmN, in the ternary complex. Analysis was done by Dr Christophe Velours (CNRS, LEBS, Gif-sur-Yvette), using an analytical ultracentrifuge XL470 (Beckman Coulter, Palo Alto, USA) and an An-50Ti rotor. I produced several samples: BAF<sub>CtoA</sub> samples at 32, 57 and 86µM, Igfold samples at 17, 26 and 40µM, a BAF<sub>CtoA</sub>/Igfold sample, with a 2/1 ratio and an EmN49/BAF<sub>CtoA</sub>/Igfold sample, with a 1/2/1 ratio. All proteins were first dialyzed in the same buffer: 50mM Tris-HCl pH8, 100mM NaCl. Then, sedimentation velocity experiments were performed on these different samples, with an optical path of 1.2cm, at 42000 rpm (128 297g), at 293K. Both complex samples were observed at an OD of 0.5, 0.8 and 1.2. Absorbance and interference were measured at 280nm, every 8 minutes. 400µl of sample were used for each experiment and 410µl of our dialysis buffer was used as a reference. At the end, results were analyzed using the Sedfit software<sup>255</sup>.

Because the same results were obtained for the different protein concentrations, I will only present results obtained with 32µM of BAF<sub>CtoA</sub>, 17µM of Igfold and both complexes (Igfold/BAF<sub>CtoA</sub> and LEM/BAF<sub>CtoA</sub>/Igfold) at an OD of 1.2 (figure 53, Table A).

After analyzing the [figures 53](#) and [table A](#), we observed that the BAF<sub>CtoA</sub>/Igfold sample contains a majority of trimers (2.8S) in solution, which could correspond to 2 BAF<sub>CtoA</sub> and one Igfold. In more details, resulting trimers possessed an elongated shape with an  $f/f_0$  value of 1.4.

Unfortunately, analysis of the EmN49/BAF<sub>CtoA</sub>/Igfold complex did not give more information than analysis of the BAF<sub>CtoA</sub>/Igfold complex, because no real modifications were observed after addition of EmN49. Indeed, same species that the one observed for the BAF<sub>CtoA</sub>/Igfold sample were observed, with majority of trimers.

Regarding the results obtained for the BAF<sub>CtoA</sub>/Igfold sample by analytical ultracentrifugation, we concluded that only one ternary complex seems to exist in solution and is composed, at least, of two BAF<sub>CtoA</sub> and one Igfold.



**Figure 53 : Analytical ultracentrifugation results obtained using absorbance at 280nm, with a XL470 ultracentrifuge (Beckman Coulter, Palo Alto, USA) and an An-50Ti rotor.**

(a) Sedimentation velocity profile of a BAF<sub>CtoA</sub> sample, in 50mM Tris-HCl pH8, 100mM NaCl, injected at 32 $\mu$ M, at 293K. (b) Sedimentation velocity profile of an Igfold sample, in 50mM Tris-HCl pH8, 100mM NaCl, injected at 17 $\mu$ M, at 293K. (c) Sedimentation velocity profile of a BAF<sub>CtoA</sub>/Igfold sample, in 50mM Tris-HCl pH8, 100mM NaCl, injected at an OD of 1.2, at 293K. (d) Sedimentation velocity profile of an EmN49/BAF<sub>CtoA</sub>/Igfold sample, in 50mM Tris-HCl pH8, 100mM NaCl, injected at an OD of 1.2, at 293K. Each corresponding species are indicated by arrows; in red are represented the majority species.

	Degradation	Monomers	Dimers	Trimers	Tetramers	Higher MW oligomers/Aggregates
Igfold alone	-	85%	12%	-	3%	2%
BAF <sub>CtoA</sub> alone	-	-	87%	-	11%	2%
BAF <sub>CtoA</sub> /Igfold	8%	-	25%	54%	11%	2%
EmN49/BAF <sub>CtoA</sub> /Igfold	14%	-	20%	55%	10%	1%

**Table 1 : Analytical ultracentrifugation results obtained using absorbance at 280nm, for four different protein samples (BAF<sub>CtoA</sub>, Igfold, BAF<sub>CtoA</sub>/Igfold and EmN49/BAF<sub>CtoA</sub>/Igfold).**

Percentage values correspond to the total of absorbance. In red is represented the percentage of the majority species for each sample; (-) no species detected.

In parallel, we tried to obtain the structure of the ternary complex, using crystallography. To achieve this goal, after size exclusion chromatography, I pooled fractions corresponding to the complex peak (fractions 2 to 4 on the [figure 52](#)) and I concentrated this sample until 3mg/ml. Then, I conserved the concentrated sample during one week at 277K. I sent one part of my complex sample to the European crystallography facility in Grenoble (HTX laboratory), in order to screen a lot of conditions to get first crystals. Six different primary crystallography sets, used for initial screening, were tested on my complex sample, at 277K (Classics Suite and PEGs-I from Qiagen/Nextal, JCSG+ and PACT *premier* from Molecular Dimensions, Salt Grid from Hampton Research and Wizard I&II from Rigaku Reagents). After one week, I obtained microcrystals in two conditions (0.2M NH<sub>4</sub>Cl and 16% of PEG3.350 pH8; 0.1M MgCl<sub>2</sub> and 16% of PEG3.350 pH8; 0.2M of NH<sub>4</sub>SO<sub>4</sub>, 20% of PEG3.350) and crystals in one condition (100mM of Bis-Tris phosphate pH5.5).

I tried to obtain similar crystals of this sample, in our laboratory, using the hanging-drop during vapor diffusion step. I prepared one crystallization screen with buffers related to conditions which gave microcrystals (listed on the [table B](#)) and one related to condition in which I got crystals (listed on the [table C](#)). After doing a crystallization screen at 277K, I got crystals in three different conditions.

The first condition contained 0.2M NH<sub>4</sub>Cl and 14% of PEG3.350 (condition 1), the second condition contained 0.1M MgCl<sub>2</sub> and 20% of PEG3.350 (condition 2) and the last condition, which was relatively different, contained 0.2M of NH<sub>4</sub>SO<sub>4</sub>, 23% of PEG3.350 and 100mM of Bis-Tris phosphate pH5,5 (condition 3). A picture of crystals obtained with the last condition is inserted below (figure 54).

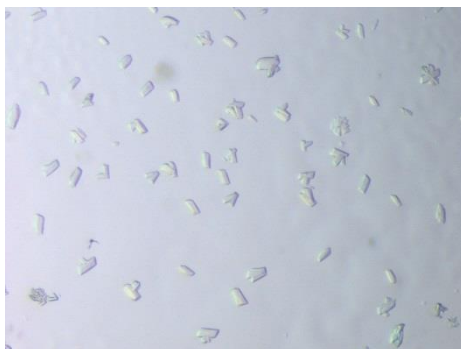


Figure 54 : Picture of crystals obtained from the purified EmN/BAF/Igfold sample, at 277K, using condition 3.

	1	2	3	4	5	6
A	PEG 3.350 12%	PEG 3.350 14%	PEG 3.350 16%	PEG 3.350 18%	PEG 3.350 20%	PEG 3.350 22%
	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM
	NH <sub>4</sub> CL 0.2M	NH <sub>4</sub> CL 0.2M	NH <sub>4</sub> CL 0.2M	NH <sub>4</sub> CL 0.2M	NH <sub>4</sub> CL 0.2M	NH <sub>4</sub> CL 0.2M
B	PEG 3.350 18%	PEG 3.350 20%	PEG 3.350 22%	PEG 3.350 18%	PEG 3.350 20%	PEG 3.350 22%
	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM
	NH <sub>4</sub> CL 0.1M	NH <sub>4</sub> CL 0.1M	NH <sub>4</sub> CL 0.1M	NH <sub>4</sub> CL 0.3M	NH <sub>4</sub> CL 0.3M	NH <sub>4</sub> CL 0.3M
C	PEG 3.350 12%	PEG 3.350 14%	PEG 3.350 16%	PEG 3.350 18%	PEG 3.350 20%	PEG 3.350 22%
	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM
	MgCl <sub>2</sub> 0.2M	MgCl <sub>2</sub> 0.2M	MgCl <sub>2</sub> 0.2M	MgCl <sub>2</sub> 0.2M	MgCl <sub>2</sub> 0.2M	MgCl <sub>2</sub> 0.2M
D	PEG 3.350 18%	PEG 3.350 20%	PEG 3.350 22%	PEG 3.350 18%	PEG 3.350 20%	PEG 3.350 22%
	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM	TrispH8 100mM
	MgCl <sub>2</sub> 0.1M	MgCl <sub>2</sub> 0.1M	MgCl <sub>2</sub> 0.1M	MgCl <sub>2</sub> 0.3M	MgCl <sub>2</sub> 0.3M	MgCl <sub>2</sub> 0.3M

Table 2 : Crystallization screen designed from conditions which led to microcrystals formation in HTX laboratory.

Conditions for which we got microcrystals are surrounded by a red rectangle.

	1	2	3	4	5	6
A	PEG 3.350 23%	PEG 3.350 25%	PEG 3.350 27%	PEG 3.350 23%	PEG 3.350 25%	PEG 3.350 27%
	Bis-Tris 5.5 100mM	Bis-Tris 5.5 100mM	Bis-Tris 5.5 100mM	Bis-Tris 5.5 100mM	Bis-Tris 5.5 100mM	Bis-Tris 5.5 100mM
	NH4SO4 0.2M	NH4SO4 0.2M	NH4SO4 0.2M	NH4SO4 0.1M	NH4SO4 0.1M	NH4SO4 0.1M
B	PEG 3.350 23%	PEG 3.350 25%	PEG 3.350 27%	PEG 3.350 23%	PEG 3.350 25%	PEG 3.350 27%
	Bis-Tris 5.5 100mM	Bis-Tris 5.5 100mM	Bis-Tris 5.5 100mM	Bis-Tris 5.5 100mM	Bis-Tris 5.5 100mM	Bis-Tris 5.5 100mM
	Li2SO4 0.2M	Li2SO4 0.2M	Li2SO4 0.2M	Li2SO4 0.1M	Li2SO4 0.1M	Li2SO4 0.1M

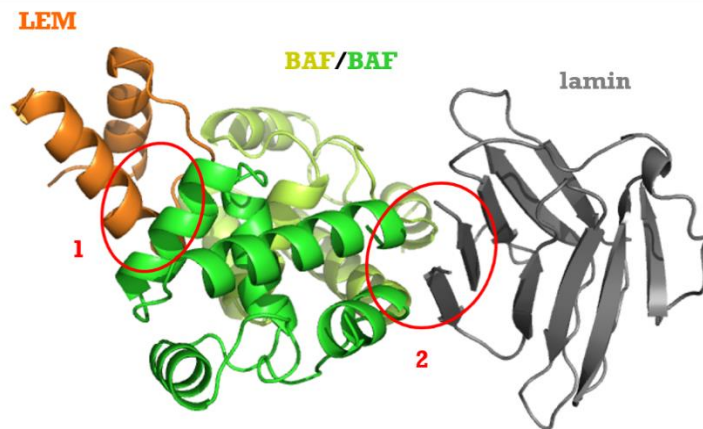
**Table 3 : Crystallization screen designed from conditions which led to crystals formation in HTX laboratory. The condition for which we got crystals is surrounded by a red rectangle.**

After shooting with X-ray on these crystals, on the PROXIMA1 beamline of synchrotron Soleil (with the help of Dr Virginie Ropars), we obtained a good diffraction pattern for crystals obtained from condition 3, with a resolution of 2.1 Angstrom (Å). By using two servers, Molrep and Phenix, and starting from structures already available for each component of the complex, I solved the 3D structure of the ternary complex. As starting structures, I used the X-ray structure of the Igfold of the lamin A/C (PDB: 1IFR), the NMR structure of the LEM domain (PDB: 2ODC) and the NMR structure of the BAF dimer in interaction with the LEM of emerin (PDB: 2ODG). Interestingly, in our crystals, only the LEM domain of emerin was present, which means that after one week at 277K, all the unfolded nucleoplasmic part of emerin was degraded in my size exclusion chromatography sample and only the LEM domain, which is the important region for binding to BAF, was conserved.

One ternary complex was present per asymmetric unit of the crystal (figure 55). I analyzed interfaces in my model, using the software PISA<sup>256</sup>, and found an interface of 594 Å<sup>2</sup> between the LEM domain and BAF and an interface of 465 Å<sup>2</sup> between BAF and the Igfold domain.



**A**



**B**

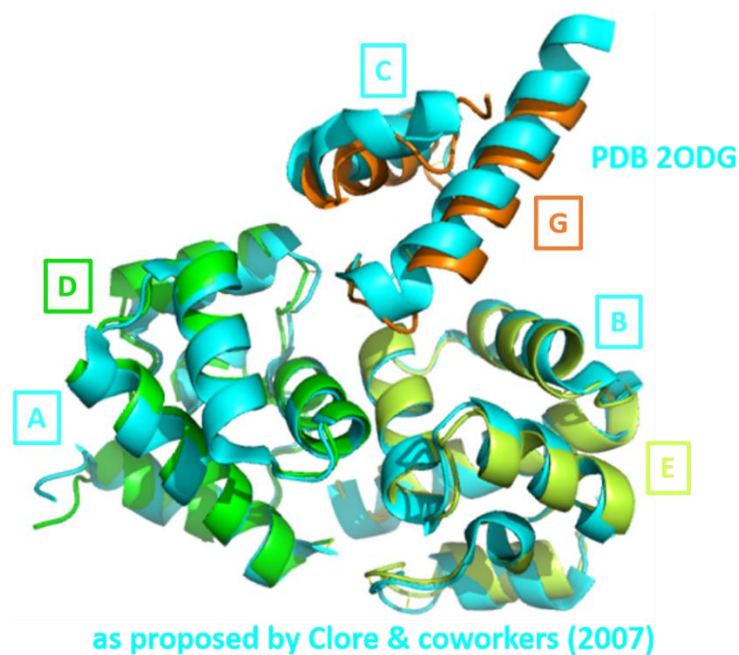
<b>Data collection</b>	
Space group	P 1
Cell dimensions	
a, b, c (Å)	57.77 62.75 64.34
a, b, $\gamma$ (°)	66.44 66.55 88.05
Resolution (Å)	49.152 - 2.1
Anisotropy direction	
Resolution where $CC_{1/2} > 0.3$	2.1
$R_{\text{meas}}$ (%)	15.9
$I/s(I)$	5.9
$CC_{1/2}$	99.1
Completeness (%)	95.67
Redundancy	3.52
B Wilson (Å <sup>2</sup> )	34.74
<b>Refinement</b>	
No. reflections	83937
$R_{\text{work}} / R_{\text{free}}$	0.1879/0.2444
No. atoms	5666
Protein	675
DNA	0
SO <sub>4</sub> <sup>2-</sup>	8
Water	279
B factors	43.38
Protein	42.97
Ligand/ion	80.03
Water	45.37
R.m.s. deviations	1.953
Bond lengths (Å)	0.008
Bond angles (°)	1.16
One crystal per structure.	

**Figure 55 : X-ray crystallography structure of the ternary complex formed between the LEM domain of the emerin (EmN49), the BAF<sub>CtoA</sub> dimer and the Igfold domain.**

**(A) 3D structure of the ternary complex with the LEM (EmN49) in orange, BAF<sub>CtoA</sub> dimer in green and the Igfold in grey. (B) Data collection and refinement statistics.**

Concerning the interface between BAF<sub>CtoA</sub> and the Igfold, my X-ray structure shows, consistently with my NMR results, that the  $\beta$ -sheet of the Igfold implicated in the interaction is the same as the one exhibiting the majority of mutations leading to progeroid syndromes.

Concerning the interface between BAF<sub>CtoA</sub> and EmN49, we superimposed our X-ray structure with the structure obtained by NMR by the group of Dr Marius Clore, in Bethesda (figure 56) and first, we measured the rmsd values between each corresponding chain (named on the figure 56) of the complex, taken alone. We found a rmsd value of 1.3 Å between chain C and G (which correspond to the LEM domain), a rmsd value of 1.42 Å between chain A and D (which correspond to the first monomer of BAF) and a rmsd value of 0.9 Å between chain B and E (which correspond to the second monomer of BAF). Then, the rmsd value was measured between the two BAF chains of the complex with the LEM domain fixed and we found a value of 1.3 Å. On the contrary, the rmsd value was measured between the two LEM domains, with the two BAF chains fixed and we found a value of 2.75 Å. This result showed that the interaction is significantly tighter in the crystal structure. We concluded that the presence of the Igfold in the complex did not change the structure of the LEM domain in interaction with BAF (and that the 3D structure of BAF<sub>CtoA</sub> was highly similar to that of wild-type BAF).



**Figure 56 : Superimposition of our 3D structure of EmN49 in interaction with the dimer of BAF<sub>CtoA</sub>, obtained by X-ray crystallography, with the 3D structure of the same complex obtained by NMR, by the group of Dr Marius Clore in 2007 (PDB: 20DG)<sup>52</sup>.**

Finally, we superimposed the 3D structure of our ternary complex with the 3D structure that was already solved by X-ray crystallography between BAF and DNA (Cf. Introduction) (figure 57). Our model suggests that BAF is able to simultaneously bind to lamin A/C, emerin and DNA and provides a first model of the interaction between the nucleoskeleton and DNA.

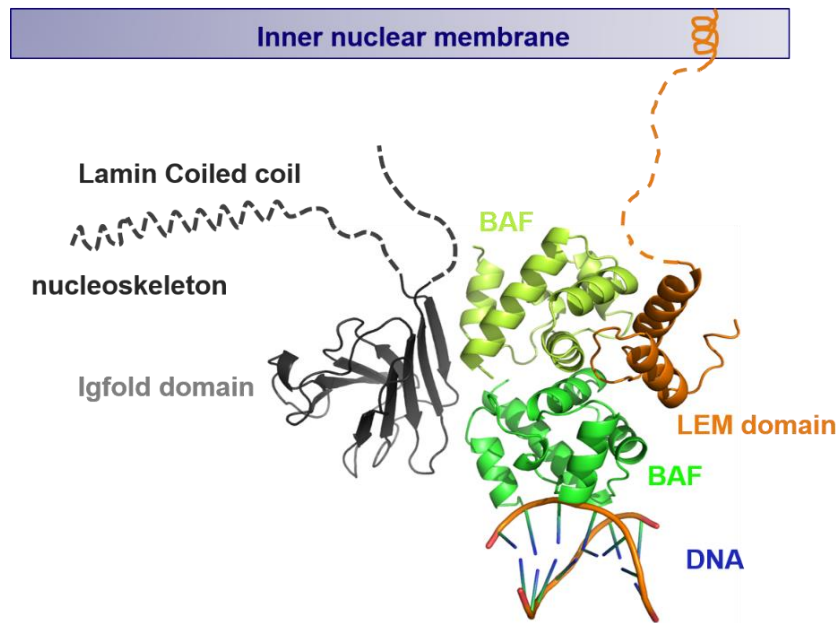


Figure 57 : A first model of the interaction between the nucleoskeleton and the chromatin.

This model results from the superimposition of our X-ray structure with that of BAF in interaction with DNA (PDB: 2BZF). The BAF dimer binds to two DNA molecules but I represented here only one DNA for simplicity.

To conclude this part, as it was shown in the literature, I confirmed the presence of an interaction between BAF and the LEM domain of emerin and between BAF and the Igfold domain of lamin A/C. Moreover, I demonstrated that this second interaction is specific to lamin A/C, because no interaction was observed, through two different techniques, between BAF<sub>CtoA</sub> and LB1. Finally, I revealed the existence of a ternary complex between EmN49, Igfold and BAF<sub>CtoA</sub> dimer and I solved the 3D structure of this complex.

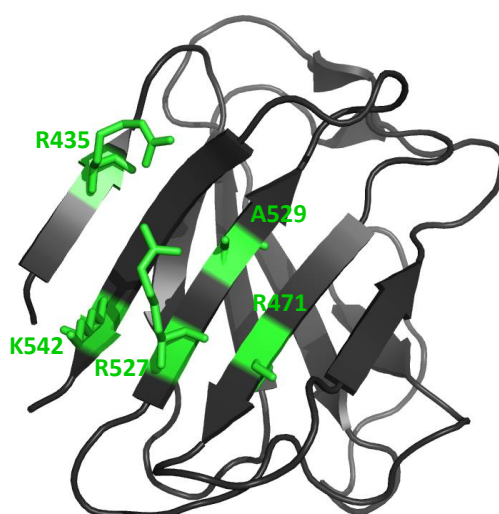
*d. Several mutants associated with progeroid syndromes disrupt the interaction between lamin A/C and BAF*

We produced a set of Igfold mutants associated with progeroid syndromes. These mutants are listed in table D: homozygous mutant R435C causes a restrictive dermopathy<sup>230</sup>, homozygous mutant K542N<sup>238</sup> causes HGPS, homozygous mutants R471C<sup>203</sup> and R527H<sup>233</sup> are associated with HGPS and/or a severe form of mandibuloacral dysplasia and homozygous mutants A529T<sup>236</sup> and A529V<sup>257</sup> cause mandibuloacral dysplasia. Localization of the mutated residues is represented on figure 58. Mutants were cloned with a mutagenesis kit (Stratagene QuikChange) based on our WT Igfold 411-566 expression vector by Ambre Petitalot in the lab.

Production and purification of each mutant was performed using the same protocol as for the WT Igfold 411-566.

Igfold Mutants	Diseases	Publications
R435C	Restrictive Dermopathy	Madej-Pilarczyk A et al., 2009
K542N	HGPS	M Plasilova et al., 2004
R471C	HGPS and/or a severe form of Mandibuloacral Dysplasia	Birgit Zirn et al., 2008
R527H	HGPS and/or a severe form of Mandibuloacral Dysplasia	Novelli G et al., 2002
A529T	Mandibuloacral Dysplasia	Kosho T et al., 2007
A529V	Mandibuloacral Dysplasia	Garg A et al., 2005

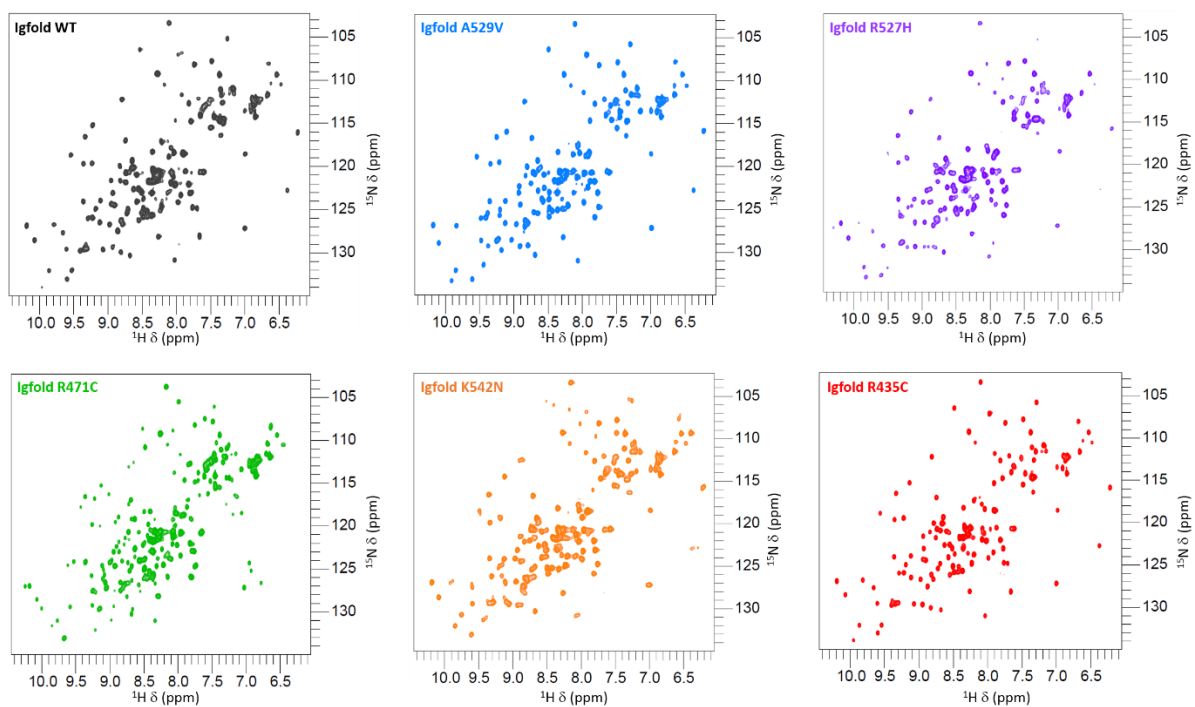
**Table 4 : Igfold mutants causing different, more or less severe, progeroid syndromes and their associated disease and publication.**



**Figure 58 : Localization of the different Igfold residues mutated in progeroid syndromes (in green) (PDB: 1IFR)<sup>43</sup>.**

First, we checked by NMR if the five mutants R435C, K542N, R471C, R527H and A529V are well-folded. After recording  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of these mutants, we confirmed that they are well-folded (figure 59), because we obtained a well-dispersed spectrum for each of them as for the Igfold WT.

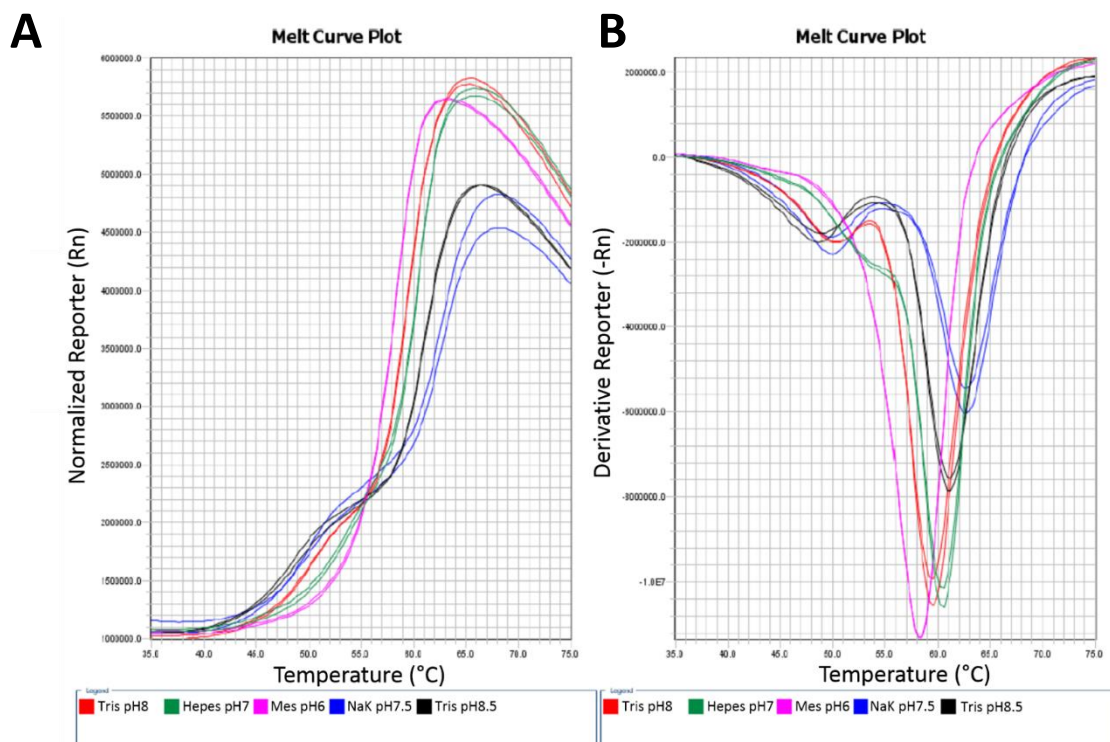
Then, we compared the stability of the Igfold WT with that of the five mutated Igfold (R435C, K542N, R471C, A529V and A529T) based on a Fluorescence-based Thermal Shift Assay performed by the group of Dr Eric Jacquet (ICSN, Gif-sur-Yvette).



**Figure 59 : Folding study of different Igfold mutant causing progeroid syndrome, by NMR.**

$^1\text{H}$ - $^{15}\text{N}$  HSQC spectra recorded on Igfold WT sample at 200 $\mu\text{M}$  (in dark grey), on Igfold A529V sample at 200 $\mu\text{M}$  (in blue), on Igfold R527H sample at 200 $\mu\text{M}$  (in purple), on Igfold R471C sample at 200 $\mu\text{M}$  (in green), on Igfold K542N sample at 200 $\mu\text{M}$  (in orange) and on Igfold R435C sample at 200 $\mu\text{M}$  (in red). All proteins were dialyzed in 20mM Tris-HCl pH8, 30mM NaCl, 2mM DTT and spectra were recorded at 303K and 700MHz spectrometer (CEA Saclay).

First, the group of Dr Eric Jacquet compared the stability of the Igfold WT in several buffers, in order to observe the stability difference between pH6 and 8.5 (figure 60). Interestingly, they found that Igfold WT shows a first unfolding event around 323K at a pH between 7 and 8.5.

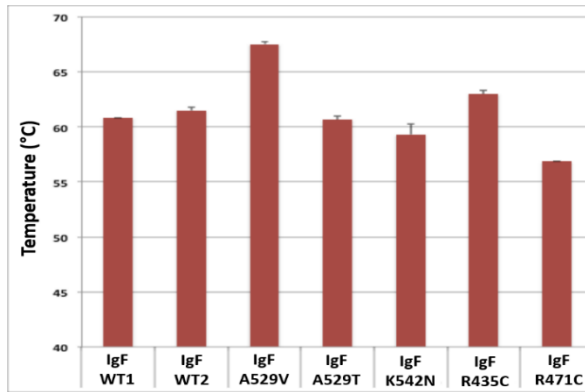


**Figure 60 : Stability test of Igfold WT in different buffers.**

**(A) Profiles of denaturation curves. (B) Derivatives of denaturation curves.** Denaturation tests were done, according to the temperature, in different buffers: Tris-HCl pH8 and NaCl 100mM (in red), Hepes 50mM pH7 and NaCl 100mM (in green), MES 50mM pH6 and NaCl 100mM (in pink), NaKP 50mM pH7.5 and NaCl 100mM (in blue) and Tris-HCl pH8.5 and NaCl 100mM (in black).

Then, the stability of the Igfold WT was compared to the stability of the 5 mutated Igfold causing progeroid syndromes (R435C, K542N, R471C, A529T and A529V) (figure 61). Stability was studied in buffer A, composed of 50mM Mes pH6 and 100mM NaCl and 10µl of each protein were used for the different measurements and diluted two times with buffer A, according to the experiment (figure 61). The tested mutants related to progeroid diseases do not show a large modification of their thermal stability. Indeed, only R471C shows a 4 degree decrease of its thermal stability relatively to WT Igfold.

Knowing that my mutants are well folded and stable, I could go further and observe the impact of the different mutations on the formation of a complex between the dimer of BAF<sub>CtoA</sub> and Igfold. First, I used the technique of ITC and worked with the same 5 mutants (R435C, K542N, R471C, R527H and A529V) (figure 62). I measured the affinity and stoichiometry of the Igfold mutants / BAF<sub>CtoA</sub> interactions.

**A**

**Figure 61 : Stability study of Igfold WT and mutants, using a fluorescence-based thermal shift assay.**

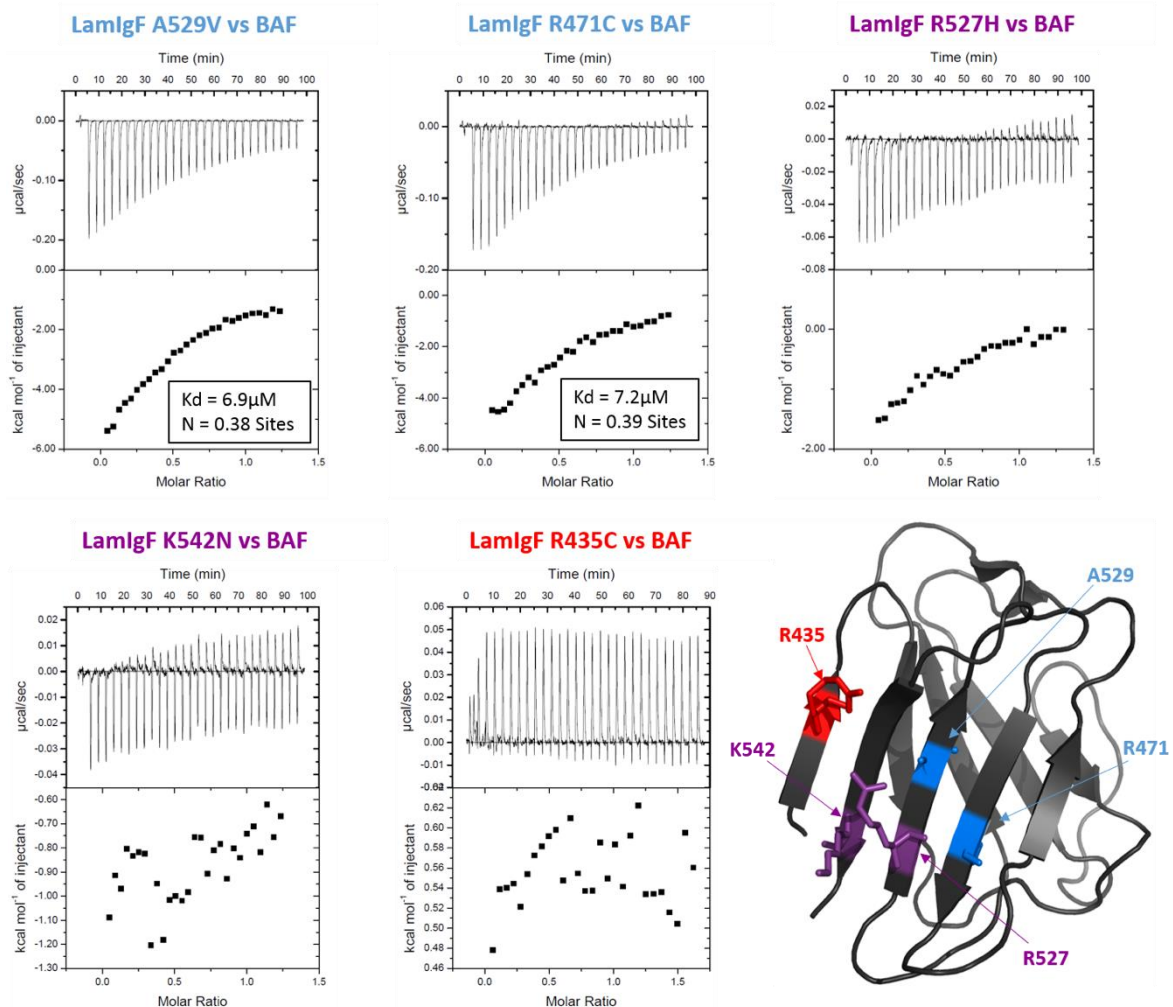
(A) Graphical representation of Igfold WT and mutant thermal stability in buffer A (50mM Mes pH6, 100mM NaCl). (B) Table of Tm and standard error values obtained for the different Igfold WT and mutants in buffer A.

**B**

Protein	Tm (°C)	Standard error (AU)
Igfold WT 1	60,8	0,0
Igfold WT 2	61,5	0,3
Igfold A529V	67,5	0,2
Igfold A529T	60,7	0,3
Igfold K542N	59,3	0,9
Igfold R435C	63,0	0,3
Igfold R471C	56,9	0,1

In conditions in which one Igfold WT can interact with a dimer of BAF<sub>CtoA</sub> with a micro molar affinity ( $K_d = 2\mu\text{M}$ , see Table 6 in Appendix 2), we observed that two mutants (R471C and A529V, in blue on the figure 62) were still able to bind a dimer of BAF<sub>CtoA</sub>, but with a lower affinity (and a decreased heat signal). Then, no affinity could be measured for two mutants (K542N and R527H, in purple on the figure 62) and no binding could be detected for one mutant (R435C, in red on the figure 62). Interestingly, the most pathogenic mutations are those which have the strongest impact on the interaction with BAF<sub>CtoA</sub>.





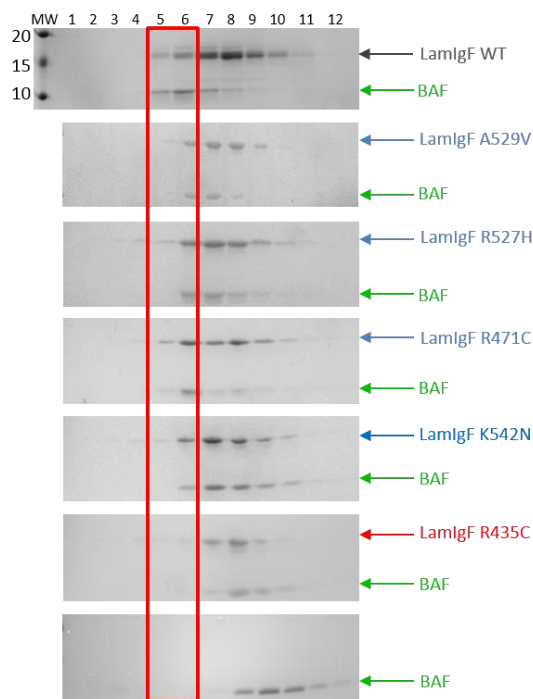
**Figure 62 : Characterization of the impact of 5 Igfold mutants on the interaction with BAF<sub>CtoA</sub>, by ITC.**

**(A) Results of ITC experiments.** For each experiments, successive injections of 10µl of Igfold concentrated at 120µM in a BAF<sub>CtoA</sub> sample concentrated at 20µM. Both proteins were dialyzed against the same buffer (20mM Tris-HCl pH8, 150mM NaCl, 10mM β-mercaptoethanol, protease inhibitors Roche). Each experiment was done two times, at 283K, on a VP-ITC. **(B) Igfold structure (PDB: 1IVT)<sup>42</sup>** with mutants which decreased affinity of the interaction represented in blue and mutants which strongly impact the interaction represented in red. All ITC values are listed in appendix 2 (Table 6).

To confirm these results, we used size-exclusion chromatography and observed which of these five mutants could still bind to BAF<sub>CtoA</sub> (figure 63). A decrease of the affinity was observed with four mutants (K542N, R471C, R527H and A529V, in blue on the figure 63) and no binding was observed with the last mutant (R435C, in red on the figure 63).

We concluded that, in vitro, the more severe is the syndrome induced by the mutant, the more decreased is the affinity of the interaction between the mutated Igfold and BAF<sub>CtoA</sub>.





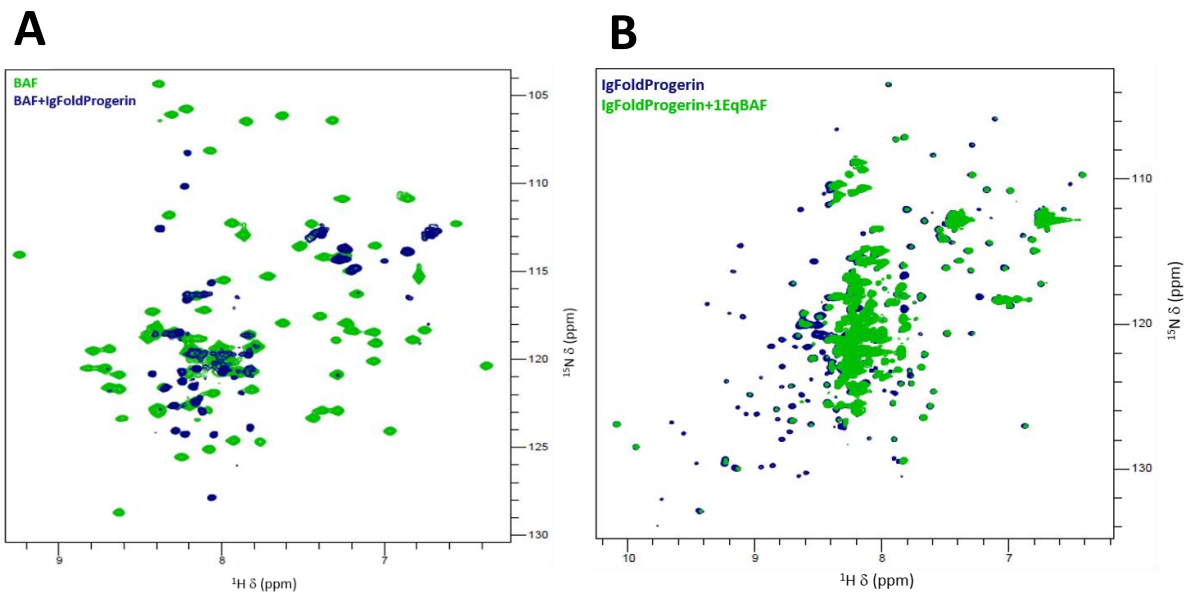
**Figure 63 : Observation of the impact of 5 Igfold mutants on the interaction with BAF<sub>CtoA</sub>, by size exclusion chromatography.**

Each gel corresponds to the result of one size exclusion chromatography. Proteins were injected in a volume of 500µl and GF column Superdex 75 10/300 GL was equilibrated in a buffer containing 20mM Tris-HCl pH8 and 150mM NaCl. Igfold WT is represented in dark grey and BAF in green. Mutants which decreased affinity of the interaction are represented in blue and mutant which strongly impact the interaction is represented in red.

In parallel, we expressed and purified a lamin A/C fragment corresponding to the tail of the mutant progerin, which causes a well-studied form of dominant HGPS. This mutant progerin, as it was explained in the introduction, is due to a mutation that triggers an alternative splicing event leading to a deletion of 50 residues including the proteolytic cleavage site important for lamin A/C maturation. We expressed a large fragment from amino acid 389 to amino acid 614, corresponding to the whole progerin tail.

To simplify, I will call this fragment IgfoldProgerin. My idea was to determine if the presence of region 567 to 614 (absent in the Igfold fragment) was impairing BAF binding.

Purification of IgfoldProgerin was performed as described for lamin A/C Igfold. Then I studied the interaction between IgfoldProgerin and the dimer of BAF<sub>CtoA</sub>. I produced a <sup>15</sup>N labeled BAF<sub>CtoA</sub> protein and added the same equivalent of non-labeled IgfoldProgerin and on the other hand, I produced a <sup>15</sup>N IgfoldProgerin and added the same equivalent of non-labeled BAF<sub>CtoA</sub> dimer (figure 64).

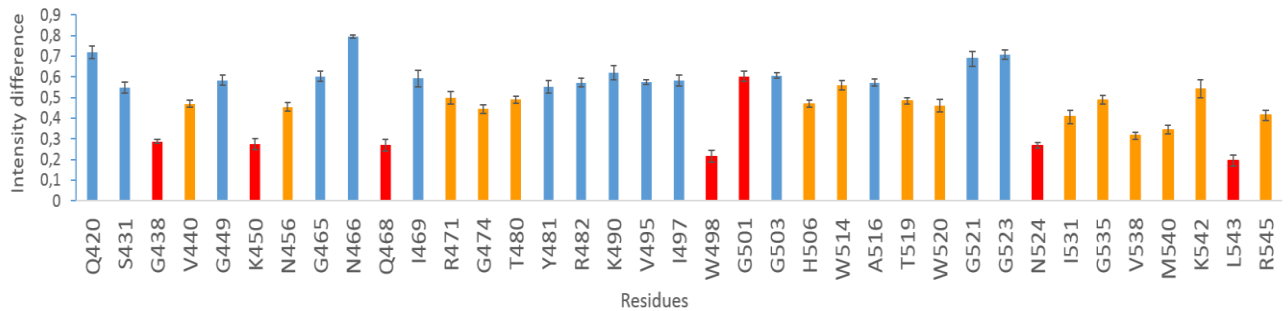


**Figure 64 : Study of BAF<sub>CtoA</sub> /IgfoldProgerin interaction, by NMR.**

**(A)** Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a BAF<sub>CtoA</sub> sample at 100 $\mu\text{M}$  alone (in green) or in presence of 100 $\mu\text{M}$  of IgfoldProgerin (in navy), in 20mM Phosphate pH6.5, 150mM NaCl, 1mM DTT, at 283K and 600MHz (CEA Saclay). **(B)** Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a IgfoldProgerin sample at 75 $\mu\text{M}$  alone (in navy) or in presence of 150 $\mu\text{M}$  of BAF<sub>CtoA</sub> (in green), in 20mM Phosphate pH6.5, 150mM NaCl, 1mM DTT, at 283K and 600MHz (CEA Saclay).

In both cases, I observed an decrease of intensity for several peaks. Based on my Igfold assignment, I did the same analysis as for the interaction between the  $^{15}\text{N}$  labeled Igfold 411-566 and the BAF<sub>CtoA</sub> dimer and I observed which NMR signals were the most impacted by the interaction with BAF<sub>CtoA</sub>.

I compared the amino acids found at the interaction surface in both cases (figure 65). I found the same interaction surface than the one obtained in presence of Igfold alone.

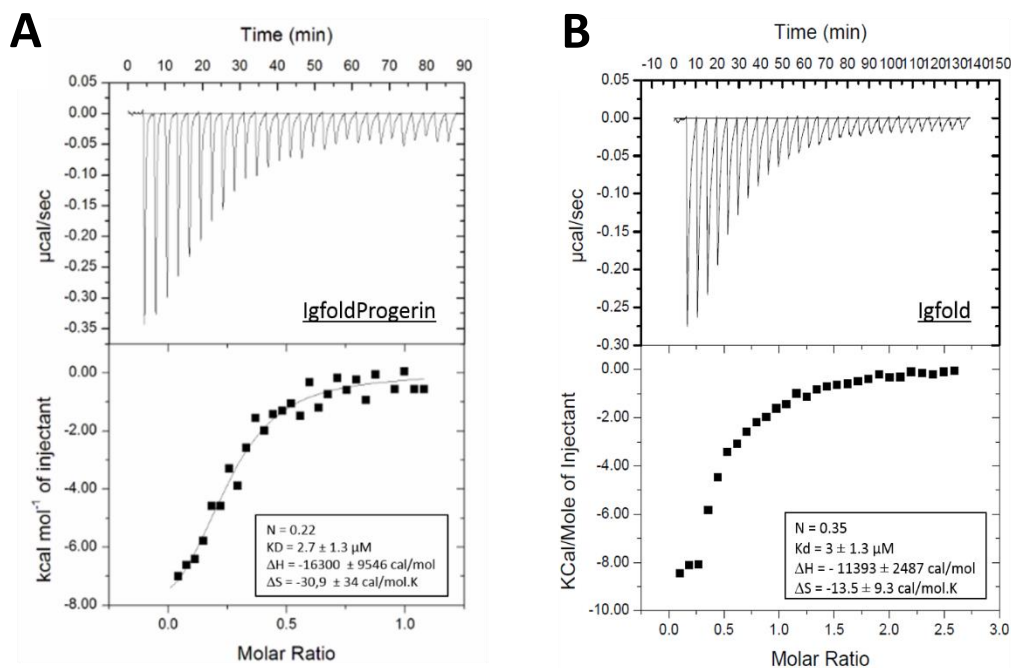
**A****B**

**Figure 65 : Intensity ratio measurement after addition of one BAF<sub>CtoA</sub> equivalent onto the <sup>15</sup>N labeled IgfoldProgerin.**

**(A) Ratio as a function of the sequence. Bars corresponding to peaks losing more than 50% and 70% of intensity after BAF<sub>CtoA</sub> addition are labeled in orange and red, respectively. (B) Three-dimensional-structure of the Igfold domain (PDB: 1IFR) with residues colored as a function of (A).**

To measure the affinity between IgfoldProgerin and BAF<sub>CtoA</sub>, I studied this interaction by ITC (figure 66). Concerning the stoichiometry, like for other ITC studies with BAF<sub>CtoA</sub>, I did not found a number of sites of 0.5, but of 0.22, certainly because of some inactive BAF<sub>CtoA</sub> in our protein sample. Concerning the affinity, I found the same range of values as found for the interaction between BAF<sub>CtoA</sub> and Igfold. On the other side, a compensation between enthalpic and entropic distribution was observed after comparison of both experiments (Interaction between BAF and Igfold or BAF and IgfoldProgerin).

Indeed, an enthalpic contribution to the binding free energy of about 11000 cal/mol and an entropic energy of about 13 cal/mol.K was found in presence of the Igfold whereas an enthalpic contribution of about 16000 cal/mol and an entropic energy of about 31 cal/mol.K was found in presence of the IgfoldProgerin, which suggests that additional contacts exist in the case of IgfoldProgerin.



**Figure 66 : Characterization of IgfoldProgerin/ BAF<sub>CtoA</sub> interaction by ITC.**

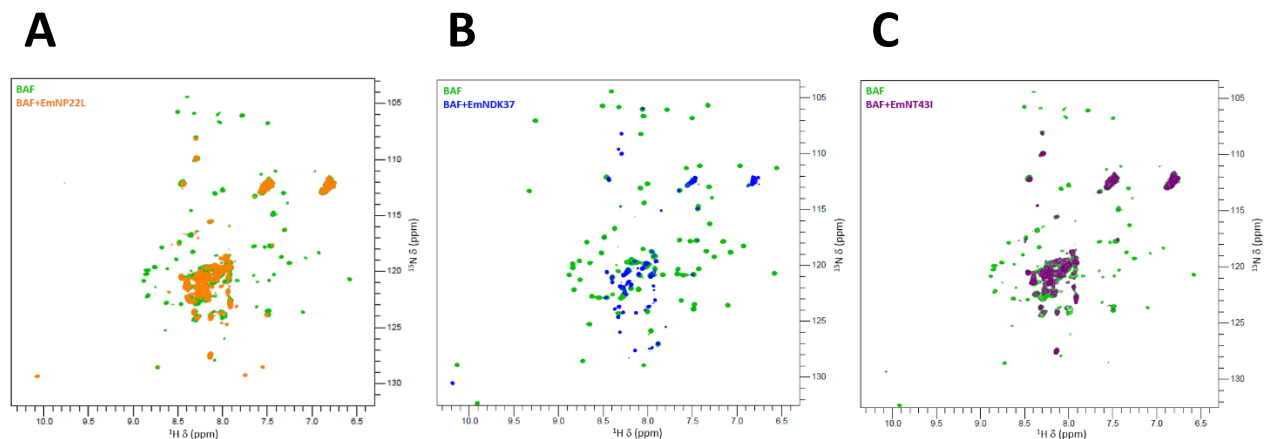
(A) Successive injections of 10 $\mu\text{l}$  of IgfoldProgerin concentrated at 120 $\mu\text{M}$  in a BAF<sub>CtoA</sub> sample concentrated at 24 $\mu\text{M}$ . A Kd of 2.7  $\mu\text{M}$  was found in average. (B) Successive injections of 10 $\mu\text{l}$  of Igfold concentrated at 270 $\mu\text{M}$  in a BAF<sub>CtoA</sub> sample concentrated at 22.5 $\mu\text{M}$ . A Kd of 3.0  $\mu\text{M}$  was found in average. All proteins were dialyzed against the same buffer (20mM Tris-HCl pH8, 150mM NaCl, 10mM  $\beta$ -mercaptoethanol, protease inhibitors Roche). Experiments were done at 288K, on a VP-ITC. All ITC values are listed in appendix 2 (Table 5).

*e. Mutants of the emerlin LEM domain associated to cardiac disease still binds to BAF*

Because the emerlin LEM domain is necessary for emerlin interaction with BAF, we hypothesized that mutations in the LEM domain, and especially  $\Delta\text{K37}$  that impacts the LEM 3D structure, impair binding of EmN to BAF.

To study this, I teamed up with Nada Essawy, a PhD student in the lab, and we first used NMR and size exclusion chromatography to determine the impact of the mutations on the EmN affinity for BAF.

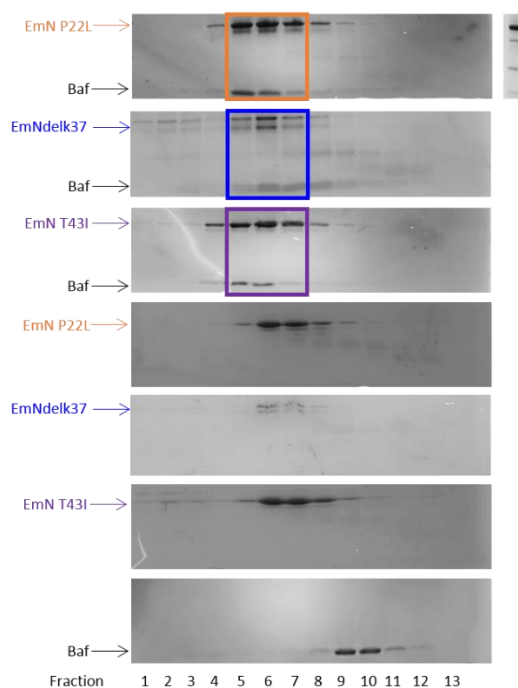
For the NMR experiments, we produced  $^{15}\text{N}$  labeled  $\text{BAF}_{\text{CtoA}}$  protein and non-labeled emerlin mutants. Then, I recorded an  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of  $\text{BAF}_{\text{CtoA}}$  alone (in green, on [figure 67](#)) and spectra of  $\text{BAF}_{\text{CtoA}}$  in contact with each LEM domain mutant, added in a 1:1 ratio ([figure 67](#), [A](#) for P22L, [B](#) for  $\Delta\text{K37}$  and [C](#) for T43I). For the three mutants, I observed that the majority of the peaks, which correspond to the amino acids of  $\text{BAF}_{\text{CtoA}}$ , lost a significant fraction of their intensity. We concluded that these three mutants seem to interact with  $\text{BAF}_{\text{CtoA}}$ . I was surprised that mutant  $\Delta\text{K37}$  could still interact, because as it was explained in the first part of my thesis results, this mutant possesses a destabilized LEM domain. Thus, we did two new hypotheses: first, this mutant could still interact but with a lower affinity than EmN WT, or this mutant could still interact by forming a new complex structure compared to EmN WT in interaction with  $\text{BAF}_{\text{CtoA}}$ .



**Figure 67 : Study of  $\text{BAF}_{\text{CtoA}}$ /EmN LEM domain mutant interaction, by NMR.**

**(A)** Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a  $\text{BAF}_{\text{CtoA}}$  sample at  $150\mu\text{M}$  alone (in green) or in presence of  $150\mu\text{M}$  of P22L (in orange), in 20mM Phosphate pH6.5, 30mM NaCl, at 303K and 700MHz (CEA Saclay). **(B)** Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a  $\text{BAF}_{\text{CtoA}}$  sample at  $150\mu\text{M}$  alone (in green) or in presence of  $150\mu\text{M}$  of  $\Delta\text{K37}$  (in blue), in 20mM Phosphate pH6.5, 30mM NaCl, at 303K and 700MHz (CEA Saclay). **(C)** Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a  $\text{BAF}_{\text{CtoA}}$  sample at  $150\mu\text{M}$  alone (in green) or in presence of  $150\mu\text{M}$  of T43I (in purple), in 20mM Phosphate pH6.5, 30mM NaCl, at 303K and 700MHz (CEA Saclay).

We confirmed our NMR results using size exclusion chromatography. [Figure 68](#) shows that the three EmN mutants still coelute with  $\text{BAF}_{\text{CtoA}}$ , demonstrating that indeed these mutants interact with  $\text{BAF}_{\text{CtoA}}$ .



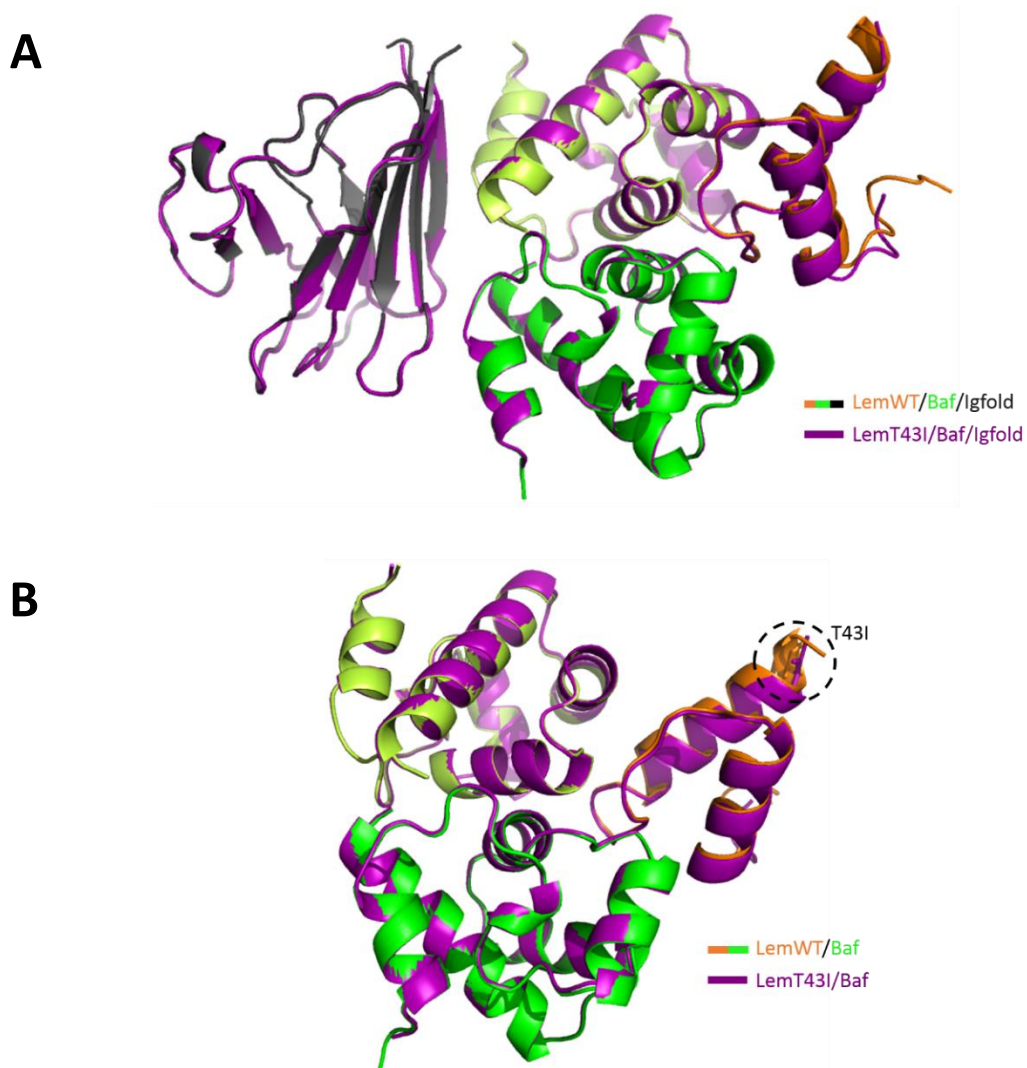
**Figure 68 : Observation of the impact of 3 emerlin LEM domain mutations on the interaction with BAF, by size exclusion chromatography.**

Each gel corresponds to the result of one size exclusion chromatography. Proteins were injected in a volume of 500µl and the GF column Superdex 75 10/300 GL was equilibrated in a buffer containing 20mM Tris-HCl pH8 and 30mM NaCl.

To see if these different mutations have an impact on the 3D structure of the EmN/BAF<sub>CtoA</sub>/Igfold complex, we did the same size exclusion chromatography experiment as presented in figure 68, but adding the Igfold to the EmN/BAF<sub>CtoA</sub> complex, and we kept the elution fractions corresponding to the ternary complex formed by the EmN mutant, BAF<sub>CtoA</sub> and the Igfold, in order to concentrate them and try to obtain crystals.

We tried to crystallize the different complexes using conditions in which we obtained crystals with the WT complex. Thus, we obtained crystals of the T43I/BAF/Igfold complex and the P22L/BAF<sub>CtoA</sub>/Igfold complex. After shooting with X-ray on these crystals, on the PROXIMA1 beamline of synchrotron Soleil (with the help of Virginie Ropars), we obtained a good diffraction pattern for the complex containing T43I, corresponding to a resolution of 2.3Å, whereas we did not obtain any diffraction pattern for the complex containing P22L, because of twinned crystals.

Concerning the complex containing the mutation T43I, I solved its 3D structure, using two servers, Molrep and Phenix, and starting from the structure of the WT ternary complex that I obtained before. After superimposition of this structure with the one obtained with EmN WT, I found that mutation T43I did not affect the structure of the ternary complex. Indeed, as it is shown on [figure 69](#), the 3D structure of the complex containing emerlin WT is superimposable to the one containing emerlin T43I and after superimposition of both complexes (EmN49 WT/BAF<sub>CtoA</sub>/Igfold and EmN49 T43I/ BAF<sub>CtoA</sub>/Igfold), the rmsd value measured between the Calpha carbons of the 3 proteins was 0.55 Å.



**Figure 69 : X-ray crystallography structure of the ternary complex formed between the mutated T43I LEM domain of the emerlin, the BAF<sub>CtoA</sub> dimer and the Igfold domain.**

**(A)** Superimposition of the X-ray crystallography structures of the ternary complexes formed between the LEM domain of emerlin WT, BAF and lamin A/C Igfold and between the LEM domain of emerlin T43I, BAF and lamin A/C Igfold. The resolution of the two complexes are 2.1Å and 2.3Å, respectively, and the rmsd measured between the Calpha carbons of the two complexes yields was 0.547Å. **(B)** Zoom on the superimposition of the BAF<sub>CtoA</sub>/LEM WT and BAF<sub>CtoA</sub>/LEM T43I complexes.

Concerning the ternary complexes containing mutants  $\Delta$ K37 and P22L, we choose to send a sample of these complexes to the European crystallography facility in Grenoble (HTX laboratory), in order to screen a lot of conditions to get crystals.

To conclude with this part of my thesis, I now propose a first interaction mechanism involving lamin A/C and emerin. Indeed, I found that this interaction could be mediated by a third partner, the BAF protein, which is a DNA-binding protein. From this conclusion and because I obtained the X-ray structure of this ternary complex, I am able to present a first structural model of the interaction between the inner nuclear membrane, the nucleoskeleton and DNA ([figure 57](#)). Moreover, I found that some mutants of the Igfold domain that cause progeroid syndromes have a decreased affinity for BAF<sub>CtoA</sub> *in vitro*. Ambre Petitalot in the lab also introduced the same mutations in a vector for expression in mammalian cells, and our collaborator Dr Brigitte Buendia at Univ. Paris Diderot will now observe *in situ* the proximities between mature lamin A and BAF as a function of the mutation.

We also reasoned that in the classical dominant HGPS syndrome, aging is caused by an incorrect processing of lamin A. If the mechanism of this dominant disease is related to the mechanism of the recessive diseases caused by homozygous mutations in the lamin A/C Igfold domain, then the incorrect processing of lamin A might also cause a loss of Igfold-BAF interaction. We observed that the progerin tail is able to bind BAF with an affinity similar to that of the lamin A/C Igfold alone. In cells, the progerin C-terminus is farnesylated. Thus it would now be interesting to test what is the impact of progerin farnesylation on these interactions.

Finally, we observed that mutants of the LEM domain of emerin, including one that exhibits a destabilized LEM domain, are capable of forming a ternary complex with BAF and the lamin A/C Igfold, as WT emerin (see for example [figure 69](#) below).



## 2. THE LAMIN A/C IGFOLD DOMAIN DIRECTLY INTERACTS WITH SELF-ASSEMBLED EMN

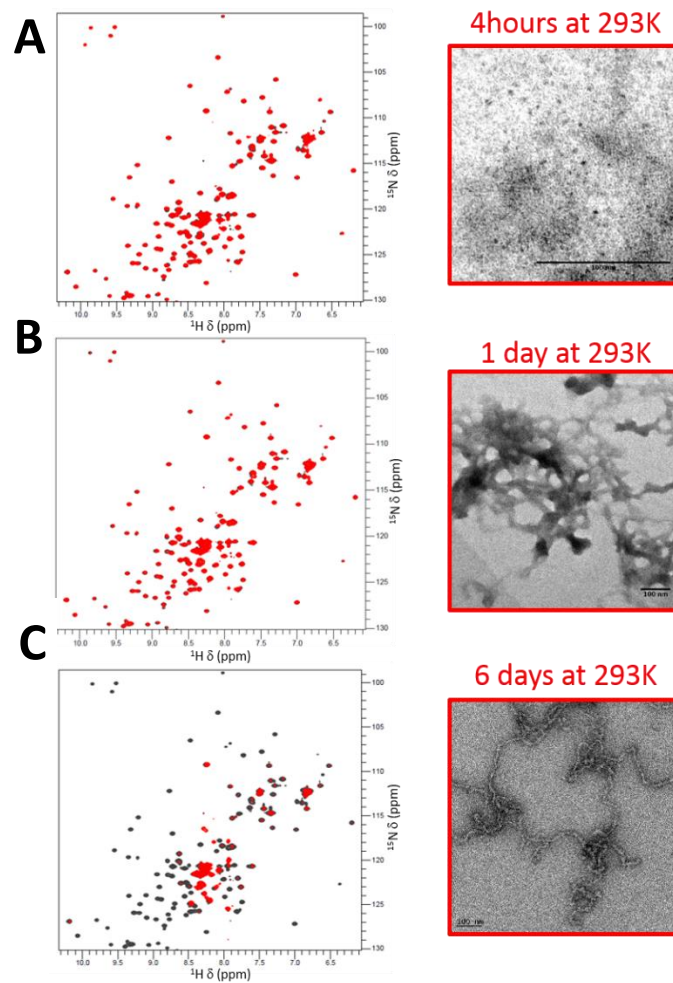
We found that A-type lamins interact indirectly with EmN, *in vitro*, whereas several studies confirmed the existence of a direct interaction between both proteins<sup>166,170</sup>. Moreover, the team of Dr K. Wilson demonstrated that mutations in emerlin region from amino acid 70 to amino acid 170 inhibit lamin binding<sup>245</sup>. We did not observe a direct interaction between the Igfold and EmN monomers and moreover, our indirect interaction does not involve region 70-170 of emerlin. We showed that EmN can form oligomers and filaments *in vitro*. For this reason, we chose to test the impact of emerlin oligomerization on the interaction with the Igfold domain of lamin A/C.

### a. *EmN oligomers bind to the globular domain of lamin A/C*

First, I studied this interaction by NMR. I produced a <sup>15</sup>N labeled Igfold and recorded its <sup>15</sup>N-<sup>1</sup>H HSQC spectrum alone, at 303K. Then, I added different oligomeric forms of non-labeled EmN. To do this, I concentrated my emerlin sample at 600μM, which was a sufficient concentration to obtain oligomers and filaments, and I stored the sample at 293K, with 5mM DTT. Finally, every day during one week, I recorded a spectrum of my <sup>15</sup>N labeled Igfold sample in the presence of the same amount of EmN protein, taken from the EmN sample stored at 293K (figure 70). In parallel, I observed each NMR sample by negative staining EM to see which oligomeric forms of emerlin were present in the sample.

I reproduced this experiment three times, and every time, I observed no change in the spectrum of the Igfold in the presence of emerlin before day 6. By EM, I also observed that before day 6, only spheres or aggregates are observed in the NMR sample, which could correspond to some emerlin inactive oligomers (figure 70, A and B). Contrariwise, after day 6, I observed that the majority of the peaks, which correspond to the amino acids of the Igfold, lost a significant fraction of their intensity (figure 70, C). Interestingly, I observed the NMR sample by EM, and found the presence of emerlin filaments.

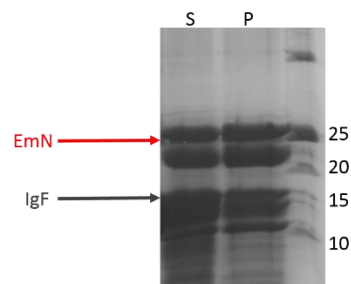
At this point, I checked if the interaction observed by NMR was not due to an aggregation of the Igfold in the presence of emerin oligomers. Therefore, I centrifuged my NMR sample, obtained after interaction, during 10 minutes at 12000 g and observed if the Igfold was still soluble or not on a SDS-PAGE gel (figure 71).



**Figure 70 : Study of Igfold/EmN oligomers interaction.**

Proteins were dialyzed in 20mM Tris-HCl pH8, 30mM NaCl, 2mM DTT and spectra were recorded at a temperature of 303K on a 700MHz spectrometer. (A) Superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$  labeled Igfold at  $250\mu\text{M}$  alone in grey, or in the presence of  $250\mu\text{M}$  of emerin incubated at  $600\mu\text{M}$  during 4h at 293K, in red. (B) Superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$  labeled Igfold at  $250\mu\text{M}$  alone in grey, or in the presence of  $250\mu\text{M}$  of emerin incubated at  $600\mu\text{M}$  during 24h at 293K, in red. (C) Superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$  labeled Igfold at  $250\mu\text{M}$  alone in grey, or in the presence of  $250\mu\text{M}$  of emerin incubated at  $600\mu\text{M}$  during 6 days at 293K, in red. EM pictures correspond to the NMR sample of each experiment.

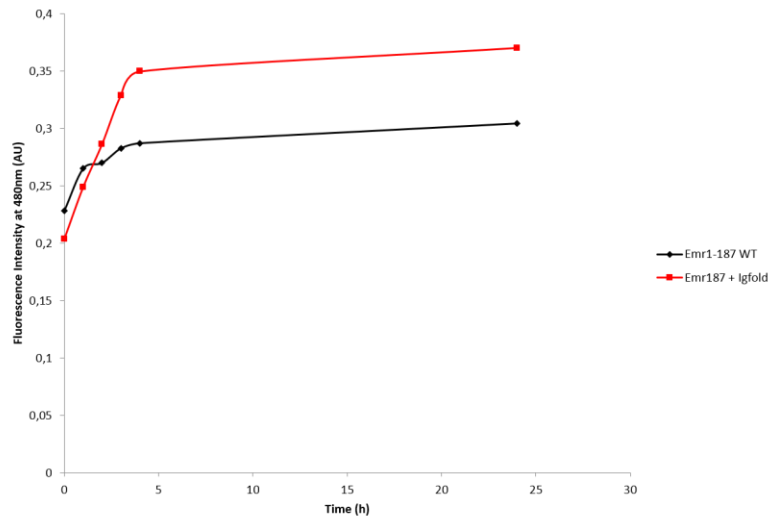
It was clearly visible on this SDS-PAGE gel that half of both proteins were still soluble whereas the other part was in the pellet. Even if we lost some Igfold because of some precipitation of the sample, the amount of Igfold that was insoluble was not sufficient to explain all the intensity decrease observed on the spectrum. We thus confirmed that the signal intensity diminution observed by NMR is due to an interaction with emerin oligomers and not to precipitation of the Igfold sample.



**Figure 71 : SDS-PAGE picture of the supernatant and the pellet obtained after centrifugation of the NMR sample corresponding to figure 69, C.**

I concluded that a direct interaction between the lamin A/C Igfold and EmN can be observed, but only after EmN self-assembly. I did not know at this moment if the Igfold interacted with globular emerin oligomers, or only with emerin filaments, and if this interaction favored emerin filament formation.

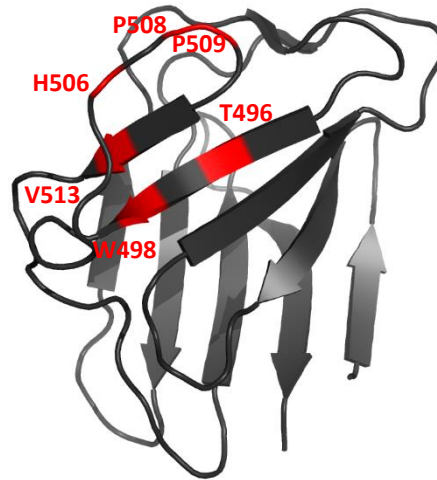
To observe the impact of the Igfold on emerin self-assembly, we used thioflavin T fluorescence, like we did to follow filament formation (figure 72). Thus, I concentrated both EmN and Igfold proteins until 600 $\mu$ M and I incubated half of the EmN sample with the Igfold to obtain a final protein concentration of 300 $\mu$ M and I incubated the other part of my EmN sample with the kinetics buffer (20mM Tris-HCl pH8, 30mM NaCl) to be also at a final concentration of 300 $\mu$ M. Afterwards, I incubated both samples (EmN alone and in the presence of Igfold) at 310K and I took a fluorescence measurement at 480nm, after excitation at 440nm, every hour during 4h and then the day after. For each measurement, I diluted my protein sample to 40 $\mu$ M with the kinetics buffer and added 10 $\mu$ M of thioflavin T. I did this experiment once and I still need to reproduce it.



**Figure 72 : Kinetics of EmN self-assembly in the absence (black) or presence (red) of the Igfold, as followed through thioflavin T fluorescence measurements.**

I observed a small increase in the self-assembly rate of EmN in the presence of the Igfold, which should now be confirmed. It is also not clear how the Igfold favors EmN self-assembly.

To confirm that the interaction between EmN oligomers and the Igfold is specific, we decided to search for some Igfold mutants that cannot bind to the EmN oligomers. Because we did not know at all the surface of the Igfold binding to the EmN oligomers, Jinchao Yu, a PhD student in the team of Dr Raphaël Guérois, in our lab, used a server called ConSurf<sup>258</sup>, to analyze a lamin multiple sequence alignment and identify a hot spot of best conserved residues, which could be implicated in lamin interactions with partners. He found a conserved patch on the Igfold surface, which contains an apolar core, and from this observation he proposed several mutants that could disrupt the Igfold/EmN interaction. He proposed mutants T496E, W498E, H506E, P508A, P509A and V513A. The mutated residues are represented on the X-ray structure of the Igfold (PDB: 1IFR) in [figure 73](#).



**Figure 73 : Localization of the different Igfold residues predicted as being important to interact with EmN oligomers (in red) (PDB: 1IFR).**

For the moment, four mutants were obtained using the QuikChange kit (Stratagene) by Ambre Petitalot in the lab. Together with Ambre, I managed to produce and purify these four mutants: T496E, H506E, P509A and V513A.

First, we observed by NMR, recording  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of each mutant, that these four mutants are well-folded (figure 74) whereas using fluorescence-based Thermal Shift Assay, we observed that the thermal stability of the Igfold is largely decreased by some of these mutations (figure 75). Indeed, only H506E shows a WT-like stability and large decreases in stability (up to 18 degrees) are observed for the others.

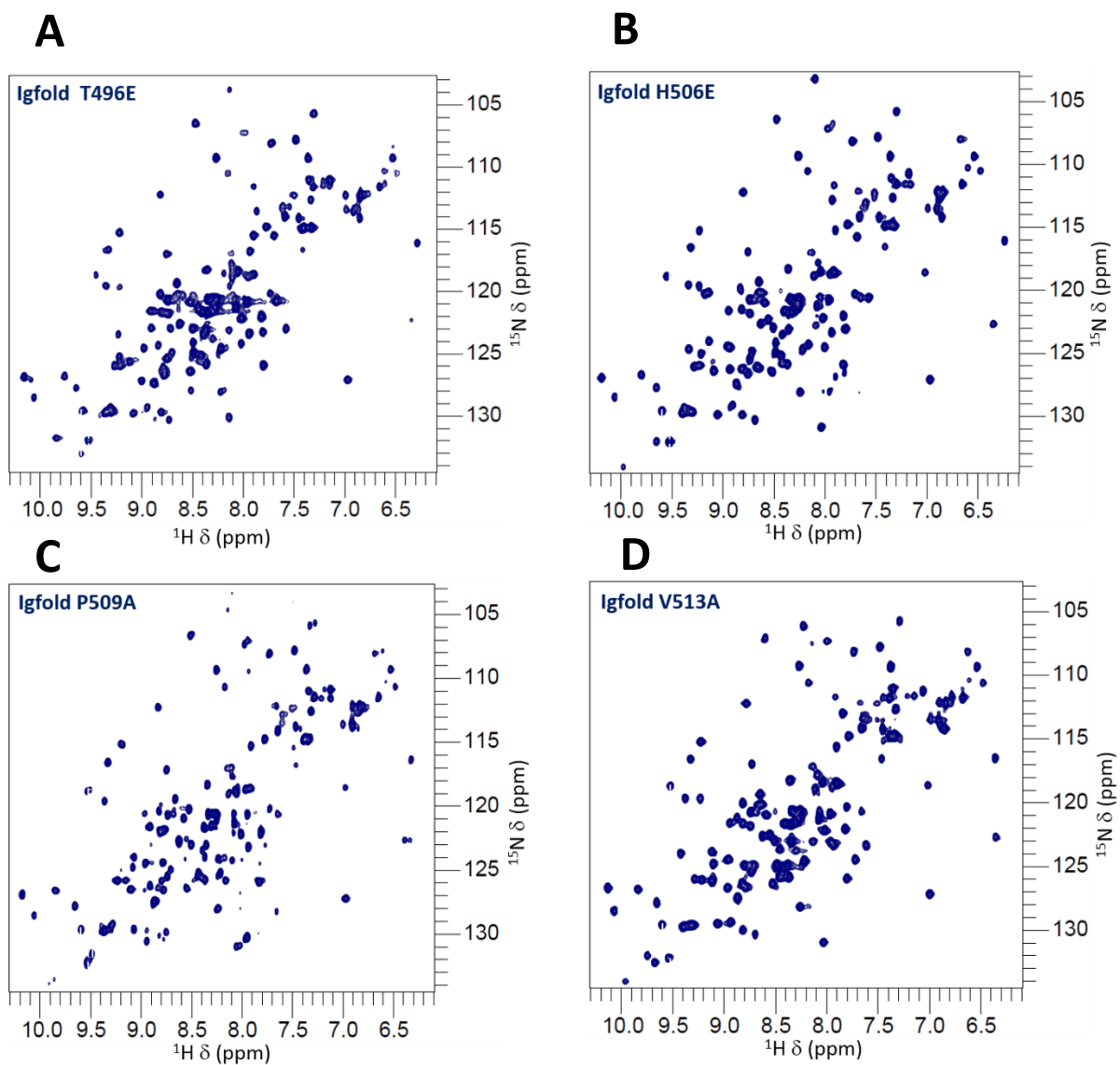
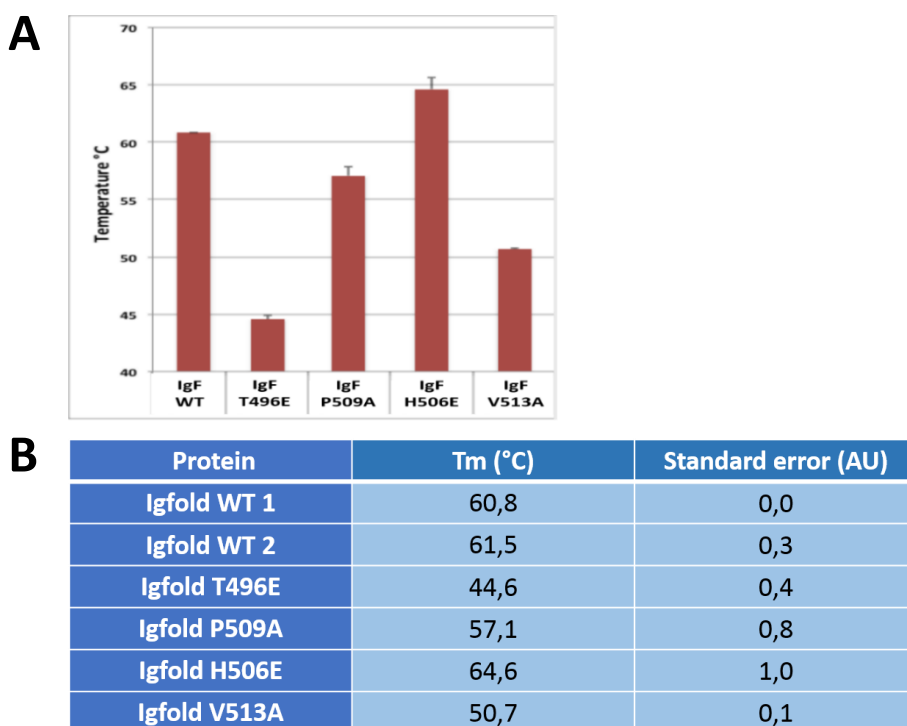


Figure 74 : NMR analysis of the different Igfold mutants.

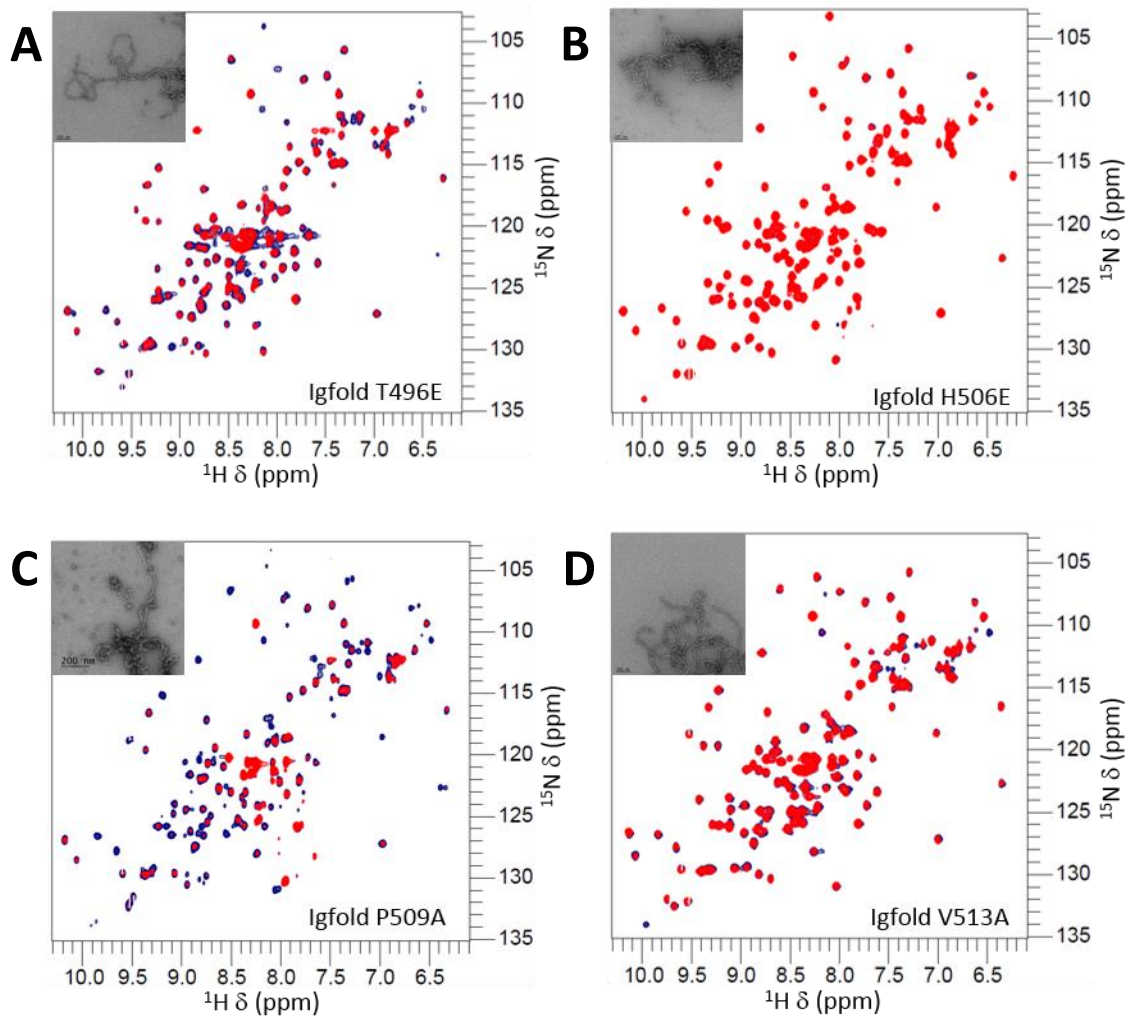
$^1\text{H}$ - $^{15}\text{N}$  HSQC spectra recorded on an Igfold T496E sample at  $200\mu\text{M}$  (A), on an Igfold H506E sample at  $200\mu\text{M}$  (B), on an Igfold P509A sample at  $200\mu\text{M}$  (C) and on an Igfold V513A sample at  $200\mu\text{M}$  (D). All proteins were dialyzed in 20mM Tris-HCl pH8, 30mM NaCl, 2mM DTT and spectra were recorded at 303K and 700MHz spectrometer (CEA Saclay).



**Figure 75 : Thermal stability of Igfold WT and mutants, measured using a fluorescence-based thermal shift assay.**

**(A)** Graphical representation of Igfold WT and mutant thermal stabilities, in Buffer A (50mM Mes pH6, 100mM NaCl). **(B)** Table of Tm and standard error values obtained for different Igfold WT and mutants, in Buffer A.

Then, I observed interaction of these  $^{15}\text{N}$  labeled mutants with EmN by NMR. For these experiments, the Igfold mutants were dialyzed in 20mM Tris-HCl pH8, 30mM NaCl, 2mM DTT and concentrated until 400 $\mu\text{M}$ . In parallel, we produced non-labeled EmN, dialyzed it in the same buffer and after concentration of the protein until 600 $\mu\text{M}$ , the EmN sample was incubated at 293K during one week in order to obtain EmN filaments. Then, we recorded HSQC spectra of each Igfold mutant at 200 $\mu\text{M}$  alone or in the presence of the same ratio of EmN oligomers (figure 76). After each NMR experiment, samples containing the Igfold and EmN oligomers were observed by EM, in order to confirm the presence of filaments. EM pictures were obtained thanks to Dr Ana Arteni (I2BC, Gif-sur-Yvette).

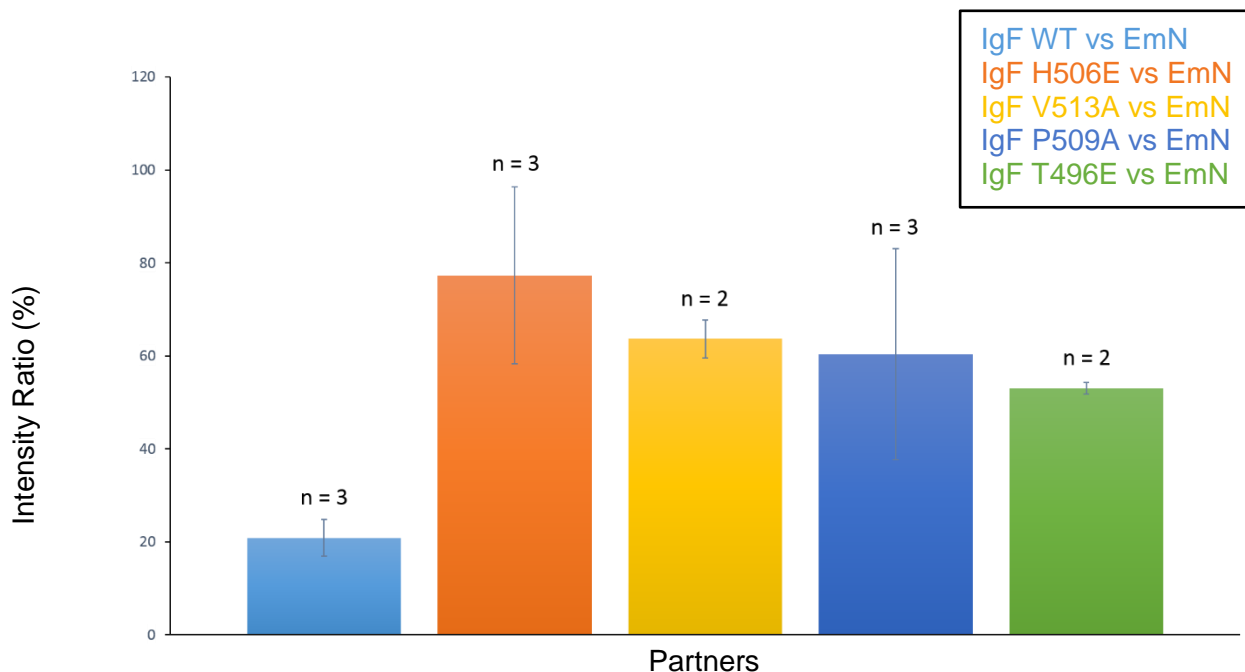


**Figure 76 : NMR and EM interaction study between different Igfold mutants and EmN oligomers.**

Proteins were dialyzed in 20mM Tris-HCl pH8, 30mM NaCl, 2mM DTT and spectra were recorded at 303K and 700MHz spectrometer (CEA Saclay). (A) Superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$  labeled T496E Igfold at 200 $\mu\text{M}$  alone in navy, or in the presence of 200 $\mu\text{M}$  of EmN oligomers, in red. (B) Superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$  labeled H506E Igfold at 200 $\mu\text{M}$  alone in navy, or in the presence of 200 $\mu\text{M}$  of EmN oligomers, in red. (C) Superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$  labeled P509A Igfold at 200 $\mu\text{M}$  alone in navy, or in the presence of 200 $\mu\text{M}$  of EmN oligomers, in red. (D) Superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$  labeled V513A Igfold at 200 $\mu\text{M}$  alone in navy, or in the presence of 200 $\mu\text{M}$  of EmN oligomers, in red. EM pictures correspond to the NMR sample of each experiment.

We reproduced these NMR experiments two times at least and then, we measured intensity differences for every peak corresponding to the bound or unbound Igfold mutant and did an average for each experiment; then values corresponding to experiments involving the same Igfold mutant were again averaged (figure 77). We observed that T496E, P509A and V513A significantly impaired binding to EmN oligomers, whereas H506E completely abolished it.

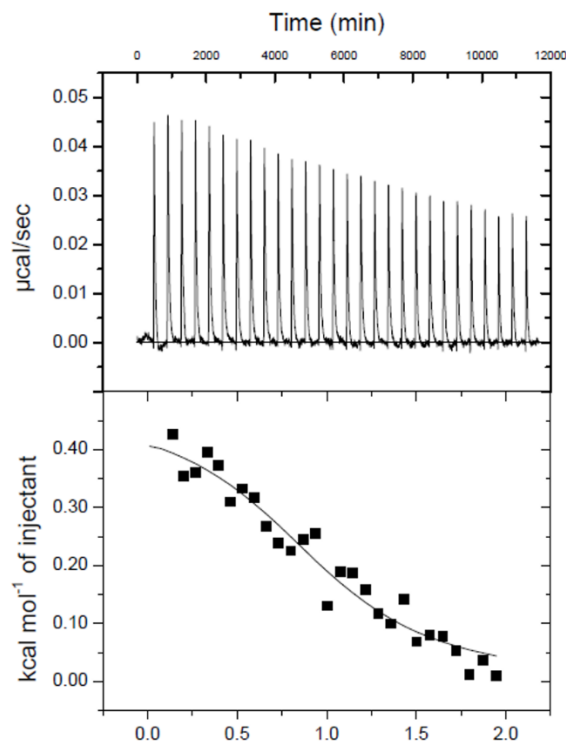




**Figure 77 : Intensity ratio between the NMR HSQC signals measured in the absence or presence of EmN oligomers on the different Igfold mutants.**

In light blue, the Igfold WT, in orange, Igfold H506E, in yellow, Igfold V513A, in dark blue, Igfold P509A and in green, Igfold T496E. “n” corresponds to the number of times the experiment was done.

As the fragment from Val494 to Trp514 (containing our four mutated positions) is mostly apolar, this lamin-emerin interaction could be driven by the hydrophobic effect. In agreement with this observation, we did preliminary isothermal titration calorimetry experiments in order to obtain stoichiometry and affinity of this lamin-emerin interaction (figure 78) and we observed that at 288K, the binding energy is provided by a favorable entropic contribution ( $7.4 \pm 0.1 \text{ kcal mol}^{-1}$ ) partially compensated by an unfavorable enthalpic contribution ( $0.3 \pm 0.1 \text{ kcal mol}^{-1}$ ). However, because we obtained a low heat signal, we did not conclude about the affinity and stoichiometry of this interaction.

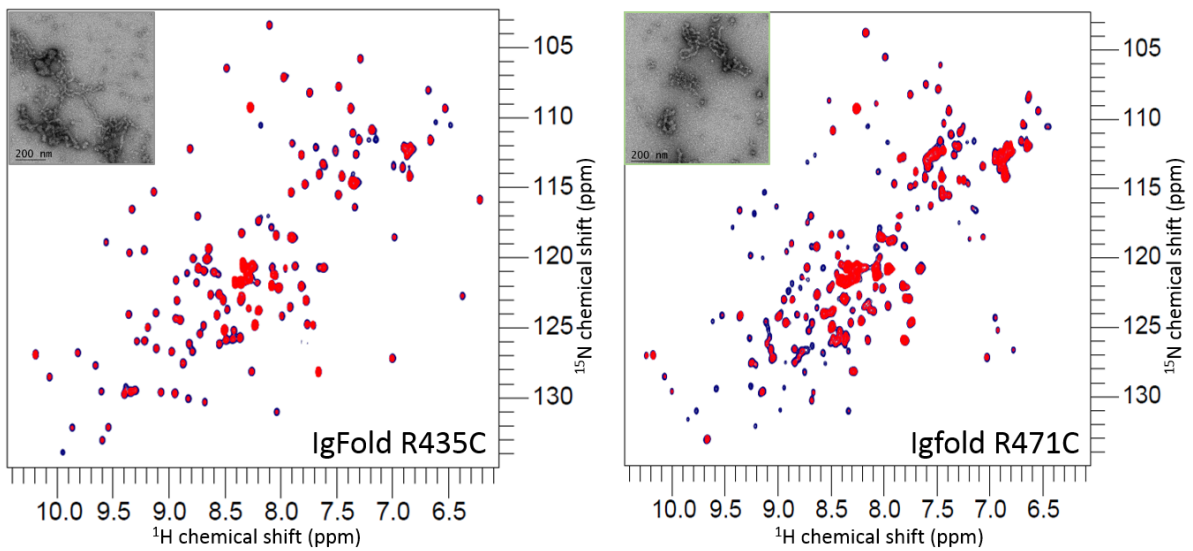
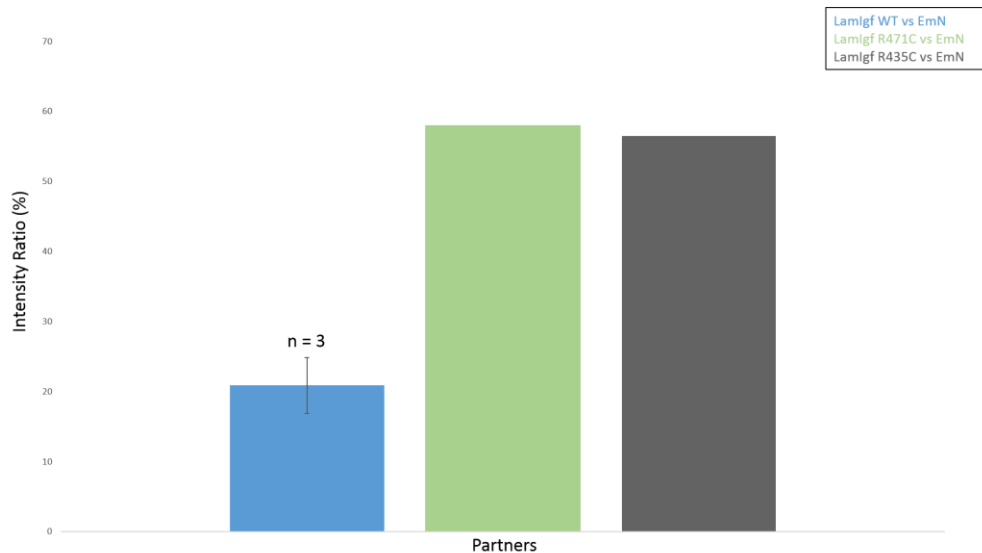


**Figure 78 : Characterization of EmN oligomers/Igfold interaction by ITC.**

Successive injections of 10µl of Igfold concentrated at 315µM in a EmN oligomer sample concentrated at 31.5µM. Both proteins were dialyzed against the same buffer (20mM Tris-HCl pH8, 30mM NaCl, 10mM β-mercaptoethanol, protease inhibitors Roche). The experiment was done at 288K, on a VP-ITC.

In parallel, we observed interactions of two <sup>15</sup>N mutants causing progeroid syndromes (R435C, which abolishes the interaction with BAF and R471C, which decreases the affinity for BAF) with EmN by NMR, in order to observe whether the mutants localized on another part of the Igfold than the one we proposed to be the interaction surface, could destabilize the interaction with EmN too. We did exactly the same experiments as the one described for the four other mutants (figure 79).

After intensity differences measurement, we observed that both mutants impaired binding to EmN oligomers, but because we only did the experiment once, we have to reproduce it to conclude.

**A****B**

**Figure 79: NMR and EM interaction study between Igfold mutants causing progeroid syndromes and EmN oligomers.**

(A) On the left, superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$  labeled R435C Igfold at  $200\mu\text{M}$  alone in navy, or in the presence of  $200\mu\text{M}$  of EmN oligomers, in red and on the right, superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$  labeled R471C Igfold at  $200\mu\text{M}$  alone in navy, or in the presence of  $200\mu\text{M}$  of EmN oligomers, in red. Proteins were dialyzed in  $20\text{mM}$  Tris-HCl pH8,  $30\text{mM}$  NaCl,  $2\text{mM}$  DTT and spectra were recorded at  $303\text{K}$  and  $700\text{MHz}$  spectrometer (CEA Saclay). EM pictures correspond to the NMR sample of each experiment. (B) Intensity ratio between the NMR HSQC signals measured in the absence or presence of EmN oligomers on, Igfold WT (in light blue), R435C Igfold mutant (in grey) or on R471C Igfold mutant (in light green). "n" corresponds to the number of times the experiment was done.

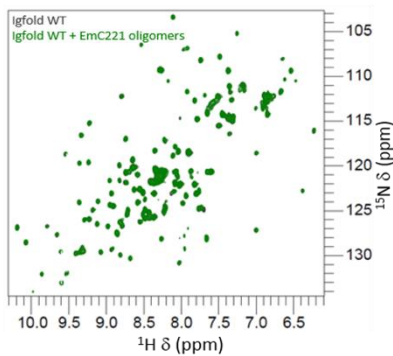
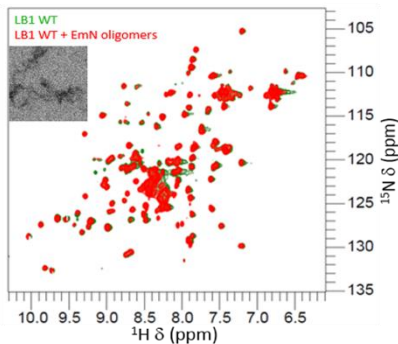
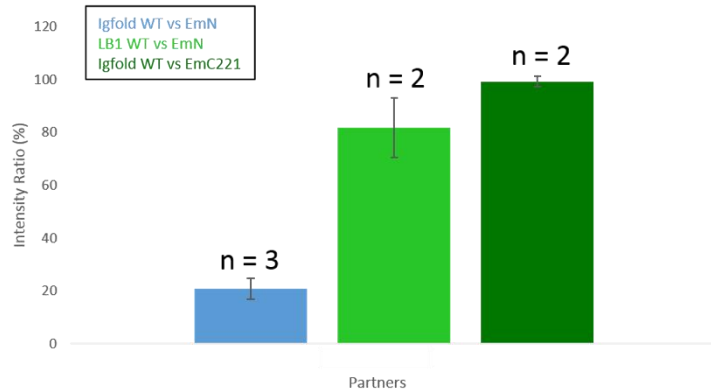
*b. The EmN / Igfold interaction is specific to the emerlin 1-187 region and A-type lamins*

In the first part of my results, I presented two mechanisms of emerlin filament formation. Indeed, I showed that EmN, which comprises a LEM domain and a region that is intrinsically disordered, is able to form monomers and oligomers and these oligomers bind to the Igfold of lamin A/C. But another PhD student of the lab, Florian Celli, found that another part of emerlin, emerlin 67-221 (EmC221), which is entirely unstructured, is able to oligomerize immediately after purification. This oligomerization necessitates region 188-221, because fragment 67-187 does not oligomerize<sup>259</sup>.

We decided to observe if the lamin A/C Igfold could also interact with the filaments formed by fragment 67-221 (EmC221). By NMR, we recorded <sup>1</sup>H-<sup>15</sup>N HSQC spectra of the <sup>15</sup>N labeled Igfold alone or in the presence of EmC221 oligomers.

Whereas addition of EmN oligomers provoked the disappearance of most NMR signals, addition of EmC221 oligomers did not modify the Igfold <sup>1</sup>H-<sup>15</sup>N HSQC spectrum (figure 80, A and C). We concluded that interaction between emerlin oligomers and lamin A/C Igfold is specific to the EmN oligomers.

In addition, because the surface identified by Jinchao Yu is also conserved in B-type lamins, we hypothesized that LB1 was able to bind to EmN oligomers, but no interaction was detected (figure 80, B and C) by NMR. We concluded that the detected emerlin-lamin interaction is specific to A-type lamins. Other Igfold residues that are not conserved in B-type lamins probably also contribute to EmN oligomer binding.

**A****B****C**

**Figure 80 : No binding was observed between EmC221 oligomers or LB1 and Igfold, by NMR.**

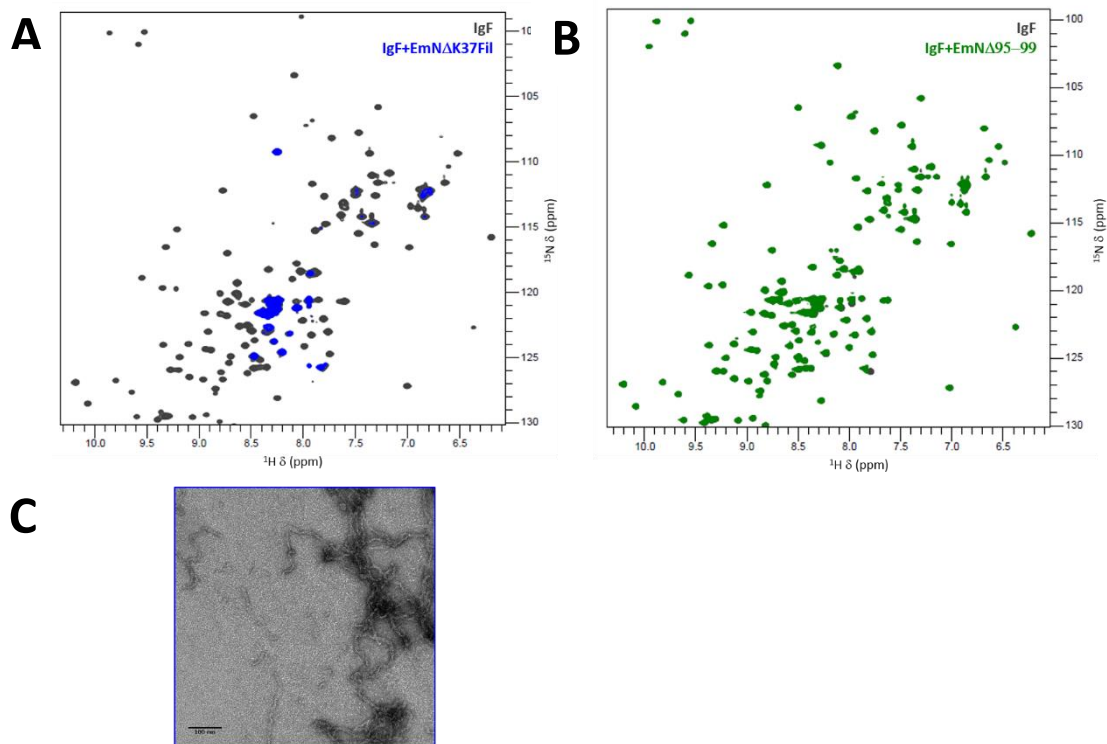
Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a Igfold sample at  $200\mu\text{M}$  alone (in grey) or in presence of  $200\mu\text{M}$  of EmC221 (in dark green), in 20mM Tris-HCl pH8, 30mM NaCl, at 303K and 700MHz (CEA Saclay). (B) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a LB1 sample at  $200\mu\text{M}$  alone (in middle green) or in presence of  $200\mu\text{M}$  of EmN (in red), in 20mM Tris-HCl pH8, 30mM NaCl, at 303K and 700MHz (CEA Saclay). (C) Intensity ratio between the NMR HSQC signals measured in the absence or presence of EmN oligomers (in blue) or EmC221 (in dark green) on Igfold or of EmN oligomers on LB1 (in middle green). “n” corresponds to the number of times the experiment was done.

Our results are consistent with the previously published results of the group of K. Wilson, who proposed that mutations around positions 70, 76, 95, 112, as well as 141 and 164, decrease binding of emerin to lamin A/C<sup>165</sup>. In addition, we did not observe any significant binding of EmN to the lamin B1 tail, and they reported only a weak binding to lamin B1 tail<sup>165</sup>. Altogether, these results demonstrate that a hydrophobic surface present on the lamin A/C Igfold domain binds to self-assembled emerin fragment EmN.

c. *Two in-frame deletion mutants of EmN have two different impacts on the direct lamin/emerin interaction*

Because we found that two emerin mutations ( $\Delta K37$  and  $\Delta 95-99$ ) which cause two different types of diseases have a different impact on EmN filament assembly, we chose to study the impact of these mutations on the interaction of EmN with the Igfold of lamin A/C. As it is explained in the first part of my results, mutant  $\Delta K37$  forms filaments faster than EmN WT, whereas mutant  $\Delta 95-99$  cannot form filaments *in vitro*.

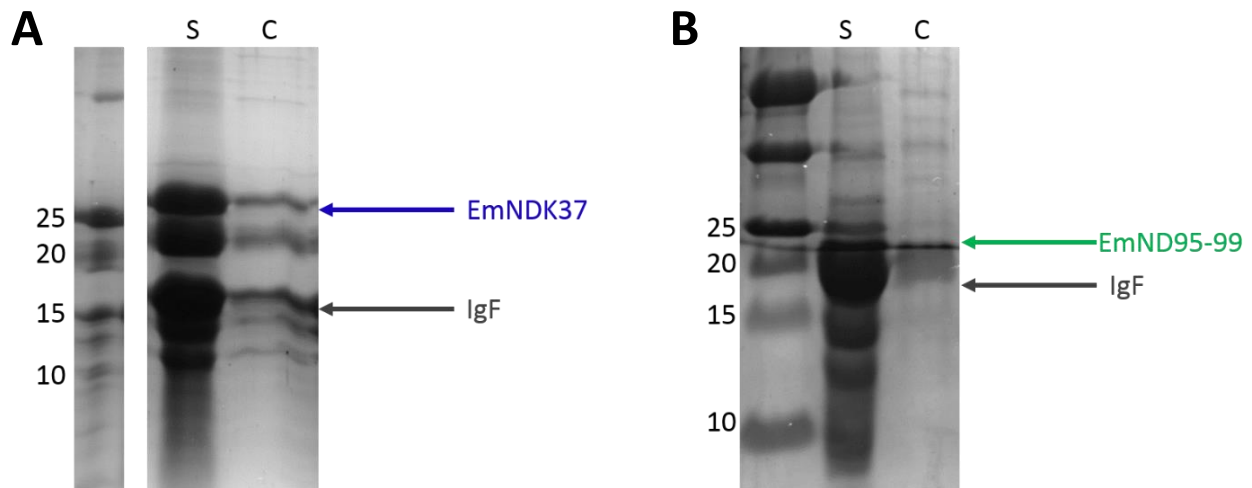
I tested these interactions by NMR. To do so, I produced the  $^{15}\text{N}$  labeled Igfold and recorded  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of this protein alone or in presence of  $\Delta K37$  or  $\Delta 95-99$  in conditions favoring oligomerization (one spectrum per day to observe evolution of mutant oligomerization) (figure 81). Concerning the mutant  $\Delta 95-99$ , after 6 days at  $600\mu\text{M}$  and at  $293\text{K}$ , which were the same conditions in which WT EmN could interact, this mutant cannot bind to the Igfold, because after superimposition of the spectra of the Igfold alone or in presence of this concentrated mutant, no intensity or chemical shift difference was observed. Contrariwise, the mutant  $\Delta K37$ , which can form filaments faster than the emerin 1-187 WT, could interact with the Igfold.



**Figure 81 : Study of Igfold/EmN  $\Delta$ K37 oligomers and Igfold/EmN  $\Delta$ 95-99 oligomers interaction.**

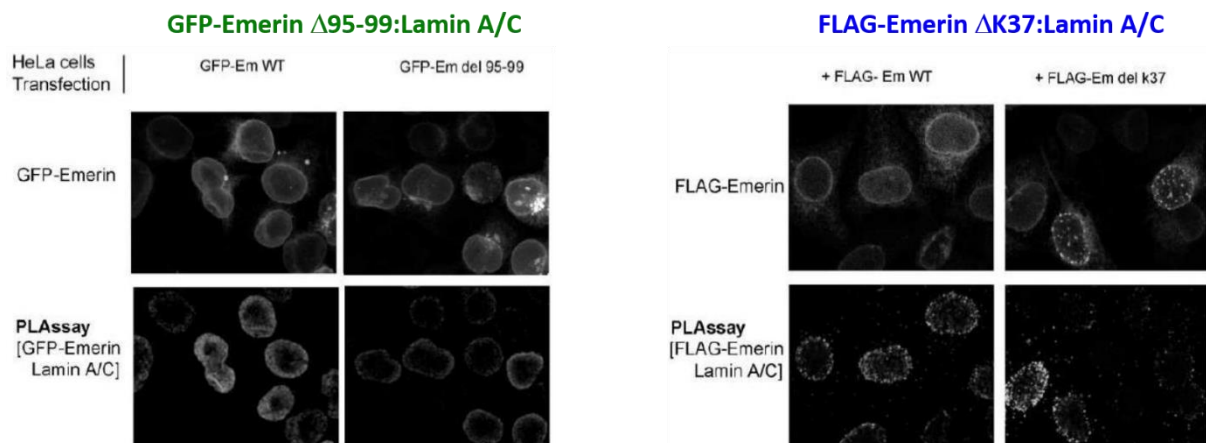
Proteins were dialyzed in a 20mM Tris-HCl pH8, 30mM NaCl, 2mM DTT and spectra were recorded at a temperature of 303K on a 700MHz spectrometer. (A) Superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$  labeled Igfold at 250 $\mu\text{M}$  alone in grey, or in the presence of 250 $\mu\text{M}$  of EmN  $\Delta$ K37 incubated at 600 $\mu\text{M}$  during 6 days at 293K, in blue. (B) Superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$  labeled Igfold at 250 $\mu\text{M}$  alone in grey, or in the presence of 250 $\mu\text{M}$  of EmN  $\Delta$ 95-99 incubated at 600 $\mu\text{M}$  during 6 days at 293K, in green. (C) EM picture of the NMR sample from experiment (A).

Like I did for EmN WT, I checked if the interaction observed by NMR was not due to aggregation of the Igfold. Again, I centrifuged my NMR sample, obtained after interaction, during 10 minutes at 12000 g and observed if the Igfold was still soluble or not on a SDS-PAGE gel (figure 82). Interestingly, all proteins were still soluble after the experiment, so we confirmed that observed NMR peak intensity decrease was not due to aggregation of the sample.



**Figure 82 : SDS-PAGE picture of the supernatant and the pellet obtained after centrifugation of the NMR samples containing the Igfold and (A) EmN  $\Delta$ K37 oligomers and (B) EmN  $\Delta$ 95-99 monomers.**

Then, we studied these interactions in HeLa cells, thanks to our collaborators from Paris Diderot University (Dr Brigitte Buendia's team), who did PLA assays to quantify the amount of closed proximities between GFP-emerin  $\Delta$ 95-99 mutant or FLAG-emerin  $\Delta$ K37 mutant and endogenous lamin A/C, in HeLa cells (figure 83). They confirmed that proximities between emerin  $\Delta$ 95-99 and lamin A/C were very much lower compared to the WT situation<sup>4</sup>, whereas for the mutant  $\Delta$ K37, proximities between both proteins were still visible in cells.



**Figure 83 : Analysis of the impact of the  $\Delta$ 95-99 and  $\Delta$ K37 mutations on Emerin/lamin A/C binding in cell.**

Localization and endogenous lamin proximity observed for GFP-emerin WT, GFP- $\Delta$ 95-99, Flag-emerin WT and Flag-emerin  $\Delta$ K37 (collaboration with Brigitte Buendia, Univ. Paris Diderot). Whereas  $\Delta$ 95-99 localization is similar to that of the WT,  $\Delta$ K37 forms nuclear foci in subpopulation of cells. On the other hand, less emerin-lamin proximities are observed in the case of  $\Delta$ 95-99 when compared to WT emerin<sup>4</sup>; in the case of  $\Delta$ K37, quantification of the observed emerin-lamin proximities is currently being performed.



To conclude, I found a second interaction mechanism involving the lamin A/C Igfold and emerin. I demonstrated that EmN oligomerization is important for direct binding to lamin A/C and to confirm this hypothesis, I observed that one emerin mutant, which cause EDMD and which cannot form EmN oligomers, was unable to bind to the Igfold *in vitro* and in cells, whereas a mutant of the emerin LEM domain, which can form filament faster than WT EmN and causes a cardiac disease, is able to bind directly to the Igfold. In addition, we found that EmN oligomers interact specifically with the lamin A/C Igfold and not with the lamin B1 tail. In the same vein, we showed that this interaction is specific to EmN oligomers and that EmC221 oligomers could not bind to the Igfold. Finally, we observed that the surface of the Igfold domain, which directly contacts emerin oligomers, contains the parallel  $\beta$ -sheet formed by strands  $\beta$ 6 and  $\beta$ 7, which was described as characteristic of the lamin Igfold structure. It is centered on lamin fragment from Val494 to Trp514 containing residues Thr496, P509, His506 and Val513. Because this fragment is mostly apolar, we did the hypothesis that the lamin-emerin interaction is driven by the hydrophobic effect.

### III. Other interaction studies involving emerin

#### 1. INTERACTION WITH ANOTHER LEM DOMAIN PROTEIN: MAN1

Emerin and MAN1 are two LEM domain proteins reported to play overlapping functional roles<sup>260</sup>. A direct interaction between the two LEM Domain proteins, MAN1 and Emerin, was observed by blot overlay and affinity purification from HeLa cell lysates<sup>261</sup>. More in details, they demonstrated that the N-terminal nucleoplasmic region of MAN1 interacts with emerin 1-222, BAF, lamin A and lamin B1. They also demonstrated that the C-terminal nucleoplasmic region of MAN1 binds to BAF. This last interaction was shown to be mediated by DNA by others<sup>57</sup>.

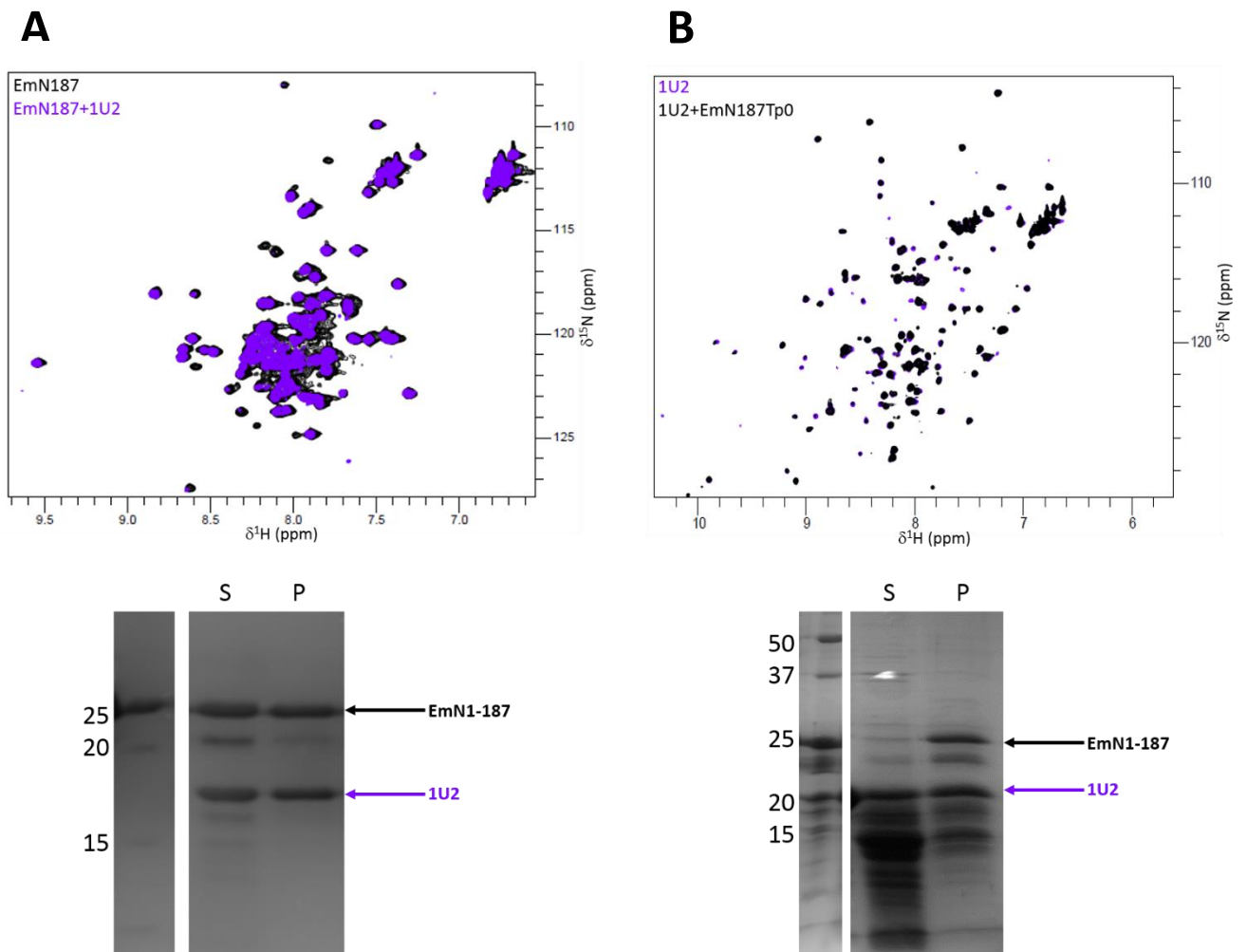
In 2012, Benjamin Bourgeois, a PhD student of the team, showed that, using a HisTag-1U2 construct (from amino acid 755 to 911) corresponding to the C-terminal domain of MAN1, it was possible to purify endogenous emerin from 293T cells. Isaline Herrada, another PhD in the team, later tried to test *in vitro* this interaction but the 2 proteins systematically precipitated when they were mixed.

As I had worked with different emerin constructs and had some experience in purifying these constructs in different oligomeric states, I decided to look at this interaction as a side project. Purification of 1U2 was already established by other students from our laboratory. This construct was cloned in a pET-M13 vector, with a histidine tag, so I purified it by nickel affinity chromatography in 50mM Tris-HCl pH7.5 and 150mM NaCl. After this first purification step, I used a heparin column in order to remove DNA and then I used a gel filtration step, in the buffer of my choice, to obtain a pure sample.

First, I studied the interaction between monomeric EmN and 1U2 by NMR. I produced <sup>15</sup>N labeled samples and after recording the NMR HSQC spectra of the free proteins, on one hand, I added non-labeled EmN onto <sup>15</sup>N labeled 1U2 and on the other hand, I added non-labeled 1U2 onto <sup>15</sup>N labeled EmN (figure 84). After spectra superimposition, I observed chemical shift differences and peak intensity decreases due to the addition of the partner. I concluded that EmN interacts with 1U2.

After the NMR experiments, I also did a SDS-PAGE gel of our NMR samples centrifuged during 5 minutes at 12000 g, in order to observe if the proteins were in the supernatant or in the pellet.

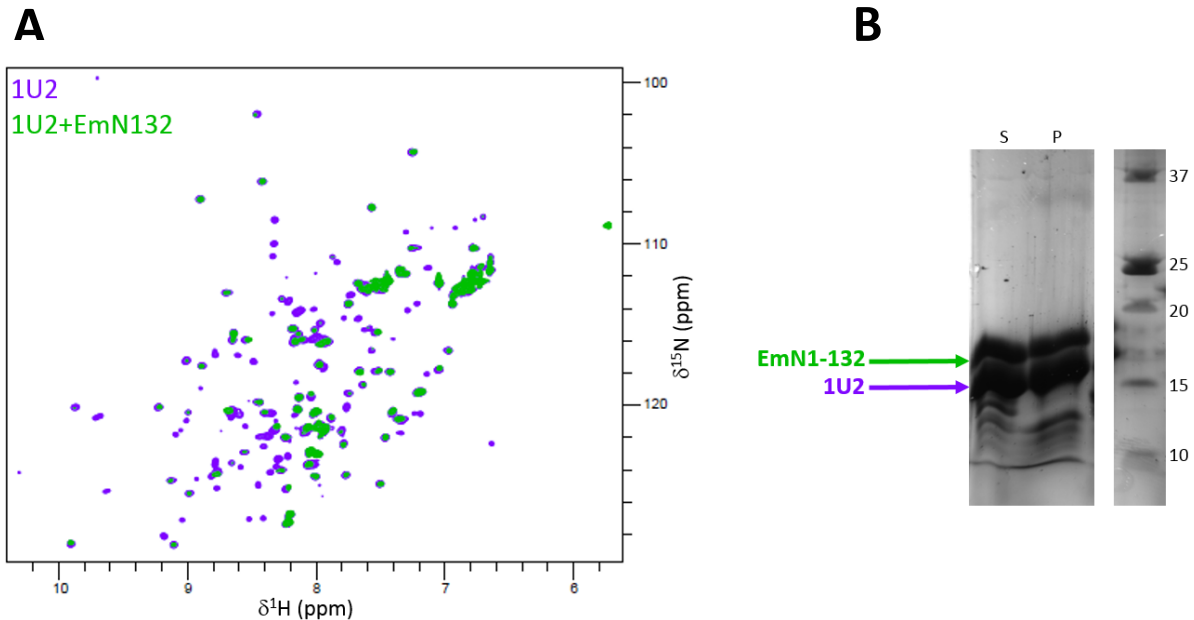
As already observed by Isaline Herrada, half of the proteins were in the pellet. Also, in one of the samples, unlabeled 1U2 seemed degraded, and in the other, EmN seemed degraded. So I need to do these experiments again.



**Figure 84 : Study of EmN/1U2 interaction, by NMR.**

(A) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a EmN sample at  $100\mu\text{M}$  alone (in dark) or in presence of  $100\mu\text{M}$  of 1U2 (in purple), in 20mM Phosphate pH6.8, 30mM NaCl, 10mM EDTA, Roche inhibitors, at 303K and 700MHz (CEA Saclay). (B) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a 1U2 sample at  $100\mu\text{M}$  alone (in purple) or in presence of  $100\mu\text{M}$  of EmN (in dark), in 20mM Phosphate pH6.8, 30mM NaCl, 10mM EDTA, Roche inhibitors, at 303K and 700MHz (CEA Saclay). SDS-PAGE gels corresponding to NMR samples (pellet and supernatant) are displayed under the NMR spectra.

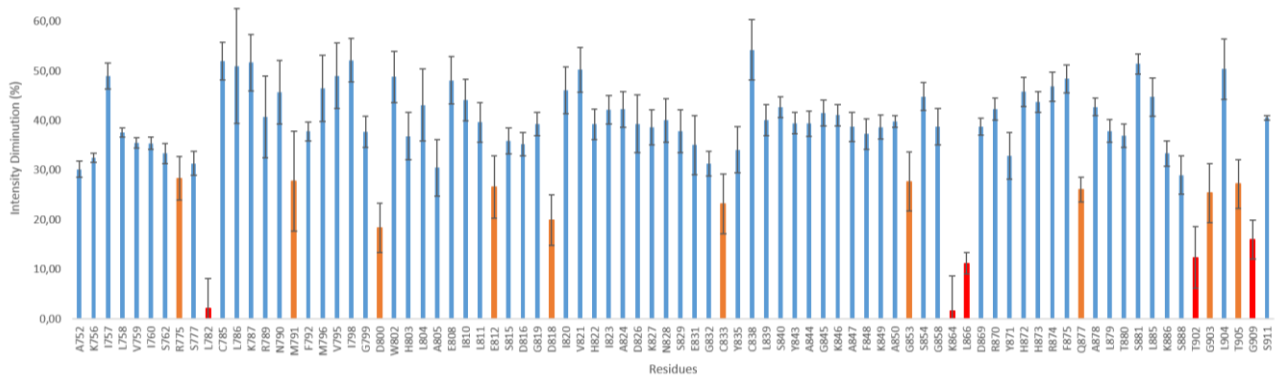
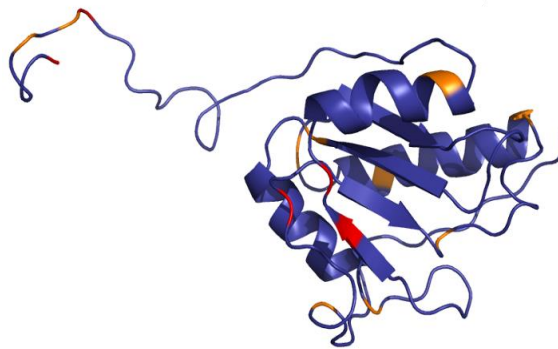
To try to stabilize this complex, I tested the interaction between 1U2 and a smaller emerlin construct, EmN132. By NMR, I looked at the interaction between  $^{15}\text{N}$  labeled 1U2 and monomeric EmN132 (figure 85). Like for EmN, chemical shift differences were observed after spectra superimposition, but half of the NMR sample was aggregated after experiment.



**Figure 85 : Study of EmN132/1U2 interaction, by NMR.**

(A) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a EmN132 sample at  $100\mu\text{M}$  alone (in green) or in presence of  $100\mu\text{M}$  of 1U2 (in purple), in 20mM Phosphate pH6.8, 30mM NaCl, 10mM EDTA, Roche inhibitors, at 303K and 700MHz (CEA Saclay). (B) SDS-PAGE gel picture of NMR sample after centrifugation (S = Supernatant, P = Pellet).

To go further on this study, I calculated peak intensity ratio like I did for other interaction analyses and thanks to the 1U2 assignment already done in the laboratory, I found which amino acids were the most implicated in this interaction (figure 86, A). Moreover, thanks to a 1U2 structure model obtained in my team<sup>58</sup>, I localized residues impacted by the interaction (figure 86, B). Finally, no clear interaction surface was observed on the 1U2 domain. Indeed, peaks found as affected by the interaction with EmN132 correspond to amino acids that are dispersed on the 1U2 structure.

**A****B**

**Figure 86 : Intensity ratio measurement after addition of EmN132 equivalent onto the <sup>15</sup>N labeled 1U2.**

**(A) Ratio as a function of the sequence. Bars corresponding to peaks losing more than 70% and 85% of intensity after EmN132 addition are labeled in orange and red, respectively. (B) Three-dimensional-structure of the 1U2 domain with residues colored as a function of (A).**

We did not conclude about this interaction. My results suggest that binding between EmN132 and the 1U2 domain of MAN1 exists *in vitro*, but we have to find a way to stabilize this complex. An idea would be to use a fragment of our emerlin protein which does not form oligomers or filaments as the mutant EmN  $\Delta$ 95-99 or the LEM domain alone.

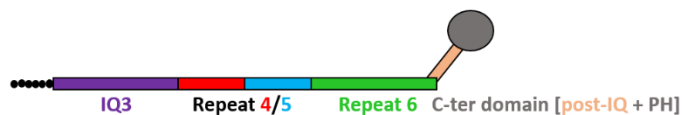
## 2. INTERACTION WITH MYOSIN 1B (IN COLLABORATION WITH THE TEAM OF DR A. HOUDUSSE, INSTITUT CURIE)

First evidence of the presence of myosin in the nucleus was reported in 1980s by a group who demonstrated that myosin, like actin, was involved in nucleoplasmic transport<sup>262</sup>. Then, ultrastructural studies were performed to confirm localization of myosin in the nucleus. In particular, 4 different myosins are in the nucleus: nuclear myosin I<sup>263</sup>, myosin VI<sup>264</sup>, myosin 16b<sup>265</sup> and myosin Va<sup>266</sup>.

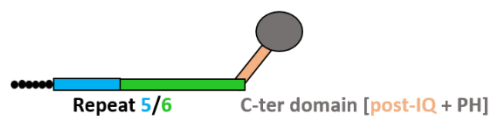
Proteomic studies revealed that emerin binds to nuclear myosin I<sup>267</sup> and we decided to work on this interaction through a collaboration with the group of Dr Anne Houdusse, from Curie Institute, who was working on the structure of myosin 1B and 1C tails.

We searched for a direct interaction between different fragments of our emerin (1-187, 1-132, 1-49 and 67-187) and 3 fragments of their myosin tails (M1b01, M1b04 and M1c01), associated with calmodulin, *in vitro*. The different myosin fragments are represented on [figure 87](#) and for each experiment, the group of Dr Anne Houdusse provided them to us.

### - M1b01 [Myosin 1b tail + neck isoform B] + 3-4 Calmodulin



### - M1b04 [Myosin 1b tail + neck isoform C] + 1-2 CaM



### - M1c01 [Myosin 1c tail + neck] + 3 CaM

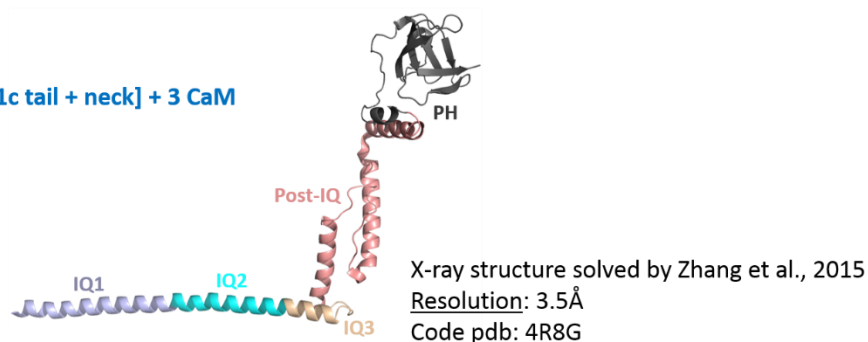
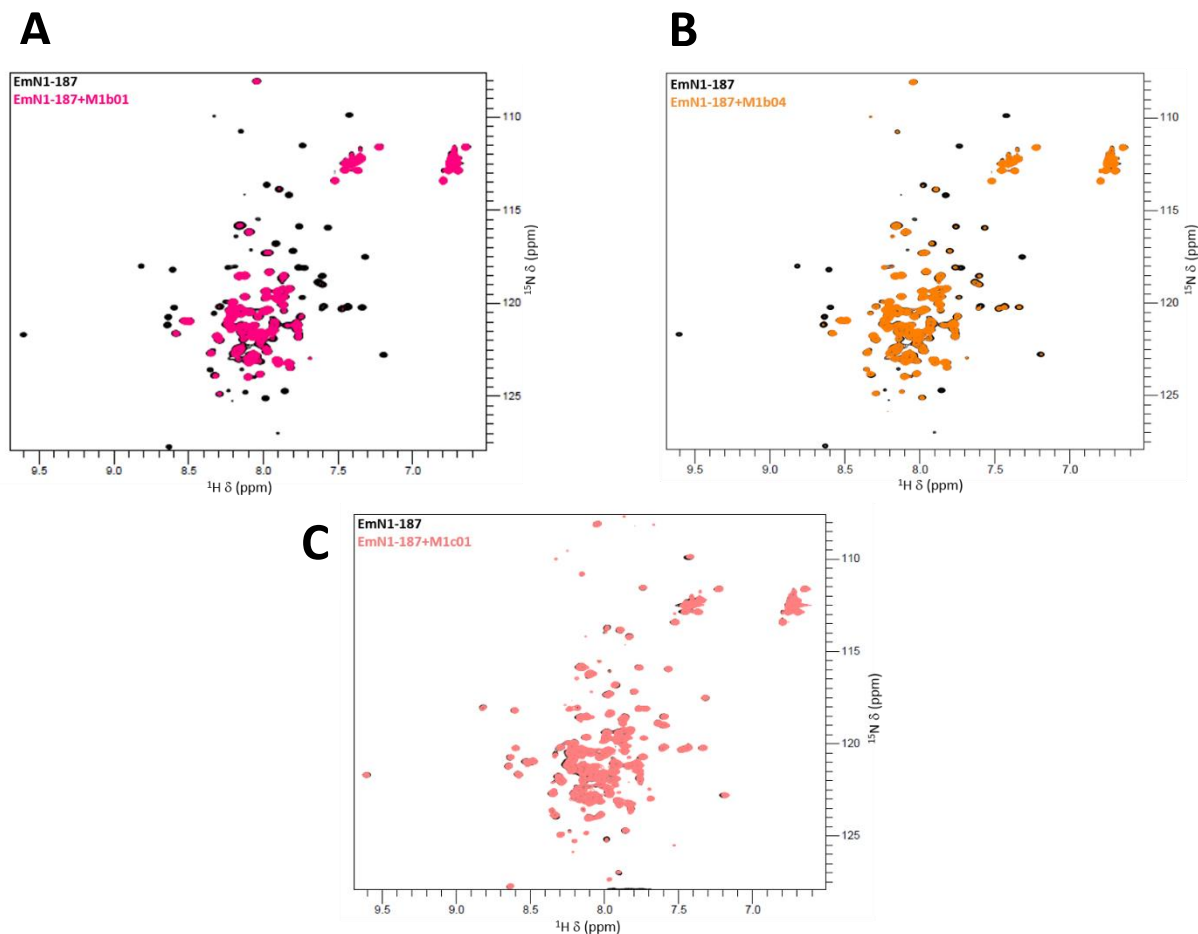


Figure 87 : Scheme of the different myosin fragments provided by the team of Dr Anne Houdusse.

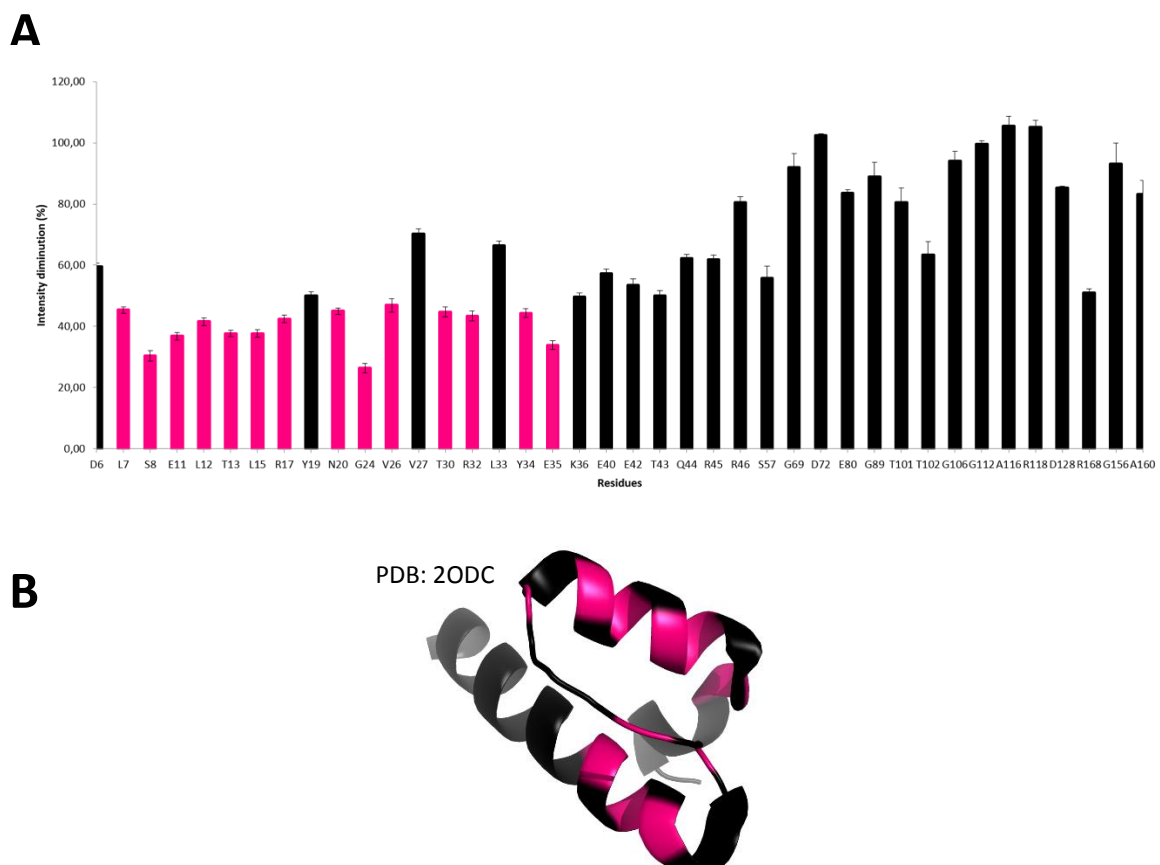
To observe if a direct interaction exists between emerin and one of the myosin tails, we used NMR. I first produced  $^{15}\text{N}$  labeled EmN in order to add the three non labelled myosin constructs one by one (figure 88). After spectra superimposition, I observed an intensity signal decrease for several EmN peaks in the presence of M1b01 and M1b04, whereas no change was observed with M1c01. I concluded that M1b tail constructs, but not the M1c tail construct, directly bind to EmN.



**Figure 88 : Study of the interaction between EmN and 3 myosin 1 fragments.**

(A) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a EmN sample at  $100\mu\text{M}$  alone (in dark) or in presence of  $100\mu\text{M}$  of M1b01 (in pink), in 50mM Tris-HCl pH7.5, 100mM NaCl, 5mM EGTA, 0.2mM DTT, at 293K and 700MHz (CEA Saclay). (B) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a EmN sample at  $100\mu\text{M}$  alone (in dark) or in presence of  $100\mu\text{M}$  of M1b04 (in orange), in 50mM Tris-HCl pH7.5, 100mM NaCl, 5mM EGTA, 0.2mM DTT, at 293K and 700MHz (CEA Saclay). (C) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a EmN sample at  $100\mu\text{M}$  alone (in dark) or in presence of  $100\mu\text{M}$  of M1c01 (in coral), in 50mM Tris-HCl pH7.5, 100mM NaCl, 5mM EGTA, 0.2mM DTT, at 293K and 700MHz (CEA Saclay).

To find which region of EmN was important for interaction with M1b tails, I assigned several peaks on the emerlin spectrum and calculated, like I did for the interaction between emerlin and Igfold, the intensity ratio after addition of one equivalent of M1b01 (figure 89). I did not assign all the emerlin spectrum because our published emerlin assignment was not done at the same pH and temperature, so it was difficult to find all the corresponding peaks and I could not reassign the spectrum without a new  $^{15}\text{N}$ ,  $^{13}\text{C}$  labeled sample, notably for the unfolded region. After this study, I observed that the region that could be the most important for this interaction was the LEM domain of emerlin and I localized residues corresponding to peaks that had lost more than 50% of their initial intensity on the known LEM domain structure solved by NMR (PDB: 2ODC).



**Figure 89** : Intensity ratio measurement after addition of one M1b01 equivalent onto  $^{15}\text{N}$  EmN.

(A) Ratio as a function of the sequence. Bars corresponding to peaks losing more than 50% of intensity after M1b01 addition are labelled in pink. (B) Three-dimensional structure of the emerlin LEM domain (PDB: 2ODC) with residues colored as in (A).



During the interaction analysis by NMR, another important information was observed. Indeed, I discovered that after 12h of contact between EmN and M1b01 or M1b04, the peak intensities on the interaction spectra continued to decrease (figure 90, A and B). This effect was really visible for the interaction with M1b01: indeed, after 12h, most peaks had totally disappeared. We have different hypotheses from this observation. A first hypothesis was that there was a precipitation of our NMR sample. A second hypothesis was that this intensity diminution after several hours could be due to an oligomerization of emerlin induced by the interaction with M1b tails. Our last hypothesis was that this diminution could be due to an oligomerization of myosin1b which eventually carried the emerlin.

To exclude our first hypothesis, I did a SDS-PAGE gel with the supernatant and the pellet of my NMR sample after a centrifugation of 10 minutes at 12000 g (figure 90, C). For M1b01, all the proteins were still in the supernatant after 12h of interaction, so protein precipitation was not the explanation for the signal decrease. Contrariwise, for M1b04, half of the sample was in the pellet and the diminution of peak intensity was not so important so we cannot exclude this hypothesis.

After these different results, I choose to study more in details this interaction through two different approaches. First, I wanted to confirm that the LEM domain was the binding site for the interaction with M1b tails and to study this, I used different emerlin constructs containing or not the LEM domain and observed the impact on the interaction. In a second part, I studied emerlin oligomerization in the presence of M1b01, using emerlin constructs forming or not oligomers and using several techniques.

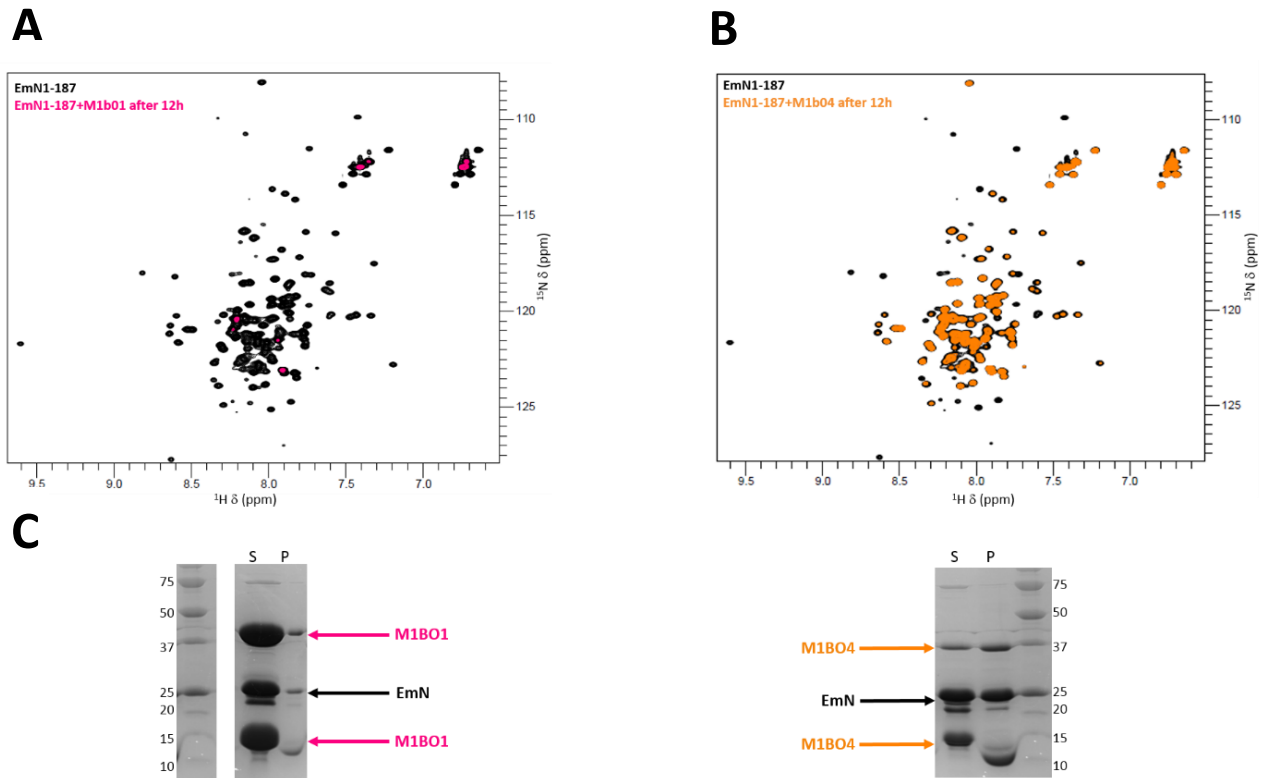
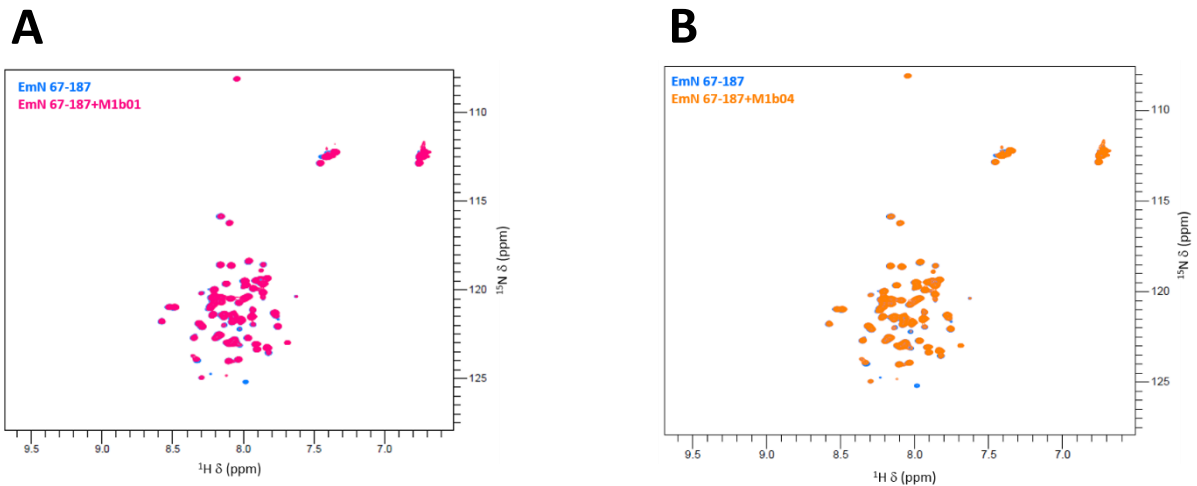


Figure 90 : Study EmN/M1b tails interaction, by NMR.

(A) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a EmN sample at  $100\mu\text{M}$  alone (in dark) or in presence of  $100\mu\text{M}$  of M1b01 during 12h (in pink), in  $50\text{mM}$  Tris-HCl pH7.5,  $100\text{mM}$  NaCl,  $5\text{mM}$  EGTA,  $0.2\text{mM}$  DTT, at  $293\text{K}$  and  $700\text{MHz}$  (CEA Saclay). (B) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a EmN sample at  $100\mu\text{M}$  alone (in dark) or in presence of  $100\mu\text{M}$  of M1b04 during 12h (in orange), in  $50\text{mM}$  Tris-HCl pH7.5,  $100\text{mM}$  NaCl,  $5\text{mM}$  EGTA,  $0.2\text{mM}$  DTT, at  $293\text{K}$  and  $700\text{MHz}$ . (C) SDS-PAGE gels of both NMR samples after 10 minutes of centrifugation.

#### a. Characterization of the emerlin region important for M1b tail binding

First, to confirm that the LEM domain was necessary for this interaction, we produced the  $^{15}\text{N}$  emerlin 67-187 fragment (EmC187), which did not contain the globular domain, and added non labelled M1b01 or M1b04 (figure 91). After spectra superimposition of emerlin protein alone or in presence of myosin, no difference was observed. We concluded that the unfolded region of EmN cannot bind M1b01 or M1b04 and that the LEM domain of emerlin is necessary to mediate the interaction with M1b tails.

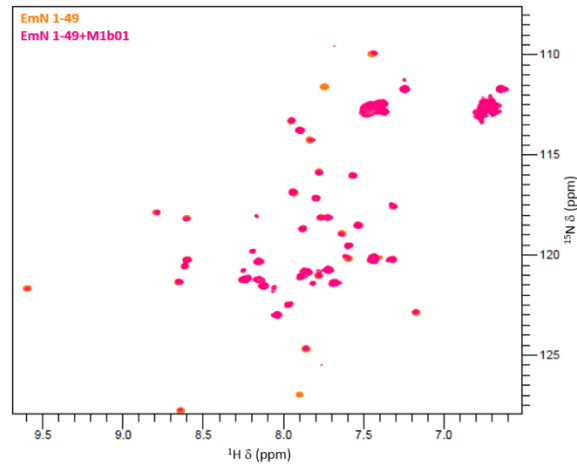
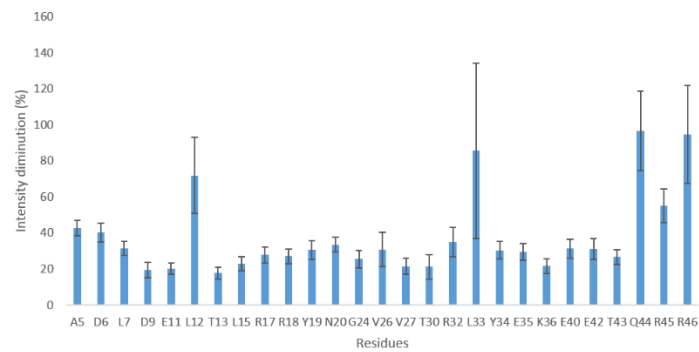


**Figure 91 : Study EmC187/M1b tails, by NMR.**

**(A)** Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a EmC187 sample at  $100\mu\text{M}$  alone (in blue) or in presence of  $100\mu\text{M}$  of M1b01 (in pink), in  $50\text{mM}$  Tris-HCl pH7.5,  $100\text{mM}$  NaCl,  $5\text{mM}$  EGTA,  $0.2\text{mM}$  DTT, at  $293\text{K}$  and  $700\text{MHz}$  (CEA Saclay). **(B)** Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a EmC187 sample at  $100\mu\text{M}$  alone (in blue) or in presence of  $100\mu\text{M}$  of M1b04 (in orange), in  $50\text{mM}$  Tris-HCl pH7.5,  $100\text{mM}$  NaCl,  $5\text{mM}$  EGTA,  $0.2\text{mM}$  DTT, at  $293\text{K}$  and  $700\text{MHz}$  (CEA Saclay).

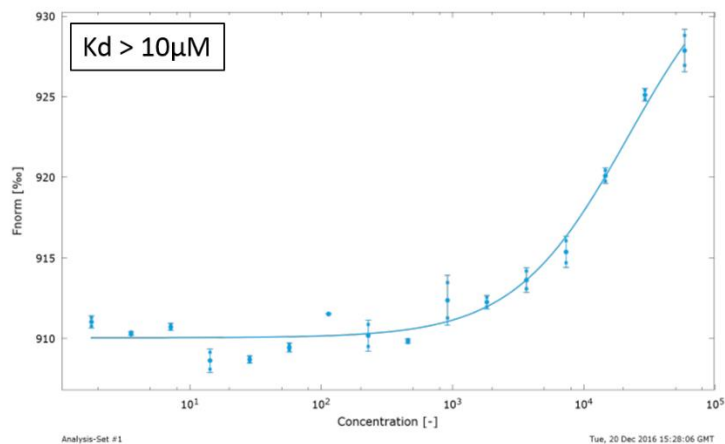
To confirm my result, I produced the LEM domain of emerin (EmN49) and tested its interaction with M1b01 by NMR (figure 92, A). After having observed an intensity decrease following addition of M1b01, I quantified this decrease (figure 92, B): it yielded more than 50% for the majority of the peaks corresponding to residues of the LEM domain. I concluded that it was not possible to identify the binding site on the LEM domain; however it was clear that the LEM domain was necessary and sufficient for binding to M1b01.

Our collaborators tried to characterize this interaction between the LEM domain and M1b01 in fusion with YFP by Microscale Thermophoresis (MST) (figure 93). They confirmed that this interaction exists but obtained just an estimation of the affinity. Indeed, the LEM interacted through a weak affinity ( $K_d > 10\mu\text{M}$ ) with M1b01.

**A****B**

**Figure 92 : Study of EmN49/M1b01 interaction, by NMR.**

(A) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a EmN49 sample at  $100\mu\text{M}$  alone (in orange) or in presence of  $100\mu\text{M}$  of M1b01 (in pink), in 50mM Tris-HCl pH7.5, 100mM NaCl, 5mM EGTA, 0.2mM DTT, at 293K and 700MHz (CEA Saclay). (B) Intensity ratio measurement after addition of one M1b01 equivalent onto  $^{15}\text{N}$  EmN49.

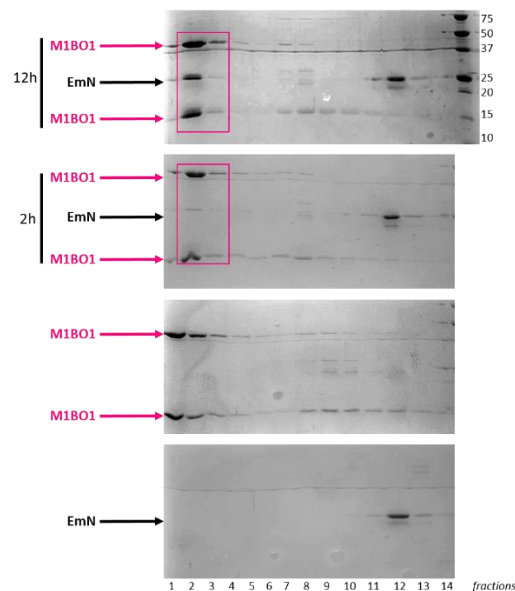


**Figure 93 : Characterization of the interaction between EmN49 and M1b01, by MST.**

Proteins were dialyzed in 50mM Tris-HCl pH7.5, 100mM NaCl, 5mM EGTA, 0.2mM DTT. MST experiments were carried with 0.05% Tween. Proteins were both concentrated at  $100\text{nM}$ .

### b. Study of protein oligomerization during interaction between EmN and M1b tails

To study this particularity of the interaction, first, we used size exclusion chromatography to observe the complex between EmN and M1b01 after 1 or 12 hours of incubation. To do this, we prepared four different samples: one containing EmN alone at 100 $\mu$ M, one with M1b01 alone at 100 $\mu$ M, one with both proteins at 100 $\mu$ M, in contact during 1h and the last one, with both proteins at 100 $\mu$ M, in contact during 12h (figure 94). Interestingly, we observed a displacement of the emerlin elution peak from its initial position, to the same position as M1b01 after 1h of contact between both proteins, and the amount of displaced emerlin seemed to increase over time.

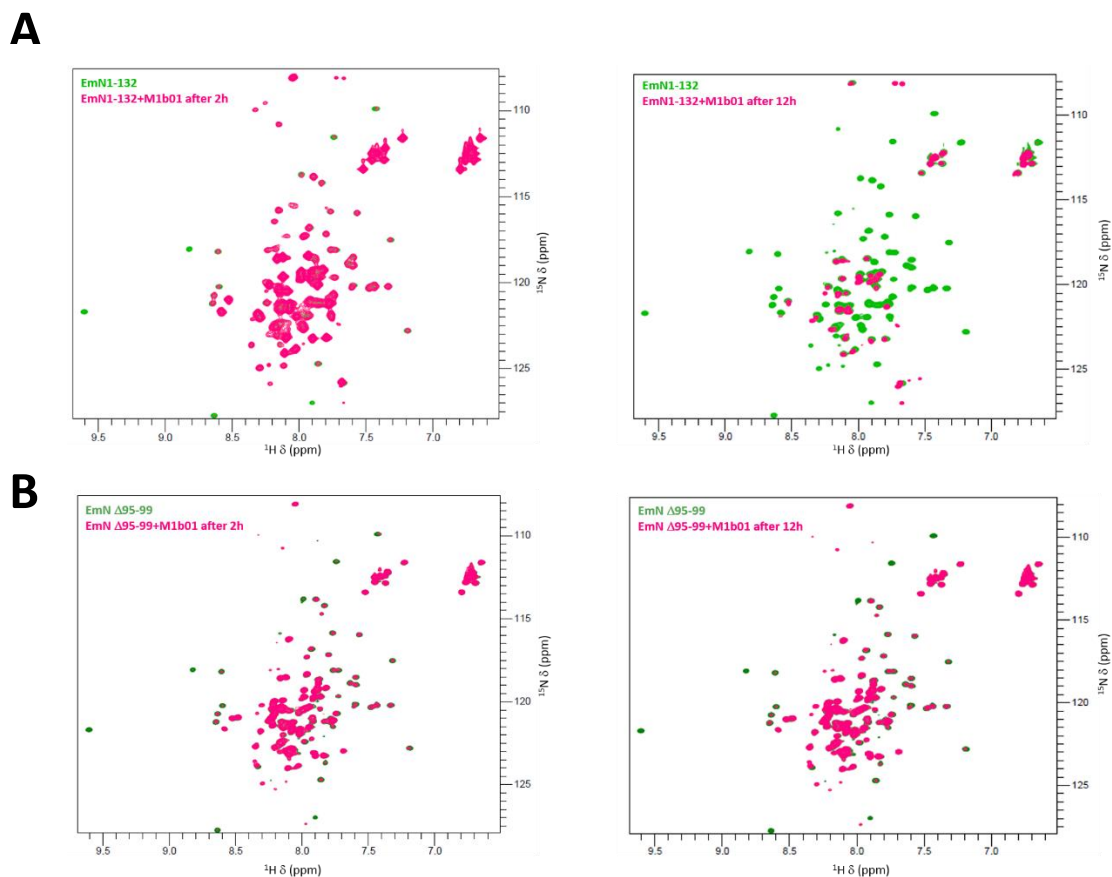


**Figure 94 : Observation of the interaction between EmN and M1b01 after 1 or 12h of contact, by size exclusion chromatography.**

Each gel corresponds to the result of one size exclusion chromatography. Proteins were injected in a volume of 500 $\mu$ l on a column GF Superdex 200 10/300GL, equilibrated in a buffer containing 50mM Tris-HCl pH7.5, 100mM NaCl, 5mM EGTA, 0.2mM DTT.

To understand this, first we observed interaction of M1b tails with an emerlin construct that can form oligomers and filaments, EmN132, and with an EmN mutant that cannot form filaments, mutant  $\Delta$ 95-99. Both emerlin constructs were able to interact (figure 95). This result was in accordance with our other results, which showed that the presence of the LEM domain is sufficient for binding.

Concerning the oligomerization question, it was interesting to observe that after 1h, signal intensity decrease was the same for both emerlin constructs, whereas after 12h of interaction, the signal decreased dramatically in presence of EmN132 but did not change in presence of EmN  $\Delta$ 95-99. We hypothesized that the presence of M1b01 could induce emerlin oligomerization.



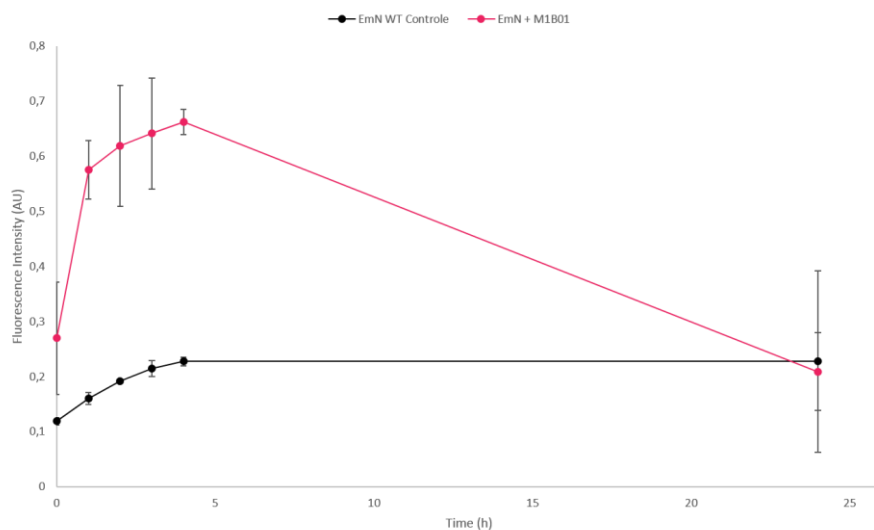
**Figure 95 : Study of EmN132 and EmN  $\Delta$ 95-99 interaction with M1b01, by NMR.**

On the left, spectrums were recorded after 1h of interaction and on the right, after 12h. (A) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a EmN132 sample at  $100\mu\text{M}$  alone (in light green) or in presence of  $100\mu\text{M}$  of M1b01 (in pink), in 50mM Tris-HCl pH7.5, 100mM NaCl, 5mM EGTA, 0.2mM DTT, at 293K and 700MHz (CEA Saclay). (B) Superimposition of 2D NMR  $^1\text{H}$ - $^{15}\text{N}$  spectra recorded on a  $\Delta$ 95-99 sample at  $100\mu\text{M}$  alone (in dark green) or in presence of  $100\mu\text{M}$  of M1b01 (in pink), in 50mM Tris-HCl pH7.5, 100mM NaCl, 5mM EGTA, 0.2mM DTT, at 293K and 700MHz (CEA Saclay).

To study the impact of M1b01 on emerlin oligomer formation, I followed the self-assembly kinetics of EmN WT alone or in the presence of M1b01, through thioflavin T fluorescence measurement (figure 96). For this experiment, both proteins were dialyzed in the kinetics buffer (50mM Tris-HCl pH7.5, 100mM NaCl, 5mM EGTA, 0.2mM DTT), and then EmN was concentrated up to  $600\mu\text{M}$  and M1b01 was concentrated up to  $200\mu\text{M}$ .

I prepared two samples: one with emerin alone at 300 $\mu$ M and one with emerin at the same concentration, in the presence of 100 $\mu$ M of M1b01. Samples were heated during one day at 310K and I took a fluorescence measurement at 480nm, after excitation at 440nm, every hour during 4h and the next day. For each measurement, we diluted the protein sample to 40 $\mu$ M in the kinetics buffer and added 10 $\mu$ M of thioflavin T.

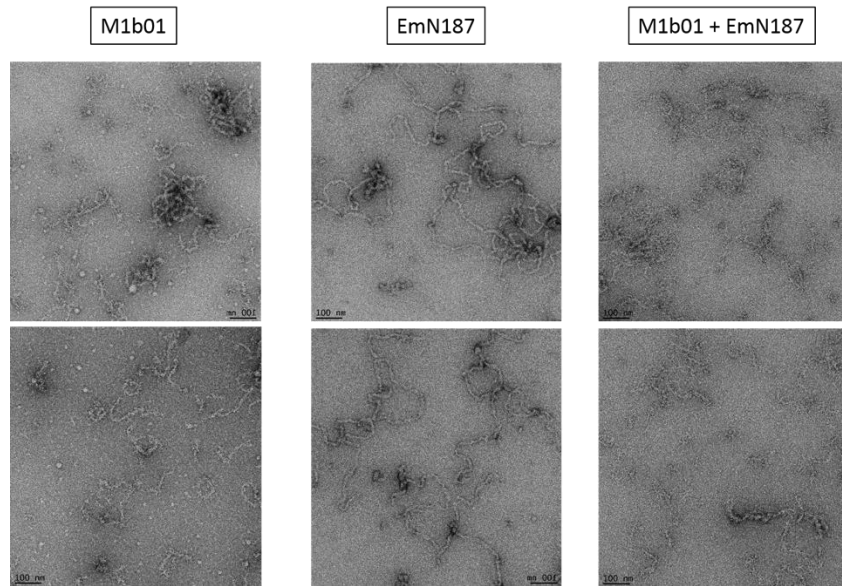
Interestingly, we observed that in presence of M1b01, EmN seemed to form oligomers faster than in absence of this partner.



**Figure 96 : Kinetics of emerin self-assembly in the absence (black) or presence (pink) of M1b01, as followed by thioflavin T fluorescence.**

To confirm our hypothesis, we observed, thanks to a collaboration with the group of Dr Jean Lepault, the impact of M1b01 on emerin oligomerization by negative staining EM. We analyzed a sample of M1b01 alone concentrated at 100 $\mu$ M, of emerin 1-187 alone concentrated at 100 $\mu$ M and of both proteins, concentrated at 100 $\mu$ M (figure 97).

We observed the presence of filaments in the EmN alone sample, and we found that M1b01 alone also seemed to form oligomers. But when emerin was in the presence of M1b01, oligomers of this myosin seemed to be present but no emerin filaments were present anymore. We concluded that the most probable hypothesis was that M1b01 could form oligomers and these kinds of oligomers could bind to EmN overtime.



**Figure 97 : Study of the interaction between emerin 1-187 and M1b01, observed by electron microscopy.**

**We used ammonium acetate to label our sample, using the technique of negative staining. All proteins were dialyzed in the same buffer (50mM Tris-HCl pH7.5, 100mM NaCl, 5mM EGTA, 0.2mM DTT) and concentrated at 100µM. 0.6mg/ml of proteins were put on the grids.**

To conclude, we found an interaction between EmN and myosin, and more specifically, we found that the LEM domain of emerin could interact with the tested M1b tail constructs (M1b01 and M1b04). Then, we found that M1b01 alone seemed to form oligomers that could carry EmN overtime, but we need to confirm this result. Finally, we can hypothesize that an interaction between emerin and myosin is possible at the nuclear envelope and contributes to the formation of a complex with actin and the LINC complex during the nucleus response to a mechanical stress.



# DISCUSSION AND CONCLUSION

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Proteins of the inner nuclear envelope have several essential functions in different cellular processes. Some of these proteins contribute to the nuclear shape and positioning; they play a role in the regulation of nuclear mechanical properties. Some contribute to genome organization and gene expression regulation. Finally, they may participate to the DNA damage response.

During my thesis, I mainly focused on the role of three proteins, emerin, lamin A and BAF, first because those three were shown as implicated in the regulation of nuclear shape and mechanical resistance. Indeed, emerin is phosphorylated during a mechanical stress, in order to increase nucleus stiffness<sup>107</sup>. In response to a mechanical stress, lamin A is post-translationally modified and exhibits a partial unfolding of its Igfold domain<sup>94</sup>. Lamin A/C deficiency is associated with both defective nuclear mechanics and impaired mechanically activated gene transcription<sup>95,97</sup>. Finally, BAF interacts with chromatin in order to regulate nuclear assembly and organization<sup>116,268</sup>. In addition, the three proteins interact together at the inner nuclear envelope<sup>55,172</sup>.

Despite of this, only few molecular details are known concerning these proteins and their functions. I chose to focus first on emerin structure, oligomerization and post-translational modifications in order to understand how this protein could play a role in the nucleus response to mechanical stress and to understand how its function is regulated in this situation. Then, I studied emerin interaction with lamin and BAF in order to understand how these interactions contribute to emerin function.

## I. Emerin nucleoplasmic region forms oligomers *in vitro*

As a large number of other inner nuclear membrane proteins, emerin exhibits large regions predicted to be intrinsically disordered<sup>4</sup>. In addition, this protein possesses an N-terminal globular domain, the LEM domain, which connects emerin to a group of proteins possessing this LEM domain and sharing the ability to bind lamins and tether repressive chromatin at the nuclear periphery<sup>269</sup>. Finally, emerin LEM domain is known to interact with the DNA binding Barrier-to-Autointegration protein (BAF)<sup>52</sup>.

When I began my thesis, a proteome study focused on liver nuclear envelope had shown, using a semi-quantitative measurement of protein abundance by mass spectrometry that the emerin/A-type lamin ratio was about 0.03<sup>270</sup>. From this ratio, it can be estimated that there is about 50,000 emerin molecules per nucleus (which corresponds to an approximate concentration of 10-100  $\mu$ M). These molecules are concentrated at the inner nuclear membrane. They were reported to self-associate, *in vitro* and *in cell*, through different mechanisms<sup>165</sup>. Several intra and/or intermolecular interactions were observed within emerin molecules, suggesting that changes in emerin oligomeric states could be responsible for the regulation of emerin binding properties at the inner nuclear envelope. Two different configurations were proposed depending on emerin LEM domain interactions. A down configuration in which the LEM domain interacts with BAF and an up configuration, in which the LEM domain is implicated in emerin self-assembly.

At the beginning of my thesis, I tried to characterize emerin structure in solution, working together with another PhD student, Dr Isaline Herrada. For this, we used different emerin constructs (1-49, 1-132, 67-170, 67-187, 67-221 and 1-187). After assignment of emerin 1-170 NMR chemical shifts, we confirmed that emerin monomers are composed of only one globular part, the LEM domain, which is followed by a large disordered fragment<sup>250</sup>. Then, we showed that more than monomers, emerin 1-187 (EmN) and emerin 67-221 (EmC221) can form higher molecular weight oligomers *in vitro*.

First, I only focused on EmN oligomers. By electron microscopy, Isaline and I observed that EmN forms some spherical particles and curvilinear filaments of 10nm of diameter. Then, using Fourier Transform Infra-Red spectroscopy and thioflavin T fluorescence, we observed that these filaments are composed of  $\beta$ -structure<sup>4</sup>. To find which part of emerin was important for filament formation and to understand the role of the LEM domain in emerin self-assembly, we followed the oligomerization kinetics of different emerin constructs (see above) and found that EmN132 (1-132) was sufficient for oligomerization whereas EmC187 (67-187) cannot form filaments anymore. In addition, using solid-state NMR as well as limited proteolysis and mass spectrometry, we demonstrated that the LEM domain, as it was suggested by the group of Dr K. Wilson, can be found either as a globular domain in a monomeric EmN fragment or as part of the structural core of long curvilinear filaments formed by EmN<sup>259</sup>.

It is interesting to observe that despite the amount of intrinsic disorder present outside of the LEM domain, a 10 kDa fragment is proteolytically protected. This could be due to an interaction between the LEM domain and a part of the unfolded region that forms a  $\beta$ -sheet with the LEM region, thus creating the EmN filament core.

Finally, we wondered if EmN oligomers are reversible. Indeed, we observed that dilution did not change our EmN oligomer structure *in vitro*, so our hypothesis is that maybe, *in cell*, their stability could be modified by the presence of the membrane or by post-translational modifications such as phosphorylations.

Another important experiment could have been done. Because thioflavine fluorescence was a good method to study our oligomers *in vitro*, we thought that it could be interesting to observe if thioflavine could bind EmN oligomers *in vivo*. Our collaborator, Brigitte Buendia, from Diderot University, tried once to observe thioflavine fluorescence *in vivo* in presence of EmN oligomers, without success. But first, it could be due to a protocol problem because no positive controls were done or it could be due to transitory interactions between thioflavine and EmN oligomers.

NMR analysis of the EmN mutant  $\Delta$ K37 - this mutation causing cardiac defects - revealed first that the LEM domain of this mutant is destabilized and, interestingly, that this mutant forms oligomers faster than EmN WT.

After confirmation that the resulting oligomers have a structural organization similar to that of EmN WT oligomers (using solid-state NMR, electron microscopy and limited proteolysis), we suggested that because the first step of emerin self-assembly is largely facilitated by deletion of K37, a rearrangement of the LEM domain structure is necessary for EmN oligomer assembly.

In parallel, together with another PhD student from the Institut de Myologie (Paris), Nada Essawy, I worked with two other mutants showing a defect in emerin LEM domain and associated with the same symptoms as  $\Delta$ K37: mutants P22L and T43I (G. Bonne, personal communication). We observed, using thioflavin T fluorescence and negative staining EM, that these two mutants form, as  $\Delta$ K37, filaments faster than EmN WT, and that their filaments are structurally similar to that of EmN WT. Contrariwise to the mutant  $\Delta$ K37, these mutants exhibit a well folded LEM domain. We concluded that the kinetics rate increase observed for the three LEM domain mutants could be due to the fact that in these mutants the interaction between the LEM domain and the unfolded part of EmN is favored.

In addition, we studied self-assembly of 5 EmN mutants causing Emery-Dreifuss Muscular Dystrophy (S54F, Q133H, P183H/T and  $\Delta$ 95-99), using thioflavin T fluorescence and negative staining EM. In these cases, mutants self-assembled similarly or less than EmN WT. Even if the mutant Q133H seemed to form oligomers slower than EmN WT, only EmN  $\Delta$ 95-99 could not form oligomers anymore, *in vitro*. Interestingly, deleted amino acids of this mutant are localized in a region predicted as partially folded by Disopred2.0 software and by NMR chemical shift analysis.

Self-assembly of emerin WT and mutants was then studied in cells, using *in situ* Proximity Ligation Assay (PLA). Consistently with our *in vitro* results, emerin WT and two mutants (P183T and  $\Delta$ K37) were found to self-associate, whereas emerin  $\Delta$ 95-99 and Q133H created less emerin-emerin proximities than emerin WT at the nuclear periphery of transfected cells.

Finally, together with another PhD student Florian Celli, I observed that another construct of emerin, which did not contain the LEM domain, EmC221 (67-221), could form oligomers *in vitro*. Based on thioflavin T fluorescence and EM studies, we found that EmC221 could form similar curvilinear filaments of 10nm of diameter as EmN, but with a faster self-assembly kinetics. Thus, two types of self-assembly mechanisms can be described for emerin. We could never purify the whole nucleoplasmic region of emerin, from aa 1 to aa 221, comprising both EmN and EmC221 fragments.

This region systematically aggregated during the dialysis against a buffer without urea. Such aggregation process might be due to a bad timing of the different self-assembly interaction steps in the absence of membrane anchoring.

## 1. EMERIN OLIGOMERS DIRECTLY BIND LAMIN A/C

Co-immunoprecipitation assays<sup>166</sup>, surface plasmon experiments<sup>170</sup> and yeast two hybrid assays<sup>166</sup> showed that a direct interaction exists between lamin A and emerin.

Then, blot overlay assays revealing emerin binding to lamin A showed that mutations in emerin region from aa 70 to aa 170 inhibit lamin A binding<sup>245</sup>. On the opposite, mutations of LEM domain residues 24 to 27 into alanine seem to favor lamin A binding. A later report from the same group revealed that two emerin regions can independently bind to mature lamin A tail: regions 1-132 and 159-220, with fragments 119-132 and 159-169 being necessary for these interactions, respectively<sup>165</sup>.

During my thesis, I studied this emerin-lamin interaction and found first, that emerin monomers could not bind directly to the lamin A/C tail. It was a very contradictory result compared to results obtained by other groups. Our first hypothesis was that the putative presence of emerin aggregates in the samples with a low concentration of urea at the end of the purifications reported in the literature<sup>165,190</sup> could favor a direct interaction with the nuclear lamina. A second hypothesis was that because one of these experiments used a GST-emerlin construct purified in urea<sup>165</sup>, the fusion protein was not well folded. Also, we tried to produce this kind of construct and it was not soluble in our conditions. Finally, our last hypothesis was that emerlin forms oligomers that could bind lamin A/C.

Finally, we confirmed that EmN oligomers can directly bind to the Igfold domain of lamin A/C. In more details, only EmN oligomers, and not EmC221 oligomers, were found to interact with the Igfold. The LEM domain is necessary for EmN self-assembly, which is essential for EmN binding to lamin A. Destabilization of the LEM domain through deletion of K37 favors EmN self-assembly. This is consistent with the observation of the group of K. Wilson that mutation of the hydrophobic core of the LEM domain (G24-P25-V26-V27 into AAAA) favors lamin A binding<sup>245</sup>. Altogether these data strongly suggest that destabilization of the LEM domain is necessary for EmN self-assembly and subsequent lamin A binding. We did not observe such emerlin – lamin interaction in the case of laminB1 tail; K. Wilson and co-workers observed only a weak binding of emerlin regions 1-132 and 159-220 to the lamin B1 tail<sup>165</sup>. I conclude that this interaction between emerlin and lamins is specific to EmN oligomers and the Igfold of lamin A/C.

As EmN mutant  $\Delta$ K37 self-assembles faster than EmN WT, I hypothesized that it still binds to the Igfold of lamin A/C. This was confirmed *in vitro* by NMR. Concerning the two other LEM domain mutants P22L and T43I, Nada Essawy will now try to characterize their interaction with the Igfold of lamin A/C.

Then, because EmN  $\Delta$ 95-99 does not self-assemble in our conditions, we thought that this mutant will not interact with lamin A/C *in vitro* and *in cell*. Concerning the *in vitro* part, we showed by NMR that this mutant cannot interact directly with the Igfold of laminA/C. Then, *in cells*, by PLA, while emerlin WT and P183T were shown to be in closed proximities with lamin A/C, for variants Q133H and  $\Delta$ 95-99, the amount of emerlin-lamin A/C proximities is decreased, suggesting that emerlin self-assembly defects impact lamin recognition in cells<sup>4</sup>.

In order to identify which part of the Igfold is involved in EmN oligomer binding, we decided to produce several <sup>15</sup>N labelled mutated Igfolds and to observe the impact of addition of EmN oligomers on their <sup>1</sup>H-<sup>15</sup>N HSQC spectrum. This idea was to find mutants that could not bind to EmN oligomers. We tested mutants that cause diseases (R435C and R471C) and mutants of the parallel  $\beta$ -sheet region that is characteristic of the lamin fold (T496E, H506E, P509A and V513A)<sup>42</sup>. We observed by NMR, that R435C, R471C, T496E, P509A and V513A significantly impaired binding to EmN oligomers, whereas H506E completely abolished the binding.

Altogether, my results demonstrate that a surface present on the lamin A/C Igfold domain binds to self-assembled emerlin fragment EmN and not EmC221 and this lamin-emerlin interaction seemed to be specific to the Igfold domain of lamin A/C.

## 2. IMPACT OF PTM ON EMN SELF-ASSEMBLY

Emerlin was proposed to be phosphorylated by the Src kinase in response to a mechanical stress<sup>107</sup>. In more details, Src kinase phosphorylates emerlin on tyrosines 74 and 95 and mutations of these two residues impair nuclear response during application of a mechanical force on the nucleus. In addition, lamin A/C is recruited to the LINC complex in order to reinforce nuclear stiffness. More recently, it was also proposed that application of a force on the nucleus increases emerlin level at the outer nuclear membrane without altering emerlin protein level<sup>249</sup>.

During the first year of my thesis, I confirmed *in vitro*, using NMR and mass spectrometry, that EmN is phosphorylated by Src on tyrosines 74 and 95. Interestingly, the emerlin region phosphorylated by Src is important for EmN self-assembly. Indeed, EmN  $\Delta$ 95-99 cannot form oligomers in our conditions. Moreover, this region is important for direct binding between emerlin and lamin A/C.

Indeed, mutation of D70-D72-Y74 into alanines as well as deletion  $\Delta$ 95-99 impair lamin A binding<sup>245</sup>. On the bases of these different observations, I expressed two opposite hypotheses:

- (1) Because the region containing tyrosine 95 is important for EmN self-assembly, because only EmN oligomers bind lamin A/C and because recently, a study suggested that during a mechanical stress, emerlin level at the outer nuclear membrane increases, I hypothesized that phosphorylation of tyrosines 74 and 95 by Src could impair emerlin oligomer assembly and thus disrupt emerlin binding to lamin A/C. In these conditions, monomeric emerlin could be displaced to the outer nuclear membrane in order to let lamin A/C free to interact with the LINC complex, and thus to reinforce nuclear stiffness.
  
- (2) I have a second hypothesis that does not take into account the fact that emerlin level at the outer nuclear membrane increases during a mechanical stress (indeed this observation was reported only in one recent paper). Because EmN  $\Delta$ 95-99 cannot self-assemble or interact with lamin A/C, and because emerlin phosphorylation by Src was shown to be important for nuclear response during a mechanical stress, Src phosphorylation could favor emerlin self-assembly in order to increase emerlin-lamin binding at the inner nuclear envelope and thus allow interaction between this complex and the LINC complex in order to increase nuclear stiffness.

To understand the impact of Src phosphorylation, I tried to observe the influence of these phosphorylations on EmN oligomer assembly, with the aim of subsequently studying their effect on lamin binding.

First, I tried to follow EmN oligomerization kinetics using thioflavin T fluorescence on both a non-phosphorylated EmN sample and an EmN sample phosphorylated by Src. I did this experiment several times, but it was difficult to reproduce and interpret it.



This can be explained by two phenomena; first, because Src phosphorylations impact tyrosines and because it was reported that thioflavin T interacts preferentially with tyrosine-rich cross- $\beta$  surfaces, we thought that maybe, phosphorylation on tyrosines could impact thioflavin T fluorescence in the presence of phosphorylated EmN. In this case, this technique is not adapted to follow impact of Src on filament assembly.

But a second idea could be that our experimental conditions are not adapted. Indeed, to do this experiment, I used every time EmN samples that were in presence of  $MgCl_2$ , ATP and then, with or without Src, during 12h at 303K and before following oligomer kinetics, I did not change the buffer nor re-purify protein samples. This means that during kinetics, other factors, such as the presence of  $MgCl_2$ , could have an impact on oligomer assembly, or Src kinase present in the sample could sometimes still be active. For this reason, Nada Essawy, who will now work on this project, will try to reproduce this experiment, but will first phosphorylate EmN before cleavage of its tag, in order to re-purify each EmN sample, after the phosphorylation kinetics and before following self-assembly by thioflavin T fluorescence.

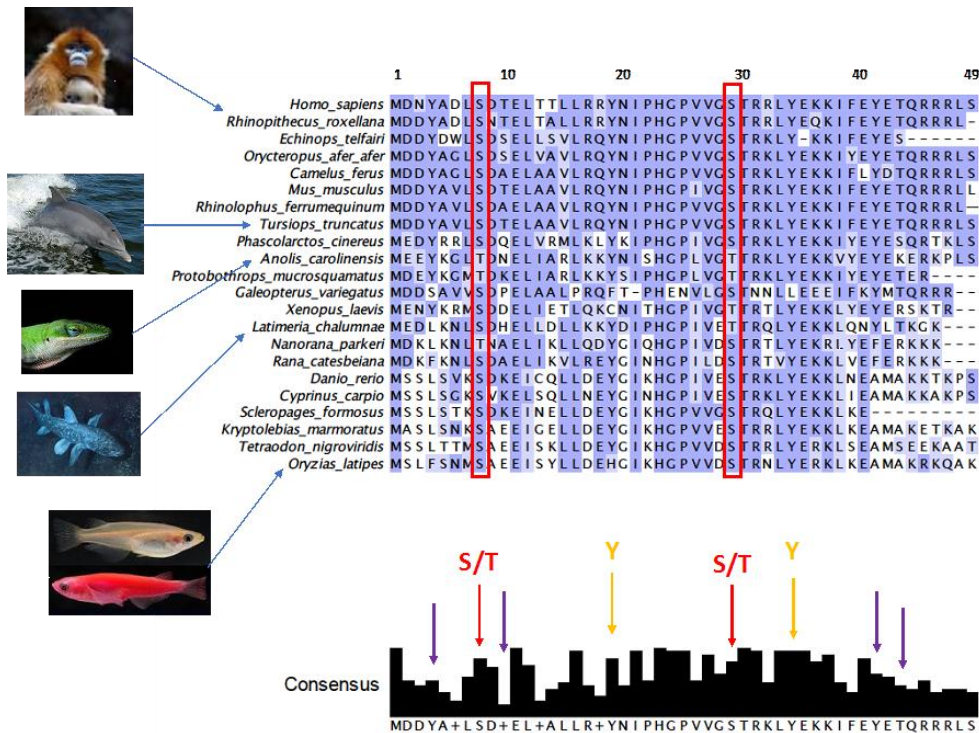
I also tried to observe the impact of Src phosphorylation on EmN self-assembly using SDS-PAGE and negative-staining EM. My first results suggested that Src phosphorylation impacts EmN oligomerization, because I observed the presence of filaments in my non-phosphorylated sample whereas aggregates were observed in the phosphorylated sample. This experiment has to be reproduced by Nada Essawy and again, re-purification of EmN after phosphorylation will be introduced in the protocol to avoid modifying the oligomerization kinetics because of the presence of additives related to the kinase in the buffer.

My preliminary results are in favor of my first hypothesis; Src phosphorylation seems to impair filament assembly, which could disrupt emerin-lamin binding during a mechanical stress and favor emerin displacement from the inner nuclear envelope to the outer nuclear envelope, in order to allow lamin A/C interaction with the LINC complex and thus reinforce nuclear stiffness.

To finish on emerin phosphorylation, I thought that in addition to their implication during a mechanical stress, phosphorylations could regulate emerin oligomerization and interactions also in a cell cycle context, like assembly or disassembly of the nuclear membrane during mitosis.

The LEM domain of emerin plays a critical role in EmN self-assembly. It is highly phosphorylated in cells (see <https://www.phosphosite.org/proteinAction.action?id=2624&showAllSites=false>) and protein sequence analysis of several emerin LEM domain from distinct animals (figure 98, A) shows that several of its serines, threonines and tyrosines are well conserved.

A



B

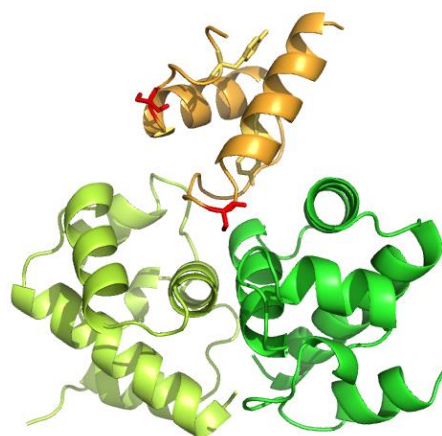


Figure 98 : Emrin LEM domain phosphorylation sites.

(A) The Phosphosite database reveals that Tyr4, Ser8, Thr10, Tyr19, Ser29, Tyr34, Tyr41 and Thr43 are phosphorylated in cells. (B) Emrin LEM domain 3D structure (PDB code: 2ODC) in orange, with conserved serines observed as phosphorylated represented in red.

In the case of emerin LEM domain, we were interested by Ser8 and Ser29, because they are conserved and phosphorylated in cells. Moreover, Ser8 is predicted to be phosphorylated by CK2; indeed, CK2 phosphorylates sites characterized by the motif S/T-X-X-E. Both serines are located at the N-terminus of a large  $\alpha$ -helix (figure 98).

Phosphoserine is the most stabilizing amino acid for  $\alpha$ -helices when located at the Ncap, N1, N2, and N3 positions, whereas it is destabilizing in the interior or at the C-terminus of the helix<sup>271</sup>. As LEM domain folding and EmN self-assembly are related, I hypothesized that phosphorylation of these serines might disfavor EmN self-assembly.

To go further, we hypothesized that EmN phosphorylation by CK2 could lead to the stabilization of the LEM domain structure, which will be in disfavor of filament assembly. Then, if CK2 phosphorylation disfavors EmN filament assembly, this phosphorylation could impair lamin-emerin binding.

During my thesis, I did preliminary studies of EmN phosphorylation by CK1 and CK2 as well as in cell extracts *in vitro* using NMR. No phosphorylation was observed *in vitro* with CK1 and CK2, in our conditions. We have to improve our phosphorylation conditions to go further into the study of the impact of phosphorylation by CK2 on emerin self-assembly and lamin binding.

Concerning emerin phosphorylation in cell extracts, I confirmed by NMR that EmN132 is modified after addition of cell extracts (293T cell extracts) and more, that the LEM domain seemed highly modified after addition of cell extracts. To go further into this study, we can imagine to test different protocols of phosphorylation in cell extracts. Because emerin mutations cause myopathies, it would be interesting to observe emerin modifications in different cell types, notably in myoblasts. Then, we can imagine to observe emerin modifications during specific cell cycle phases or after a mechanical stress.

## II. Nuclear envelope interactions that could be important for structure and mechanical properties of the nucleus

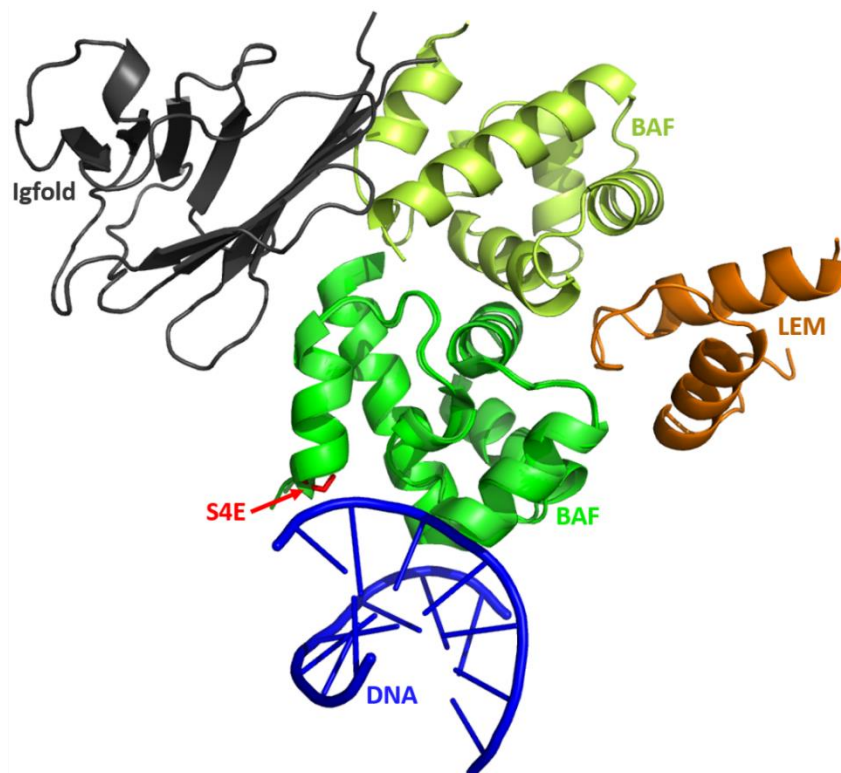
### 1. A FIRST MODEL OF THE INTERACTION BETWEEN NUCLEAR ENVELOPE AND DNA

An important part of my thesis was focused on the emerin-lamin interaction. Indeed, it was well established that both proteins were interacting directly *in vitro* and in cells and but no molecular details were known. As I already explained, we confirmed that a direct interaction exists between emerin oligomers and the Igfold of lamin A/C. In addition, it was reported that the two proteins could interact through a third one, BAF, in order to form a ternary complex<sup>175,253,254</sup>. Concerning the emerin-BAF interaction, the 3D structure of this complex was already solved by NMR<sup>52</sup>; it was shown that the LEM domain of emerin can bind a dimer of BAF.

We decided to work on these interactions using several techniques such as NMR, ITC or gel filtration and we confirmed first that the LEM domain of emerin is able to bind BAF with a ratio 1:2. Then, we found that the Igfold of lamin A/C can bind a dimer of BAF too and more, we observed that the three proteins form a ternary complex. In addition, no interaction between the lamin B1 tail and BAF was observed, which demonstrated that the interaction between lamin and BAF is specific to the Igfold of lamin A/C. Finally, we obtained the X-ray structure of the ternary complex by crystallography and confirmed that the lamin A/C Igfold domain, in addition to directly interact with EmN oligomers, could also interact with the emerin LEM domain through a dimer of BAF. Our result are consistent with the observation by the group of K. Wilson that binding of <sup>35</sup>S-emerin to prelamin A tail-containing strips was enhanced fourfold by wild-type BAF<sup>172</sup>.

I superimposed our X-ray structure with the one already available for BAF in interaction with DNA (PDB code: 2BZF)<sup>111</sup> in order to propose a first model of the interaction between the nuclear envelope and chromatin (figure 98). This model suggests that the BAF dimer is able to simultaneously bind to DNA, emerin and lamin A/C. In addition, it led me to another observation. Indeed, whereas BAF WT increases binding of <sup>35</sup>S-emerin to prelamin A tail, BAF S4E has no effect on this binding<sup>172</sup>.

In addition, binding of BAF to the prelamin A tail is also significantly reduced in the case of BAF mutant S4E. And finally, in cells that overexpress BAF (that is phosphorylated by endogenous kinases) or BAF-S4E, but not BAF-S4A, emerin fails to localize at the nuclear envelope. Because of these different results. I observed the position of Ser4 on BAF to define its localization compared to the emerin and lamin binding surfaces (figure 99). Surprisingly, this serine did not localize on BAF binding surfaces to the emerin LEM domain or the Igfold of lamin A/C but on the BAF binding surface to DNA. Consistently, it was reported that phosphorylation of BAF Ser4 impairs BAF binding to DNA<sup>172</sup>. I now wonder why a phosphorylation that affects BAF binding to DNA can disrupt formation of our ternary complex. In addition, BAF interacts directly with histones H3 and H4 and more, three methyl marks (H3-K4-Me2, H3-K9-Me3, H3-K79-Me2) are increased in BAF-overexpressing cells<sup>272,273</sup>. To go further into our structural analysis of the interaction between the nuclear envelope and DNA, it would be important to understand how our ternary complex lamin/BAF/emerin interacts with DNA and histones.



**Figure 99 : A first model of the interaction between nucleoskeleton and DNA at the inner nuclear envelope.**

To obtain this model, I superimposed my X-ray structure to the 3D structure of BAF in interaction with DNA (PDB: 2BZF). Here, only one DNA fragment is represented, whereas in the crystal, both BAF monomers interact with DNA. In red is represented BAF Ser4 that is phosphorylated during the cell cycle.

My structural study also revealed that the Igfold of lamin A/C interacts with BAF through a surface containing residues mutated in progeroid syndromes. During the last part of my thesis, working with Ambre Petitalot, we tried to characterize the impact of these Igfold mutants, which cause different progeroid syndromes (R435C, K542N, R471C, R527C and A529V) on BAF binding *in vitro*. In parallel, Dr Brigitte Buendia (Uni Paris Diderot) was working on the impact of these mutations in cells, using *in situ* Proximity Ligation Assays. We observed *in vitro* that four mutations (R471C, R527H and A529V and K542N) decreased the affinity of the BAF/Igfold interaction whereas one mutation (R435C) seemed to completely disrupt the interaction. *In vivo*, less proximities between BAF and each mature lamin A mutants (R435C, K542N, R527C and A529V) were observed compared to lamin A WT.

To go further, in the well-studied progeria syndrome called Hutchinson-Gilford Progeria Syndrome (HGPS), because of a deletion of 50 amino acids in the C-terminal region of prelamin A, containing a critical site for maturation<sup>37,38</sup> or because of a mutation in the ZMPSTE24 enzyme, involved in lamin A maturation<sup>36,274</sup>, lamin A exhibits a maturation defect. The presence of a permanently farnesylated prelamin A mutant called progerin is then observed in cells. The lamin mutation causing progerin is heterozygous, which means that only part of the lamin A proteins possesses a maturation defect in HGPS, but this is sufficient to cause the accelerated aging disease. After my structural study, I thought that maybe a common mechanism exists between autosomal recessive progeroid syndromes due to a homozygous mutation in the BAF binding region and the autosomal dominant HGPS disease. First, I hypothesized that (1) the farnesylated prelamin A (progerin) could bind to BAF, which could induce a defect in mature lamin A binding to BAF. Consistent with this hypothesis, different prelamin A forms were shown to interact with BAF *in vivo*<sup>218</sup>. In addition, it was demonstrated that progerin could bind BAF in HEK293 cells<sup>219</sup>. Finally, it was shown that a mutation in ZMPSTE24 (which normally cleaves the farnesylated lamin C-terminus) favors BAF nuclear localization<sup>275</sup>. It was also shown that the presence of progerin induces a loss of heterochromatin and H3K9 trimethylation<sup>151,152,213,214</sup>, whereas BAF was shown to bind H3 in order to increase methyl marks<sup>272,273</sup>. We deduced that presence of progerin could impair, in addition to BAF-lamin A/C binding, BAF binding to histone H3.

But a second hypothesis is possible. (2) Indeed, a defect in lamin-BAF interaction could induce a partial defect in prelamin A maturation that would provoke the accelerating aging syndrome.

Consistently with this idea, it was observed that prelamin A is accumulated in cells from a patient with mutation R527H causing mandibuloacral dysplasia<sup>275</sup>.

During the end of my thesis, we did preliminary studies about the impact of lamin maturation defects on the BAF-lamin interaction. I produced two different prelamin A constructs (IgfoldProgerin [389-614] and ProgerinCter [567-614]) in order to test their ability to bind BAF *in vitro*, using ITC and NMR. First, I observed that the fragment IgfoldProgerin, corresponding to the lamin A tail in HGPS, could bind BAF with the same affinity and stoichiometry than the Igfold alone and more, the same binding surfaces were identified. Then, I tried to characterize the interaction between BAF and the C-terminus of the progerin alone (ProgerinCter), by NMR and ITC, but no clear and strong interaction was identified. These experiments have to be reproduced. In addition, both progerin peptides that I used were not farnesylated, so Florian Celli will try to co-express the two constructs of progerin with a farnesyltransferase, in order to understand the impact of farnesylation on progerin-BAF binding and then, to observe if farnesylation induces a defect in BAF-histone binding.

To conclude about emerin-lamin binding, I showed that the lamin A/C Igfold domain interacts with the inner nuclear membrane protein emerin through two mechanisms: it either binds directly to self-assemble EmN or it interacts with monomeric EmN through the chromatin-associated protein BAF (figure 100). We hypothesized that both mechanisms could be regulated by post-translational modifications.



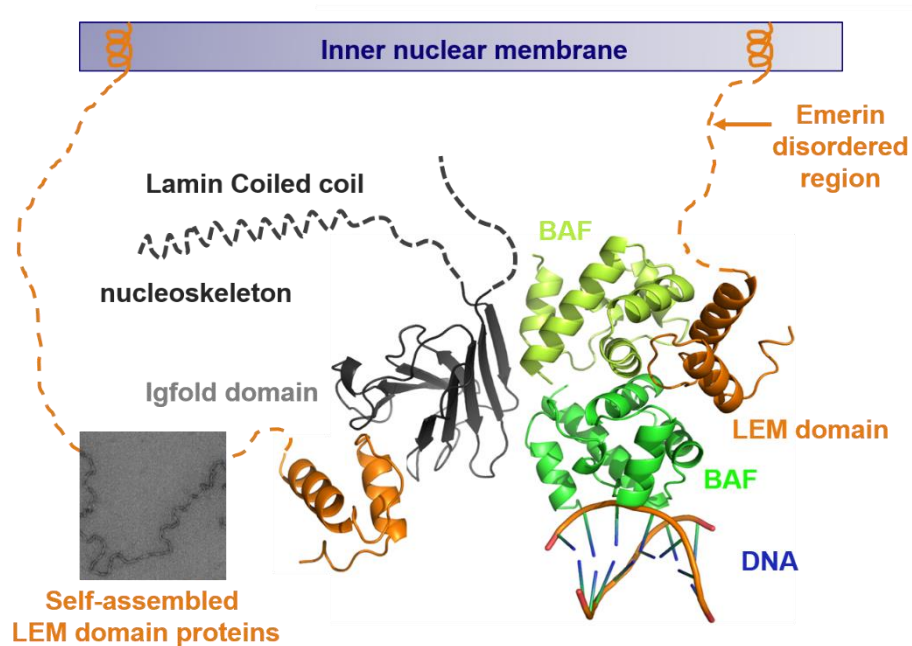


Figure 100 : Lamin A/C Igfold domain interacts with the inner nuclear membrane protein emerin through two mechanisms.

## 2. EMERIN INTERACTS WITH MYOSIN1B

During my thesis, I also did a collaboration with the group of Dr Anne Houdusse (Institut Curie, Paris), in order to observe if emerin could bind myosin 1 tail *in vitro*. Several results have prompted us to be interested in this possible interaction. First, nuclear myosin I was shown to be localized in the nucleus, by ultrastructural studies<sup>263</sup>. Then, proteomic studies revealed that emerin binds to nuclear myosin I<sup>267</sup>.

We searched for a direct interaction between different constructs of our emerin (1-187, 1-132, 1-49 and 67-187) and 3 constructs of their myosin tails (M1b01, M1b04 and M1c01), associated with calmodulin, *in vitro*. First, we found a direct interaction between emerin and myosin 1 tail, and more, we observed that this interaction was specific to myosin 1b; indeed, no interaction was observed between M1c01 and emerin. In addition, we found that the LEM domain was necessary and sufficient for binding to myosin 1b tail. Furthermore, we observed protein oligomerization during emerin binding to myosin1b. Two hypotheses were possible. (1) First, we thought that myosin 1b tail binding to emerin could favor emerin oligomerization. (2) Then, we supposed that instead of emerin, maybe myosin 1b tail could form oligomers that could carry emerin overtime.



After several *in vitro* studies, using thioflavin T fluorescence, NMR and electron microscopy, it seemed that the second hypothesis is the real one, but we have to confirm our results.

Our hypothesis concerning the existence of an interaction between emerin and myosin at the nuclear envelope is that this complex could contribute to the formation of a higher molecular weight complex with actin in order to maintain the structural integrity of the nucleus. Consistent with our hypothesis, a very recent study demonstrated that emerin binds to non-muscle myosin IIA at the outer nuclear membrane in order to increase F-actin polymerization at this site<sup>249</sup>.

# MATERIAL AND METHODS

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# I. PLASMID CONSTRUCTS

## 1. EMERIN NUCLEOPLASMIC FRAGMENTS

All emerlin fragments (1-187, 67-221, 67-187 and 1-49) were expressed as N-terminal octahistidine fusions with a TEV cleavage site between the tag and the emerlin fragment. Expression vectors coding for these fragments were purchased from Genscript, who cloned an optimized emerlin gene into a pETM-13 vector provided by the lab. All emerlin mutant expression vectors ( $\Delta$ K37, P22L, T43I, S54F, Q133H, P183H, P183T and  $\Delta$ 95-99) were obtained by mutagenesis using the Quikchange (Agilent) kit from the WT emerlin 1-187 expression vector. Only mutant Y4C-E35C was expressed by mutating the vector coding for WT emerlin 1-132.

## 2. LAMIN A/C IGFOLD FRAGMENT (TAIL REGION COMMON TO LAMINS A AND C)

The pGEX-4T vector coding for the Igfold of WT lamin A/C (411-566) was a gift from the lab of Prof Howard J. Worman (Department of Medicine, College of Physicians & Surgeons, Columbia University, New York). The resulting protein contained a GST tag, a thrombin cleavage site and a lamin A/C fragment from amino acid 411 to amino acid 566. All mutant expression vectors (A529V, A529T, K542N, M540T, T528M, R435C, R471C, T496E, W498E, P508A, P509A, H506E, V513A and R482W) were obtained by mutagenesis using the Quikchange (Agilent) kit from the WT expression vector.

## 3. IGFOLDPROGERIN (PROGERIN TAIL)

The tail region from progerin was also expressed using a pGEX-2T expression vector provided by the laboratory of Dr Brigitte Buendia from Université Paris Diderot. The resulting protein contained a GST tag, a thrombin cleavage site and the C-terminal progerin fragment from amino acid 389 to amino acid 614.

## 4. LAMINB1 TAIL

The tail region of lamin B1 was also expressed using a pGEX-4T expression vector provided by the laboratory of Prof Howard J. Worman (Department of Medicine, College of Physicians & Surgeons, Columbia University, New York). The resulting protein contained a GST tag, a thrombin cleavage site and the C-terminal progerin fragment from amino acid 409 to amino acid 586.

## 5. BAF

BAF<sub>CtoA</sub> was expressed using a pET-M13 vector. The BAF<sub>CtoA</sub> gene was optimized for *E. coli* production (Genscript). The resulting protein contained an N-terminal octa-histidine tag, a TEV cleavage site and BAF<sub>CtoA</sub>. All BAF mutants (S4E, A12T and G47E) were expressed after mutagenesis with the Quikchange (Agilent) kit of the BAF<sub>CtoA</sub> expression vector.

## 6. 1U2

The 1U2 domain of Man1 was expressed using a pTEM10 vector, optimized for *E. coli* production (Genscript). The resulting protein contained an N-terminal octa-histidine tag, a TEV cleavage site and 1U2.

## 7. MYOSIN

The different myosin fragments were produced by the team of Dr Anne Houdusse (Institut Curie, Paris).

# II. BACTERIAL CULTURES

## 1. IN RICH MEDIUM

For all proteins, expression was performed in *E. coli* BL21 (DE3) Star. The starter culture was done at 310K overnight in LB medium containing antibiotics.

For Igfold, IgfoldProgerin, LaminB1 and 1U2 fragments, bacterial cultures were grown at 310K in LB medium with antibiotics, and after addition of the starter culture and induction at an OD of 0.8 with 0.5mM IPTG, cultures were grown at 293K overnight. For the ProgerinCter construct, bacterial cultures were induced at an OD of 0.8 with 0.5mM IPTG, and were grown at 310K during 4 hours. For the other fragments, bacterial cultures were induced at an OD of 1, with 0.5mM IPTG, and grown at 293K overnight.

Afterwards, cultures were centrifuged at 3500 rpm during 30 minutes and each pellet was resuspended in 20ml of Lysis buffer (50mM Tris-HCl pH 8, 300mM NaCl, 5% glycerol, 1% Triton TX-100 and 10mM PMSF) before being frozen at -80°C.

## 2. IN MINIMAL MEDIUM

This production protocol is used in two cases: to increase protein solubility and to label the protein with  $^{15}\text{N}$  or  $^{13}\text{C}$  for NMR experiments.

The production protocol was the same as in rich medium, except that the LB medium was replaced by a minimal medium which is composed of:

- 100ml M9 10X (60g  $\text{Na}_2\text{HPO}_4$ , 30g  $\text{KH}_2\text{PO}_4$ , 5g NaCl)
- 2ml Trace Element 500X (EDTA 5 g /L 13.4mM,  $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$  0.83 g/L 3.1mM,  $\text{ZnCl}_2$  84 mg/L 0.62mM,  $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$  13 mg/L 76 $\mu\text{M}$ ,  $\text{CoCl}_2\cdot 2\text{H}_2\text{O}$  10mg/L 42 $\mu\text{M}$ ,  $\text{H}_3\text{BO}_3$  10mg/L 162 $\mu\text{M}$ ,  $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$  1.6 mg/L 8.1  $\mu\text{M}$ )
- 1ml  $\text{MgSO}_4$  1M
- 0.3ml  $\text{CaCl}_2$  1M
- 1mM of Biotine
- 1mM of Thiamine
- 2g Glucose
- 0.5g  $^{15}\text{NH}_4\text{Cl}$

### III. PROTEIN PURIFICATIONS

#### 1. EMERIN AND BAF PURIFICATION

Both proteins were produced in inclusion bodies. Therefore, purification protocols for the different emerin and BAF fragments are almost the same.

The first step of purification consisted of a lysis step.

##### 1) Lysis step

- Pellet thawing.
- Sonication in 3 cycles of 30 seconds, separated by 1 second, with a power of 70% and without any temperature constraint.
- Centrifugation at 20 000 rpm during 20 minutes.
- Pellet solubilization in buffer C8 (50mM Tris-HCl pH8, 150mM NaCl, 20mM Imidazole and 8M urea) during one hour at room temperature on a wheel.
- Centrifugation at 20 000 rpm during 20 minutes.
- Recovery of the supernatant (which contains urea).

##### 2) Purification

After the lysis step, the soluble extract is loaded onto a Ni-NTA column (GE-Healthcare) equilibrated with buffer C8. After two hours of incubation at room temperature, the column is washed with buffer C8 and then, proteins are eluted with buffer E8 (50mM Tris-HCl pH8, 150mM NaCl, 1M Imidazole and 8M urea). Proteins are then diluted at an OD of 1 for emerin fragments and an OD of 0.5 for BAF fragments, before being refolded by dialysis in D1 buffer (50mM Tris-HCl pH8, 30mM NaCl) for emerin fragments and buffer D2 for BAF fragments (50mM Tris-HCl pH8, 150mM NaCl), overnight.

The next day, proteins are dialyzed twice during two hours in the same buffer as before. Then they are concentrated to obtain 20ml. After addition of 2mM DTT and one TEV aliquot at 1mg/ml, proteins are incubated during 2h at room temperature in a falcon tube and deposited onto a Ni-NTA column. After column incubation during 30 minutes at room temperature, proteins are recovered in the flow-through and dialyzed in the selected buffer depending on the experiments.

## 2. IGFOLD PURIFICATION

Every Igfold construct (WT, mutants and IgfoldProgerin) was soluble in bacteria. Like for all proteins, the first purification step consists of a lysis step.

### 1) Lysis step

- Pellet thawing.
- Sonication in 3 cycles of 30 seconds, separated by 1 second, with a power of 70% and at 277K.
- Incubation with 2 $\mu$ l of benzonase (Sigma) and 100mM MgCl<sub>2</sub>, during 20 minutes at room temperature.
- Centrifugation at 20 000 rpm during 20 minutes.
- Recovery of the supernatant.

### 2) Purification

Because of their GST-Tag, the Igfold fragments were purified on a GSTrap FF column (GE Healthcare). The column is first washed with buffer A (50mM Tris-HCl pH8, 150mM NaCl, 1mM DTT). Then, proteins are added onto on the column and incubated during 2h at 277K on a wheel. After incubation, the column is washed with buffer A, followed by buffer B (50mM Tris-HCl pH8, 1M NaCl, 1mM DTT) to remove in particular DNA and again with buffer A. Finally, proteins are incubated in 10ml of buffer A and the thrombin protease is added overnight at 277K.

The next day, proteins are eluted in the flow-through, dialyzed in a selected buffer corresponding to further experiments and kept at 277K.

### 3. PROGERINCTER PURIFICATION

In the case of this construct, the lysis step is performed using the protocol described for the Igfold construct.

After the lysis step, the supernatant is loaded onto a GSTrap FF column (GE-Healthcare) equilibrated with buffer A. Then, proteins are incubated during 2h at 277K, on a wheel. After incubation, column is washed with buffer A, followed by buffer B (50mM Tris-HCl pH8, 1M NaCl, 1mM DTT) to remove DNA and again with buffer A. Afterwards, proteins are incubated in 10ml of buffer A and the TEV protease is added during 2h at 277K. Finally, proteins are recovered in the flow-through, dialyzed in a selected buffer corresponding to further experiments and kept at 277K.

### 4. LAMINB1 AND 1U2 PURIFICATION

In the case of these two constructs again, the lysis step is performed using the protocol described for the Igfold construct.

After the lysis step, the supernatant is loaded onto a Ni-NTA column (GE-Healthcare) equilibrated with buffer A. Then, proteins are incubated during 2h at 277K, on a wheel. After incubation, column is washed with buffer A, followed by buffer B (50mM Tris-HCl pH8, 1M NaCl, 1mM DTT) to remove DNA and again with buffer A. Afterwards, proteins are eluted with buffer E (50mM Tris-HCl pH8, 150mM NaCl, 1M Imidazole and 1mM DTT) and dialyzed against buffer A 3 times during 2 hours. The next day, the TEV protease is added during 2h at room temperature in a falcon tube, and the protein sample is loaded onto a Ni-NTA column during 30 minutes at room temperature. After column incubation, proteins are eluted in the flow-through and dialyzed in the selected buffer depending on experiments.



## IV. LIQUID NMR

NMR experiments were performed on 600, 700 and 750 MHz Bruker Avance spectrometers, all equipped with a triple resonance cryogenic probe. Two-dimensional  $^1\text{H}$ - $^{15}\text{N}$  correlation spectra were acquired using a HSQC pulse sequence at a temperature of 283K to 303K, on a 3-mm-diameter NMR sample tube, containing 80:20%  $\text{H}_2\text{O}/\text{D}_2\text{O}$ . All NMR data were processed using Topspin3.1 (Bruker) and analyzed with CCPNMR<sup>276</sup>.

### 1. ASSIGNMENTS OF THE NMR SIGNALS

#### *a. Emerin 1-170*

We assigned the NMR signals of the emerin fragment from residues 1 to 170 using two approaches. We acquired three-dimensional  $^1\text{H}$ - $^{15}\text{N}$ - $^{13}\text{C}$  correlation spectra, using 3D HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO and HN(CO)(CA)NH experiments (table 5) at 303 K, on a 5-mm-diameter NMR sample tubes containing, on one hand, 500  $\mu\text{M}$  uniformly  $^{15}\text{N}/^{13}\text{C}$ -labeled Emerin67-170, in 20mM Tris-HCl pH8, 30mM NaCl, 5mM DTT and 80:20%  $\text{H}_2\text{O}/\text{D}_2\text{O}$  and on the other hand, 500  $\mu\text{M}$  uniformly  $^{15}\text{N}/^{13}\text{C}$ -labeled EmN132, in 20mM Tris-HCl pH 8, 30mM NaCl, 5mM DTT and 80:20%  $\text{H}_2\text{O}/\text{D}_2\text{O}$ .

Spectrum	Dimensions	Observed atoms
HSQC	2D	N-H
HNCACB	3D	N-H+C $\alpha$ +C $\beta$ +(C $\alpha$ )-1+ (C $\beta$ )-1
CBCA(CO)NH	3D	N-H+(C $\alpha$ )-1+(C $\beta$ )-1
HNCO	3D	N-H+C from liaison (C=O)-1
HN(CA)CO	3D	N-H + C from liaison C=O and (C=O)-1
HN(CO)(CA)NH	3D	N-H+(N-H)+1

**Table 5 : List of spectra used for emerin 67-170 and 1-132 backbone assignment.**

Using our 3D experiment combination and using the emerlin 67-170 sample, we success to assign 96% of  $^1\text{H}$ - $^{15}\text{N}$  pairs (95 out of 99), 99% of  $^{13}\text{C}\alpha$  (104 out of 105), 99% of  $^{13}\text{C}\beta$  (98 out of 99) and 98% of  $^{13}\text{CO}$  (103 out of 105) resonances of emerlin 67-170 sequence.

The chemical shift data have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under the accession number 26654 for the emerlin 67-170 fragment. And the assignment of this unstructured peptide is further described in Samson et al., *Biomol NMR Assign.* 2016 (Appendix 1).

### *b. Igfold*

For the assignment of the NMR signals of the lamin A/C Igfold fragment, three-dimensional  $^1\text{H}$ - $^{15}\text{N}$ - $^{13}\text{C}$  correlation spectra were acquired using 3D HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO and HN(CO)(CA)NH experiments at 303 K, on a 3-mm-diameter NMR sample tube containing 500  $\mu\text{M}$  uniformly  $^{15}\text{N}/^{13}\text{C}$ -labeled IgFold411-566, in 20mM sodium phosphate pH 7, 30mM NaCl and 80:20%  $\text{H}_2\text{O}/\text{D}_2\text{O}$ . The assignment procedure took advantage of the already published assignment of lamin A/C fragment 428-547 at pH 6.3 and 303K (BMRB 5224)<sup>42</sup>.

## 2. PHOSPHORYLATION KINETICS

### *a. In vitro*

To study emerlin phosphorylation kinetics, the protocol was the same, regardless the kinase. The only differences were the pH value of the NMR buffer and the temperature of the NMR spectra acquisition, which were chosen according to the kinase. Here are reported the conditions used for phosphorylation by the Src kinase: the first step was to produce  $^{15}\text{N}$  labelled emerlin (1-187 or 1-132) and to dialyze it, at the end of purification, in the NMR buffer (20mM Phosphate pH7, 30mM NaCl, 10mM  $\beta$ -mercaptoethanol, 2mM ATP and 5mM  $\text{MgCl}_2$ ). Then, emerlin protein was concentrated until 200 $\mu\text{M}$  and the sample was stored at 277K.

The first NMR step was to record a  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of the emerlin protein alone, at 200 $\mu\text{M}$ , in presence of all kinase additives (5mM  $\text{MgCl}_2$ , 2mM ATP, 2mM DTT), at 303K. This spectrum served as a reference for the rest of the study.

Afterwards, a certain amount of kinase (depending of its activity) was added to the reference NMR tube and the pH was adjusted again to 7.0. Finally, the phosphorylation kinetics was followed by acquisition of  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra every hour, at 303K.

### *b. In cell extracts*

To study emerin phosphorylation kinetics in cell extracts, the first important condition was to purify the protein in two steps. First, emerin was purified in 8M urea and refolded by dialysis. Then, instead of cutting the His-Tag with the TEV protease, the protein was directly modified in cell extracts and repurified by affinity chromatography on an Ni-NTA column.

To prepare cell extracts, the protocol was:

- Extracts were obtained using 200 $\mu\text{l}$  of lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1% Triton X-100, 1mM EDTA, 1mM DTT, 10mM  $\text{MgCl}_2$ ), and protease inhibitors (we used SigmaFAST pastille) as well as phosphatase inhibitors (we used Phosphostop pastille from Roche) were added to these extracts.
- Extracts were incubated during 15 minutes on ice.
- They were centrifuged during one hour at 13000g.
- The protein amount present in the cell extracts was quantified using a Bradford test.
- The extracts were diluted to reach 15mg/ml.
- 175 $\mu\text{l}$  of cell extracts concentrated at 15mg/ml were added in 25 $\mu\text{l}$  of protein concentrated at 600 $\mu\text{M}$ , which already contained 25 $\mu\text{l}$  of ATP 1M and 25 $\mu\text{l}$  of  $\text{D}_2\text{O}$ .

After addition of cell extracts to our protein, the sample was let at 303K during 4 hours. Then, as I already explained, the protein was purified again on a nickel column, equilibrated with the buffer of our choice, and after elution, the buffer was changed to remove imidazole. We choose to change the buffer during protein concentration. Then, an  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum was recorded, and another one was recorded on the same His-Tag-emerin but that was no modified by cell extracts, in order to superimpose both spectra and observed changes due to modifications in the cell extracts.

## V. SOLID STATE NMR

After purification and His-Tag cleavage, proteins were dialyzed against 20mM Tris-HCl pH 8, 30mM NaCl and 5mM DTT and concentrated up to 600 $\mu$ M to initiate self-assembly. Then, filaments were observed by EM before to realize solid-state NMR experiments. The solid-state experiments were performed by Max Zinke, Kitty Hendricks and Prof Adam Lange at FMP Berlin. In the case of EmN and EmN132, solid-state NMR experiments were recorded on a Bruker Avance III HD 800 MHz spectrometer (18.8 T external magnetic field) or on a Bruker Avance III HD 700 MHz spectrometer (16.4 T external magnetic field). All solid-state NMR experiments for EmN delK37 mutant were recorded on a Bruker Avance III HD 700 MHz spectrometer. Temperature was determined using the water-proton signal referenced to DSS and kept at 283 K. Double quantum – single quantum spectra were recorded with magic-angle spinning at 8 kHz and C-C correlation spectra with 11 kHz MAS rate. High-power  $^1\text{H}$  decoupling was performed during evolution and detection periods using SPINAL-64 at a radio frequency strength of 83 kHz. All spectra were processed using Bruker TopSpin 3.2 and analyzed with CcpNmr.

## VI. ELECTRON MICROSCOPY

To observe self-assembled proteins, EmN, EmN49, EmN132, EmC221 and EmN delK37 were dialyzed against 20mM Tris-HCl pH 8.0, 30mM NaCl and 5mM DTT. They were concentrated up to 600  $\mu$ M and incubated at 293 K during 1 week. Specimens were prepared by negative staining with 2 % uranyl acetate on glow-discharged carbon-coated copper grids. Data collection was performed using a Tecnai Spirit transmission electron microscope (FEI) equipped with a LaB6 filament, operating at 100 kV. Images were recorded on a K2 Base camera (Gatan, 4kx4k) at 15,000 or 4,400 magnification (pixel size at specimen level – 0.25 & 0.83 nm, respectively), at I2BC, by Dr Ana Arteni.

## VII. X-RAY CRYSTALLOGRAPHY

### 1. CRYSTALLIZATION AND DATA COLLECTION

The Emerin/BAF/Igfold complex was purified by gel filtration (Superdex 75, 10/300 GL) in 50mM Tris-HCl pH8, 100mM NaCl and concentrated to 3mg/ml. Crystallization was initiated one week after the gel filtration and in these conditions, EmN was proteolysis and the final complex was composed of LEM/BAF/IgFold. For crystallization, 1  $\mu$ L of the complex was mixed with 1  $\mu$ L of the reservoir solution and equilibrated against a 500- $\mu$ L reservoir by hanging drop at 277K. Crystals were grown in 25% (w/v) polyethylene glycol monomethyl ether (PEG) 3.350, 20mM Bis-Tris pH5.5, 30mM NaCl and 0.2M NH<sub>4</sub>SO<sub>4</sub>. Crystals were transferred into a solution composed of 25% PEG 3.350, 20mM Bis-Tris pH5.5 and 0.2M NH<sub>4</sub>SO<sub>4</sub>, supplemented with 27% (V/V) ethylene glycol, before being flash-cooled in liquid nitrogen.

### 2. STRUCTURE DETERMINATION AND DATA REFINEMENT

The 3D structure of the complex was determined by molecular replacement using Molrep in CCP4<sup>277</sup>. The coordinates of BAF dimer with DNA (PDB entry 2BZF), the coordinates of laminA/C globular domain (PDB entry 1IFR) and the coordinates of the emerin LEM domain (PDB entry 2ODC) were used as template. The resulting model was rebuilt using PHENIX<sup>278</sup>, manual correction was performed with Coot<sup>279</sup> according to  $|F_o| - |F_c|$  and  $2|F_o| - |F_c|$  maps, and further refinement was carried out with phenix.refine. All structure figures were prepared with PyMOL (Schrödinger, LLC).

## VIII. SELF-ASSEMBLY

Self-assembly was initiated using proteins that were dialyzed in a buffer containing 50mM Tris-HCl pH8 and 30mM NaCl. After dialysis, proteins were concentrated until 600 $\mu$ M, reduced with 5mM DTT and left at room temperature during one week.

## IX. THIOFLAVIN KINETICS

After purification and His-Tag cleavage, proteins were dialyzed against 20mM Tris-HCl pH 8.0, 30mM NaCl and 5mM DTT and concentrated until 300 $\mu$ M. Oligomer assembly was followed with time by incubating proteins at 300  $\mu$ M and 310 K and by regularly taking protein aliquots to be analyzed by fluorescence. These aliquots were diluted in a thioflavin T (ThT) containing buffer so as to obtain 20  $\mu$ M protein and 2.5  $\mu$ M ThT in 20mM Tris-HCl (pH 8.0), 30mM NaCl, and 5mM DTT. The fluorescence measurements were carried out in a 60  $\mu$ l cuvette at 293 K using a fluorimeter JASCO ADP-303T. ThT fluorescence was monitored using an excitation wavelength of 440 nm and fluorescence emission was read at 480 nm.

## X. SIZE-EXCLUSION CHROMATOGRAPHY

Size-exclusion chromatography experiments aiming at identifying interactions between emerin, BAF and lamin fragments (WT and mutants) were performed using a Superdex-75 10/300 GL column (GE Healthcare) pre-equilibrated with buffer G (20mM Tris-HCl pH8, 30mM NaCl, 2mM DTT). 500 $\mu$ l of proteins concentrated at 150 $\mu$ M were injected for each experiment at a flow rate of 0.5ml/min at 277K.

## XI. ISOTHERMAL TITRATION CALORIMETRY (ITC)

ITC was performed using a high-precision VP-ITC calorimetry system. To characterize interactions between BAF dimer and the different lamin A/C fragments (Igfold WT and mutants, IgfoldProgerin), all proteins were dialyzed against 50mM Tris-HCl pH8, 150mM NaCl, 10mM  $\beta$ -mercaptoethanol and protease inhibitor (Roche). For each experiment using BAF, BAF dimer in the calorimetric cell at 288K was titrated with the lamin A/C fragment in the injection syringe.

To characterize interactions between BAF dimer and EmN, proteins were dialyzed against 50mM Tris-HCl pH8, 100mM NaCl, 10mM  $\beta$ -mercaptoethanol and protease inhibitor (Roche). BAF dimer (40 $\mu$ M) in the calorimetric cell at 288K was titrated with EmN187 (at a concentration of 200 $\mu$ M in the injection syringe).

Analysis of the data were performed using the Origin software provided with the instrument.

## XII. ANALYTICAL ULTRACENTRIFUGATION

Sedimentation velocity experiments were realized by measuring the absorbance at 280nm, every 8 minutes, with an optical path of 1.2cm, at 42000 rpm (128 297g), at 293K. An analytical ultracentrifuge XL470 (Beckman Coulter, Palo Alto, USA) and an An-50Ti rotor were used. 400 $\mu$ l of protein sample were useful for each experiment and 410 $\mu$ l of our dialysis buffer was used as reference. Results were analyzed using Sedfit software<sup>255</sup>.

### XIII. FLUORESCENCE-BASED THERMAL SHIFT ASSAY

The thermal stability of LamIgF proteins was monitored by a fluorescence-based thermal shift assay performed with a QuantStudio 12K Flex instrument (LifeTechnologies). 10 µg of purified protein was mixed with the SYPRO Orange dye (diluted 800-fold from a 5000-fold stock solution, Invitrogen) in 50mM Tris pH 8.0, 150mM NaCl, 10% glycerol. Reactions were carried out in duplicate in a 96-well fast PCR plate at a final volume of 20 µl and each experiment was repeated at least twice independently. The samples were submitted to a denaturation kinetic from 283 to 368K at a rate of 276K/min and fluorescence of Sypro Orange dye was recorded in real time. The protein denaturation temperature ( $T_d \pm$  s.e.m) was calculated using the Protein Thermal Shift software v1.3 (LifeTechnologies) as the maximum of the derivative of the resulting fluorescence curves.



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# APPENDIX 1

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Article published in *Biomol NMR Assign*:

Samson *et al.*,  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$  backbone resonance assignment of the intrinsically disordered region of the nuclear envelope protein emerin.

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<https://doi.org/10.1007/s12104-015-9662-7>

# APPENDIX 2

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## Additional Figures

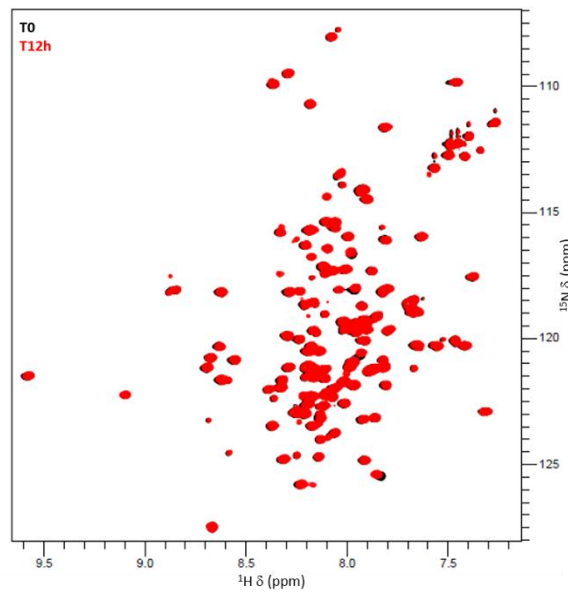


Figure 101 : EmN132 phosphorylation kinetics, in the presence of CK1, by NMR.

EmN132 sample (in black) or an EmN132 sample phosphorylated by CK1 during 12h (in red) at 100 $\mu$ M in 20mM Phosphate pH7, 30mM NaCl, 10mM  $\beta$ -mercaptoethanol, 2mM ATP, 5mM MgCl<sub>2</sub>, at 303K and 750MHz (FMP Berlin).

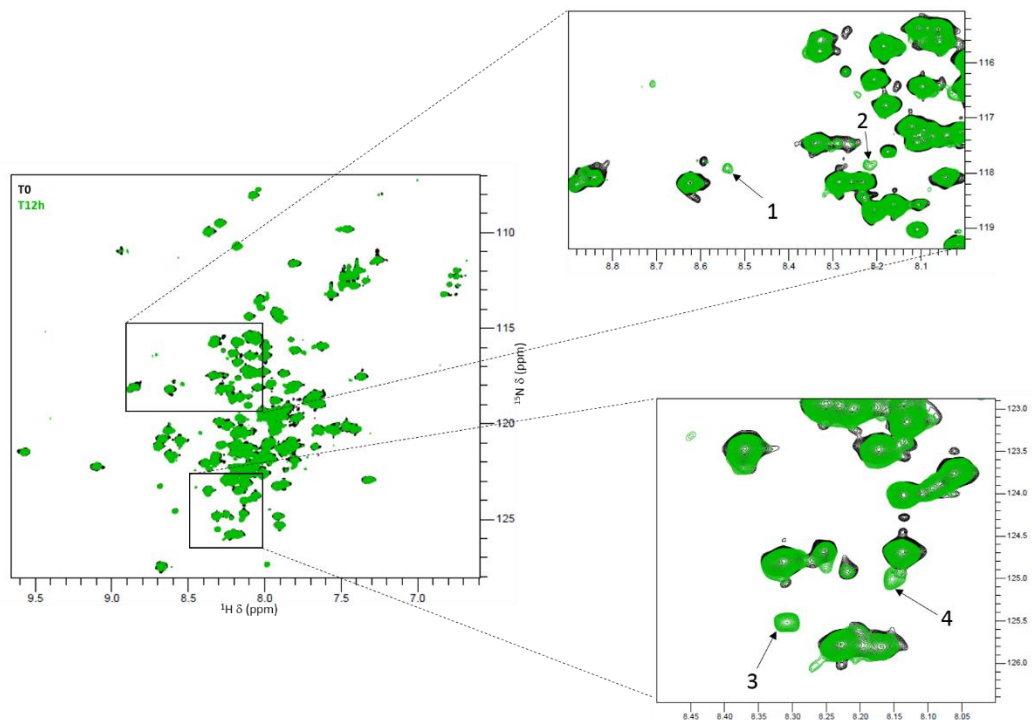


Figure 102 : EmN132 phosphorylation kinetics, in the presence of CK2, by NMR

EmN132 sample (in black) or an EmN132 sample phosphorylated by CK2 during 12h (in green) at 100 $\mu$ M in 20mM Phosphate pH7, 30mM NaCl, 10mM  $\beta$ -mercaptoethanol, 2mM ATP, 5mM MgCl<sub>2</sub>, at 303K and 750MHz (FMP Berlin).

Experiments done at 15°C	BAF / Igfold		BAF / EmN		BAF / IgfoldProgerin	
	N = 5		N = 3		N = 2	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Ns	0.35	0.09	0.25	0.02	0.22	0.04
Ka (M <sup>-1</sup> )	3.7E5	2.1E5	1.4E6	2.9E5	4.2E5	2.1E5
Kd (μM)	3.15	1.16	0.7	0.17	2.7	1.3
ΔH (cal/mol)	-1.15E4	2.1E3	-4.1E3	9.1E2	-1.6E4	9.5E3
ΔS (cal/mol/deg)	-13.5	9.3	-13.9	3.1	-30.9	34

**Table 6 : Table containing the mean and the standard deviation of all ITC experiments done at 288K.**

**N = number of data points, Ns = number of sites, Ka = the association rate constant, Kd = the dissociation rate constant, ΔH = enthalpy and ΔS = entropy.**

Experiments done at 10°C	Igfold WT		Igfold A529V		Igfold R471C	
	N = 2		N = 2		N = 2	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Ns	0.39	0.02	0.38	0.05	0.39	0.16
Ka (M <sup>-1</sup> )	7.2E5	2.6E5	1.5E5	1.4E4	1.4E5	1.6E4
Kd (μM)	1.5	0.54	6.9	1.6	7.2	0.8
ΔH (cal/mol)	-1.3E4	5.9E2	-8E3	1.6E3	-1.1E4	3E3
ΔS (cal/mol/deg)	-20.5	2.75	-4.8	5.9	-14	10

**Table 7 : Table containing the mean and the standard deviation of all ITC experiments done at 283K.**

**N = number of data points, Ns = number of sites, Ka = the association rate constant, Kd = the dissociation rate constant, DH = enthalpy and DS = entropy.**

# APPENDIX 3

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Article submitted in *Nature Structural and Molecular Biology*:

Samson *et al.*, Structural analysis of the ternary complex between lamin A/C, BAF and emerin identifies an interface disrupted in autosomal recessive progeroid diseases

**Structural analysis of the ternary complex between lamin A/C, BAF and emerin identifies  
an interface disrupted in autosomal recessive progeroid diseases**

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**Abstract**

Lamins are the main components of the nucleoskeleton. Whereas their 3D organization was recently described using cryoelectron tomography, no structural data highlights how they interact with their partners at the interface between the inner nuclear envelope and chromatin. A large number of mutations causing rare genetic disorders called laminopathies were identified in the C-terminal globular Igfold domain of lamins A and C. We here present a first structural description of the interaction between the lamin A/C immunoglobulin-like domain and a nuclear envelope protein. We reveal that this lamin A/C domain both directly binds self-assembled emerin and interacts with monomeric emerin LEM domain through the dimeric chromatin-associated Barrier-to-Autointegration Factor (BAF) protein. Mutations causing autosomal recessive progeroid syndromes specifically impair proper binding of lamin A/C domain to BAF, thus destabilizing the interface between lamin A/C and BAF in cells. Our results suggest that these mutations could perturb the BAF-mediated interaction between lamin A/C and chromatin, thus leading to a dysregulation of chromatin organization and gene expression.

Laminopathies are rare genetic disorders caused by mutations in genes encoding lamins or by abnormalities in the processing of lamin A <sup>1</sup>. They display a large variety of clinical symptoms including cardiomyopathy, muscular dystrophy, lipodystrophy, mandibuloacral dysplasia, restrictive dermopathy, peripheral neuropathy, and premature ageing. A major fraction of the disease-causing mutations comprises single point mutations with poorly understood functional implications. Most of them are distributed all along the *LMNA* gene coding for both prelamin A and lamin C. These two proteins are, together with lamins B1 and B2, essential components of the nucleoskeleton. They share an N-terminal region of 566 residues, and whereas lamin C only possesses 6 additional specific C-terminal residues, prelamin A presents 98 additional residues, is farnesylated, carboxymethylated and then cleaved to become mature lamin A. Disease-causing mutations are observed in the large region common to lamins A and C (lamin A/C) as well as in the lamin A specific C-terminal region <sup>2</sup>. At the cellular level, lamins A and C form filaments that are mainly present at the nuclear periphery but are also observed in the nucleoplasm <sup>3,4</sup>. Laminopathies are characterized by nuclear morphological abnormalities and an altered pattern of heterochromatin distribution that is more severe in progeroid syndromes, including Hutchinson-Gilford progeria syndrome (HGPS), mandibuloacral dysplasia (MAD), atypical-Werner syndrome (WS) and restrictive dermopathy (RD) <sup>5,6</sup>. The lamin filament network interacts with chromatin at different stages of the cell cycle: in anaphase, lamin A and C dimers are recruited at the core regions of sister chromosomes; in interphase cells, they relocalize both at the nuclear envelope and within the nucleoplasm, where lamins A and C provide anchor points for chromatin <sup>7-10</sup>. The complex molecular organization of the nuclear envelope, nucleoskeleton and their interface with chromatin was recently described using cryo-electron tomography <sup>11,12</sup>. Within the densely packed environment observed close to the nuclear envelope, only nuclear pores, lamin filaments and chromatin could be recognized. Lack of high resolution 3D structures for proteins anchored at the inner nuclear membrane as well as complexes between these proteins, the lamina and chromatin still precludes any further description of the nuclear periphery architecture and of the structural defects caused by mutations in the *LMNA* gene coding for lamins A and C.

We set out to understand how the C-terminal immunoglobulin-like (Igfold) domain of lamin A/C interacts with the inner nuclear membrane protein emerin and the chromatin-associated protein Barrier-to-Autointegration Factor (BAF). Indeed, a large number of disease-causing mutations were identified in this lamin Igfold domain<sup>13,14</sup>. Mutations causing muscle diseases affect residues of the hydrophobic core, suggesting that destabilization of the lamin A/C Igfold domain is responsible for these diseases<sup>14</sup>. Mutations causing either lipodystrophies or progeroid syndromes involve residues localized on two different solvent-exposed sites of the lamin A/C Igfold domain, suggesting that these accessible regions are binding sites for unknown lamin partners and that disruption of these binding events cause the diseases<sup>13-15</sup>. Lamin A/C binds to the inner nuclear membrane protein emerin, as shown by blot overlay, coimmunoprecipitation and yeast 2-hybrid assays<sup>16-18</sup>. Moreover, the tail region of lamin A/C (residues 384 to 566, comprising the Igfold domain) is responsible for emerin binding<sup>18,19</sup>. The central region of emerin (residues 70 to 178) is essential for lamin binding<sup>16,19</sup>, and its N-terminal domain (residues 1 to 45, the LEM domain) interacts with BAF, as shown by a panel of techniques going from blot overlay and coimmunoprecipitation assays to X-ray crystallography analyses<sup>16,20</sup>.

The structural inter-dependence of lamins, emerin and BAF was revealed by downregulating either lamins A and C or BAF or the LEM domain proteins emerin and LEM2 in *C. elegans* embryos. If any one component was missing, the other two failed to co-assemble, with severe consequences for mitotic spindle assembly and positioning<sup>21</sup>, mitotic chromosome segregation and postmitotic nuclear assembly<sup>22,23</sup>. *In vitro* experiments revealed that human lamin A binds weakly ( $K_d$  of 1  $\mu$ M) but directly to BAF and that lamin A and BAF simultaneously bind to emerin<sup>24</sup>. Moreover, BAF enhances binding of prelamin A tails to emerin<sup>25</sup>.

We now present the 3D structure of a complex involving lamin A/C, emerin and BAF. We show that emerin oligomerization regulates direct lamin A/C binding. We discuss the role of these lamin-emerin interactions during the cell cycle. We further show that BAF assembly within these complexes is impaired by lamin mutations causing autosomal recessive progeroid syndromes, thus highlighting the essential functional role of interactions between nuclear envelope and chromatin-associated proteins in human cells.



## RESULTS

### The lamin A/C Igfold domain interacts with the self-assembled emerin fragment EmN

To determine how the lamin A/C tail interacts with emerin, we generated two fragments of the emerin nucleoplasmic region (Figure 1A). We tested binding between the tail region common to lamins A and C (LamIgF comprising aa 411 to aa 566) and these emerin fragments. As previously published, the whole emerin nucleoplasmic region from aa 1 to aa 221 is poorly soluble<sup>26</sup>. We thus produced two overlapping emerin peptides, which we called EmN<sup>27</sup> and EmC (Figure 1A). EmN (aa 1 to aa 187) comprises a LEM domain and a region that is intrinsically disordered<sup>26,28</sup>. We previously showed that, *in vitro*, it can be observed either as a monomer or as an oligomer<sup>27</sup> (Figure 1B, left panel). EmC (aa 67 to aa 221) is entirely unstructured as observed by NMR (Suppl. Fig. 1A), and after purification immediately oligomerizes as observed by fluorescence (Suppl. Fig 1B) and negative-staining electron microscopy (Figure 1B, right panel). In order to identify an interaction between lamin A/C and emerin, we produced a <sup>15</sup>N labeled LamIgF peptide, and mixed the labeled peptide with either monomeric EmN, oligomeric EmN or oligomeric EmC (Figure 1C). Nuclear Magnetic Resonance (NMR) analysis of these samples revealed that LamIgF only interacts with oligomeric EmN in these conditions. This interaction is specific to lamin A/C as NMR analysis of a sample containing <sup>15</sup>N labeled lamin B1 tail and oligomeric EmN did not demonstrate any binding (Figure 1C, lower and right panel).

### The Igfold domain of lamin A/C forms a ternary complex with the LEM domain of emerin and BAF

The chromatin-associated protein BAF binds to both lamin A/C and emerin<sup>29,30</sup>. We examined the role of BAF in mediating an interaction between LamIgF, EmN and BAF by Size-Exclusion Chromatography (SEC). Consistent with our previous NMR results, we observed that LamIgF and monomeric EmN did not co-elute under the conditions used (Figure 2A). However, they co-eluted in the presence of BAF. Similarly, we tested the binding between the lamin B1 tail fragment, EmN and BAF. In this case, no binding could be observed between lamin B1 and BAF, precluding the formation of a ternary complex with EmN. NMR further confirmed that the lamin B1 tail fragment did not interact with BAF (Suppl. Fig. 2).

Isothermal Titration Calorimetry (ITC) experiments showed that LamIgf and EmN bind to BAF with an affinity of  $3.2 \pm 1.2 \mu\text{M}$  and  $0.7 \pm 0.2 \mu\text{M}$ , respectively (Figure 2B; Suppl. Table 1). Crystal structure of the ternary complex was solved at 1.9 Å resolution (Figure 3A; Suppl. Table 2). One BAF dimer interacts on one side with the LEM domain of emerin (interface 1) and on the other side with the Igfold domain of lamin A/C (interface 2). The emerin fragment outside of the LEM domain is not visible in the electron density map. SDS-PAGE revealed that it was proteolyzed between protein purification and crystallization.

### **Dimerization of BAF is essential for both lamin and emerin binding**

The 3D structure of the ternary complex highlights that BAF dimerization is essential for emerin and lamin A/C binding. First, BAF monomers 1 and 2 both interact with the LEM domain of emerin (interfaces of  $395 \text{ \AA}^2$  and  $211 \text{ \AA}^2$ , respectively) (Figure 3B). BAF monomer 1 loop  $\alpha 2\alpha 3$  (Phe39), helix  $\alpha 3$  (Gln48, Val51, Leu52) and helix  $\alpha 4$  (Leu58, Glu61, Trp62) contact a LEM surface comprising the  $\beta$ -strand (Gly24, Pro25), loop  $\beta 1\alpha 2$  (Ser29, Thr30) and helix  $\alpha 2$  (Leu33, Tyr34, Lys36, Lys37). BAF monomer 2 loop  $\alpha 2\alpha 3$  (Arg37, Phe39) and helix  $\alpha 3$  (Glu48) contact an overlapping LEM surface comprising helix  $\alpha 1$  (Thr13), the  $\beta$ -strand (Pro25) and loop  $\beta 1\alpha 2$  (Val27). A similar 3D structure between BAF dimer and emerin LEM domain was previously modeled based on NMR data (Figure 3C; PDB reference 2ODG<sup>20</sup>). The surface buried at the LEM/BAF interface yields  $510 \text{ \AA}^2$  in the NMR structure compared to  $606 \text{ \AA}^2$  in the X-ray structure, showing that the interaction is significantly tighter in the crystal structure.

Both BAF monomers also contribute to lamin A/C binding, through interfaces of  $270 \text{ \AA}^2$  and  $192 \text{ \AA}^2$ , respectively (Figure 4A). BAF monomer 1 helix  $\alpha 1$  (Val11, Ala12), loop  $\alpha 1\alpha 2$  (Pro14) and helix  $\alpha 5$  (Glu83, Ala87, Phe88) contact lamin A/C strand  $\beta 1$  (His433, Ala434, Arg435) and strand  $\beta 9$  (Met540, Lys542), whereas BAF monomer 2 helix  $\alpha 1$  (Ala12), loop  $\alpha 1\alpha 2$  (Pro14) and helix  $\alpha 5$  (Ala87, Phe88) contact lamin A/C strand  $\beta 1$  (Arg435) and loop  $\beta 8\beta 9$  (Glu537). It was reported that BAF mutant G47E lost its ability to bind to lamin A as well as prelamin A in cells<sup>6</sup>. Gly47 is located at the center of the dimerization interface (in magenta on Figure 4A). Mutation G47E might destabilize the BAF dimer, therefore hindering BAF interaction with both emerin LEM domain and lamin A/C Igfold.

## **The interface between lamin A/C and BAF involves residues mutated in progeroid syndromes**

By solving the 3D structure of a ternary complex formed by BAF, EmN and LamIgf, we revealed a yet undescribed interface between the BAF dimer and the lamin A/C Igfold domain. On the BAF side, Ala12, Pro14 and Phe88 of both monomers are more than 50% buried in the complex (Figure 4A). An Ala12 to Thr amino acid substitution occurs in patients with a Nestor-Guillermo progeria syndrome<sup>31</sup>. Our 3D structure predicts that this substitution causes a defect in BAF/lamin binding. Consistently, in cells, it significantly decreases BAF binding to lamin A and prelamin A<sup>6</sup>.

On the lamin side, Arg435, Met540 and K542 are more than 50% buried at the lamin/BAF monomer 2 interface (Figure 4A). Arg435 side-chain is hydrogen-bonded to Phe88 backbone oxygens of both BAF monomers, while Lys542 side-chain is bonded to BAF monomer 2 Asp86 backbone oxygen. These lamin residues are mutated in patients with severe autosomal recessive progeroid syndromes (RD and HGPS<sup>15,32-34</sup>). They belong to a hot spot for mutations causing progeroid diseases<sup>15</sup>. In order to verify that in solution, this hot spot surface is contacting BAF, we followed the NMR <sup>1</sup>H and <sup>15</sup>N signals of LamIgf while adding BAF to the NMR sample. We observed that the intensities of the NMR signals corresponding to several residues of strand  $\beta$ 1 (as Arg435), loop  $\beta$ 1 $\beta$ 2, strand  $\beta$ 4 (as Arg471), loop  $\beta$ 8 $\beta$ 9 and strand  $\beta$ 9 (as Met540 and Lys542) were significantly decreased (Figure 4B). This demonstrated that the  $\beta$ -sheet formed by strands  $\beta$ 1 and  $\beta$ 9 is indeed involved in BAF binding in solution.

## **Lamin A/C Igfold mutations causing progeroid syndromes decrease binding affinity for BAF**

To identify the consequences of the LamIgf mutations causing progeroid diseases on the formation of the lamin-BAF complex, we produced a set of LamIgf peptides mutated on sites responsible for severe diseases. We focused our interest on 5 homozygous LamIgf mutations, R435C (strand  $\beta$ 1)<sup>32,33,35</sup>, R471C (strand  $\beta$ 4)<sup>36</sup>, R527H<sup>37-40</sup>, A529V<sup>41</sup> (strand  $\beta$ 8) and K542N (strand  $\beta$ 9)<sup>34</sup>, which were identified in patients with atypical progeroid syndromes. Two of these mutations cause syndromes generally appearing during early childhood as RD (R435C) and HGPS (K542N), characterized by severe premature aging features, whereas mutations R471C, R527H and A529V cause a disease generally appearing later, called MAD, characterized by growth retardation and skeletal abnormalities.

In the case of R471C, the disease was particularly severe and the phenotype combined MAD, progeria, and rigid spine muscular dystrophy<sup>36</sup>. We first verified that the protein variants are well folded and have a thermal stability close to that of WT LamIgf (i.e.  $\pm 5^\circ$  relatively to WT; Suppl. Fig. 3). We then measured their affinity for BAF by ITC (Figure 5A; Suppl. Table 1). Compared to WT LamIgf and to mutants R453W and R482W causing Emery-Dreifuss Muscular Dystrophy (EDMD) and Dunnigan-type Familial Partial Lipodystrophy (FPLD), respectively, mutant R435C showed a complete loss of detectable binding to BAF in our conditions. Mutants K542N and R527H bind BAF but the corresponding heat release is so weak that no affinity could be measured. R471C and A529V exhibit a 5-fold decreased affinity for BAF. We also tested the binding of LamIgf mutants to BAF by SEC (Figure 5B). We observed that mutants R453W and R482W coeluted together with BAF as WT LamIgf. However, mutant R435C did not induce any elution volume shift of BAF, and mutants R471C, A529V, R527H and K542N only weakly shifted BAF elution volume. We concluded that the 5 tested mutations causing atypical progeroid syndromes decrease the affinity of lamin A/C for BAF. Moreover, the binding defect seems to globally correlate with the disease severity (Figures 5A,B).

Finally, we transfected HeLa cells with plasmids coding for GFP-BAF and FLAG-mature lamin A (aa 1 to aa 646) WT or variants and detected using an *in situ* Proximity Ligation Assay the proximities between BAF and lamin A in these cells. We thus tested the impact of mutations related to RD (R435C), HGPS (K542N) and MAD (R527H, A529V) on these proximities. FLAG-lamin variants were expressed at similar protein levels as FLAG-lamin WT; they localized both at the nuclear periphery and in the nucleoplasm (Figure 6A,B). We observed that all 4 lamin A mutations associated with atypical progeroid syndromes similarly reduced the frequency of the proximity events between BAF and lamin A (Figure 6C, D).

## DISCUSSION

The mechanical roles of lamins and their functions in gene regulation are often viewed as independent activities, but recent findings suggest a highly cross-linked and interdependent regulation of these different functions<sup>42-44</sup>. Lamins interact with inner nuclear membrane proteins to mediate mechanical signaling but also contact chromatin-associated proteins that contribute to gene transcription regulation. Most of these interactions depend on the lamin ability to assemble into higher-order structures<sup>45,46</sup>. Therefore, lamin assembly has been studied intensively *in vitro* and the resulting filaments have been observed in cells<sup>12</sup>. In contrast, little is known about the contacts of lamins with their partners *in vivo*. Lamins are extensively modified and interact with a large number of proteins<sup>47,48</sup>, the inner nuclear membrane<sup>49</sup> and chromatin<sup>50</sup>. Their tail region is responsible for association with partners. It contains a globular Igfold domain that is mutated in several laminopathies<sup>14</sup>. Until this study, it was not known how this domain interacted with partners anchored at the inner nuclear membrane and established contacts with chromatin.

### **The lamin A/C Igfold domain directly binds to self-assembled emerin but also recognizes monomeric emerin through BAF**

We showed that the lamin A/C Igfold domain interacts with the inner nuclear membrane protein emerin through two mechanisms: it either directly binds to self-assembled EmN or interacts with monomeric EmN through the chromatin-associated protein BAF. It was previously published that direct lamin A tail interaction with emerin is disrupted not by mutations in the LEM domain, but by mutations in emerin region from aa 70 to aa 164: mutations around positions 70, 76, 95, 112, as well as 141 and 164, decreased binding of lamin A to emerin<sup>16,30</sup>. These results are consistent with our analysis. Indeed, LamIgF only binds directly to self-assembled emerin and the disordered region of emerin between aa 50 and aa 132 is necessary for emerin self-assembly<sup>27</sup>. Similarly, GST fusions of an emerin fragment from aa 1 to aa 132 is sufficient to bind mature lamin A tails<sup>19</sup>, and we showed that emerin aa 1 to aa 132 is sufficient for self-assembly<sup>27</sup>. Finally, the EDMD-associated emerin deletion mutation del95-99, which impairs EmN self-assembly *in vitro*, causes a decrease of lamin A/C binding in cells<sup>27</sup>.

Altogether these results strongly suggest that emerin self-assembly regulates direct lamin A/C binding in cells. On the other hand, we showed that the monomeric LEM domain (emerin aa 1 to aa 45) interacts with the lamin A/C Igfold domain through a dimer of BAF, consistent with previous data showing that BAF enhanced binding of prelamin A tails to emerin <sup>25</sup>.

### **A defect at the interface between lamin A/C and the chromatin-associated BAF protein is observed in atypical progeroid syndromes**

By analyzing the 3D structure of our ternary complex comprising domains of lamin A/C, BAF and emerin, we noticed that one of the interfaces coincides with a hot spot of lamin A/C residues mutated in progeroid syndromes <sup>15</sup>. We measured *in vitro* and in cells the impact of several homozygous mutations causing autosomal recessive progeroid syndromes and showed that these mutations disrupt the interaction between the lamin A/C Igfold and BAF. In HeLa cells, all tested mutations consistently reduced the frequency of the proximity events between lamin A and BAF. As endogeneous lamin A probably forms filaments together with the FLAG-tagged lamin A, a number of PLA events were expected due to interactions between endogeneous lamin A and BAF that create proximities between associated FLAG-tagged lamin and BAF. Consistently, the mutations reduced the number of PLA signals to about 50% of the signals measured for WT lamin A and BAF. *In vitro*, a range of affinity decreases were observed. In particular, SEC experiments revealed that, in our conditions, LamIgF control mutants R453W and R482W bind BAF as WT LamIgF, mutants R471C, A529V, R527H and K542N weakly bind BAF, and mutant R435C did not bind BAF. ITC experiments provided only estimations for affinity decreases, due to the weak affinities of the mutants. However, an affinity decrease of about 5 fold could be calculated for mutants R471C and A529T, whereas essentially no binding could be detected for R527H, K542N and R435C. Interestingly, this affinity scale matches with the severity of the associated diseases: R471C and A529V cause MAD that generally appears during late childhood <sup>36,41</sup>, whereas K542N causes a HGPS with an onset between 18 and 24 months <sup>34</sup> and R435C causes the most severe disease, RD, which appears during the first months after birth <sup>32,33,35</sup>; only mutant R527H, which causes a MAD <sup>37-40</sup>, also shows a strong BAF binding defect under the conditions we used. We conclude that a destabilization of the interface between lamin A/C and BAF might mediate the disease-mechanism in atypical progeroid syndromes due to homozygous mutations in LamIgF.

Another progeroid syndrome called the Nestor-Guillermo Progeria Syndrome occurs in patients older than the average lifespan of progeroid patients. This syndrome is due to the homozygous BAF mutation A12T<sup>31</sup>. The mutated protein is expressed and stable<sup>51</sup>. In cells, it still co-immunoprecipitates with endogenous emerin, lamin A and histone H3. Our preliminary data show that, *in vitro*, BAF mutant A12T consistently interacts with LamIgf, but with an affinity lower than BAF WT and BAF S4E (Suppl. Fig. 5). Immunoprecipitation experiments performed in co-transfected cells showed a reduced interaction between BAF mutant A12T and lamin A<sup>6</sup>. BAF mutant A12T also exhibits a marked defect in double-stranded DNA binding compared to WT BAF<sup>51</sup>. Altogether, these data strongly suggest that the complex between BAF, lamin A/C, DNA and emerin is destabilized by the mutation A12T, highlighting the similarity between the mechanisms of autosomal recessive progeroid syndromes due to mutations in LamIgf and BAF.

#### **Complexes including lamin A/C and emerin are assembled at different stages of the cell cycle**

In cells, formation and localization of complexes including lamin A/C, BAF and emerin depend on the cell cycle, in accord with the disassembly/reformation of the nuclear envelope that delimits the interphase nuclear compartment. While in interphase cells, lamins assemble into polymers within the nuclei, they disassemble at mitosis, leading to cytoplasmic lamin A/C dimers. At that stage, pools of lamin A/C and BAF form stable protein complexes with the LEM domain protein LAP2 $\alpha$ ; these complexes contribute to microtubule spindle assembly and orientation<sup>21</sup>. Interestingly, knocking down lamin A severely affects binding of LAP2 $\alpha$  to GFP-BAF and knocking down BAF disrupts binding of LAP2 $\alpha$  to GFP-lamin A, suggesting that formation of a lamin A-BAF complex is essential for the recruitment of the LEM domain LAP2 $\alpha$  protein. Later, in ana-telophase, first BAF, and then emerin and lamin A/C accumulate at the core region of chromosomes<sup>52</sup>. On the BAF side, mutation G25E, at the interface with DNA, impairs localization of BAF at the core region of chromosomes, emerin localization during telophase and further lamin A accumulation at reforming nuclear envelopes. On the emerin side, the LEM domain is necessary and sufficient for the core localization of emerin. Superimposition of our 3D structure with that of a complex between BAF dimer and DNA (PDB reference 2BZF<sup>53</sup>) revealed that the BAF dimer can simultaneously bind to emerin, lamin and DNA (Figure 7, left panel).

We propose that BAF mediates the interaction between emerin and lamin A at the core region. The fact that emerin must interact with BAF at the core region of chromosomes before being assembled at the nuclear envelope<sup>54,52</sup> also suggests that post-translational modification events occur at the core that further enable emerin assembly at the nuclear envelope.

In interphase cells, emerin binds to lamin A/C<sup>30</sup>. Moreover, an emerin protein mutated in its LEM domain (residues 24-27 being changed into alanines), which does not interact with BAF<sup>16</sup>, localizes normally at the nuclear envelope<sup>54</sup>, consistent with its ability to directly bind lamin A/C *in vitro*<sup>16</sup>. Emerin self-assembles at the nuclear envelope<sup>19,55</sup>. From our NMR results, we propose that emerin self-assembly creates a high concentration of binding sites for the lamin filaments at the nuclear periphery of cells (Figure 7, right panel). During *in vitro* self-assembly, emerin exhibits a large LEM domain conformational change, strongly suggesting that self-assembled emerin does not bind BAF<sup>26</sup>. Altogether, previous studies as well as our data support a model in which, in interphase cells, emerin exists in two conformations, a self-assembled conformation that interacts with lamin A/C and a conformation in which the LEM domain is free to bind BAF<sup>19,55</sup>. Whether trimeric emerin-BAF-lamin A/C complexes also form at the nuclear envelope of interphase cells remains an open question.

### **A defective interaction between lamin A/C and BAF could impact chromatin organization of interphase cells**

Fluorescence resonance energy transfer experiments showed that YFP-emerin and CFP-BAF interact at the inner nuclear membrane of interphase HeLa cells<sup>56</sup>. In this case, emerin is still relatively immobile, but BAF is dynamic and mobile: it binds to emerin frequently but transiently using a “touch-and-go” mechanism. We also observed proximities between lamin A and BAF in interphase HeLa cells. These proximities detected *in situ* using a proximity assay are particularly enriched at the nuclear periphery (Suppl. Fig. 6). They are less frequent in cells expressing lamin A variants that are defective for BAF binding *in vitro*. Interestingly, defects of the nuclear envelope-chromatin interface were revealed using electron microscopy in the case of progeroid syndromes caused by lamin A or BAF mutations.



A loss of nuclear peripheral heterochromatin was observed both in cells of patients with autosomal dominant HGPS due to a mutation G608G in prelamin A and in HEK293 cells overexpressing lamin A and BAF A12T<sup>6,57,58,59</sup>. Moreover, lamin A and BAF were shown to directly bind histones and to influence histone epigenetic marks, as H3K9me3, H3K27me3, H3K4me2 and H3K79me2<sup>60,61,62</sup>. We conclude that defect in the recruitment of BAF to the lamina via the immunoglobulin-like domain of lamin A/C could be responsible for abnormal heterochromatin distribution at the nuclear envelope of interphase cells. More work is needed to understand how the lamin-BAF complex interacts with DNA and histones, and how a defect in this complex compromises the interactions between lamina and chromatin at the nuclear periphery in autosomal recessive progeroid syndromes.

In conclusion, the Igfold domain of lamin A/C can both directly interact with self-assembled emerin and bind to monomeric emerin through the chromatin-associated protein BAF. A defect in the interaction between lamin A/C and BAF might destabilize ternary complexes with emerin, thus leading to a less efficient targeting of emerin at the reforming nuclear envelope. However, diseases associated with mutations disrupting the lamin A/C-BAF interaction are much more severe than diseases associated with emerin loss of function. This strongly suggests that the impact of these lamin A/C and BAF mutations goes beyond LEM-domain protein mislocalization and loss of function. We propose that the disease-causing mutations perturb the BAF-mediated interaction between lamin A/C and chromatin, thus leading to a dysregulation of chromatin organization and gene expression.

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**Author contributions** – S.Z.J. conceived and designed new experiments reported in this paper. C.S., A.P., F.C., I.H., V.R., N.N, E.J., A.A.A. and B.B. performed the experiments. C.S., A.P., E.J., B.B. and S.Z.J. analyzed data. M.H.L. supervised the analysis of the X-ray crystallography data. C.S., B.B. and S.Z.J. wrote the manuscript.

**Competing financial interests** – The authors declare no competing financial interests.

## **Methods.**

### **Protein Expression and Purification.**

Human WT emerlin fragments from amino acid 1 to amino acid 187 (EmN), from amino acid 67 to 170 (EmC170), from amino acid 67 to 187 (EmC187) and from amino acid 67 to amino acid 221 (EmC), human BAF with all cysteines mutated in alanine and human lamin B1 tail from amino acid 409 to amino acid 586 (LB1) were expressed using a pETM13 vector as N-terminal octa-histidine fusions in *Escherichia coli* BL21 DE3 Star (Novagen). The emerlin and BAF cDNAs were optimized for expression in *Escherichia coli* (Genscript). Human wild-type lamin fragment from amino acid 411 to amino acid 566 (LamIgF) was expressed using a pGEX vector as an N-terminal GST fusion in *Escherichia coli* BL21 DE3 Star (Novagen). All LamIgF mutant expression vectors (R435C, R453W, R471C, R482W, R527H, A529V, and K542N) were obtained by mutagenesis using the Quikchange (Agilent) kit from the LamIgF expression vector. Bacteria were cultured in rich medium (*lysogeny broth, LB*) or <sup>15</sup>N-labeled minimum medium, induced at an optimal density of 1 with 0.5mM isopropyl β-D-1-thiogalactopyranoside, grown overnight at 293K, and lysed in 50mM Tris-HCl pH 8.0, 300mM NaCl, 40mM imidazole, 5% glycerol, 1% Triton X-100, and 1mM phenylmethanesulfonylfluoride.

For EmN, EmC170, EmC187, EmC and BAF, after sonication at room temperature and centrifugation at 20 000 g, for 20 min, at 277K, the pellet was resuspended in buffer C8 (50mM Tris-HCl pH8.0, 150mM NaCl, 20mM imidazole, 8M urea). A second centrifugation step was performed at 20 000 g, for 20 min, at 293K. The soluble extract was then loaded onto Ni-NTA beads (GE-Healthcare) equilibrated with buffer C8. Proteins were eluted directly with buffer E8 (50mM Tris-HCl pH 8.0, 150mM NaCl, 1M imidazole, 8M urea). Then, proteins were refolded by dialysis in buffer D1 (20mM Tris-HCl pH8.0, 30mM NaCl, 2mM DTT) for EmN, EmC170, EmC187 and EmC and buffer D2 (50mM Tris-HCl pH8.0, 150mM NaCl, 2mM DTT) for BAF. After refolding, they were incubated with the TEV protease during 3 h at room temperature and finally dialyzed into the selected buffer. The final yield was typically 10 mg of purified protein per liter of bacterial culture

Self-assembly was initiated using proteins that were dialyzed in a buffer containing 20mM Tris-HCl pH8 and 30mM NaCl. After dialysis, proteins were concentrated until 600 $\mu$ M, reduced with 5mM DTT and stored at room temperature during one week.

For LB1, after sonication at 277K, benzonase (SigmaAldrich) addition and centrifugation at 20 000 g, for 30 min, at 277K, the soluble extract was loaded onto Ni-NTA beads (GE-Healthcare) equilibrated with buffer C (50mM Tris-HCl pH8.0, 150mM NaCl and 20mM imidazole). The protein was then eluted directly with buffer E (50mM Tris-HCl pH 8.0, 150mM NaCl and 1M imidazole) and dialyzed in buffer C. It was incubated with the TEV protease during 3h at room temperature, loaded again onto Ni-NTA beads and finally, after flow-through recovery, dialyzed into the selected buffer. The final yield was typically 20 mg of purified protein per liter of bacterial culture.

For Lam1gF (WT and mutants), after sonication at 277K, benzonase (SigmaAldrich) addition and centrifugation at 20 000 g, for 30 min, at 277K, the soluble extract was loaded onto GST-beads (GE-Healthcare) equilibrated with buffer T2 (50mM Tris-HCl pH7.5, 150mM NaCl, 2mM DTT). After 2h of incubation at 277K, GST-beads were washed with buffer T2 and 1M NaCl, and then thrombin was added and incubated with the beads overnight. The protein was recovered in the flow-through and finally dialyzed into the selected buffer. The final yield was typically 2 mg of purified protein per liter of bacterial culture.

#### **Liquid-State NMR Spectroscopy.**

NMR experiments were performed on a 700 MHz Bruker Avance spectrometer equipped with a triple resonance cryogenic probe. To study interaction between the lamin fragments and BAF, two-dimensional  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra were acquired at 288 K, on a 3-mm-diameter NMR sample tube containing 100-200 $\mu$ M uniformly  $^{15}\text{N}$ -labeled lamin peptides and non-labeled BAF, in 20mM sodium phosphate pH 7, 100mM NaCl and 80:20%  $\text{H}_2\text{O}/\text{D}_2\text{O}$ . To study interaction between lamin fragments and EmN or EmC, two-dimensional  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra were acquired at 303 K, on a 3-mm-diameter NMR sample tube containing 150  $\mu$ M uniformly  $^{15}\text{N}$ -labeled lamin peptides and non-labeled EmN or EmC, in 20mM Tris-HCl pH8, 30mM NaCl and 80:20%  $\text{H}_2\text{O}/\text{D}_2\text{O}$ .

In order to reassign LamIgF NMR signals in our conditions, three-dimensional  $^1\text{H}$ - $^{15}\text{N}$ - $^{13}\text{C}$  correlation spectra were acquired using 3D HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO and HN(CO)(CA)NH pulse sequences at 303 K, on a 3-mm-diameter NMR sample tube containing 500  $\mu\text{M}$  uniformly  $^{15}\text{N}/^{13}\text{C}$ -labeled LamIgF, in 20mM sodium phosphate pH 7, 30mM NaCl and 80:20%  $\text{H}_2\text{O}/\text{D}_2\text{O}$ . The data were processed using Topspin3.1 (Bruker) and analyzed using CCPNMR<sup>63</sup>.

#### **Size-exclusion chromatography.**

Size-exclusion chromatography experiments aiming at identifying interactions between emerin, BAF and lamin fragments (LamIgF WT and mutants, LB1) were performed using a Superdex-75 10/300 GL column (GE Healthcare) pre-equilibrated with buffer G (20mM Tris-HCl pH8, 30mM NaCl, 2mM DTT). 500 $\mu\text{l}$  of proteins concentrated at 150 $\mu\text{M}$  were injected for each experiment at a flow rate of 0.5ml/min at 277K.

#### **Isothermal Titration Calorimetry.**

ITC was performed using a high-precision VP-ITC calorimetry system. To characterize interactions between the BAF dimer and LamIgF (WT and mutants), all proteins were dialyzed against 50mM Tris-HCl pH8, 150mM NaCl, 10mM  $\beta$ -mercaptoethanol and protease inhibitors (Roche). BAF (20 $\mu\text{M}$ ) in the calorimetric cell at 288 K was titrated with LamIgF WT (at a concentration of 100  $\mu\text{M}$  in the injection syringe) or BAF in the calorimetric cell at 283 K was titrated with LamIgF mutants (at a concentration of 100 $\mu\text{M}$  in the injection syringe). To characterize interactions between the BAF dimer and EmN, all proteins were dialyzed against 50mM Tris-HCl pH8, 150mM NaCl, 10mM  $\beta$ -mercaptoethanol and protease inhibitors (Roche). BAF (30 $\mu\text{M}$ ) in the calorimetric cell at 288 K was titrated with EmN (at a concentration of 150 $\mu\text{M}$  in the injection syringe). Analyses of the data were performed using the Origin software provided with the instrument.

### **Negative-staining Electron Microscopy.**

To observe self-assembled EmN and EmC proteins, with or without LamIgF WT or LB1, protein samples containing 2 % uranyl acetate were deposited on glow-discharged carbon-coated copper grids. Data collection was performed using a Tecnai Spirit transmission electron microscope (FEI) equipped with a LaB6 filament, operating at 100 keV. Images were recorded using a K2 Base camera (Gatan, 4kx4k) at 15,000 magnification (at the level of the microscope) with a pixel size of 0.25 nm at the specimen level.

### **X-ray crystallography.**

**Crystallization and data collection** - The EmN-Baf-LamIgF complex was purified by gel filtration (Superdex 75, 10/300 GL) in 50mM Tris-HCl pH8, 100mM NaCl and concentrated to 3mg/ml. Crystallization was initiated one week after the gel filtration and in these conditions, EmN was proteolyzed and the final complex was composed of LEM-BAF-LamIgF. For crystallization, 1  $\mu$ L of the complex was mixed with 1  $\mu$ L of the reservoir solution and equilibrated against a 500- $\mu$ L reservoir by hanging drop at 277K. Crystals were grown in 25% (w/v) polyethylene glycol monomethyl ether (PEG) 3.350, 20mM Bis-Tris pH5.5, 30mM NaCl and 0.2M NH<sub>4</sub>SO<sub>4</sub>. They were flash-cooled in liquid nitrogen, using a cryo-protection solution of 25% PEG 3.350, 20mM Bis-Tris pH5.5 and 0.2M NH<sub>4</sub>SO<sub>4</sub>, supplemented with 27% (V/V) ethylene glycol.

**Structure determination and refinement** - The 3D structure of the complex was solved by molecular replacement using Molrep in CCP4<sup>64</sup>. The coordinates of the BAF dimer with DNA (PDB entry 2BZF), the coordinates of lamin A/C globular domain (PDB entry 1IFR) and the coordinates of the emerin LEM domain (PDB entry 2ODC) were used as templates. The resulting model was rebuilt using PHENIX<sup>65</sup>, manual correction was performed with Coot<sup>66</sup> according to  $|F_o| - |F_c|$  and  $2|F_o| - |F_c|$  maps, and further refinement was carried out with phenix.refine. All structure figures were prepared using PyMOL (Schrödinger, LLC).

### **Fluorescence-based thermal shift assay.**

The thermal stability of LamIgF proteins was monitored by a fluorescence-based thermal shift assay performed with a QuantStudio 12K Flex instrument (LifeTechnologies). 10 µg of purified protein was mixed with the SYPRO Orange dye (diluted 800-fold from a 5000-fold stock solution, Invitrogen) in 50mM Tris pH 8.0, 150mM NaCl, 10% glycerol. Reactions were carried out in duplicate in a 96-well fast PCR plate at a final volume of 20 µl and each experiment was repeated at least twice independently. The samples were submitted to a denaturation kinetic from 10 to 95°C at a rate of 3°C/min and fluorescence of Sypro Orange dye was recorded in real time. The protein denaturation temperature ( $T_d \pm$  s.e.m) was calculated using the Protein Thermal Shift software v1.3 (LifeTechnologies) as the maximum of the derivative of the resulting fluorescence curves.

### **Proximity Ligation Assays.**

**Transfection** - HeLa cells were obtained from American Type Culture Collection and cultured in Minimum Essential Medium containing Glutamax (Gibco), 1% non-essential amino acids and 10% fetal bovine serum. HeLa cells were transfected using XtremeGene 9 (Roche). After 24 h, cells were processed for either immunoblotting, immunofluorescence or Proximity ligation assay.

**Immunoblotting** - Whole cell protein extracts were suspended in Laemmli sample buffer, separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1.5 h in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 5% dry milk, incubated with mouse anti FLAG antibody (Sigma; 1:600) and rabbit anti-BAF (Santacruz, 1:200) for 1 h in TBST/1% milk, washed 4 times and incubated with HRP-conjugated secondary antibodies. After 4 washes in TBST, proteins were detected by enhanced chemiluminescence.

**Immunofluorescence microscopy** - Cells were fixed with 3% paraformaldehyde for 12 min at R.T, permeabilized with phosphate buffered saline containing 0.5 % Triton for 5 min at R.T, and quenched with 2% bovine serum albumin diluted in phosphate buffered saline containing 0.1% Triton. Primary antibodies were rabbit anti-FLAG (Sigma, 1:300) and mouse anti-GFP (Roche, 1:200). Fluorescent labeled secondary antibodies (donkey anti-mouse Cy2 1:60 and donkey anti-rabbit 594 1:200) were from Jackson ImmunoResearch. DNA was stained with Hoechst 33258 (1 µg/ml).

**Proximity ligation assay** - PLA was used to detect interactions between GFP-BAF and FLAG-Lamin A, either WT or mutant (R435C, R527H, A529V, K542N), based on proximity (< 40 nm) of two secondary antibodies directed against these tagged proteins. After cell fixation, cell permeabilisation and quenching (as above), pairs of primary antibodies, rabbit anti-FLAG 1:300 and mouse anti-GFP 1:200, were added on HeLa cells expressing GFP-BAF together with FLAG-Lamin A, for 40 min at R.T. Next, Duolink PLA probe anti-rabbit plus, Duolink PLA probe anti-mouse minus and Duolink detection reagents orange (detected with a Cy3 filter) were used according to manufacturer's instructions (Olink, Bioscience). At last, cells were mounted in Duolink mounting medium with Dapi (Olink, Bioscience). Confocal microscopy image acquisition was performed using a LSM 700 Laser scanning microscope (Zeiss) at the imaging facility of the BFA institute. Quantitative analysis of PLA signals was done on images using Image J. Data were then analyzed by comparing median values for the total number of pixels showing a signal (Cy3) per nucleus. Data of the three independent experiments were normalized to 1 (100%) for cells expressing FLAG-lamin A WT. Statistical analysis were performed using Kruskal-Wallis tests.



## Legends

### **Figure 1. The lamin A/C Igfold domain directly binds to emerin self-assembled nucleoplasmic region. (A)**

Architecture of the emerin protein, highlighting its LEM domain (in orange) and its transmembrane domain (in dark grey), as well as the 2 fragments of the nucleoplasmic region that could be obtained as soluble peptides (EmN and EmC). **(B)** Negative staining EM images of EmN and EmC fragments obtained after purification, concentration and incubation at 293K (white bar: 100 nm). EmN filaments were observed at 600  $\mu$ M after 1 week<sup>27</sup>, whereas EmC filaments were already visible at 300  $\mu$ M after 1 day. The fragment corresponding to the region common to EmN and EmC did not self-assemble in these conditions (Suppl. Figure 1B). **(C)** Superimposition of the solution-state NMR <sup>1</sup>H-<sup>15</sup>N HSQC spectra of the lamin Igfold domain (lamin A/C in grey or lamin B1 in green) recorded in the absence or presence of emerin fragments EmN or EmC (EmN in red, EmC in purple; 1:1 ratio). The emerin fragments were EmN filaments (upper left and lower right views), EmN monomers (lower left view) and EmC filaments (upper right view). All these spectra were recorded at 303K on a 700 MHz spectrometer.

### **Figure 2. The lamin A/C Igfold domain binds to emerin monomeric LEM domain through BAF. (A)**

Detection of a ternary complex including LamIgF, EmN and BAF by size exclusion chromatography using a Superdex 75 10/300GL column. Each gel corresponds to the elution of a different set of proteins, using the same column, the same protein concentrations and the same buffer: from the bottom EmN, LamIgF, BAF, Lamin B1 tail, BAF and lamin B1 tail, EmN and LamIgF, EmN and BAF, LamIgF and BAF and finally EmN, LamIgF and BAF. The bands corresponding to the different complexes are boxed: EmN and BAF in orange, LamIgF and BAF in green and the ternary complex in blue. **(B)** ITC titration of BAF onto LamIgF (upper panel) and BAF onto EmN (lower panel). 200  $\mu$ M LamIgF or EmN were injected in a cell containing 40  $\mu$ M of BAF at a temperature of 288K. The affinities deduced from the curves are  $3.2 \pm 1.2 \mu$ M and  $0.7 \pm 0.2 \mu$ M, respectively. The LamIgF / BAF interaction is enthalpy driven whereas the EmM/BAF interaction is both enthalpy and entropy driven (Suppl. Table 1).

**Figure 3. Three-dimensional structure of the complex between LamIgF, emerin LEM domain and BAF. (A)**

Cartoon representation of the complex, with emerin LEM domain (residues 3 to 44) in orange, BAF dimer in yellow-green and green (residues 4 to 89), and lamin A/C Igfold domain (residues 432 to 544) in grey. The interfaces corresponding to the emerin / BAF and BAF / lamin interactions are indicated by red circles and numbered as 1 and 2, respectively. **(B)** Superimposition of the 3D structure of the BAF dimer bound to the emerin LEM domain, as determined in this work (same colors as in (A)), and as revealed using NMR by Clore and co-workers (PDB reference 2ODG<sup>20</sup>; in cyan). **(C)** Zoom on the EmN/BAF interface, with residues more than 30% buried within the interface displayed in sticks. On the emerin side, the interface is mainly formed by residues Gly24 to Lys37 (labeled residues on loop  $\alpha 1\alpha 2$  and helix  $\alpha 2$ ). On the BAF side, it is mainly formed by helices  $\alpha 3$  and  $\alpha 4$  of one monomer and loop  $\alpha 2\alpha 3$  and helix  $\alpha 3$  of the other monomer.

**Figure 4. Lamin A/C Igfold domain binds BAF through a  $\beta$ -sheet including residues mutated in recessive progeroid syndromes. (A)**

Cartoon representation of the complex between the lamin A/C Igfold domain and the BAF dimer (main chain colors as in Fig. 3). On the lamin side, the interface is mainly polar: it is formed by residues His433, Ala434, Arg435, Glu537, Met540 and Lys542 (in red sticks). On the BAF side, it is formed by residues from helix  $\alpha 1$ , loop  $\alpha 1\alpha 2$  and helix  $\alpha 5$  from both monomers. At the BAF monomer-monomer interface, Gly47 is displayed in magenta. **(B)** <sup>1</sup>H-<sup>15</sup>N HSQC spectrum of LamIgF in the absence (grey) and presence (green) of BAF (1:1 ratio). The labelled peaks exhibit a significant intensity decrease after addition of BAF. The peaks labelled in italics correspond to residues mutated in patients with progeroid diseases (R435<sup>32,33,35</sup>, R471C<sup>36</sup>, M540<sup>67,15</sup>, K542<sup>34</sup>).

**Figure 5. Impact of mutants causing recessive progeroid syndromes on BAF binding depends on the disease severity. (A)**

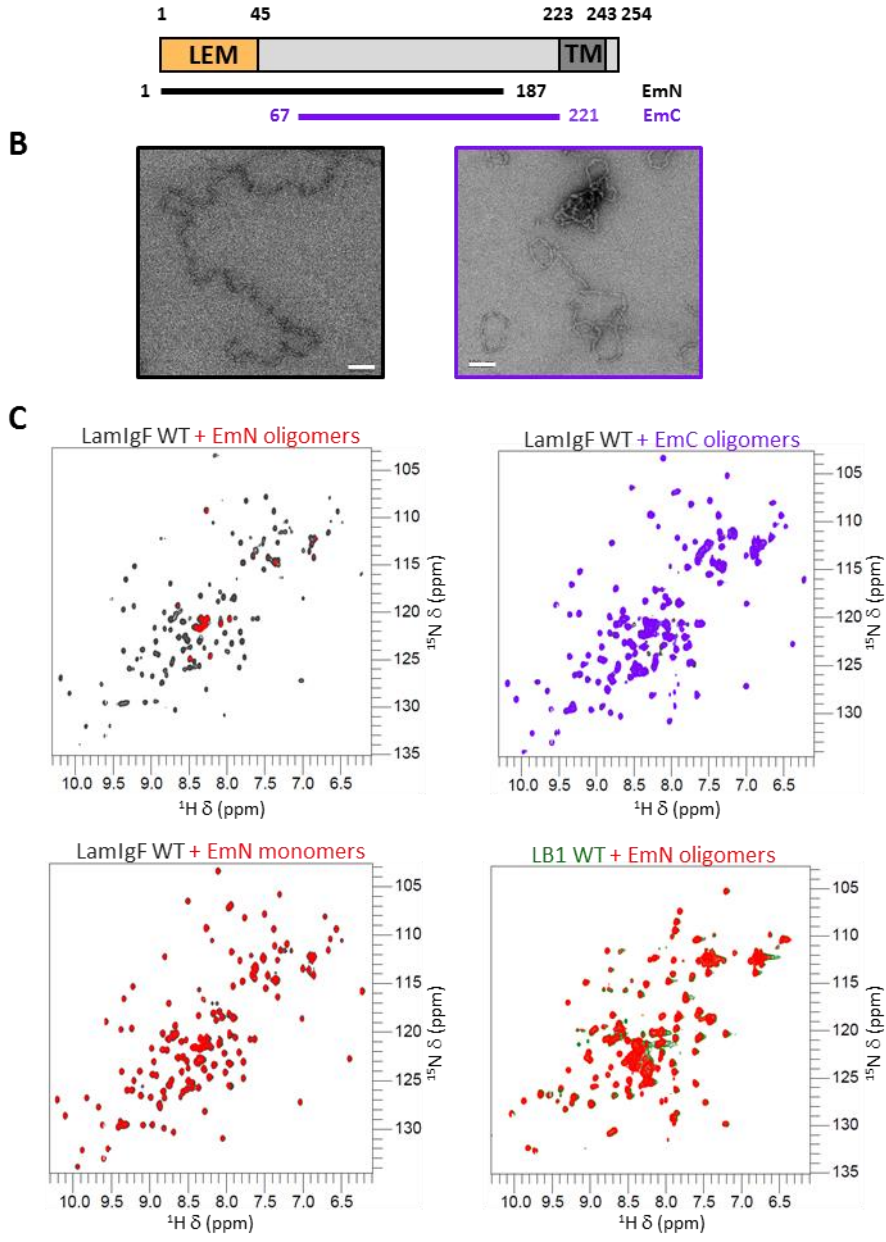
ITC titration of BAF onto LamIgF, as measured at 283K. Whereas variants R453W and R482W show WT binding, variants A529V and R471C exhibit a significantly lower binding enthalpy and affinity as compared to LamIgF WT. No affinity could be measured for R527H and K542N and no binding could be detected for R435C. In the lower right panel, the spatial distribution of residues mutated in (A) is displayed on the lamin A/C Igfold structure (PDB reference 1IFR<sup>13</sup>).

The 2 residues mutated in control variants, causing muscle and adipose tissue diseases respectively, are colored in green, whereas the 5 residues mutated in variants with a lower affinity for BAF are colored in cyan, purple and red (for weak binding, no measurable affinity and no detected binding, respectively). **(B)** Size exclusion chromatography of LamIgF mutants mixed to BAF (Superdex 75 10/300GL). Each gel corresponds to the elution of a different set of proteins, using the same column, the same concentrations and the same buffer: from bottom BAF, BAF and R435C, BAF and K542N, BAF and R527H, BAF and R471C, BAF and A529V, BAF and R482W, BAF and R453W and BAF and WT LamIgF. The bands corresponding to the BAF-LamIgF complex are boxed in red.

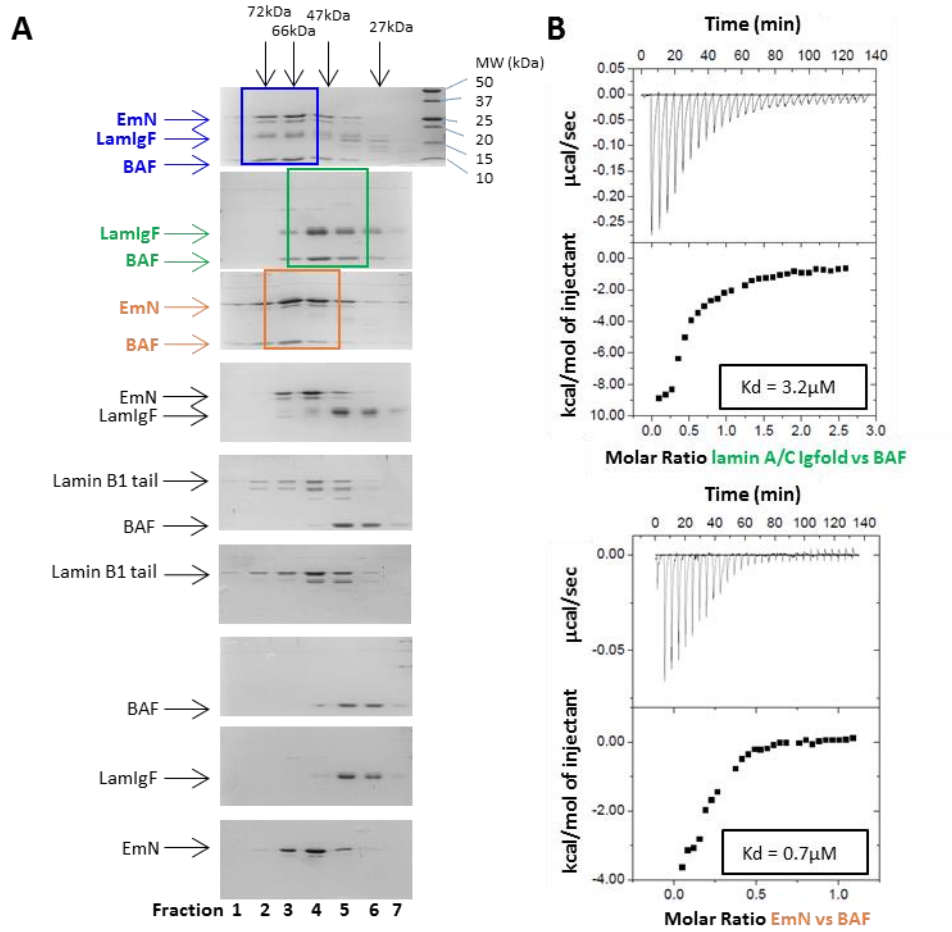
**Figure 6. Mature lamin mutants with mutations causing recessive progeroid syndromes impair BAF binding in cells. (A)** HeLa cells expressing GFP-BAF (WT) together with either WT, R435C, R527H, A529V or K542N FLAG-LAm were fixed, and processed for immunofluorescence using as primary antibodies, a mix of anti GFP and anti-FLAG antibodies. Observation was done at the confocal microscope. Are shown in the left panels, merges for GFP-BAF (green), FLAG-LAm (red) and DNA (blue), and in the right panels, the FLAG-LAm alone. Scale bar: 20  $\mu$ M. **(B)** Whole cell protein extracts prepared from HeLa cells control (ctrl) or expressing GFP-BAF (WT) together with either WT, R435C, R527H, A529V or K542N FLAG-LAm were analyzed by western blot using anti FLAG and anti BAF antibodies. **(C)** HeLa cells, expressing GFP-BAF (WT) together with either WT, R435C, R527H, A529V or K542N FLAG-LAm were fixed, labeled with a mix of anti GFP and anti-FLAG antibodies and further processed for PLA before observation at the confocal microscope. The PLA signals (red) are shown merged with DNA (blue) and GFP-BAF (green) or alone (PLAssay). Signals for GFP-BAF and DNA are shown alone in the two right panels, respectively. Scale bar: 15  $\mu$ M. **(D)** Quantification of PLA signals per nucleus as shown in C. The graph represents the mean values  $\pm$  stdevpa (n = 3 experiments; \*  $p < 0.05$  with Kruskal and Wallis test).

**Figure 7. A first model of the interface between the LEM-domain proteins, the nucleoskeleton and the chromatin-associated protein BAF. (Left)** The lamin A/C Igfold is able to recognize emerlin through BAF. Superimposition of the X-ray structure of the LamIgF-LEM-BAF complex onto the X-ray structure of the BAF protein complexed to DNA (PDB reference 2BZF<sup>53</sup>) suggests that BAF can simultaneously interact with lamin, emerlin and DNA. The lamin-BAF interface is disrupted by mutations causing autosomal recessive progeroid syndromes, as symbolized by the red cross. **(Right)** In interphase cells, the lamin A/C Igfold domain recognizes self-assembled emerlin at the nuclear envelope. It may also binds to monomeric emerlin through BAF. The lamin A/C Igfold domain is able to recognize BAF and histones, thus creating an interface between the lamina and chromatin. This interface is disrupted by mutations causing autosomal recessive progeroid syndromes, as symbolized by the red cross.

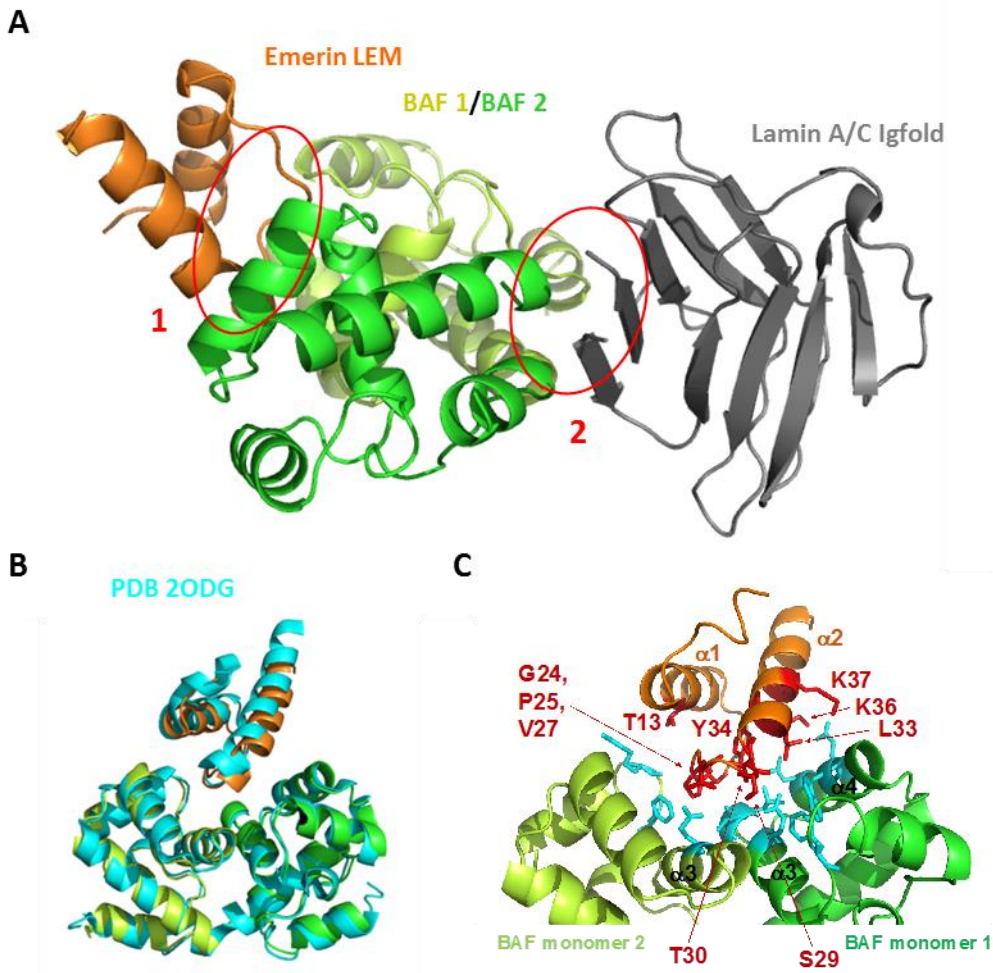
**Figure 1.**



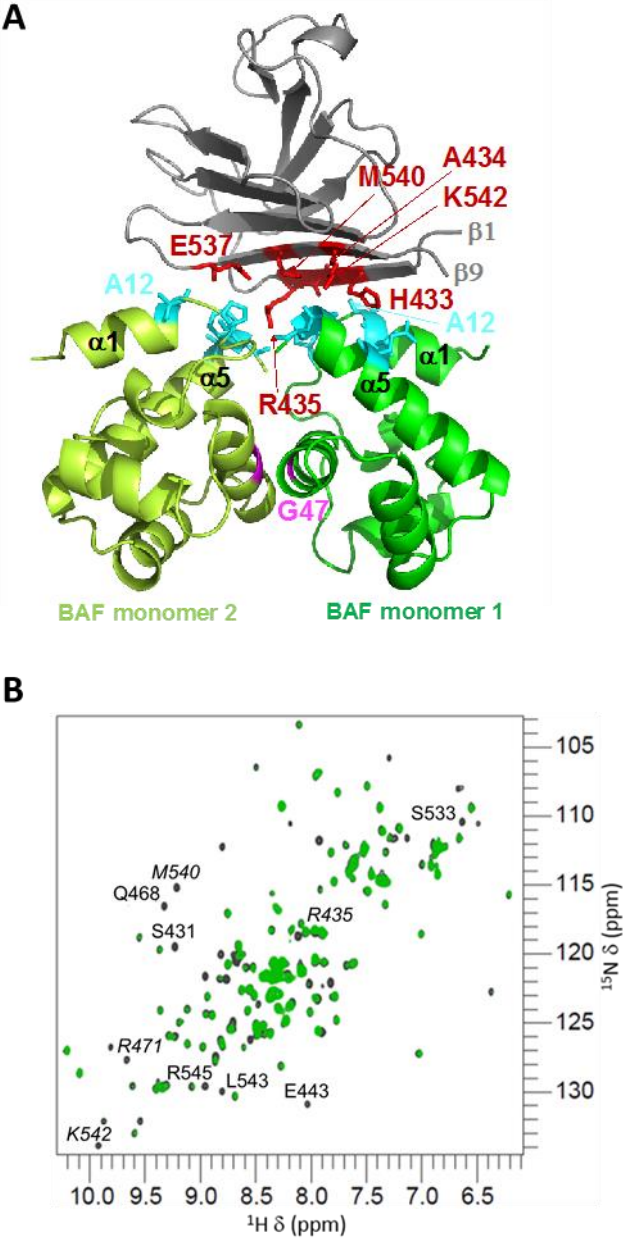
**Figure 2.**



**Figure 3.**

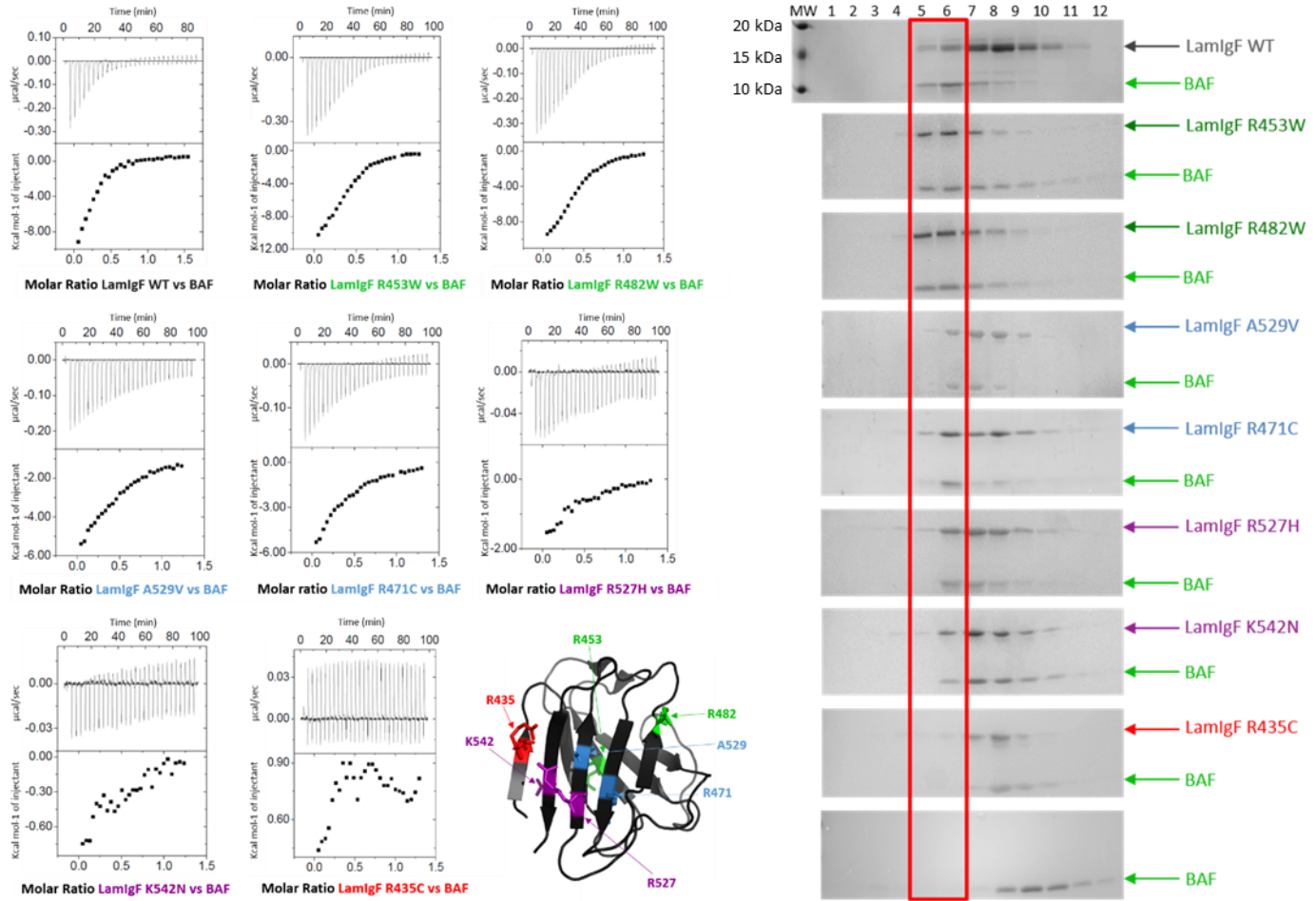


**Figure 4.**

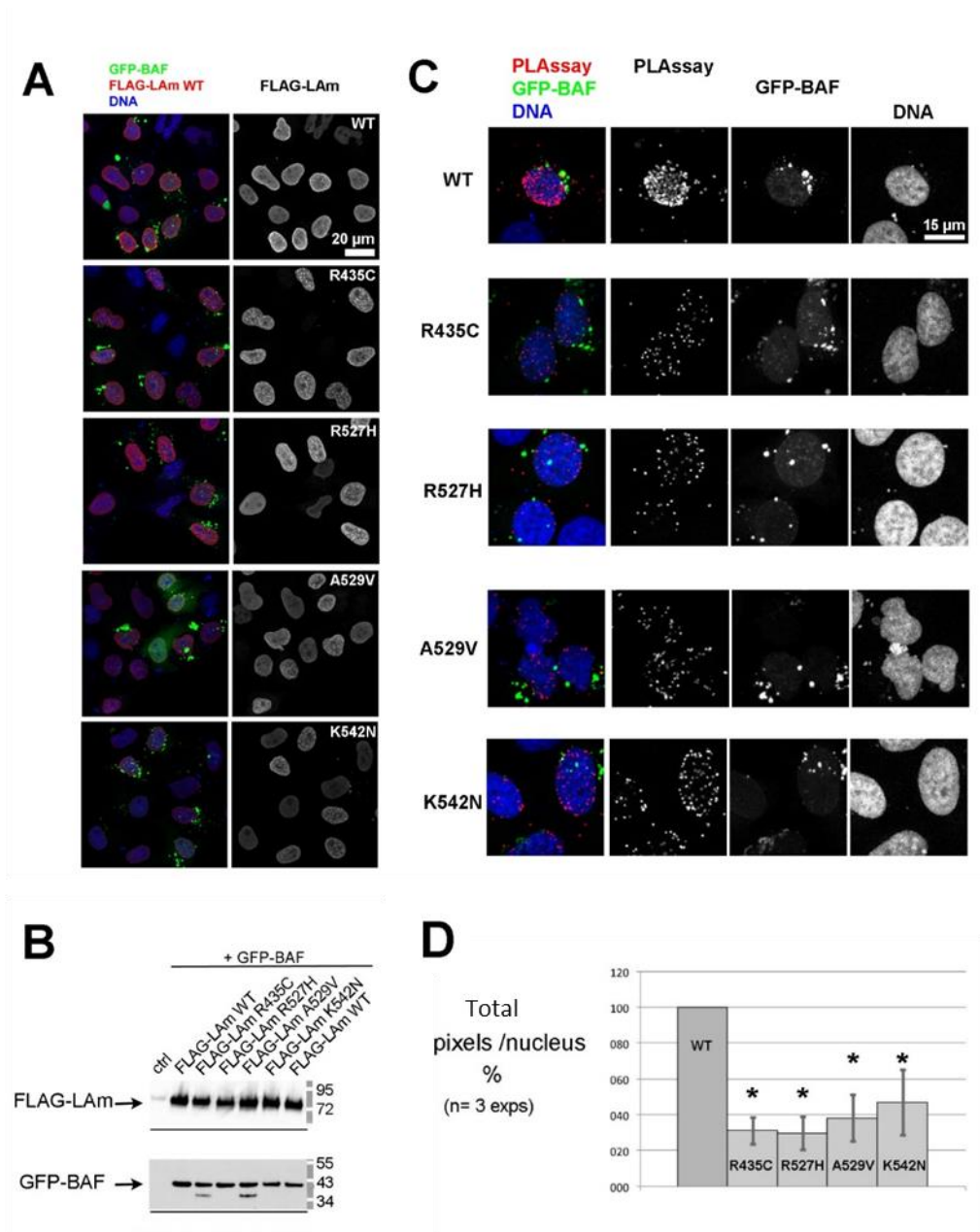




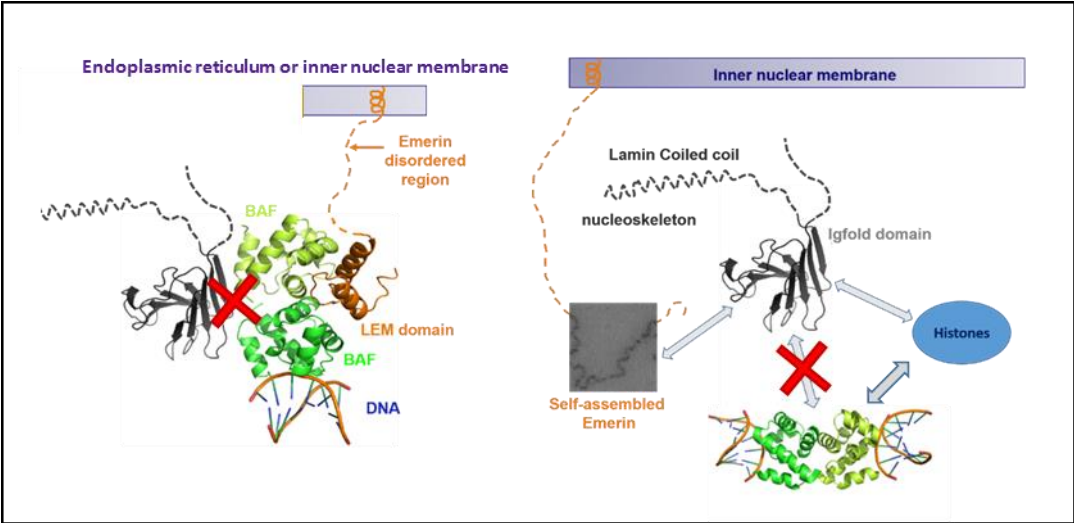
**Figure 5.**



**Figure 6.**



**Figure 7.**



## Supplementary information

### **Suppl. Fig. 1. Monomeric structure of fragments EmN49, EmC170, EmC187 and EmC, as observed by NMR.**

**(A)** Superimposition of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of different emerin fragments is shown. This superimposition reveals that, whereas the EmN49 fragment, corresponding to the LEM domain (3D structure in the upper left corner; PDB reference 1ODG<sup>68</sup>), is well folded, the 3 other fragments are entirely unstructured. **(B)** ThT fluorescence as a function of the incubation time at 310 K, measured on 3 emerin fragments (EmN, Em67C and EmC) concentrated at 300 $\mu\text{M}$  (n=2; error bars correspond to standard deviations). Only EmN and EmC are able to self-assemble. Em67C does not form filaments.

### **Suppl. Fig. 2. Structure of the LamIgF variants, as observed by NMR and fluorescence. (A)**

**(A)**  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra were recorded at 303K on different LamIgF variants (LamIgF WT in dark grey, LamIgF R453W and R482W in green, LamIgF A529V, R471C and R527H in blue and LamIgF K542N and R435C in red). All proteins were concentrated to 200 $\mu\text{M}$ , dialyzed in 20mM Tris-HCl pH 8.0, 30mM NaCl, 2mM DTT and NMR spectra were recorded on a 700MHz spectrometer. **(B)** Thermal stability of the different LamIgF variants was measured using a fluorescence thermal shift assay in 50mM Tris pH 8.0, 150mM NaCl, 10% glycerol. Error bars correspond to the standard deviation calculated from at least 4 measurements.

### **Suppl. Fig. 3. The lamin B1 tail domain does not bind to BAF, as observed by NMR.**

Addition of BAF onto the  $^{15}\text{N}$  labeled lamin B1 tail (residues 395 to 586) does not affect the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of the lamin B1 tail. The spectra were recorded at 283K, on a 600MHz, in phosphate pH 6.5, 20mM, 150mM NaCl, 1mM DTT.

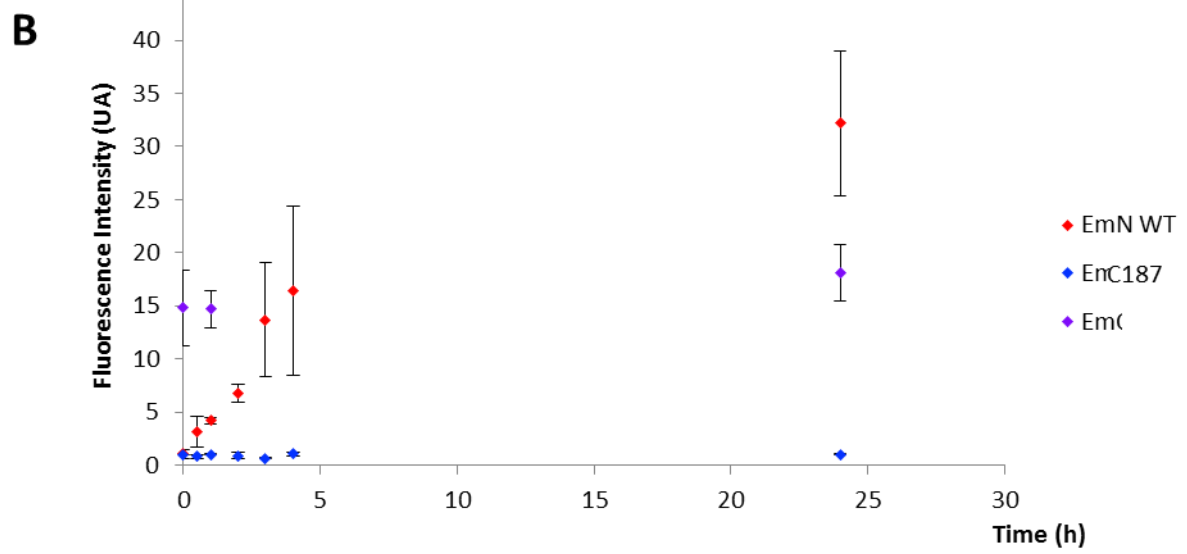
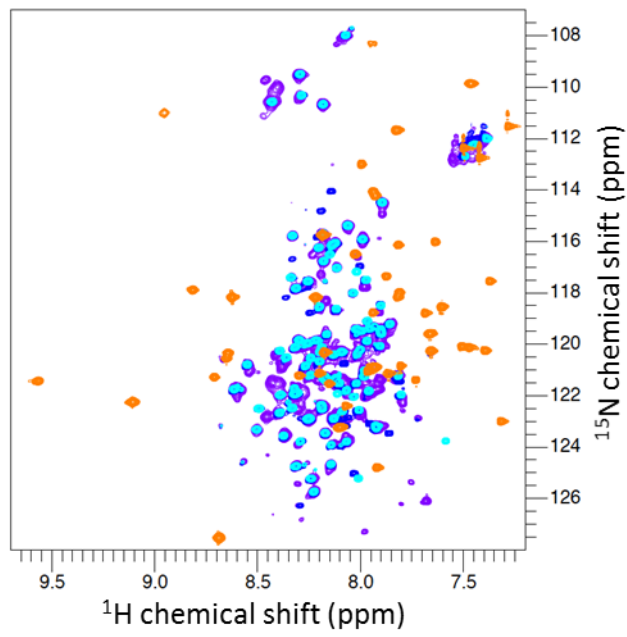
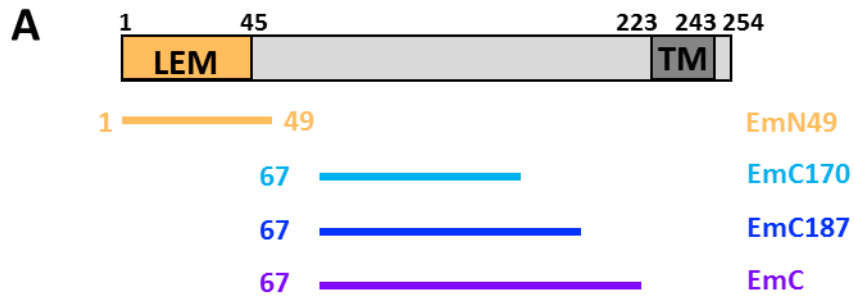
### **Suppl. Fig. 4. Quantification of the PLA signals per nucleus (related to Figure 6C).**

Graphs represent the median values for the total signal expressed in number of pixels per nucleus for three independent experiments (Exp1, Exp2, Exp3; n= 105 to 230 nuclei per sample). Boxes show first and third quartiles (\* p<0.005 with Kruskal and Wallis test).

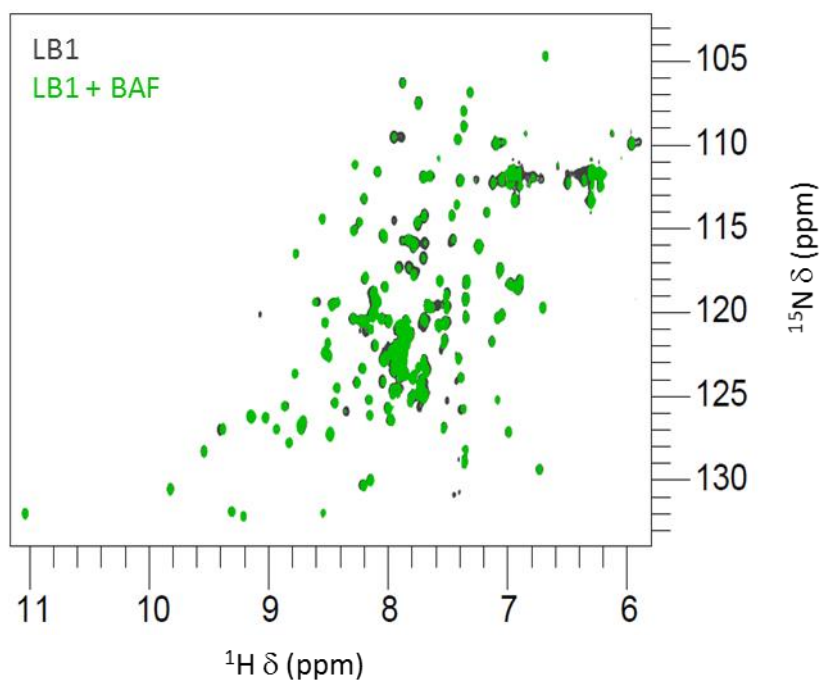
**Suppl. Fig. 5. The WT LamIgf still binds to BAF A12T but with a lower affinity.** Size exclusion chromatography experiments revealed an interaction between LamIgf and either BAF WT, BAF S4E or BAF A12T (Superdex 75 10/300GL). Each gel corresponds to the elution of a different set of proteins, using the same column, the same concentrations and the same buffer: from the bottom BAF A12T, LamIgf and BAF A12T, BAF S4E, LamIgf and BAF S4E, BAF WT, LamIgf, LamIgf and BAF WT. The bands corresponding to the ternary complex are boxed in red. Whereas BAF S4E binds as BAF WT to LamIgf, BAF A12T shows a weaker affinity for LamIgf.

**Suppl. Fig. 6. Lamin A – BAF proximities are enriched at the nuclear periphery of interphase HeLa cells.** HeLa cells transfected to express FLAG-LAm together with GFP-BAF were fixed, labeled with a mix of anti-GFP and anti-FLAG antibodies and further processed for PLA before observation at the confocal microscope. Are shown in the left panels the PLA signals (red) merged with GFP-BAF (green) and DNA (blue). Are shown in the right panels the PLA signals alone. Scale bar: 10  $\mu$ M.

**Suppl. Fig. 1.**

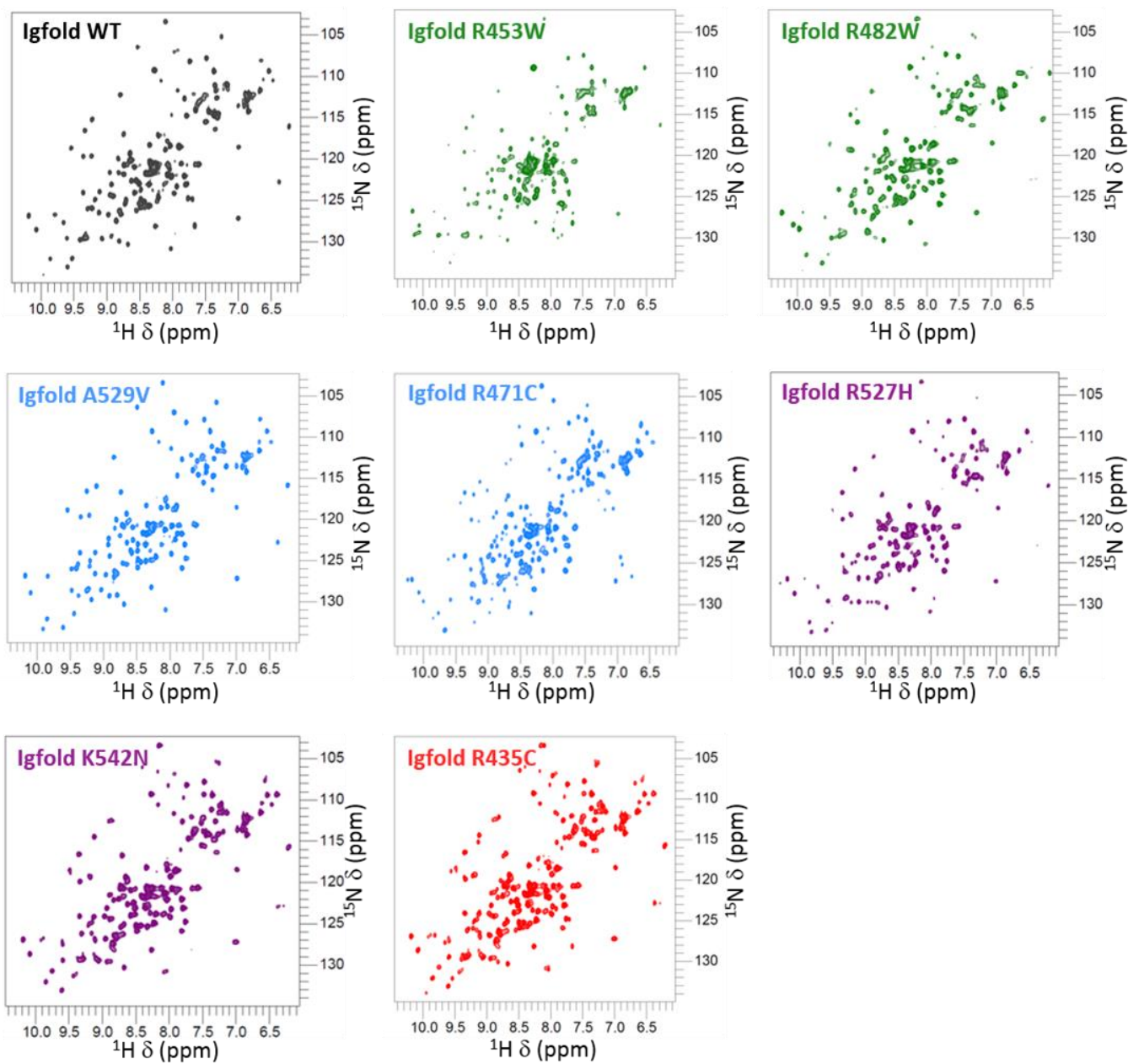


**Suppl. Fig. 2.**

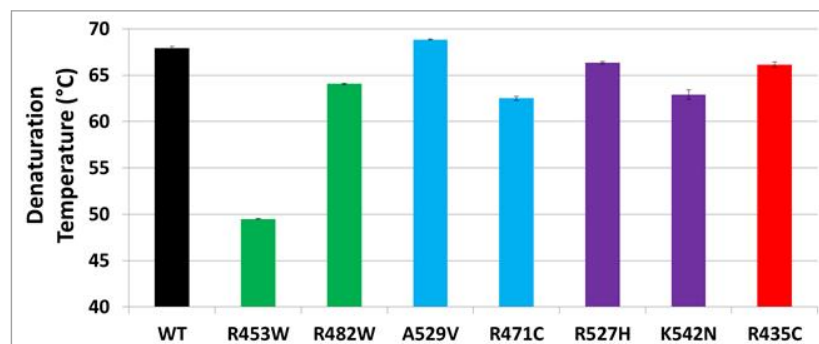


Suppl. Fig. 3.

A

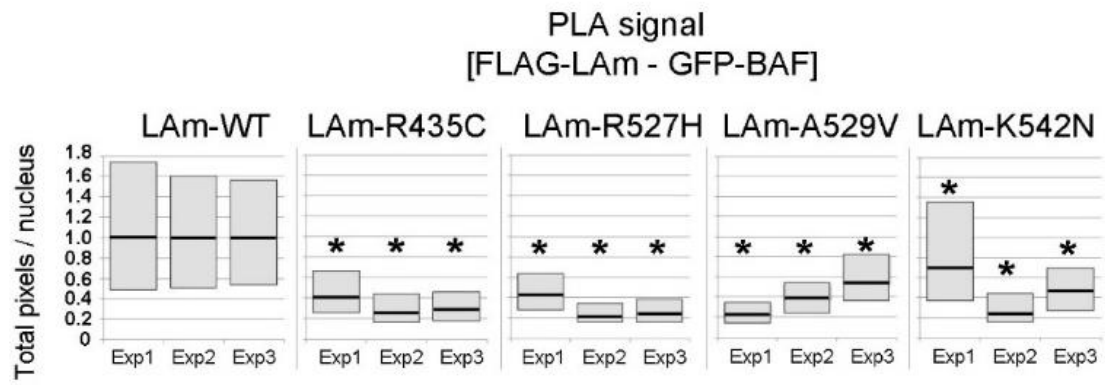


B

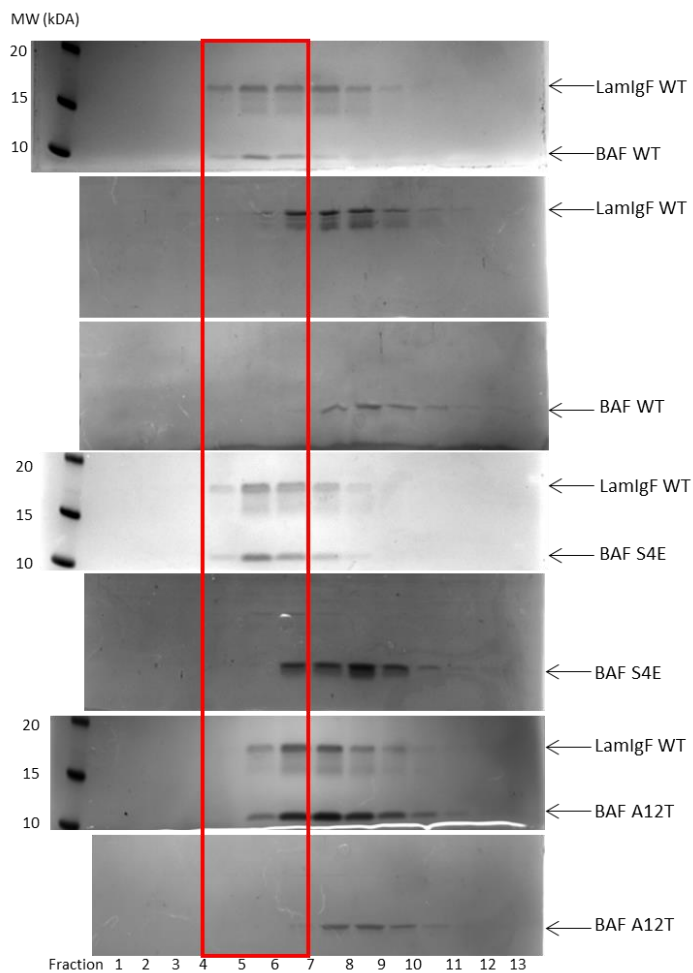




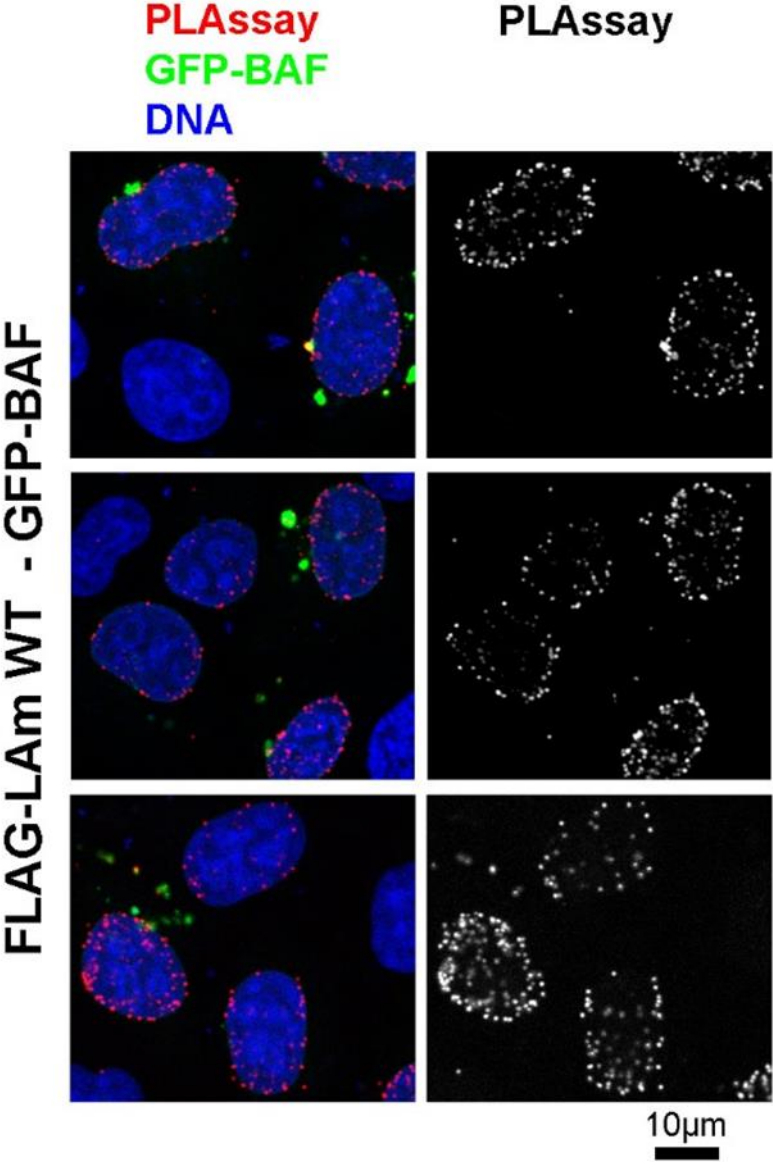
**Suppl. Fig. 4.**



**Suppl. Fig. 5.**



Suppl. Fig. 6.



**Suppl. Table 1. Summary of Isothermal Titration Calorimetry results. (A)** Table containing parameters deduced from all ITC experiments performed at 288K. **(B)** Table containing parameters deduced from all ITC experiments performed at 283K. N is the number of experiments, N<sub>s</sub> the stoichiometry, K<sub>a</sub> the association rate constant, K<sub>d</sub> the dissociation rate constant, ΔH the measured enthalpy and ΔS the resulting entropy.

Experiments done at 15°C	BAF / Igfold		BAF / EmN	
	N = 5		N = 3	
	Mean	Standard deviation	Mean	Standard deviation
N <sub>s</sub>	0.35	0.09	0.25	0.02
K <sub>a</sub> (M <sup>-1</sup> )	3.7E5	2.1E5	1.4E6	2.9E5
K <sub>d</sub> (μM)	3.15	1.16	0.7	0.17
ΔH (cal/mol)	-1.15E4	2.1E3	-4.1E3	9.1E2
ΔS (cal/mol/deg)	-13.5	9.3	-13.9	3.1

Experiments done at 10°C	Igfold WT		Igfold R453W		Igfold R482W		Igfold A529V		Igfold R471C	
	N = 2		N = 2		N = 2		N = 2		N = 2	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
N <sub>s</sub>	0.39	0.02	0.39	0.03	0.43	0.04	0.38	0.05	0.39	0.16
K <sub>a</sub> (M <sup>-1</sup> )	7.2E5	2.6E5	6.5E5	5.3E4	5.6E5	2.6E4	1.5E5	1.4E4	1.4E5	1.6E4
K <sub>d</sub> (μM)	1.5	0.54	1.5	0.12	1.7	0.08	6.9	1.6	7.2	0.8
ΔH (cal/mol)	-1.3E4	5.9E2	-1.3E4	6.4E2	-1E4	2E3	-8E3	1.6E3	-1.1E4	3E3
ΔS (cal/mol/deg)	-20.5	2.75	-18.8	2.12	-10.6	7	-4.8	5.9	-14	10

**Suppl. Table 2.** Data collection and refinement statistics.

<b>Data collection</b>	
Space group	P 1
Cell dimensions	
a, b, c (Å)	57.77 62.75 64.34
a, b, $\gamma$ (°)	66.44 66.55 88.05
Resolution (Å)	49.152 – 2.1
Anisotropy direction	
Resolution where $CC_{1/2} > 0.3$	2.1
$R_{\text{meas}}$ (%)	15.9
I/s(I)	5.9
$CC_{1/2}$	99.1
Completeness (%)	95.67
Redundancy	3.52
B Wilson (Å <sup>2</sup> )	34.74
<b>Refinement</b>	
No. reflections	83937
$R_{\text{work}} / R_{\text{free}}$	0.1879/0.2444
No. atoms	5666
Protein	675
DNA	0
SO <sub>4</sub> <sup>2-</sup>	8
Water	279
B factors	43.38
Protein	42.97
Ligand/ion	80.03
Water	45.37
R.m.s. deviations	1.953
Bond lengths (Å)	0.008
Bond angles (°)	1.16
One crystal per structure.	

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# FRENCH SYNTHESIS

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Ma thèse portait sur la caractérisation structurale et fonctionnelle de l'enveloppe nucléaire. Cette enveloppe est formée d'une bicouche lipidique (membranes nucléaire interne et externe) et de différentes protéines associées à ces membranes. Elle est impliquée dans:

- la forme et la position du noyau
- la structure et la mobilité cellulaire car elle est reliée au cytosquelette via le complexe LINC
- l'organisation du génome, en interagissant avec la chromatine
- la régulation de voies de signalisation en interagissant avec des facteurs de transcriptions et différentes enzymes.

Des mutations au niveau des gènes codant pour les protéines de l'enveloppe nucléaire interne sont à l'origine d'un grand nombre de maladies humaines, appelées les envelopathies, incluant des dystrophies musculaires, des lipodystrophies et des syndromes de vieillissement prématuré. Toutefois, les données structurales sont rares pour ces protéines, entravant ainsi la description de leurs mécanismes fonctionnels normaux et pathogènes. Dans mon projet de thèse, nous avons proposé de caractériser structurellement le complexe émerine-lamine et d'observer les conséquences de 6 mutations pathogènes de l'émerine, à l'origine de la dystrophie d'Emery-Dreifuss. qui n'empêchent pas l'expression et la bonne localisation de la protéine (delK37, S54F, Q133H, del95-99, P183T, P183H). L'une des nouveautés de ce projet était de réussir à stabiliser des oligomères de l'émerine et de la lamine, afin de reconstituer le complexe fonctionnel impliqué dans l'architecture de l'enveloppe nucléaire.

Lorsque j'ai débuté ma thèse, nous avions des preuves préliminaires que l'émerine dimérise in vitro, forme des oligomères in vitro et dans les cellules, et que cette oligomérisation a une influence sur la liaison de cette protéine à la lamine. Des publications récentes avaient également montré que l'émerine est phosphorylée par Src après l'application d'une force mécanique sur le noyau et que le complexe émerine-lamine est au centre de la réponse nucléaire face à une force mécanique (Ho et al, Nature 2013; Guilluy et al., Nat Cell Biol 2014).



Au cours de ma première année, j'ai caractérisé les oligomères formés par le fragment de l'émerine comprenant les résidus 1 à 187 (protocole de production déjà établi au laboratoire) à l'aide de différentes techniques telles que la microscopie électronique, la fluorescence et la spectroscopie infrarouge. J'ai observé que l'émerine 1-187 est capable de former des filaments in vitro et que ces filaments sont formés de feuillets  $\beta$ . J'ai également mis au point un protocole robuste de production de ces filaments. J'ai caractérisé l'état oligomérique de 6 mutants de l'émerine à l'origine de la dystrophie musculaire d'Emery-Dreifuss (S54F, del95-99, Q133H, P183T et P183H). Pendant une deuxième partie de ma première année de thèse, j'ai également commencé une étude des conséquences structurales de la phosphorylation de l'émerine par la kinase Src (voir introduction) et en particulier l'analyse de l'impact de la phosphorylation sur la formation des filaments. Pour mener à bien ce projet, notre équipe s'est rendue pendant 9 mois (d'Avril à Décembre 2015) dans le laboratoire du Dr Phillip Selenko, au FMP de Berlin-Buch.

Je me suis ensuite intéressée au mécanisme d'assemblage des filaments in vitro et j'ai déterminé quelles parties de l'émerine étaient indispensables à cet assemblage. Pour ce faire, la première approche fut de produire différentes parties de l'émerine et de tester leur capacité à former des filaments. J'ai d'abord produit les fragments 67-170 puis 67-187, afin de savoir si le domaine LEM était indispensable à la formation des filaments et j'ai pu constater que ces deux fragments ne formaient pas ce type d'oligomères. J'ai ensuite voulu savoir si les régions 1-146 puis 1-132 étaient suffisantes pour former des filaments. J'ai choisi ces régions car elles ne comportaient pas l'unique cystéine présente dans l'émerine (en position 147), sachant que nous avons prouvé auparavant que les filaments étaient formés à partir du monomère et non du dimère d'émerine, et car elles comportaient la région 95-99 qui semblait être importante pour la formation des filaments. J'ai pu observer la formation de nos oligomères dans les deux cas. J'ai également produit la région du domaine LEM 1-50 seule et observé l'absence de filaments. Donc la région minimale suffisante pour former des filaments est 1-132. Enfin, j'ai étudié la cinétique de formation d'un mutant du domaine LEM de l'émerine, le mutant delK37, et observé que ce dernier possède un domaine LEM déstructuré et est capable de former des filaments plus rapidement que l'émerine 1-187 sauvage.

De manière consistante, j'ai fait la protéolyse ménagée des filaments d'émerine 1-187 sauvage et delK37, et par spectrométrie de masse (collaboration avec l'équipe du Dr Jean Armengaud, CEA Marcoule), j'ai observé que les régions restantes après coupure par la chymotrypsine, la trypsine et l'endoprotéinase GluC correspondaient en majeure partie au domaine LEM, mais également à la région autour des résidus 95-99.

Enfin nous avons étudié nos filaments à l'aide de la technique de la RMN du solide, à travers une collaboration avec l'équipe du Prof Adam Lange, au FMP de Berlin-Buch. Nous avons obtenu des premiers spectres des filaments de l'émerine 1-187 et des filaments de l'émerine 1-132. Ces spectres montrent que la région structurée est la même dans les deux types de filaments et comprend une vingtaine de résidus. Puis, nous avons également obtenu des spectres du mutant 1-187 delK37 de l'émerine et observé que les filaments formés par ce mutant étaient identiques à ceux de l'émerine 1-187 WT. Cette analyse par RMN du solide ne nous a pas permis d'identifier la structure 3D du cœur des filaments à cause de l'hétérogénéité conformationnelle présente dans ces filaments, mais nous a permis de conclure, avec les données obtenues en spectrométrie de masse, que l'interaction entre le domaine LEM et la région autour des résidus 95-99 de l'émerine est à l'origine de la formation des filaments.

La dernière grande partie de mon projet de thèse concernait l'analyse structurale des interactions entre l'émerine et ses partenaires. Dans un premier temps, nous nous sommes intéressés à l'interaction entre l'émerine et la lamine A, notamment son domaine Igfold (domaine globulaire de la lamine). Lorsque que je suis arrivée au laboratoire, plusieurs expériences avaient déjà été menées sur ce projet, mais l'interaction entre ces deux protéines, connue dans la littérature, n'avait été observée qu'une fois par RMN et n'avait pas été reproduite. Grâce aux différentes études menées pendant ces deux années, j'ai amélioré notre connaissance des déterminants de l'état oligomérique de l'émerine et j'ai pu montrer l'interaction par RMN, ITC et microscopie électronique, entre le monomère du domaine Igfold de la lamine A et les filaments d'émerine 1-187 sauvage et delK37.

Nous avons étudié d'autres interactions lors de ma deuxième année de thèse. Tout d'abord l'interaction entre l'émerine et la queue de la myosine 1B (M1BO1). Cette étude a été possible grâce à une collaboration avec le groupe du Dr Anne Houdusse, de l'institut Curie. Nous avons d'abord caractérisé l'interaction par résonance magnétique nucléaire (RMN), puis nous avons fait des études préliminaires par titration calorimétrique isotherme (ITC) et par thermophorèse à micro-échelle (MST). Après avoir observé que l'interaction de l'émerine 1-187 WT avec M1BO1 semblait induire la formation des filaments d'émerine, nous avons fait des premières études par fluorescence, soit des cinétiques de formation de filaments à l'aide de la thioflavine et avons eu des premières confirmations de notre hypothèse.

Nous avons aussi étudié le complexe LEM/BAF/IgFold. En effet, la liaison entre le domaine globulaire LEM de l'émerine et la protéine BAF avait déjà été bien caractérisée (Cai M et al., 2007) et l'interaction entre l'IgFold de la lamine et la protéine BAF avait été plusieurs fois suggérée. Nous avons donc caractérisé et certifié l'interaction entre la protéine BAF et l'Igfold par RMN, ITC et Gel Filtration. Puis nous avons caractérisé l'interaction entre ces deux protéines et le domaine LEM de l'émerine, démontrant ainsi que les trois protéines peuvent former un complexe ternaire. Enfin nous avons cristallisé le complexe ternaire, obtenu des cristaux et une première structure de ce complexe, résolue à 2.8 Å. Finalement, nous avons étudié l'impact sur l'affinité entre l'Igfold et BAF de mutations causant des pathologies et impliquant des résidus se trouvant à l'interface IgFold/BAF.

**Titre :** Représentation en trois dimensions de l'interface entre l'enveloppe nucléaire et la chromatine

**Mots clés :** Interactions protéine-protéine, enveloppe nucléaire, Emery-Dreifuss Muscular Dystrophy

**Résumé:** Le noyau est un organelle caractéristique des cellules eucaryotes et les propriétés mécaniques de ce dernier jouent un rôle essentiel dans le comportement de la cellule, notamment sa motilité, sa polarité et sa survie. Le noyau est entouré par une enveloppe comprenant une membrane interne et une membrane externe, ainsi que de nombreuses protéines. Mes objectifs de thèse étaient de comprendre des mécanismes moléculaires déficients dans deux types de maladies génétiques causées par des mutations dans les lamines: la dystrophie musculaire d'Emery-Dreifuss et les syndromes de type progéroïde.

Dans un premier temps, nous avons montré que l'émerine s'auto-associe *in vitro* et en cellules (Herrada et al. *ACS Chem. Biol.* 2015). J'ai ensuite étudié la structure des oligomères d'émerine, déterminé le fragment protéique minimal nécessaire à la formation de ces oligomères et décrit l'impact de mutations de l'émerine, causant une dystrophie musculaire d'Emery-Dreifuss, sur son auto-assemblage (Samson et al. *Biomol NMR Assign.* 2016 ; Samson et al. *FEBS J.* 2016).

Puis, j'ai montré que seule cette forme auto-assemblée de l'émerine est capable d'interagir avec la lamine A et que la phosphorylation de l'émerine par la kinase Src, observée suite à un stress mécanique, régule cette interaction entre l'enveloppe nucléaire et le nucléosquelette.

Pour finir, j'ai montré que la forme monomérique de l'émerine est capable de former un complexe ternaire avec BAF et la lamine A. Après avoir mesuré les affinités protéine-protéine au sein de ce complexe, identifié les fragments minimaux des différentes protéines permettant de former ce complexe et mis au point un protocole robuste de purification de ce complexe, j'ai pu obtenir des cristaux de ce complexe dans plusieurs conditions. Par la suite, nous avons pu résoudre la structure de ce complexe par remplacement moléculaire avec une résolution de 2 Å. Enfin, j'ai montré que les mutations dans les lamines de type A provoquant des syndromes de type progéroïde pouvaient altérer l'interaction avec BAF *in vitro*, et nos collaborateurs, l'équipe du Dr B. Buendia (Paris Diderot), ont montré que ces mêmes mutations induisaient une diminution significative de la proximité entre la lamine A et BAF dans les cellules HeLa.

**Title :** A three-dimensional view of the interface between nuclear envelope and chromatin

**Keywords :** Protein-protein interactions, nuclear envelope, Emery-Dreifuss Muscular Dystrophy

**Abstract:** The nucleus is an organelle characteristic of eukaryotic cells and its mechanical properties play an essential role in the behavior of the cell, in particular its motility, polarity and survival. It is surrounded by an envelope comprising an inner membrane and an outer membrane, as well as a large number of proteins. These proteins are either anchored at the nuclear membrane, as emerin, or form a filament meshwork lining the inner nuclear membrane, as lamins. My thesis objectives were to understand molecular mechanisms deficient in two types of genetic diseases caused by mutations in inner nuclear envelope proteins: Emery-Dreifuss muscular dystrophy, associated to mutations in emerin and A-type lamins, and progeroid syndromes caused by mutations in A-type lamins.

First, we showed that the emerin protein self-assembles *in vitro* and in cells (Herrada, Samson et al., *ACS Chem. Biol.*, 2015). I then studied the structure of emerin oligomers, determined the minimal protein fragment necessary for the formation of these oligomers, identify residues forming the structural core of these oligomers by solid-state NMR in collaboration with the group of Prof A. Lange (FMP Berlin).

And described the impact of emerin mutations causing Emery-Dreifuss muscular dystrophy on emerin self-assembly (Samson et al., *Biomol. NMR Assign.* 2016, Samson et al., *FEBS J.* 2017). Then, I observed, mainly using solution-state NMR, that only the self-assembled form of emerin is able to interact with A-type lamin tail, and that mutants causing Emery-Dreifuss muscular dystrophy and unable to self-assemble are also defective in A-type lamin binding. I also obtained preliminary data showing that phosphorylation of emerin by the Src kinase, observed after a mechanical stress in purified nuclei, regulates the interaction between self-assembled emerin and A-type lamins. Finally, I showed that the monomeric form of emerin is able to form a ternary complex with A-type lamin tail through the chromatin-associated protein Barrier-to-Autointegration Factor (BAF). After having measured the protein-protein affinities within this complex, identified the minimal protein fragments involved in the complex and developed a robust protocol for purification of this complex, I was able to obtain crystals under several conditions. Subsequently, I solved the 3D structure of this complex by molecular replacement at a resolution of 2 Å. Finally, I showed that mutations in A-type lamins causing autosomal recessive progeroid syndromes impair interaction with BAF *in vitro*, and our collaborators at Univ. Paris Diderot, the team of Dr B. Buendia, showed that these same mutations induce a significant decrease in the proximity between lamin A and BAF in HeLa cells.

