Roles of ERK1/2 signaling in *LMNA*-cardiomyopathy

A Dissertation

Submitted in Partial Fulfilment of the

Requirements for the Degree of “Doctor rerum naturalium (Dr. rer. nat.)”

to the Department of Biology, Chemistry and Pharmacy of Freie Universität Berlin

by

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Berlin, 2016

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Date of the defense: 08/11/2016

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Abbreviations

ACE, angiotensin II converting enzyme
ADF, actin-depolymerizing factor
ALK, activin receptor-like kinases
ALS, amyotrophic lateral sclerosis
AMH/MIS, anti-Müllerian hormone/Müllerian inhibiting substance
AP-1 activator protein-1
ATF-2, activated transcription factor-2
aWRN, atypical Werner syndrome
BAF, Barrier to Autointegration Factor
BMP, bone-morphogenetic protein
CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II,
CBD, chromatin binding domain
Cdc42, cell division cycle 42,
CDK, cyclin dependent kinases
Cdk1, cyclin dependent kinase-1
CH, Calponin-homology domain
CIN, chronophin
CMD, Congenital muscular dystrophy
CMT2B1, Charcot-Marie Tooth type 2B1
Cobl, cordon-bleu
Co-Smads, common Smads,
CP, capping protein
CTGF, Connective Tissue Growth Factor
DAG, diaglycerol
DCM, dilated cardiomyopathy
DCM-CD, dilated cardiomyopathy with conduction system disease
ECG, electrocardiogram
ECM, extracellular matrix
EDMD, Emery Dreifuss muscular dystrophy
EF, ejection fraction
ERK, extracellular sign-regulated kinases
ERK1/2 extracellular signal-regulated kinase 1/2
ESCs, embryonic stem cells
F-actin, filamentous actin
FAK, focal adhesion kinase
FH1, formin-homology 1;
FH2, formin-homology 2
FPLD, familial lipodystrophy of Dunnigan type
FR, fractional shortening
FRAP, fluorescence-recovery after photobleaching
FS, Fractional shortening
G-actin, globular actin
GDF, growth and differentiation factors family
GPCR, G-protein-coupled receptors
GSK, Glycogen synthase kinase 3
HGPS, Hutchinson-Gilford progeria syndrome
HP1, heterochromatin-associated protein-1
ICD, intracardiac cardioverter defibrillator
ICMT, isoprenylcysteine carboxyl methyltransferase
INM, inner nuclear membrane
iPSCs, induced pluripotent stem cells
I-Smads, inhibitory Smads,
JNK, c-Jun N terminal kinase
JNK, c-JUN N-terminal kinase
KASH, Klarsicht/ANC-1/Syne-1 homologue
LAD, lamina-associated domains
LAP, lamina-associated polypeptide
LAP, latency-associated peptide

LBR, lamin B receptor
LEM, Lamina-associated polypeptide, Emerin and-MAN1
LGMD1B, limb-girdle muscular dystrophy type 1B
LIMK, Lin-11/Isl-1/Mec-3 kinase
LINC, LInker of Nucleoskeleton and Cytoskeleton
Lmod, leiomodin
LV, left ventricule
MAPK, mitogen-activated protein kinase
MCLK, myosin light chain kinase
MEK, MAP/ERK kinase
MLP, LIM protein
MRTF-A, myocardin-related transcription factor A
NE, nuclear envelope
NEBD, nuclear envelope breakdown
Nesprin, nuclear envelope spectrin repeat
NF-Kb, nuclear factor-κB
NLS, nuclear localization signal
NPC, nuclear pore complexes
NPFs, Nucleation Promoting Factors
NRK, NCK-interacting kinase (NIK)-related kinase
N-WASP, neural WASP
ONM, outer nuclear membrane
PAK, p21-activated kinase
PI(4,5)P2, phosphatidylinositol *4*,*5*-bisphosphate
PKC, protein kinase C
PP1, protein phosphatase 1
PP2A, protein phosphatase 2A
Rce1, Ras-converting enzyme 1
ROCK, RHO-associated coiled-coil-containing protein kinase
R-Smads, regulatory Smads
RTK, receptor tyrosine kinase
RTK, receptors with intrinsic tyrosine kinase activity
SARA, Smad anchor for receptor activation
Smurf2, Smad ubiquitination-related factor
SRF, serum-response factor
SSH, slingshot
SUN, Sad1, UNC-84 homology
TAK, TGF-β-activated kinase
TAN, transmembrane actin-associated nuclear
TAZ, transcriptional coactivator with PDZ-binding motif
TESK, testis-specicific kinase
TGF-β, transforming-growth factor
Tmod, tropomodulin
TβR, TGF-β receptor
WH2, Wiskott-Aldrich Syndrome protein (WASp) homology 2
YAP, yes-associated protein (YAP)
Zmpst24, Zinc metalloprotease related to Ste24p
α-SMA, α-smooth muscle

Abstract

Dilated cardiomyopathy is one of the leading causes of heart failure in Europe. Despite of the conventional medical care, there is no definitive and satisfactory treatment for the progressive cardiac dilatation and loss of contractility in *LMNA* cardiomyopathy often leading to sudden death or heart transplantation. *LMNA* gene encodes nuclear A-type lamins, which are the main constituents of the nuclear lamina. To explain how mutations in proteins of the nuclear envelope can cause a disease of the heart, it has been proposed that nuclear envelope abnormalities bring about cellular fragility and a decrease in the mechanical resistance to stress, which could partially explain the cardiac muscle disease, considering that the heart muscle is constantly subjected to mechanical force. In previous work, it has been showed that there is an abnormal activation of stress-activated ERK1/2 signaling in hearts that carry *LMNA* mutations. Administration of drugs inhibiting ERK1/2 signaling improves cardiac ejection fraction in mice, and blunts further increase in left ventricular dilatation. These studies clearly show that the abnormal ERK1/2 activation is involved in the pathophysiology of *LMNA* dilated cardiomyopathy. However, its role in the development of cardiac dysfunction remains unclear.

Inhibition of ERK1/2 signaling also slows progression of myocardial fibrosis, which is prominent in humans with dilated cardiomyopathy. I suggested that aberrant TGF-β signaling activity could participate to the abnormal ERK1/2 activation and be involved is the pathophysiology of left-ventricular contractile dysfunction in *LMNA* cardiomyopathy. My work led us to describe the TGF-β/ERK1/2/CTGF axis as a key player for the onset of myocardial fibrosis, which impairs left ventricular function, a major symptom of *LMNA* cardiomyopathy.

Given that the understanding of molecular and cellular mechanisms underlying the modulation of ERK1/2 signaling in the heart caused by *LMNA* mutation remains totally unclear, I tested the hypothesis that ERK1/2 abnormal modulation leads to alteration of cytosolic targets and alter cardiac cytoskeleton network. This may lead to *LMNA* cardiomyopathy. My work highlighted a novel partner of activated (phosphorylated) ERK1/2, ADF/cofilin-1. Cofilin promotes debranching of actin filaments. I showed that disrupted actin dynamics leads to abnormal destructuration of sarcomere and ADF/cofilin accumulation in the heart from a mouse model of *LMNA* cardiomyopathy, suggesting a defect in actin depolymerization. This project unraveled an unexpected role played by ERK1/2 signaling in actin dynamics and in the development of left-ventricular dysfunction in *LMNA* cardiomyopathy.

Rèsumè

La cardiomyopathie dilatée est l'une des principales causes d'insuffisance cardiaque en Europe. Dans le cadre de la cardiomyopathie liée aux mutations du gène *LMNA*, en dépit des soins médicaux conventionnels et de la transplantation cardiaque, aucun traitement satisfaisant ne permet de pallier à la dilatation cardiaque progressive et à la perte de la contractilité. Le gène *LMNA* code pour les lamines nucléaires de type A, qui sont les principaux constituants de la lamina nucléaire. L'impact des mutations dans un gène codant pour des protéines de l'enveloppe nucléaire sur le développement de maladies cardiaques demeure non élucidé. Afin d'expliquer cette corrélation, il a été proposé que des anomalies de l'enveloppe nucléaire pourrait engendrer une fragilité cellulaire et une diminution de la résistance mécanique aux contraintes. Ce phénomène pourrait- en partie - expliquer la pathologie musculaire cardiaque, en considérant le cœur comme constamment soumis à des forces mécaniques. De précédents travaux ont montré qu'il existe une activation anormale de la signalisation *de réponse au stress* ERK1/2 dans le cœur des patients porteurs de mutations du gène *LMNA*. L'administration médicamenteuse d'inhibiteurs de la voie de signalisation ERK 1/2 améliore la fraction d'éjection cardiaque chez la souris et atténue la progression de la dilatation ventriculaire gauche. Ces études démontrent que l'activation anormale de ERK 1/2 est impliquée dans la pathophysiologie de la cardiomyopathie dilatée liée aux mutations du gène *LMNA*. Cependant, son rôle dans le développement de la dysfonction cardiaque reste incertain.

L'inhibition de la voie ERK 1/2 ralentit également la progression de la fibrose myocardique, relativement développée chez l'homme, en cas de cardiomyopathie dilatée. Dans le cadre de ma thèse, j'ai suggéré que l'activité aberrante de la voie TGF-β pourrait participer à l'activation anormale de ERK 1/2 et être impliquée dans la physiopathologie de dysfonction contractile ventriculaire gauche dans la cardiomyopathie liée aux mutations du gène *LMNA*. Mon travail nous a conduits à décrire l'axe TGF-β/ERK1/2/CTGF comme un acteur clé de l'apparition de la fibrose myocardique, altérant la fonction ventriculaire gauche, symptôme majeur de la cardiomyopathie liée aux mutations du gène *LMNA*.

Les mécanismes moléculaires et cellulaires sous-jacents à la modulation de la signalisation ERK1/2, causée dans le cœur, par les mutations du gène LMNA, restent totalement incertains. De ce fait, j'ai testé l'hypothèse selon laquelle la modulation anormale de ERK1/2 induirait l'altération des cibles cytosoliques et modifierait le réseau du cytosquelette cardiaque. Cela conduirait ainsi à

la cardiomyopathie. Mon travail a mis en évidence un nouveau partenaire de la forme active (phosphorylée) d'ERK1/2 : cofiline-1. La cofiline induit la déramification des filaments d'actine. J'ai montré qu'une perturbation de la dynamique du réseau d'actine induisait une déstructuration anormale des sarcomères et une accumulation de la protéine ADF/cofiline dans le coeur d'un modèle murin de cardiomyopathie liée aux mutations du gène *LMNA* ; suggèrant ainsi un défaut de dépolymerisation de l'actine. Ce projet met en lumière un rôle inattendu joué par la signalisation ERK1/2 dans la dynamique de l'actine et dans le développement de la dysfonction ventriculaire gauche de la cardiomyopathie liée aux mutations du gène *LMNA*.

Zusammenfassung

Dilatative Kardiomyopathie ist eine der häufigsten Ursachen der Herzinsuffizienz in Europa. Neben einer konventionellen medizinischen Versorgung gibt es, außer der Herztransplantation, keine kurative Behandlung gegen die progressive Herzdilatation und den Verlust der Kontraktilität, die in der *LMNA* Kardiomyopathie auftreten. Das *LMNA* Gen kodiert die nuklearen A-typ Lamine, die den Hauptbestandteil der nuklearen Lamina bilden. Um zu erklären wie Mutationen in Proteinen der nuklearen Lamina zu Krankheiten des Herzens führen können, wird vermutet, dass die Anomalien der Kernhülle zu Fragilität und einer Abnahme der mechanischen Widerstandsfähigkeit der Zelle gegen mechanischen Stress führt. In der Erwägung, dass der Herzmuskel ständig mechanischen Kräften ausgesetzt ist, könnte dies zur Entstehung der Herzmuskelerkrankung beitragen. In früheren Arbeiten wurde bereits eine abnormale Aktivierung des stress-aktivierten ERK1/2 Signalwegs im Herzmuskelzellen, die eine *LMNA* Mutationen tragen, gezeigt. Die Verabreichung von Arzneimitteln, die die ERK1/2 Aktivierung hemmen, verbessert die Herzauswurffraktion und verhindert das weitere Fortschreiten der Dilatation des linken Ventrikels im Mausexperiment. Diese Studien zeigen deutlich, dass die abnormale ERK1/2 Aktivierung in der Pathophysiologie der dilatativen Kardiomyopathie beteiligt ist. Die genaue Rolle von ERK1/2 bei der Entstehung der Herzdysfunktion bleibt jedoch unklar.

Die Hemmung der ERK1/2 Aktivität verlangsamt außerdem das Fortschreiten der myokardialen Fibrose, die häufig bei Menschen mit dilatativer Kardiomyopathie beobachtet wird. Ich habe vermutet, dass eine anomale TGF-Beta Signalaktivität zu der abnormalen ERK1/2 Aktivität beiträgt und daher in die Pathophysiologie der linksventrikulären kontraktilen Dysfunktion der LMNA Kardiomyopathie involviert ist. Meine Arbeit zeigt den TGF-Beta/ERK1/2/CTGF Signalweg als einen wichtigen Akteur in der Entstehung der myokardialen Fibrose, welche die linksventrikuläre Funktion beeinträchtigt und ein Hauptsymptom der Kardiomyopathie darstellt. Da der molekulare und zelluläre Mechanismus der ERK1/2 Signaltransduktion in Herzen, versucht durch die *LMNA* mutation, völlig ungeklärt ist, habe ich die Hypothese getestet, dass die abnormale ERK1/2 Modulation zu Veränderungen der zytosolischen Interaktionspartner sowie zu einer Veränderung des Herz-Zytoskelettes führt. Diese Veränderungen wiederum könnten zur Kardiomyopathie führen.

Meine Arbeit zeigt einen neuen Interaktionspartner von aktiviertem ERK1/2, Cofilin1. Cofilin1 reguliert das Entzweigen von Aktin-filamenten. Ich konnte zeigen, dass eine gestörte Aktindynamik zu einer abnormalen Desorganisation des Sarkomers und einer Akkumulation von ADF/Cofilin im Herzen des Mausmodells der *LMNA* Kardiomyopathie führt. Dies weist auf einen Defekt in der Aktinpolymerisierung hin. Dieses Projekt deckt eine unerwartete Rolle der ERK1/2 Signaltransduktion in der Regulierung der Aktindynamik und in der Entstehung der linksventrikulären Dysfunktion in der *LMNA* Kardiomyopathie auf.

Preamble

Mutations in *LMNA*, encoding A-type lamins, intermediate-filament proteins of the inner nuclear membrane, cause a variety of diseases (i.e. 'laminopathies') that primarily affect striated muscles, and more particularly the heart. Despite our gaps in understanding many of their fundamental functions, much of the current research on the A-type lamins is focused on how mutations leading to alterations in these proteins cause striated muscle diseases. We previously demonstrated that the mechanically activated extracellular signal-regulated kinase (ERK) 1/2 is hyper-activated in striated muscle diseases caused by *LMNA* mutations (Muchir et al. 2007a). However, insights in the molecular mechanisms bridging ERK1/2 modulation and depressed muscle function are lacking. The main goal of the present work was to study the role of ERK1/2 signaling pathway in the pathogenesis and development of *LMNA* cardiomyopathy.

This doctoral thesis comprises three parts:

i/ a general introduction to the scientific topics addressed in the dissertation;

ii/ four manuscripts: two reviews and two peer-reviewed articles, which document and discuss the scientific work that has been conducted. One of the peer-reviewed manuscripts investigates the role of ERK1/2 and TGF-β signaling pathway in the development of fibrosis while the second manuscript focused on the role of ERK1/2 signaling on actin dynamics in the context of *LMNA* cardiomyopathy

iii/ a concluding chapter summarizing the principal outcomes of my thesis.

Chapter 1 Introduction

1.1 Nucleus, nuclear lamina and laminopathies

The cell nucleus can be structurally and functionally divided into at least 2 separate regions, the nuclear envelope and the nuclear interior. The nuclear envelope separates the cytoplasm from the nucleus in eukaryotic cells. It is composed of the nuclear membrane, the nuclear lamina and the nuclear pore complexes (NPCs) (**Figure 1**). The nuclear membrane consists of 2 phospholipid bilayer membranes, the outer nuclear membrane (ONM), which is continuous with the endoplasmic reticulum, and the inner nuclear membrane (INM). The NPCs are large protein complexes made up of multiple copies of approximately 30 different proteins, called nucleoporins, that span through both the INM and ONM as well as the lamina and mediate the exchange of macromolecules such as nucleic acids and proteins with molecular weights exceeding ~40 kDa between the nucleus and the cytoplasm (Schermelleh et al. 2008; Wente & Rout 2010). Nuclear lamina is a dense fibrous three-dimensional meshwork underlining the nucleoplasmic face of the INM.

The main function of the nuclear envelope is to provide protection to the genetic information as well as compartmentalization to concentrate enzymes and substrates for biochemical processes in the nucleus such as DNA replication, transcription, RNA processing and ribosome synthesis.

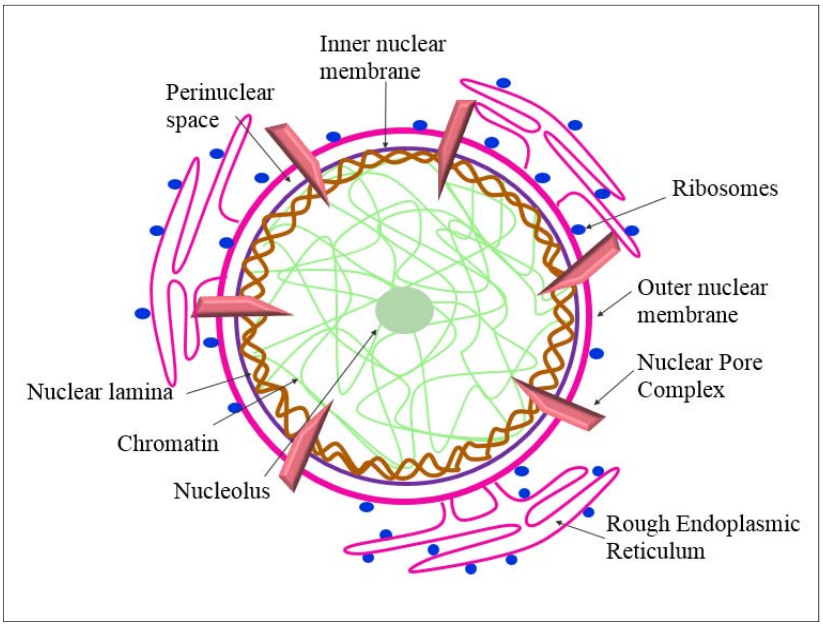

Figure 1: The eukaryotic nucleus. The nucleus of a cell eukaryotic cell contains the DNA, the nuclear envelope, the nucleolus and ribosomes. The nuclear envelope surrounds the nucleus with a double membrane (outer and inner) with nuclear pores. The ONM is continued with the rough ER. The nucleolus is a dense region of the nucleus where the ribosomal RNA is made.

1.1.1 Nuclear lamins

Nuclear lamins are the major structural components of the nuclear envelope that are implicated in a plethora of essential cellular processes, such as chromatin organization, nuclear positioning, spacing of the nuclear pore complexes, nuclear disassembly and reassembly during mitosis, DNA replication, RNA polymerase II-dependent gene expression, cell proliferation and differentiation, cell signaling, and cell division (Burke & Stewart 2012; Choi & Worman 2014; Collas et al. 2014; Kennedy & Pennypacker 2014; Shimi & Goldman 2014). Lamins are type V intermediate filament proteins that polymerize to form the nuclear lamina (Fisher et al. 1986; McKeon et al. 1986; Aebi et al. 1986) and divided into A-type lamins (lamins A and C) and B-type lamins (lamin B1 and lamin B2) (**Figure 2**). In addition to the major lamins A and B, mammalian cells express the minor isoforms lamin AΔ10 (Machiels et al. 1996) and germ cell-specific lamins C2 (Furukawa et al. 1994) and B3 (Furukawa & Hotta 1993). All metazoans examined to date, ranging from hydra to human, express at least one B-type lamin, but lamins are absent from unicellular organisms and plants (Cohen et al. 2001; Höger et al. 1990; Vorburger et al. 1989). Although plant cells have lamina, other proteins substitute for lamins (Ciska & Moreno DÃ-az de la Espina 2014).

A-type lamins

Lamins A and C, the main A-type lamins, arise by alternative splicing of a single gene, *LMNA* (Daniel Z Fisher et al. 1986; McKeon et al. 1986). The *LMNA* gene (Online inheritance in man (OMIM): #150330) is located on chromosome 1q21.2-q21.3 (Lin & Worman 1993; Wydner et al. 1996) and contains 12 exons encompassing around 58 kb. Lamin A is produced by alternative splicing of exon 10. It is 664 amino acids in length and the longest isoform of A-type lamins. The lamin C protein ends at exon 10 which makes it identical to lamin A up to codon 566 (Fisher et al. 1986; McKeon et al. 1986). The last six amino acids (567-572) are unique to lamin C (Lin & Worman 1993). Lamin AΔ10 is identical to lamin A but lacks exon 10, shortening the tail domain by 30 amino acids (Machiels et al. 1996). In mouse, the *LMNA* gene has been shown to give rise to a fourth splice variant termed lamin C2 (Furukawa et al. 1994). Lamin C2 is expressed exclusively in germ cells and is identical to lamin C, except an alternative exon, 1C2, located in intron 1 of *LMNA*, codes for the N-terminal head domain (Jahn et al. 2010; Alsheimer & Benavente 1996). A-type lamins play a pivotal role in the maintenance of nuclear shape (Scaffidi 2006; Dahl et al. 2008; Lammerding et al. 2006), structure (Broers et al. 2005; Lammerding et al. 2006;

Stewart et al. 2007) and stability (Lammerding et al. 2004; Dahl 2004). In contrast to B-type lamins, A-type lamins are also found in a dynamic pool (Moir et al. 2000) in the nucleoplasm (Dechat et al. 2010), where interacts with chromatin. Numerous lines of evidence showed that lamins bind not only to heterochromatin as it was thought for a long time but also to euchromatin (Amendola & van Steensel 2014; Gruenbaum & Foisner 2015; Gesson et al. 2016).

A-type lamins are expressed at specific developmental stages or differentiated cells with high levels in skeletal and cardiac muscle (Krohne & Benavente 1986; Takamori et al. 2007; Lin & Worman 1997). Recent findings showed that small amounts of A-type lamins are also present in mouse ESCs (embryonic stem cells) and in preimplantation embryos (Eckersley-Maslin et al. 2013; Kim et al. 2013). In most tissues, lamin A and C are expressed at equal levels except in the brain where lamin C is the predominant form of A-type lamin (Yang et al. 2011).

In human, mutations in *LMNA* gene results in a range of phenotypic alterations, collectively termed laminopathies (Wilson & Foisner 2010; Zastrow et al. 2004). The pathogenic mechanism for the majority of *LMNA* mutations is still unclear. However, proposed disease mechanisms for laminopathies are closely linked to the function of lamins and lamin-binding proteins (Broers et al. 2006; Worman & Bonne 2007). Lamin-binding proteins include structural proteins: nesprin-1α (Mislow et al. 2002), nesprin-2 (Zhang et al. 2005), SUN1/2 (Haque et al. 2006; Haque et al. 2010), emerin (Lee et al. 2001; Sakaki et al. 2001), actin (Sasseville & Langelier 1998; Simon et al. 2010) and Nup88 (Lussi et al. 2011); proteins involved in gene transcription and cell signalling: c-Fos, Erk1/2 (González et al. 2008), MOK2 (Dreuillet 2002), PKCα (Martelli et al. 2002), SREBP1 (Lloyd 2002) and MAN1 (Mansharamani & Wilson 2005) and proteins involved in cell cycle regulation: pRb (Ozaki et al. 1994), cyclin D3 (Mariappan et al. 2007), PCNA (Shumaker et al. 2008) and Lap2α (Dechat et al. 2000).

B-type lamins

B-type lamins are encoded by either the *LMNB1* gene on chromosome 5q23.3-q31.1 which encode lamin B1 or *LMNB2* gene on chromosome 19p13.3, which encode lamin B2 and lamin B3 (Furukawa & Hotta 1993; Peter et al. 1989; Vorburger et al. 1989). Lamins B are constitutively expressed in essentially all somatic types including undifferentiated cells in early stages of embryonic development (Liu et al. 2011; Stewart & Burke 1987).

Mice deficient in lamin B1, lamin B2, or both are smaller than wild-type littermates at birth, have major defects in the lungs and brain, and die within a few minutes after birth (Vergnes et al. 2004; Coffinier et al. 2010; Kim et al. 2011). Fibroblasts from lamin B1 mutant mice display severely dysmorphic nuclei, impaired differentiation and premature senescence (Vergnes et al. 2004). B-type lamins have been associated with the assembly of the mitotic spindle matrix as well as DNA replication, genome organisation and apoptosis and are therefore thought to be essential for proliferation and cell survival (Guelen et al. 2008; Liu et al. 2000; Tsai et al. 2006). Another study showed that forebrain-specific removal of lamin B1 and B2 results in severe atrophy of the cortex, demonstrating an essential role in brain development (Coffinier et al. 2011; Yang et al. 2011).

In contrast to A-type lamins, only a few disease-causing mutations have been identified in *LMNB1* or *LMNB2* such as leukodystrophy demyelinating autosomal dominant adult-onset (ADLD) for *LMNB1* defects and partial acquired lipodystrophy associated with susceptibility variants of *LMNB2* (Padiath et al. 2006; Zuela et al. 2012; Schreiber & Kennedy 2013; Brussino et al. 2010; Molloy et al. 2012; Schuster et al. 2011).

1.1.2 Structure of lamins

Similar to cytoplasmic intermediate filaments, lamins have a tripartite structure consisting of a helical rod domain flanked by globular N-terminal head and C-terminal tail domains (Aebi et al. 1986). Lamin A resembles lamin B in the head and rod domains, but has an expanded tail domain that comprises an extra 90 amino acid segment (Dittmer & Misteli 2011). The central rod domain of the lamins consists mainly of heptad repeats that are characteristic of α-helical proteins (McKeon et al. 1986; Stuurman et al. 1998). The tail domain contains a chromatin binding domain (CBD), a nuclear localization signal (NLS), an immunoglobulin (Ig Fold) sequence and a conserved CAAX box (Loewinger & McKeon 1988; Dhe-Paganon et al. 2002; Krimm et al. 2002). The NLS is located between the central rod and the Ig-fold and is required for the transport of lamins into the nucleus (Loewinger & McKeon 1988). The Ig fold domain is common to many proteins and mediates various protein interactions, and the CAAX box, a motif of serves as a substrate for extensive post-translational processing (Rusiñol & Sinensky 2006). Lamin C does not have a CaaX box, so it does not undergo modifications (Dechat et al. 2010) (**Figure 2**).

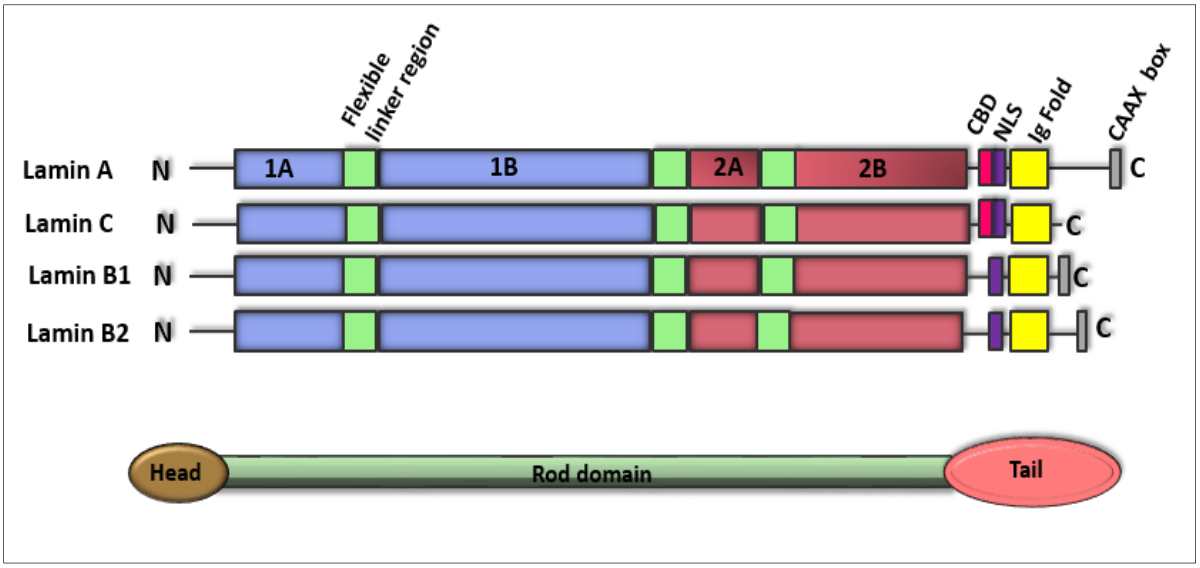

Figure 2: Schematic structure of lamin family members. Main characteristics are four central rod domains (1A, 1B, 2A, 2B), flanked by a globular head and a globular tail domain. In the globular tail domain, a nuclear localization signal (NLS; purple) can be identified as well as a chromatin-binding domain (CBD; pink) and a Caax motif, which is absent in lamin C.

1.1.3 Posttranslational Processing and Modifications of the Nuclear Lamins

Posttranslational modifications of the head and tail domains of lamins are required to control lamin assembly. In the first step, the C-terminal CaaX motif of prelamin A, lamin B1 and B2 undergoes farnesylation, and subsequently the terminal –AAX amino acids are cleaved by an endopeptidase, most likely Rce1 (Ras-converting enzyme 1) or FACE1. In the third step the cysteine residue undergoes a methylation step at the ER, a process catalyzed by the enzyme isoprenylcysteine carboxyl methyltransferase (ICMT) (Nigg 1992; Maske et al. 2003; Varela et al. 2005). Once incorporated into the nuclear lamina, only prelamin A is further cleaved 15 amino acids away (in human lamin A after Tyr646) from its farnesylated/carboxymethylated cysteine of the C terminus by the protease Zmpst24 (Zinc metalloprotease related to Ste24p)/FACE1 (Pendas et al. 2002; Corrigan et al. 2005). This cleavage event occurs 30–60 minutes post-synthesis—after assembly into the nuclear lamina (Gerace et al. 1984; Pendas et al. 2002; Bergo et al. 2002). This final modification step completes the post-translational modification of prelamin A to mature lamin A and it is thought to aid localization of lamin A to the nuclear envelope.

1.1.4 Lamin filament assembly and disassembly

Similarly to other intermediate filament proteins, both A-type and B-type lamins can self-associate to form filaments *in vitro* and i*n vivo* (Foeger et al. 2006). The first step in this assembly involves a polar head-to-tail polymerization of the lamin dimers in parallel association of two α-helical rod domains into a left handed superhelix (Heitlinger et al. 1991; Stuurman et al. 1996) and these polarized arrays associate laterally in an antiparallel fashion to build tetrameric protofilaments. The interaction between four protofilaments form the characteristic ∼10-nm-diameter lamin filament (Heitlinger et al. 1991; Stuurman et al. 1996; Ben-Harush et al. 2009). In contrast, the lamin filaments assembly *in vitro* produces large paracrystalline arrays which do not exist *in vivo* under normal conditions (Stuurman et al. 1998). The apparent difference between lamin assembly *in vivo* (10-nm filaments) and *in vitro* (paracrystals) demonstrates that lamin organization *in vivo* is regulated by interactions with other molecules.

The nuclear lamins are rapidly disassembled during the prophase/metaphase transition in vertebrate cells, in a process called nuclear envelope breakdown (NEBD) (Fields & Thompson

1995). The disassembly of A-type lamins is triggered in a phosphorylation-dependent manner by cyclin dependent kinase-1 (Cdk1), protein kinase C (PKC) and other mitotic kinases leading to their depolymerization and solubilization (Heald & McKeon 1990). In contrast, B-type lamins maintain proximity with the nuclear membrane. After mitosis, the A-type lamins are dephosphorylated and reassembled into the nuclear envelope along to B-type lamins.

1.1.5 **Connections of A-type lamins with other nuclear envelope proteins**

At the nuclear membrane, several proteins form connections with the underlying lamina structure on the one hand or the cytoskeleton on the other. In particular, the lamin proteins interact with INM proteins, like emerin, LBR (Lamin B receptor) and LAP (lamina-associated polypeptide), and chromatin-binding proteins, like BAF (Barrier to Autointegration Factor), at the nuclear periphery to form a stable network that supports the membrane and links the INM to the chromatin (Ellenberg & Siggia 1997; Moir et al. 2000; Wilson & Foisner 2010).

Emerin is a serine-rich transmembrane protein encoded by the *EMD* gene and is located at the inner nuclear membrane (Bione, Maestrini, Rivella, et al. 1994; Nagano et al. 1996). The N-terminal of emerin contains a ~45-residue structural motif known as LEM (for Lamina-associated polypeptide, Emerin and-MAN1) domain which binds BAF, a small chromatin-binding molecule (Lee et al. 2001) (**Figure 3**). Emerin interacts with several proteins such as lamin A/C (Clements et al. 2000; Lee et al. 2001; Sakaki et al. 2001; Dreger et al. 2001), lamin B receptor (LBR) (Martins et al. 2000), actin (Fairley et al. 1999) and nesprins (Warren et al. 2005; J. M. K. Mislow et al. 2002; J. M. . Mislow et al. 2002). The localization of emerin to the nuclear envelope is lamin A-dependent (Sullivan et al. 1999; Vaughan et al. 2001). Mutations on *EMD* are associated with X-linked Emery-Dreifuss muscular dystrophy (X-EDMD) and are mostly nonsense mutations resulting in a loss of emerin (Bione, Maestrini, Rivella, et al. 1994; Nagano et al. 1996).

Lamin-associated polypeptide (LAP) family is encoded by two genes *LAP1* and *LAP2* (Martin et al. 1995; Harris et al. 1995; Furukawa et al. 1995; Berger et al. 1996). The mammalian *LAP2* gene encodes six alternative spliced isoforms (LAP2α, β, γ, δ, ε, ζ) that play an important role in the regulation of nuclear architecture by binding lamin B1 and chromosomes in a manner regulated by phosphorylation during mitosis (Berger et al. 1996; Dechat et al. 2000; Furukawa et al. 1994).

LAP2α is linked to a laminopathy-type dilated cardiomyopathy and Lap2α knock out mice showed delayed satellite cells differentiation, although delayed muscle regeneration was not observed (Taylor et al. 2005; Gotic et al. 2010; Gotic et al. 2010).

LAP1A and LAP1B have been shown to bind to A and B-type lamins, whereas LAP2C only binds lamin B (Foisner & Gerace 1993). Mutations on the gene encoding for LAP1B protein has been found in patients with dystonia associated with dilated cardiomyopathy as well as in individuals with muscular dystrophy and cardiomyopathy (Kayman-Kurekci et al. 2014; Dorboz et al. 2014).

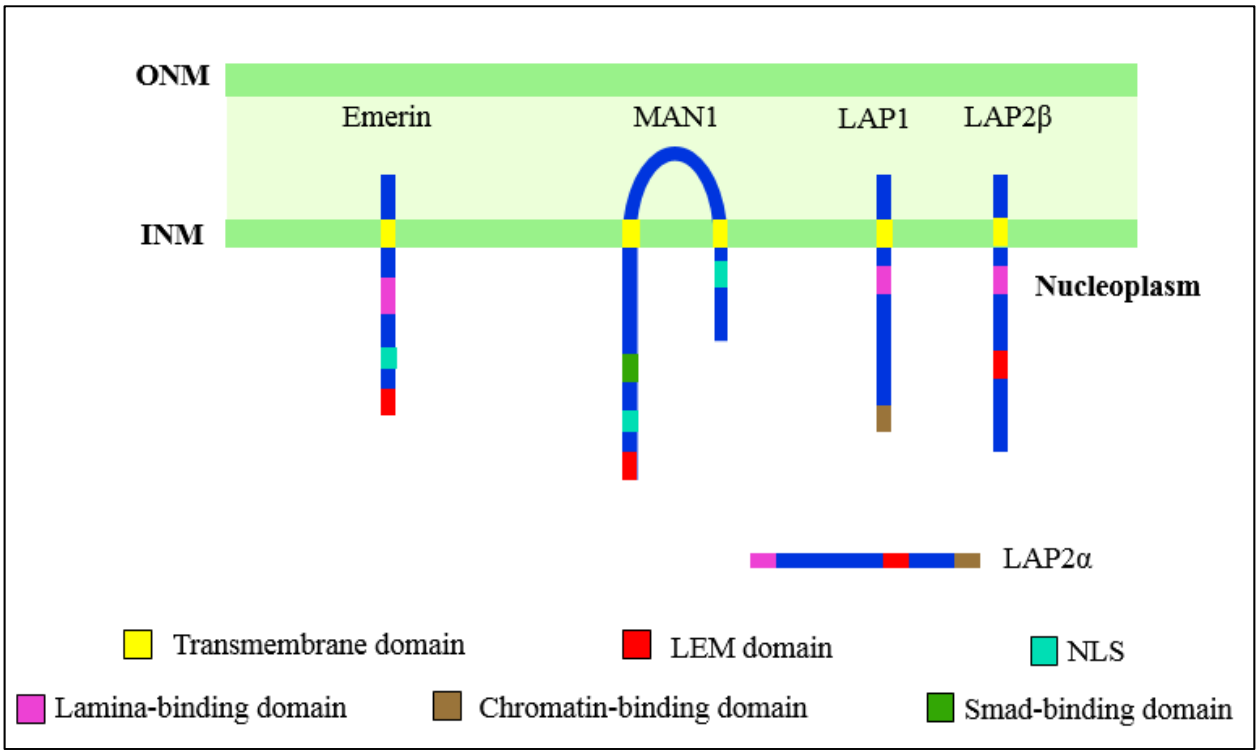

Figure 3: Schematic illustration of the LEM-domain proteins. LAP2α, LAP2β, emerin and MAN1 contain the LEM domain, which interacts with the DNA-binding protein barrier-to-autointegration factor (BAF). LAP, lamina-associated polypeptide; NLS, nuclear localization signal; INM, inner nuclear membrane; ONM, outer nuclear membrane

MAN1 is an integral protein of the INM (Bengtsson 2007; Paulin-Levasseur et al. 1996). The N-terminal domain of MAN1 contains a LEM domain which binds Lamin A/C and chromatin via BAF and C-terminal region has an RNA-recognition motif (RR-motif) which is a binding site for regulatory Smads (R-Smads) (Bengtsson 2007). MAN1 binds R-Smads but not inhibitory Smads, and therefore acts as an inhibitor of BMP (bone-morphogenetic protein) and TGF-β (transforming-growth factor) signaling (Lin et al. 2005). Overexpression of MAN1 has been shown to block transcription of BMP and TGF-β targets upon cytokine stimulation and loss of MAN1 at the INM results in increased TGF-β signaling (Lin et al. 2005). Loss of-function mutations in MAN1 result in bone-related diseases such as osteopoikilosis and Buschke-Ollendorff syndrome (Mumm et al. 2007), phenotypes characterised by increased bone density and associated with increased TGF-β signaling.

Lamin B receptor (LBR) is an inner nuclear membrane protein and belongs to the ERG4/ERG24 family. *LBR* is targeted to the INM via 8 putative transmembrane domains (Worman et al. 1990). The LBR anchors B-type lamins to the INM and has been shown to interact with emerin (Martins et al. 2000), lamin associated protein-2β (LAP2β) (Ye & Worman 1994), double strand DNA, histones and the heterochromatin-associated protein-1 (HP1) (Ye & Worman 1996). Mutations in the *LBR* gene cause Pelger-Huet anomaly, an hematology particularity and HEM/Greenberg skeletal dysplasia, a bone disease (Hoffmann et al. 2002; Waterham et al. 2003).

1.1.6 Connections between nucleus and cytoskeleton

Besides the connection between the inner nuclear membrane and the nuclear lamina, the nucleus is also connected to the cytoskeletal filaments through linker proteins (Dittmer & Misteli 2011) (**Figure 4**). This link between nucleus and cytoskeleton has been implicated in a variety of functions important for nuclear positioning, migration, morphology and mechanics (Starr & Han 2003).

Nesprins (Nuclear envelope spectrin repeat proteins) comprise a large protein family of spectrin-repeat-containing proteins localized to the ONM and INM depending on their size and interaction partners (Razafsky & Hodzic 2009). Nesprins are encoded by four genes (*SYNE1-4*) (Mellad et al. 2011), that give rise to multiple isoforms. Their structure contains a central rod region with several

spectrin repeats, variable in length, and a C-terminal KASH (Klarsicht/ANC-1/Syne-1 homologue) transmembrane domain that mediates nuclear membrane localization and

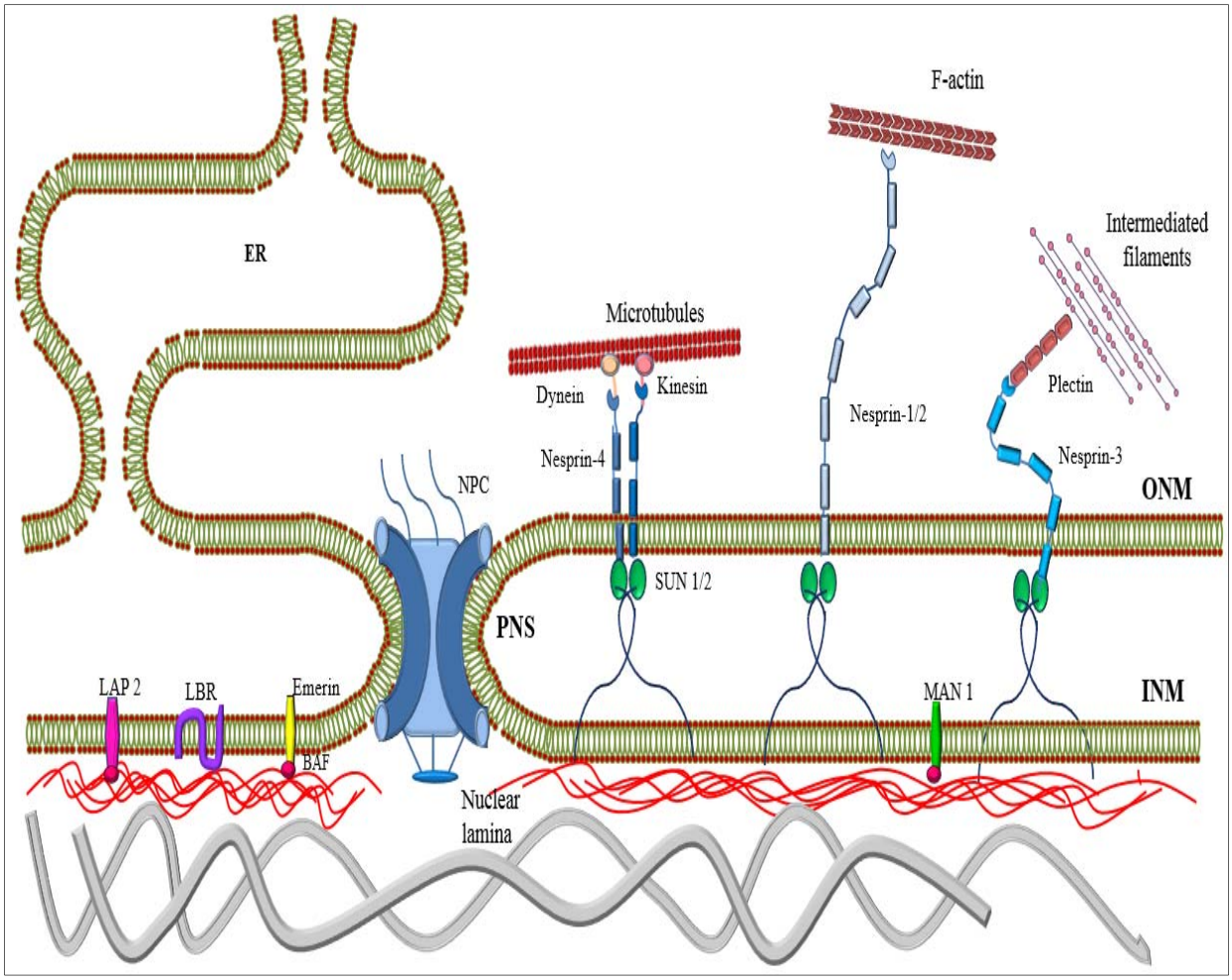

Figure 4: Schematic diagram of the nucleocytoskeltal interactions. The nuclear envelope consists the nuclear membranes, nuclear lamina, and pore complexes**.** The nuclear lamina lies on the inner surface of the inner nuclear membrane (INM), where it serves to maintain nuclear stability, organize chromatin and bind nuclear pore complexes (NPCs) Nuclear envelope proteins that are bound to the lamina include nesprin, emerin, lamina-associated proteins 1 and 2 (LAP1 and LAP2), the lamin B receptor (LBR) and MAN1.

interacts with the SUN-domain in the perinuclear space (Crisp et al. 2006; Zhang et al. 2001; J. M. K. Mislow et al. 2002) (**Figure 4**). Nesprin-1 and -2 (giant isoforms) harbor an N-terminal actin-binding Calponin-homology domain (CH) (Zhen et al. 2002; Padmakumar et al. 2004) (**Figure 5**).

Nesprin-3 contains an N-terminal binding domain for plectin which interacts with intermediate filament proteins (Wilhelmsen et al. 2005; Ketema et al. 2007) and nesprin-4 binds to kinesin-1, a microtubule motor protein participating in the nuclear positioning process (Roux et al. 2009) (**Figure 4**). Missense mutations in *SYNE1* and *SYNE2* (Zhang et al. 2007) genes in humans as well as mice harbouring KASH-domain deletions (Puckelwartz et al. 2009; Zhang et al. 2007), are associated with a skeletal muscle phenotype resembling EDMD, whereas other mutations in *SYNE1* are associated with non-muscle pathologies, including arthrogryposis and cerebellar ataxia (Gros-Louis et al. 2007; Voit et al. 2002).

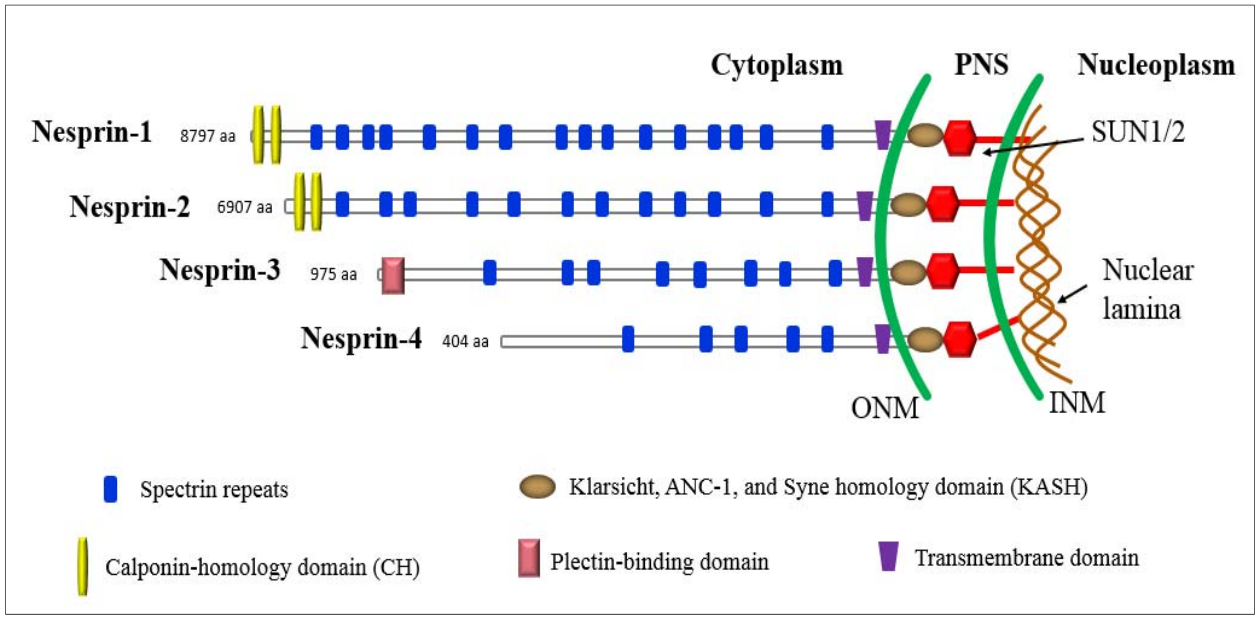

Figure 5: Domain structure of the four main nesprin isoforms. The nesprins-1 and -2 contain two N-terminal actin-binding Calponin homology (CH) domains, a central region comprised of multiple Spectrin repeats (the number depends on the isoform) and a C-terminal KASH domain that is required for their nuclear envelope localization. Nesprin-3 and nesprin-4 are smaller isoforms which anchor to plectin and microtubules, respectively. The length of each protein in amino acids is indicated.

SUN (Sad1, UNC-84 homology) domain proteins SUN1 and SUN2 are located to the INM. SUN proteins have a central helical coil domain that mediates dimerization (Tzur et al. 2006), an N-terminal domain which interacts directly with A-type lamins (Haque et al. 2006), emerin and short nesprin-2 isoforms (Haque et al. 2010) and a C-terminal domain. The latter interacts with the KASH domain found in giant nesprins 1 and 2 in the luminal space between the INM and the ONM (Padmakumar et al. 2005; Crisp et al. 2006; Ketema et al. 2007), forming a mechanical link, called LINC (LInker of Nucleoskeleton and Cytoskeleton) complex (Stewart et al. 2007; Crisp et al. 2006; Padmakumar et al. 2005). SUN proteins, bind to A-type lamins (Padmakumar et al. 2005; Worman & Gundersen 2006; Hodzic et al. 2004; Crisp et al. 2006; Haque et al. 2006) as well as to nuclear pore complexes (NPCs).

LINC complex is essential for a multitude of cellular functions including nucleocytoskeletal connection and organization, nuclear morphology, nuclear positioning, cell polarity, cell differentiation, organelle positioning and mechanotransduction (Lombardi et al. 2011; Zhang et al. 2010). **Table 1** summarizes the cellular functions in which LINC plays role. The first experimental clues for the role of LINC complex in mechanotransduction come from a seminal study in which magnetic tweezers were used to pull on nesprin-1 antibody-coated beads attached to isolated nuclei (Guilluy et al. 2014). Pulling on nesprin-1 resulted in local nuclear stiffening accompanied by an enhanced recruitment of A-type lamins to the LINC complex and Src-dependent phosphorylation of emerin (Tyr74 and Tyr95). This phosphorylation reinforces the connection between lamin A/C and the LINC complex. In support of their role in the direct force transmission to the nucleus, the nuclear deformation response to mechanical strain was found abrogated upon LINC disruption using dominant-negative nesprin and SUN constructs (Lombardi et al. 2011). Disrupting the LINC complex and the nucleo-cytoskeletal linkage can perturb normal gene expression in both myoblasts and cardiomyocytes (Nikolova-Krstevski et al. 2011; Brosig et al. 2010).

Table 1: Functions of LINC complex

Functions	References
Association with actin and microtubules networks	(Starr & Han 2003; Starr & Fridolfsson 2010; Münter et al. 2006)
Formation of TAN lines in migrating cells; cell migration; cell polarization	(Luxton et al. 2011; Chancellor et al. 2010)
Force propagation from FA and nucleus	(Lombardi et al. 2011; Crisp et al. 2006)
Centrosome association	(Dawe et al. 2009)
Chromatin separation from the nuclear envelope and organization of the mitotic spindle	(Turgay et al. 2014)
Nuclear positioning at NMJ	(Grady et al. 2005)
Cell division via interaction with telomeres	(Ding et al. 2007)
Formation of actin cap	(Khatau et al. 2009)
Stretch-induced nuclear rotation and stretch-induced inhibition of differentiation	(Brosig et al. 2010)

A remarkable finding showed that, LINC complexes also participate in the formation of the transmembrane actin-associated nuclear (TAN) lines at specialized apical regions of the nucleus (Luxton et al. 2010) (**Figure 6**). In a series of papers, Gundersen and coworkers showed that Nesprin-2 giant, Sun-2 and myosin II are essential components of the TAN lines. TAN lines play a major role in nuclear movement and polarity in migrating fibroblasts and myoblasts (Luxton et al. 2010; Chang et al. 2015). The INM proteins Samp and emerin were also found in these complexes (Borrego-Pinto et al. 2012; Chang et al. 2013).

A subset of cytoskeletal actin filaments are directly attached to the nucleus, forming the perinuclear actin cap (Khatau et al. 2009). These specialized actin fibers cover the top of the nucleus, as opposed to conventional basal stress fibers lying at the basal surface of the cell. Although both TAN lines and actin cap are connected to the surface of the nuclear envelope through the lamin A/C and LINC complex, actin cap stress fibers are oriented parallel and TAN lines perpendicularly with the direction of migration (**Figure 6**). Apart from the role of the actin cap as nuclear shape regulator, it has been also proposed to serve rapid transduction of information about the

mechanical properties of the cellular environment into the nucleus (Chambliss et al. 2013; Khatau et al. 2009). The absence of LINC complexes in cellular models carrying *LMNA* mutations results in the disappearance of actin caps (Hotulainen & Lappalainen 2006).

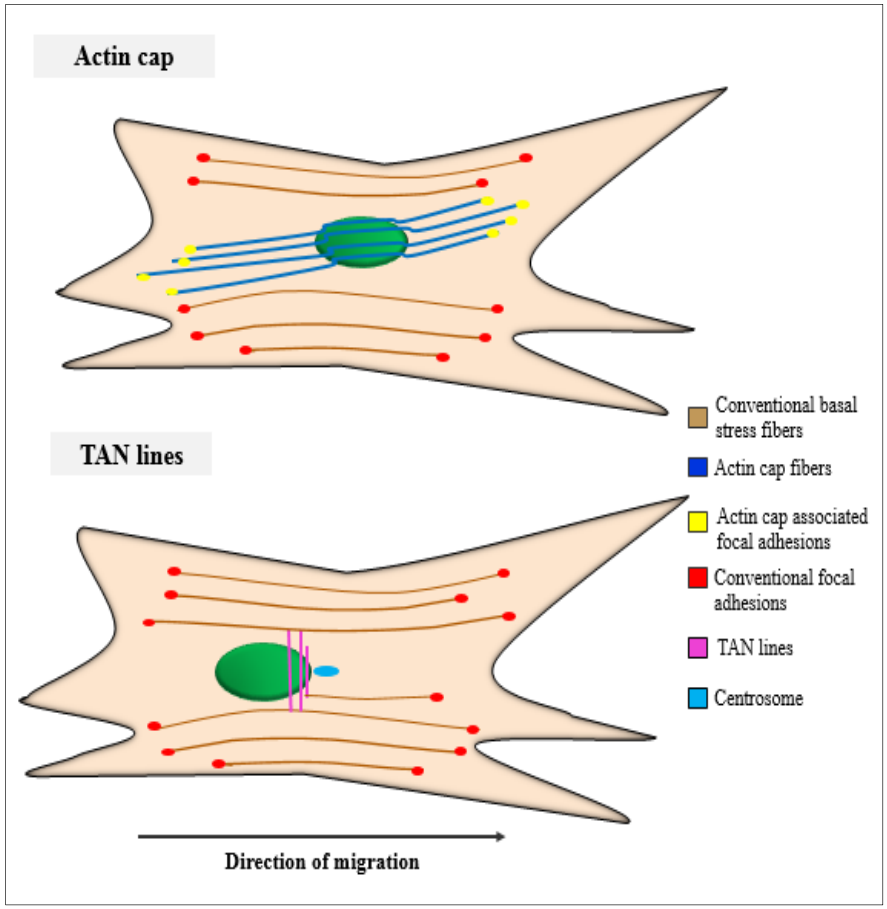

Figure 6: Schematic representation of the two perinuclear actin structures: actin cap and TAN lines. TAN lines are composed of linear arrays of nesprin-2G/SUN2, on the surface of the nuclear envelope along actin cables. Both actin caps and TAN lines are linked to nucleus via LINC complex. However, actin caps are oriented parallel while TAN lines perpendicularly to the direction of migration.

1.1.7 Laminopathies

Lamin A/C alterations/substitutions result in a vast range of tissue-specific disorders broadly termed laminopathies (Worman 2012; Butin-Israeli et al. 2012) and can be separated into primary and secondary laminopathies. To date, more than 450 mutations (see http://www.umd.be/LMNA/) in the *LMNA* gene have been identified in so called primary laminopathies; the majority of these

mutations are linked to muscular dystrophies, but some mutations have little or no effect on muscle tissue. These include point mutations, frameshift mutations, deletions, and two nonsense mutations.

The spectrum of ~14 distinct primary *LMNA* laminopathies can be broadly classified into 1) diseases that affecting the striated muscles include *LMNA*-related congenital muscular dystrophy (L-CMD), Emery-Dreifuss muscular dystrophy (EDMD), limb-girdle muscular dystrophy type 1B (LGMD1B) and dilated cardiomyopathy with conduction system disease(DCM-CD) (**Figure 7**), 2) diseases affecting the adipose tissue include familial lipodystrophy of Dunnigan type (FPLD), 3) peripheral neuropathy associated with demyelination of motor neurons such as axonal neuropathy Charcot-Marie Tooth type 2B1 (CMT2B1), and 4) premature aging syndromes which include Hutchinson-Gilford progeria syndrome (HGPS) and atypical Werner syndrome (aWRN) (**Table 2**) (De Sandre-Giovannoli et al. 2002; Shackleton et al. 2000; Muchir et al. 2000; Fatkin et al. 1999; Eriksson et al. 2003; Chen et al. 2003). The mechanism whereby different mutations in a single gene that is widely expressed cause such a broad spectrum of diseases remains a puzzle. The clinical profile of the same *LMNA* mutation often varies in severity among members of the same family (Fatkin et al. 1999; Cao & Hegele 2000; Brown et al. 2011).

Secondary laminopathies are caused by mutations in *ZMPSTE24* gene, leading to defects in posttranslational modification of A-type lamins and include restrictive dermopathy (Navarro et al. 2004) and Mandibuloacral dysplasia (Agarwal et al. 2003). Description of secondary laminopathies is beyond the scope of this thesis.

Emery-Dreifuss Muscular Dystrophy (EDMD)

One of the tissues most frequently affected by changes in genes encoding lamin A/C is striated muscle (**Figure 7**). Emery-Dreifuss muscular dystrophy (EDMD) is the most prevalent disorder of nuclear envelopathies, a group of diseases caused by mutations of genes that encode proteins of the nuclear envelope. EDMD is a genetically heterogenous disorder with X-linked (XL-EDMD: OMIM #310300), autosomal dominant (AD-EDMD: OMIM #181350) and autosomal recessive (AR-EDMD: OMIM #604929) forms. The genes responsible for the three genetic forms were identified. In 1994, Bione et al. identified emerin (*EMD*) as the gene on chromosome Xq28 that is mutated in X-linked EDMD (Bione, Maestrini, Rivella, et al. 1994; Raffaele Di Barletta et al. 2000). Most mutations in the X-EDMD gene are point mutations or small deletions/insertions of

emerin that cause interruption of transcription or translation (Bione, Maestrini & Rivella 1994; Klauck et al. 1995; Nigro et al. 1995; Wulff et al. 1997). Later, Bonne et al. demonstrated that mutations in *LMNA* gene cause AD-EDMD and AR-EDMD (Bonne et al. 1999). Missense mutations introducing amino acid changes in the coding sequence appear to be the most common mutations in the *LMNA* gene (Bonne et al. 1999; Raffaele Di Barletta et al. 2000).

Table 2: Diseases caused by mutations in *LMNA* gene

Laminopathies	Reference
Affecting striated muscles	
AD-Emery Dreifuss Muscular Dystrophy (AD-EDMD)	(Bonne et al. 1999)
AR-Emery Dreifuss Muscular Dystrophy (AR-EDMD)	(Raffaele Di Barletta et al. 2000)
Limb-girdle muscular dystrophy type 1B (LGMD1B)	(Muchir et al. 2000)
LMNA-associated congenital muscular dystrophy (L-CMD)	(Quijano-Roy et al. 2008)
Dilated-cardiomyopathy with conduction defects (DCM-CD)	(Fatkin et al. 1999)
Lipodystrophy syndromes	
Dunnigan-type familial partial lipodystrophy (FPLD2)	(Shackleton et al. 2000)
Peripheral neuropathy	
Charcot Marie Tooth Disease type 2B1 (CMT-2B1)	(De Sandre-Giovannoli et al. 2002)
Accelerating aging syndromes	
Hutchinson-Gilford Progeria Syndrome (HGPS)	(Eriksson et al. 2003)
Atypical Werner Syndrome (aWRN)	(Chen et al. 2003)
Restricted Dermopathy (RD)	(Navarro et al. 2004)

The three forms of EDMD are characterized clinically by slowly progressive muscle weakness and wasting in a scapulo-humeroperoneal distribution, early contractures of the elbows, ankles, Achilles tendons and posterior neck. Cardiac conduction defecits and arrythmias may occur, resulting in life-threatening dilated cardiomyopathy. Cardiac disease, in most cases, begins at the end of the second decade with no direct relationship to the severity of the skeletal muscle involvement.

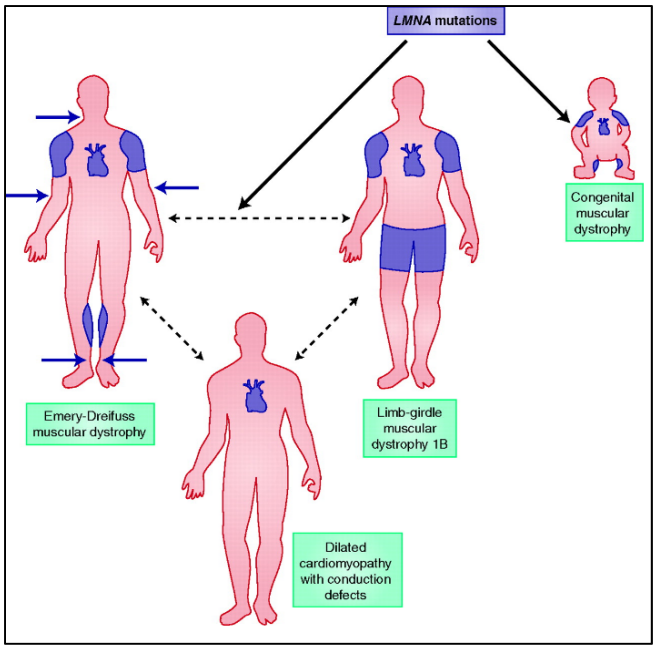

Figure 7: Laminopathies affecting striated muscles present in childhood or adulthood (Emery-Dreifuss muscular dystrophy, limb-girdle muscular dystrophy 1B and dilated cardiomyopathy with conduction defects) are shown with affected muscle groups illustrated in blue. Adapted from Lu et al. 2011

Dilated cardiomyopathy with conduction system disease (DCM-CD)

Soon after the identification of the first *LMNA* mutations in EDMD, Fatkin et al. reported that *LMNA* mutations can cause dilated cardiomyopathy associated with conduction system disease (DCM-CD: OMIM #115200) with minimal to no skeletal muscle involvement (Fatkin et al. 1999). Dilated cardiomyopathy is characterized by progressive weakening of the heart muscles, thinning of the left ventricle wall and insufficient pumping that typically leads to heart failure. Similar to other dilated cardiomyopathies, *LMNA* cardiomyopathy is characterized by chamber enlargement and systolic dysfunction of one or both ventricles (**Figure 8**). Several reports emphasized that different lamin A/C mutations cause very similar cardiac phenotypes, characterized by atrial fibrillation, conduction system disease, sudden death and heart failure (Fatkin et al. 1999; Bécane et al. 2000; Taylor et al. 2003; Kärkkäinen et al. 2004; van Berlo et al. 2004). In addition, both atrial and ventricular arrhythmias are common among *LMNA* mutation carriers. Some studies have pointed out that sudden death may be the first disease manifestation, which could be due to ventricular arrhythmias and therefore occur despite the implantation of pacemaker (Bécane et al. 2000; Sanna et al. 2003; van Berlo et al. 2004; Van Berlo et al. 2005; Kärkkäinen et al. 2004).

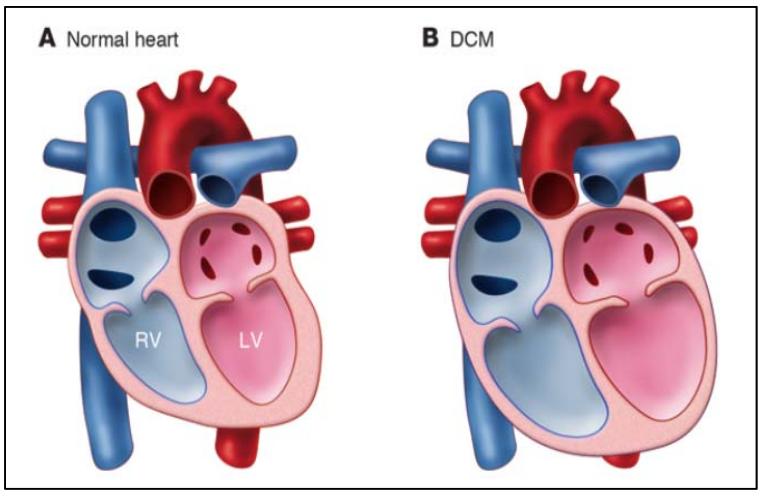

Figure 8: Morphological changes of the heart in cardiomyopathy. (A) Normal heart. (B) DCM heart. Adapted from (Mcnally et al. 2013)

Thus, implantation of cardioverter-defibrillator (ICD) is required for primary prevention of ventricular arrhythmias and sudden cardiac death (Meune et al. 2006; Charron et al. 2012).

The course of *LMNA* cardiomyopathy is aggressive due to a high rate of malignant ventricular arrhythmias and end-stage heart failure, often leading to premature death or cardiac transplantation (Taylor et al. 2003; Van Berlo et al. 2005). By the age of 60 years, 55% of *LMNA* mutation carriers die of cardiovascular death or receive a heart transplant, compared to 11% of patients with idiopathic cardiomyopathy without *LMNA* mutation (Taylor et al. 2003). Diagnosis of *LMNA*-related DCM relies on sequence analysis. Conduction system disease and arrhythmias are detected by electrocardiogram (ECG) and left ventricular dilatation and reduced systolic function are most commonly identified with two-dimensional echocardiography.

Limb-Girdle muscular dystrophy type 1B

Limb-girdle muscular dystrophy (LGMD) is a heterogeneous group of disorders characterized by a limb girdle distribution of weakness. Patients with limb-girdle muscular dystrophy can also develop cardiomyopathy, although at a lesser frequency than those with EDMD. LGMD1B (OMIM #159001) is a form of LGMD inherited as an autosomal dominant trait. It is slowly progressive muscle dystrophy displaying a classic limb-girdle pattern of muscle atrophy with age related atrioventricular cardiac conduction defect and dilated cardiomyopathy (van der Kooi et al, 1996). Mutations in the *LMNA* gene were identified in LGMD1B (Muchir et al. 2000).

Clinical terms

Cardiomyopathy: disease or dysfunction of heart muscle.
Conduction system disease: reduction of electrical signal through the heart conduction system
Arrhythmia: abnormal electrical impulses leading to defects in heart rhythm; can be too fast (tachyarrhythmia), too slow (bradyarrhythmia) or irregular.
Systolic function: estimated by a measure of the LV ejection fraction or fractional shortening. Reduced systolic function is defined as ejection fraction of less than 50% or a fractional shortening of less than 25%-30%.
Ejection fraction (EF): percentage of blood that heart pumps with each contraction.
Fractional shortening (FS): the change in the diameter of the left ventricle between the contracted and relaxed states. It is calculated as (FS = [LVEDd − LVEDs]/LVEDd) where LVEDd is LV dimension in end diastole and LVEDs is LV end dimension in systole
Electrocardiogram: electrical activity of the heart recorded by electrodes attached to the patient's chest, arm or leg.
Echocardiogram: ultrasound of the heart, allowing visualization of chamber size, pumping function and velocity of blood flow of the heart.
Implantable pacemaker: device that uses electrical impulses to regulate the heart rhythm
Intracardiac cardioverter defibrillator (ICD): device placed under the skin that keeps track of the heart rate.

The recurrence of common DCM-causing mutations is low and the level of pleiotropy of some mutations is unusual. For instance, the mutation R644C displays extreme pleiotropic diversity. As such, the mechanism through which mutated lamins cause cardiac dysfunction remains obscure. DCM can also be caused by mutations in emerin, nesprin-1 and -2, LUMA, an inner nuclear membrane protein that binds emerin, and LAP2α (Bengtsson & Otto 2008; Schreiber & Kennedy 2013). Many of the disease mechanism may mirror those for muscular dystrophy described above and it remains unclear why some mutations specifically affect cardiac but not skeletal muscle.

LMNA-associated congenital muscular dystrophy (L-CMC)

Congenital muscular dystrophies (CMDs) are rare genetic myopathies characterized by dystrophic features on muscle biopsy as well as severe hypotonia, diffuse limb and axial muscle weakness and atrophy or delayed motor development from the first few months of life. CMDs are genetically and phenotypically heterogeneous disorders caused by several different genes; among them is *LMNA* (L-CMD: OMIM #613205) (Finsterer et al. 2010). All children have a progressive course with an initial rapid decline in cervical/axial strength followed by a period of slower progression (Quijano-Roy et al. 2008). Cardiac involvement was reported in some cases (i.e., severe ventricular arrhythmias associated with sudden cardiac death).

1.1.8 Pathophysiology

Many hypotheses have been proposed attempting to link the pathophysiology of laminopathies to known or emerging functions of A-type lamins. Prominent among these hypotheses include those based on functions that A-type likely have in maintaining the mechanical integrity of cells subject to stress (i.e. the 'mechanical stress hypothesis') or in regulating tissue-selective transcription or signal transduction (i.e. the 'gene expression hypothesis'). Recent studies have also led some investigators to hypothesize that abnormalities in cell proliferation or differentiation underlie the pathogenesis of laminopathies (Favreau et al. 2004).

1.1.8.1 Mechanical hypothesis

The '*mechanical stress hypothesis'* posits that striated muscles are constantly subjected to mechanical forces. Alteration of A-type lamins expression disrupts the nuclear integrity leading to decreased ability of the nucleus to resist stress. This is supported by the findings that muscle cells lacking A-type lamins or expressing A-type lamins with amino acid substitutions found in individuals with striated muscle diseases, have visible abnormalities in nuclear architecture and abnormalities in the peripheral chromatin (Favreau et al. 2004; Ostlund et al. 2001; Fidziańska & Hausmanowa-Petrusewicz 2003; Sylvius et al. 2005). Additionally, impaired mechano-transduction and reduced resistance under repetitive mechanical strain as indicated by the weakened activity of the mechanical stress-responsive transcription factor NF-κB (Nuclear factor-κB), yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) signaling pathway and mechanosensitive transcription factor MRTF-A (myocardin-related transcription factor A) signaling, have also been reported in cells lacking or carrying mutations on *Lmna* gene (Broers et al. 2004; Lammerding et al. 2004; Lammerding et al. 2006; Bertrand et al. 2014; Ho et al. 2013). We can, thus, speculate that some features of the striated muscle diseases caused by *LMNA* mutations arise from defective nuclear mechanics.

1.1.8.2 Gene expression hypothesis

The '*gene expression hypothesis*' is based on the notion that A-type lamins expression is crucial for tissue-specific gene expression, as evidenced by the findings that A-type lamins bind to

chromatin (Glass et al. 1993), transcription factors such as SREBP1 and HOK2 (Dreuillet 2002; Mancini et al. 1994; Lloyd 2002) and members of cell signaling cascades including ERK/12 and TGF-β (Emerson et al. 2009; González et al. 2008; Muchir et al. 2007a; Muchir et al. 2007b). However, there are few direct data showing that abnormalities in A-type lamins lead to altered gene expression and regulation. A recent interesting study claimed that mutation in *LMNA* interfere with the formation of heterochromatic lamina-associated domains (LADs) that disrupt developmental epigenetic programming (Perovanovic et al. 2016).

More studies support the notion that A-type lamins interact with proteins involved in cell cycle progression and control. Nucleoplasmic lamins A/C directly interact with the cell cycle regulator pRb in complex with LAP2α, tethering pRb to the nucleoskeleton where the cyclin D/cdk4 and 6 complex hyperphosphorylates pRb resulting in the release of E2F and the activation of genes involved in G1/S transition (Markiewicz 2002; Markiewicz et al. 2005). Other studies showed that A-type lamins interact with cyclin D3 which is involved in G1/S transition and the DNA elongation factor PCNA (proliferating cell nuclear antigen)(Mariappan et al. 2007; Shumaker et al. 2008).

1.1.9 Animal models

Several mouse models of the striated muscle laminopathies have been extremely helpful in studying the pathogenic mechanisms as well as potential innovative pharmacological therapies. These include mice that are null for either *Lmna* or *Emd* and mice carrying *Lmna* missense mutations such as p.H222P (Arimura et al. 2005), p.N195K (Mounkes et al. 2005) and p.M371K (Wang et al. 2006).

Lmna$^{-/-}$ mouse

The first lamin A mouse model was lamin A and C knockout (*Lmna*$^{-/-}$) (Sullivan et al. 1999). However, there is no report of human patient completely lacking lamins A/C apart from an individual who has haploinsufficiency of *LMNA* and a fetus homozygous for a premature stop codon in *LMNA* that died in gestation (Bonne et al. 1999; van Engelen et al. 2005; Muchir et al. 2003). While this model was considered as 'null' for many years, it has been recently shown that the mice express a very low levels of the truncated (deletion of exons 8-11) *Lmna* gene product at mRNA and protein level. The resulting truncated lamin D8-11 could act as hypoactive protein

(loss-of-function) or a toxic molecule (gain-of-function) to explain the phenotype of the *Lmna*$^{-/-}$ mice. These mice showed post-natal growth defects by 2 weeks of age and die at 6-7 weeks. *Lmna*$^{-/-}$ mice have a reduced lifespan and exhibit regional skeletal myopathy that resemble human EDMD associated with the development of DCM-CD with hallmark characteristics such as ventricular dilation, decreased fractional shortening as measured by echocardiogram, and decreased heart conductivity as measured by electrocardiogram (Cohen et al. 2013; Nikolova et al. 2004; Sullivan et al. 1999). Heterozygous mice are normal at early age but develop atrioventricular conduction defects with atrial and ventricular arrhythmias (Wolf et al. 2008).

Lmna$^{H222P/H222P}$ mouse

A knock-in mouse line carrying a missense mutation (histidine-to-proline substitution at amino acid 222 (H222P) which causes AD-EDMD in humans, was established in 2005 (Arimura et al. 2005). The *Lmna*$^{H222P/H222P}$ mice develop normally without growth defects and live significantly longer than *Lmna*$^{-/-}$ mice, dying at 9 months of age. Adult male homozygous mice develop dilated cardiomyopathy, showing decreased fractional shortening, ventricular dilation, and increased fibrosis in the myocardium. These mice show decreased locomotion and an abnormal stiff walking posture, which in combination with the wide variation in muscle fiber size and centrally located nuclei, are classical signs of muscular dystrophy. This was the first *Lmna* mouse model mimicking human laminopathy from the gene mutation to the clinical characteristics.

Lmna$^{N195K/N195K}$ mouse

A second knock-in mouse model was created, that carries the missense mutation N195K (asparagine-to-lysine substitution at amino acid 195) (Mounkes et al. 2005). The introduction of *Lmna* N195K missense mutation resulted in the expression of a mutant protein, which caused death in homozygous mutant mice at 3 months of age due to a heart-specific pathology reminiscent of dilated cardiomyopathy type 1A. Phenotypes observed in the *Lmna*$^{N195K/N195K}$ mice consistent with dilated cardiomyopathy included dilation of heart chambers, increased heart weight, increased interstitial fibrosis, upregulation of a fetal gene expression profile and conduction defects. Through the implantation of continuous electrocardiographic monitoring transmitters, they demonstrated that the *Lmna*$^{N195K/N195K}$ mice have severe arrhythmic events, which became progressively worse and eventually resulted in death. These mice showed minimal or no indication of muscular dystrophy.

***Lmna*$^{\Delta K32}$**

An Lmna mutant mouse model deleted for lysine 32 of lamin A/C (*Lmna*$^{\Delta K32}$ mouse) was engineered in 2012 (Bertrand et al. 2012). Homozygous *Lmna*$^{\Delta K32/\Delta K32}$ mice exhibit maturation defects of skeletal and cardiac muscles, and severe metabolic disorders responsible for premature death at 2 weeks of age. Interestingly, at this age, heterozygous *Lmna*$^{\Delta K32/+}$ mice did not have an obvious pathological phenotype but later develop a cardiac specific phenotype caused by the lamin A/C haploinsufficiency, which is in part due to delK32 lamin A/C degradation through the ubiquitin-proteasome system (Cattin et al. 2013). A-type lamins are absent from the NE and found exclusively in the nucleoplasm and this fact is thought to affect the cell proliferation and differentiation via the regulation of Rb function. Heterozygous *Lmna*$^{\Delta K32/+}$ mice die between 10 and 20 months of age from heart failure.

***Lmna* M317K mouse**

A model of overexpression of mutant A-type lamin in the heart has also been created and studied. In an alternative approach, cardiac-specific expression in transgenic mice (with two endogenous wildtype *Lmna* alleles) of human lamin-A M371K (methionine-to-lysine substitution at amino acid 371), which is encoded by an *LMNA* mutant that causes AD-EDMD in humans, leads to acute or subacute heart damage without fibrosis or severe inflammation, whereas similar overexpression of wildtype human lamin A does not cause significant abnormalities (Wang et al. 2006). Heterozygous mice expressing the mutant M371K lamin A showed a low birth rate and died by 2-7 weeks of age.

1.1.10 Current therapeutic options for striated muscle laminopathies

To date, no specific cure exists for human patients with striated muscle laminopathies. However, cardiac defects remain the most life-threatening clinical manifestation of striated muscle laminopathies. The management of *LMNA*-related DCM is focused on treatment of conduction system disease, arrhythmia, and heart failure. The conduction system abnormalities often necessitate the implantation of a permanent pacemaker. Current treatments for heart failure include conventional medications such as angiotensin II converting enzyme (ACE) inhibitors, angiotensin receptor blockers, beta blockers and aldosterone antagonists. Even though these approaches are able to reduce the risk of heart failure, patients still experience left ventricular dysfunction. In most cases of cardiomyopathy caused by *LMNA* mutations, implantation of an ICD is required to prevent arrhythmias and sudden cardiac death (Meune et al. 2006). However, this invasive procedure carries a risk of infection, can be traumatic to patients, and is technically more demanding and liable to complication in young children and infants. Therefore, cardiac transplantation is frequently the definitive treatment for DCM.

1.1.11 Potential new treamtents

Importantly, findings in animal models of the disease have yielded promising results that might be translated into novel pharmacological therapy. The mechanism through which mutated A-type lamins cause cardiac disease remains still obscure. To explore the pathogenesis of *LMNA* cardiomyopathy, a genome-wide expression analysis on the heart tissue from emerin null and *Lmna*H222P knockin mice, has been carried out. This analysis showed that increased extracellular signal-regulated kinase 1/2 (ERK1/2) branch of the mitogen-activated protein kinase (MAPK) signaling cascade and activation of downstream target genes (Muchir et al. 2007a; Muchir, et al. 2007b) and that this activation of MAPK pathway plays role in the pathogenesis of cardiac disease in EDMD.

Inhibition of MAPK pathways

MAPK pathways belong to a family of serine/threonine protein kinases and are major ubiquitously expressed signaling cascades, which transduce signal from extracellular mitogens, growth factors and cytokines at the cell surface to the nucleus. MAPK pathways include MAP kinases, MAP

kinase kinases (MKKs) and MAP kinase kinase kinases (MKKKs) (Johnson et al. 2005). MKKKs phosphorylate and activate MKKs, which in turn phosphorylate and activate MAP kinases. MAP kinases can activate downstream proteins in the cytoplasm or a number of transcription factors in the nucleus which regulate key cellular activities processes. The kinase immediately upstream of the MAP kinase is a member of the MAP/ERK kinase (MEK) family (Crews et al. 1992; Kosako et al. 1992; Ashworth et al. 1992; Wu et al. 1993), which are dual specificity enzymes that can phosphorylate serine/threonine and tyrosine residues. There are four well-known MAPK pathways in mammalian cells: ERK1/2, ERK5, c-JUN N-terminal kinase (JNK) and p38 (**Figure 9**).

The classical ERK1/2 pathway is initiated either by the stimulation of different receptors such as receptors with intrinsic tyrosine kinase activity (RTKs), cytokine receptors and G-protein-coupled receptors (GPCR) or by many extracellular ligands and cellular perturbations (e.g., mechanical stress, osmotic shock), with cell-type specificity. ERK1/2 are activated by closely related MEKs, MEK1 and MEK2 which phosphorylate ERK1/2 at both threonine and tyrosine residues within the TEY motif (Crews et al. 1992; Seger et al. 1992; Zheng & Guan 1993). It has been postulated that MEK1/2 have no other substrates and hence, this specificity of MEK1/2 has been used by several pharmacological companies to design potent inhibitors of ERK1/2 signaling (Akinleye et al. 2013). A variety of MEK1/2 inhibitors are ATP non-competitive that bind to a unique allosteric site adjacent to the ATP site.

Pharmacological inhibition of ERK1/2 cascade with a MEK1/2 selective inhibitor (Selumetinib) treatment of *Lmna*$^{H222P/H222P}$ mice, prevents left ventricular dilation and deterioration in cardiac contractility, decreases the cardiac fibrosis and prolonges the lifespan (Muchir et al. 2009; Wu et al. 2011; Muchir et al. 2010). Germline deletion of ERK1 from *Lmna*$^{H222P/H222P}$ mice leads to ameliorated heart function at early stage (i.e., 16 weeks) but this improvement disappeared at 20 weeks of age concurrent with an increase greater than 2-fold in ERK2 activation (Wu et al. 2014). Blocking cardiac ERK2 activity by treatment with Selumetinib leads to significant improvement in ejection fraction at 20 weeks, strongly suggesting that the increased ERK2 activity to compensate the loss of ERK1 leads to deterioration in heart function in *Lmna*^{H222P} mice lacking ERK1. Combined treatment of *Lmna*$^{H222P/H222P}$ mice with the MEK1/2 inhibitor selumetinib and the ACE inhibitor benazepril has beneficial effects on left ventricular function in *Lmna*$^{H222P/H222P}$ mice and both drugs together have a synergistic benefit (Muchir et al. 2014). These mouse studies

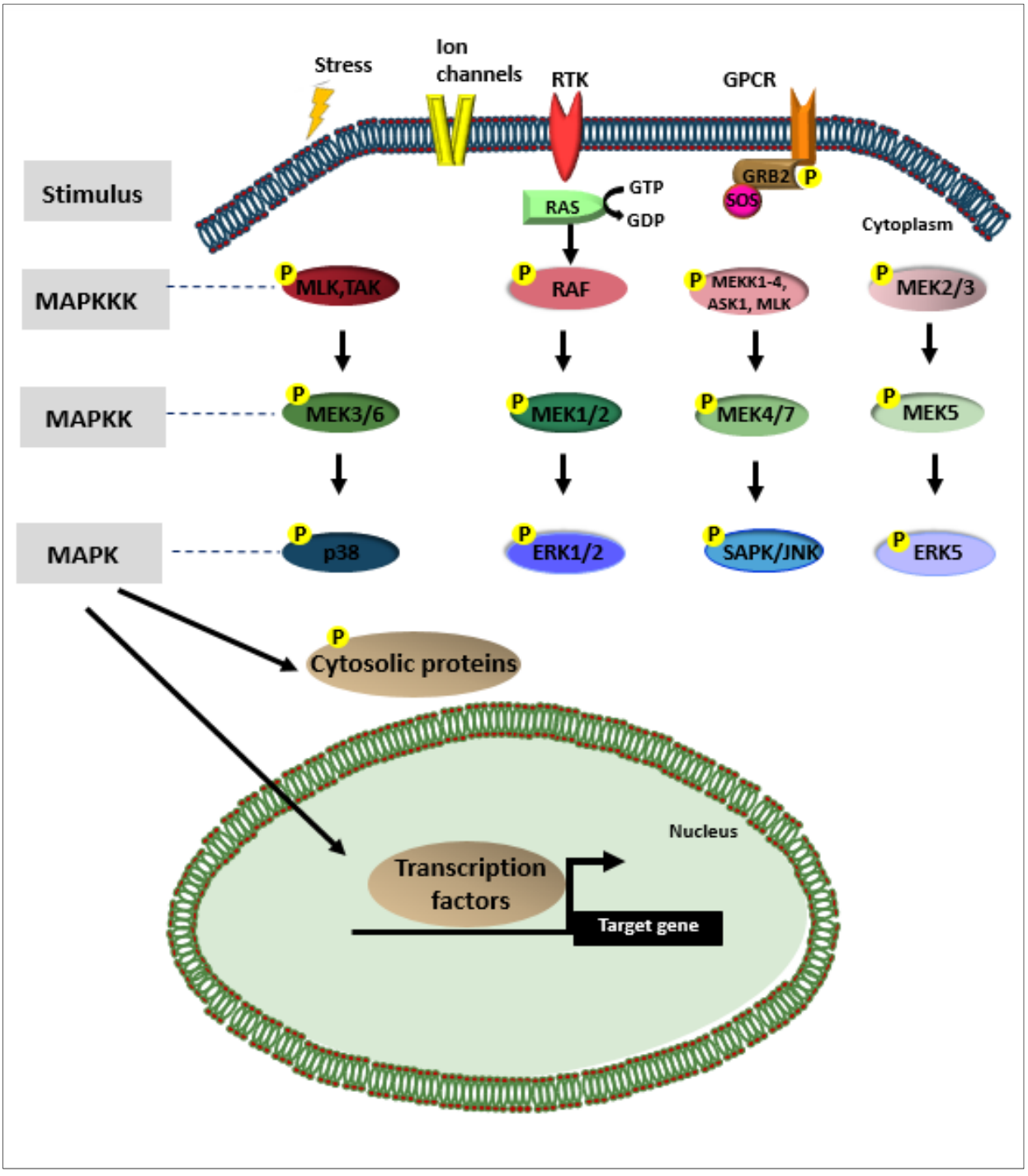

Figure 9: A simplified overview of MAPK pathways in mammals. MAPK signaling pathways mediate intracellular signaling initiated by extracellular or intracellular stimuli. MAPKKKs directly phosphorylate MAPKKs, which, once activated, phosphorylate MAPKs. Activated MAPKs phosphorylate numerous cytoplasmic substrates and modulate transcription factors that drive context-specific gene expression.

suggest that ERK inhibition might hold promise for treating cardiomyopathy in human subjects with *LMNA* and *EDM* mutations.

Remarkably, cardiac tissue from *Lmna*$^{H222P/H222P}$ mice displayed enhanced activity of JNK and p38 and inhibition of these cascades (i.e., SP600125 and ARRY797, respectively) has benefits for the left ventricular dysfunction (Muchir et al. 2007a; Muchir et al. 2012; Wu et al. 2010).

Induction of autophagy

In both *Lmna*^{H222P} and Lamin A/C-null mice, the AKT signaling pathway is activated, which in turn activates the mammalian target of rapamycin complex 1 (mTORC1). TOR is a serine/threonine protein kinase that belongs to the phosphatidylinositol 3-kinase related kinase family and regulates cell growth and protein synthesis. Inhibition of mTOR pathway by rapamycin enhances the heart function of the *LMNA* mutants and blocks molecular cardiac remodeling but fails to lower the degree of cardiac fibrosis (Choi et al. 2012; Ramos et al. 2012). The rapamycin binds and inhibits TOR in an allosteric and specific manner. However, inhibition of both ERK1/2 and AKT pathways did not result in an improvement, although the number of mice tested was limited (Choi et al. 2012). The exact mechanisms by which defects in A-type lamins lead to dysregulation of ERK1/2 and AKT pathways, remain unknown.

1.1.12 Other therapeutic options

Arimura and co-workers used Ca^{2+} sensitizers, novel cardiotonic agents that increase Ca^{2+} sensitivity of muscle contraction, as a therapeutic strategy for heart failure and showed improved systolic dysfunction, prolonged life expectancy and decreased cardiac interstitial fibrosis of *Lmna*^{H222P} mice (Arimura et al. 2010). Although the underlying mechanism remains unknown, these findings implied that the Ca^{2+} sensitizers could be an option for preventing the progression of *LMNA* cardiomyoapthy.

Importantly, the development of cell-based therapy is progressively emerging as a promising tool for the treatment of DCM and heart failure. In a study, the authors point out the functional benefit produced by myoblasts (Mb) transplantation to the dilated myocardium of *Lmna*$^{H222P/H222P}$ mice (Catelain et al. 2013). Moreover, the use of patient-derived induced pluripotent stem cells (iPSCs)

that can be differentiated into various cell types provide an important tool that allows better prediction of the therapeutic responses induced by newly developed drugs and offers insight into the underlying mechanisms. Based on this, disease-specific iPSC lines derived from patients with *LMNA* mutation (p.R225X) have been generated and differentiated into cardiomyocytes (Siu et al. 2012). When these cardiomyocytes subjected to electrical stimulation to mimic their *in vivo* host environment, they exhibited nuclear abnormalities and increased susceptibility to apoptosis. Pharmacological blockade of ERK1/2 signaling abolished the electrostimulation-mediated apoptosis, providing further evidence that ERK1/2 may be a potential therapeutic target in *LMNA*-cardiomyopathy.

1.2 Actin

The mammalian cytoskeleton is an internal framework of actin, tubulin, and intermediate filament proteins. Actin is the most abundant protein in most eukaryotic cells (Dominguez and Holmes, 2011) and is mainly located in the cytoplasm, but there is also a considerable amount in the nucleus. Cytoplasmic actin forms a range of structures, such as filopodia, lamellipodia or actin stress fibers and contributes to an even broader range of processes, including cell shaping, polarity and motility (Ridley & Hall 1992; Kozma et al. 1995), cell division, vesicle and organelle movement, cell signaling and muscle contraction.

Cellular actin is found in two forms: G-actin (globular actin) and F-actin (filamentous actin) (Pollard & Borisy 2003). G-actin is monomeric while F-actin is incorporated into polymers. Actin filaments are structurally polarized, with one dynamic end called the barbed end and the other end called the pointed end (Pollard & Borisy 2003). Actin monomers preferentially add to the barbed end (designated plus) and disocciate from the pointed end (designated minus). The process in which filament length remains approximately constant, while actin monomers add at the (+) end and dissociate from the (-) end at the same rate, is described as treadmilling.

Actin filament polymerization occurs over three sequential phases: (1) A nucleation phase, where polymerization is slow, (2) an elongation phase, when polymerization is rapid and (3) a steady state phase, when there is no net polymerization. During the nucleation phase the formation of an 'actin nucleus' occurs, which is usually a complex of three actin monomers. In the elongation phase monomers are rapidly added to the filament at the barbed end and this is often facilitated by additional elongation factors. In the steady state phase, the filament dynamics enter a state of equilibrium where monomer disassembly from the (-) end and polymerization at the (+) end is balanced.

Dynamic remodeling of the actin cytoskeleton is critical for many cellular processes. The dynamics of actin cytoskeleton is regulated by a large set of proteins including among others, nucleators factors, proteins involved in the filament severing, polymerization and depolymerization. **Figure 10** illustrates the main actin-binding factors that play role in the actin turnover process.

1.2.1 Nucleator proteins

Spontaneous nucleation of actin filaments is inhibited *in vivo* by factors like profilin and thymosin β4. To overcome this inhibition, actin nucleation requires additional proteins known as 'actin nucleators' to promote the formation of a stable actin nucleus. These proteins include the Arp2/3 complex, formins, and the tandem-monomer-binding nucleators (Campellone & Welch 2010; Kerkhoff 2006; Renault et al. 2008).

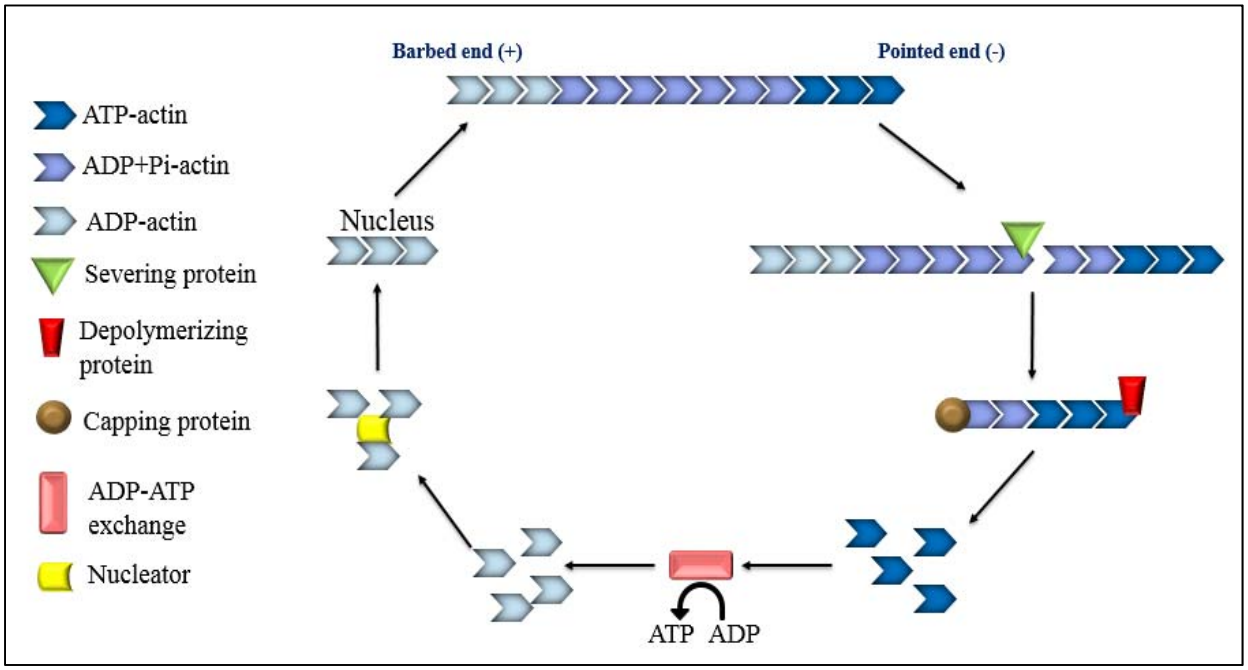

Figure 10: Schematic representation of the actin-binding proteins (ABPs) that influence the actin treadmilling. ABPs can affect F-actin by promoting its depolymerization, its severing or its polymerization (nucleators). Capping proteins bind to filament ends and can modify filament turnover to affect their length. ATP-actin monomers associate to the barbed end whereas ADP-actin dissociates from the pointed end of an actin filament.

The Arp 2/3 complex is a seven-subunit multiprotein complex (Arp2, Arp3, ARPC1-C5) with two 'actin-related proteins', Arp2 and Arp3, and five 'Arp complex' (Arc) (Goley & Welch 2006). The two major subunits, Arp 2 and Arp 3, are structurally similar to monomeric actin (Robinson et al. 2001). Arp2/3 complex binds to the side or pointed-end of an existing actin filament (Schoumacher et al. 2010) and nucleates assembly of a new filament branch, oriented at a 70° angle relative to the mother actin filament (**Figure 11**). By itself, Arp 2/3 complex is inactive and must be activated

by accessory proteins, described as NPFs (Nucleation Promoting Factors) (Goley & Welch 2006; Pollard 2007; Campellone & Welch 2010). The most abundant NPFs are described as class I NPFs which is composed of five groups: WASP and neural WASP (N-WASP), WASP-family verprolin homologues (WAVE or SCAR), WASP and SCAR homologues (WASH), and WASP homologue associated with actin, membranes, and microtubules (WHAMM). All WASP family proteins have an acidic carboxyl terminal WCA domain (WH2 domains, Connector region, Acidic domain) and an amino-terminal basic domain (**Figure 11**). The WH2 (Wiskott-Aldrich Syndrome protein (WASp) homology 2) domain binds G-actin, and the CA domains collectively interact with the Arp 2/3 complex and with filamentous actin. The Arp2/3 complex nucleates branched actin networks and is involved in the myoblast fusion and in the formation of the lamellipodium at the leading edge of migrating cells (Richardson et al. 2008; Rochlin et al. 2010).

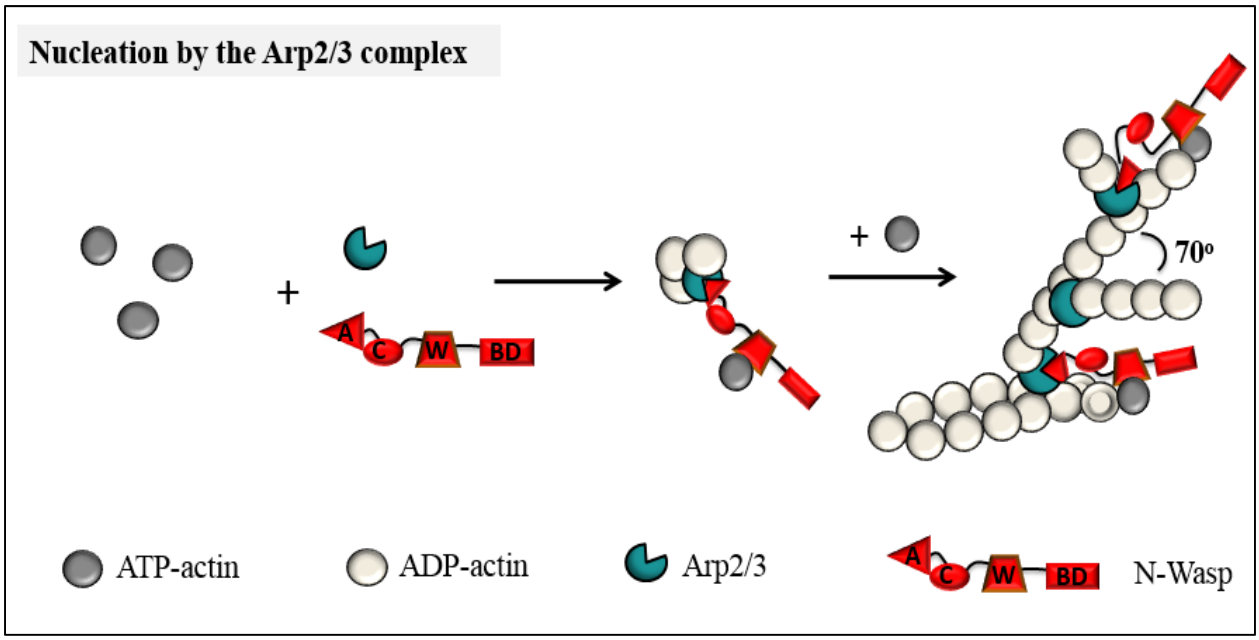

Figure 11: Arp2/3-mediated actin nucleation. The Arp2/3 complex, once activated by an NPF such as N-WASP nucleates a new filament at a 70° angle relative to an existing actin filament. The N-WASP uses its WCA domain to stimulate Arp2/3-mediated actin polymerization. The C and A regions of this domain bind the Arp2/3 complex, whereas the WH2 domain interacts with G-actin. A, acidic region; C, central connector region; L, linker region; W, WASP Homology 2 (WH2) domain BD: basic domain

Actin monomers are also nucleated by the formin proteins. Formins (formin homology proteins), unlike Arp2/3, promote the nucleation and elongation of unbranched filaments, forming linear actin polymers (Higgs & Peterson 2005; Pruyne et al. 2002; Zigmond et al. 2003). In mammals, formins are classified into seven subfamilies (DIA, DAAM, FMN, INF, FHOD, FMNL and Delphilin) and characterized by the presence of two conserved domains: FH1 and FH2 (Higgs 2005; Higgs & Peterson 2005). The FH2 (formin-homology domain 2) domain which is ~400 amino acids in length, is responsible for the actin nucleation at the barbed-end. During actin polymerization, formin functions as homodimer. After nucleation and elongation, the formin dimer moves processively with the growing barbed end of the actin in contrast to Arp 2/3 which remains bound to the non-growing pointed filament end and is immobile relative to the mother and daughter filaments **(Figure 12**). The proline-rich FH1 domain also regulates actin polymerization through binding with the profilin protein.

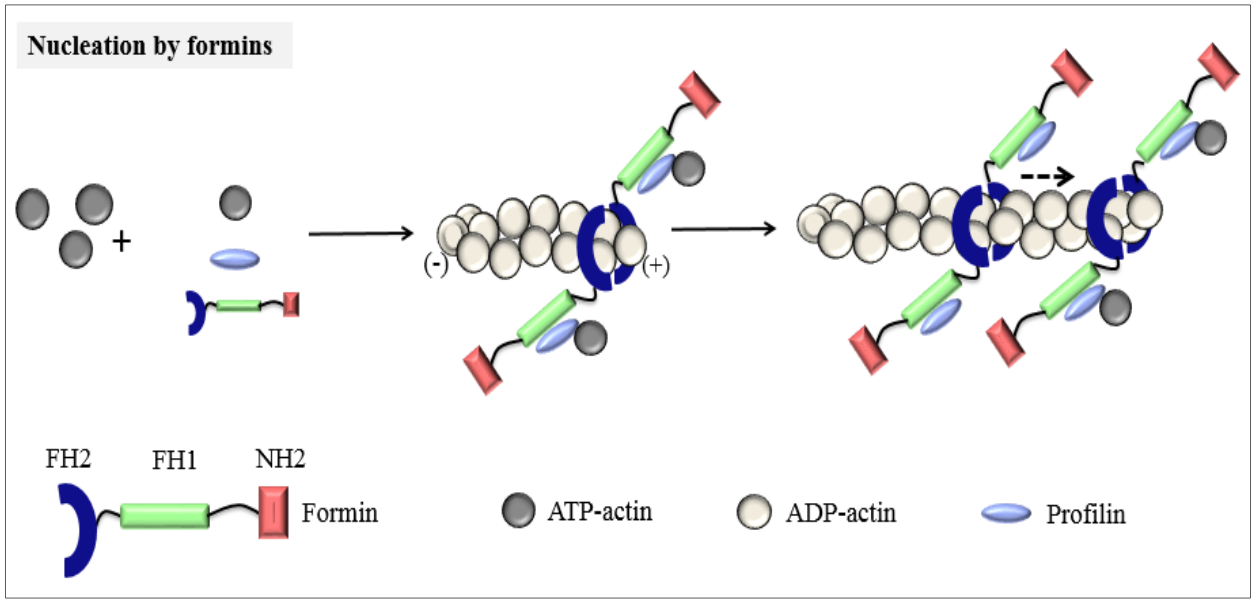

Figure 12: Actin nucleation and elongation by the formin family proteins. A formin FH2-domain dimer binds the barbed end of an actin filament and the proline-rich FH1 domain recruits the ATP-actin binding protein profilin to the barbed end. The formin moves processively with the barbed end as the actin filament elongates. FH1, formin-homology 1; FH2, formin-homology 2

The third group of actin nucleators includes Spire (Quinlan et al. 2005), cordon-bleu (Cobl)(Ahuja et al. 2007), VopL/VopF (Liverman et al. 2007; Tam et al. 2007), leiomodin (Lmod) (Chereau et al. 2008), the junction-mediating and regulatory protein JMY (Zuchero et al. 2009) and adenomatous polyposis coli (APC)(Okada et al. 2010). Although different mechanisms have been proposed for each, these nuleators share a common feature which is an actin-binding WH2 [Wiskott-Aldrich Syndrome protein (WASp) homology 2] motif that binds three to four actin subunits to form an actin nucleus (**Figure 13).**

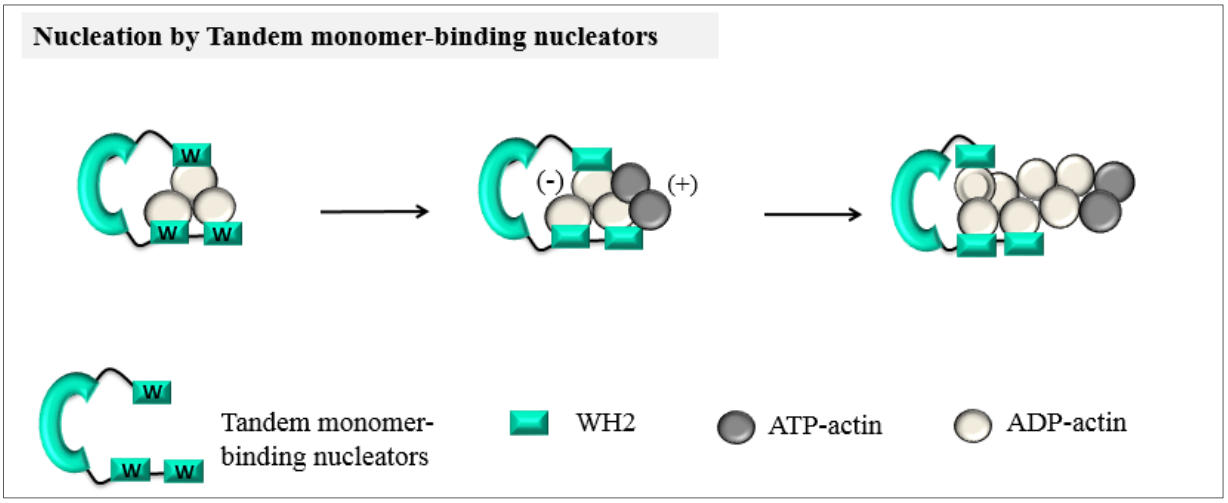

Figure 13: Actin nucleation by tandem monomer-binding nucleators which bring together actin monomers through G-actin-binding motifs to form a trimeric actin nucleus (e.g. cordon-bleu or leiomodin) and remain associated with the pointed end of the actin filament as the filament elongates. WH2, Wiskott-Aldrich syndrome protein (WASp) homology 2.

1.2.2 Profilin

Profilin is a monomer-sequestering protein that binds ATP-actin monomers (Kd = 0.1μM) with higher affinity than ADP-actin (Kd = 0.5 μM) (Goldschmidt-Clermont et al. 1991; Pantaloni & Carlier 1993; Vinson et al. 1998). In mammalian cells, four profiling isoforms have been identified; profilin-1 is expressed in most tissues whereas profilin-2 is predominant in the brain, kidney and muscle. The importance of profilin function has been initially shown in *Listeria* actin-based motility assay (Grenklo et al. 2003). Profilins promote filament elongation at barbed ends through several mechanisms, including enhancing ADP/ATP exchange on G-actin (**Figure 14**), displacing monomers from sequestering proteins, and thus increasing the local concentration of

actin monomers available for assembly (Ressad et al. 1999; Yarmola et al. 2008). Notably, when the barbed end is capped, profilin acts as an inhibitor of pointed-end actin polymerization.

Listeria monocytogenes is a Gram-positive intracellular pathogenic bacterium that recruit the host cell actin cytoskeleton both to move within the cytoplasm of infected cells and to spread from cell to cell. *L. monocytogenes* encodes the actin assembly-inducing protein (ActA) protein which interacts with the Arp2/3 and actin monomers on the bacterial surface to induce actin polymerization. This, subsequently is reorganized into a 'comet tail', located at one pole of the bacterial surface. This structure provides the locomotive force in intracellular bacterial motility. The proteins that are required for motility include: Arp2/3-complex, cofilin, capping protein and a nucleation-promoting factor. However, the movement is more effective when profilin, α-actinin and VASP (Vasodilator-stimulated phosphoprotein) are included.

1.2.3 Capping protein (CP)

The barbed end of actin filaments are capped by the capping protein (CP) which prevents actin subunit addition and loss (**Figure 14**). Heterodimeric CP is found in almost all eukaryotic cells and consists of α and β subunits. Vertebrates contain three α subunit isoforms encoded by three different genes, and three β subunit isoforms (β1, β2, and β3) that are produced by alternative splicing from a single mRNA transcript display tissue specific distribution; β1 isoform (aka CapZ) is predominantly expressed in muscle cells, whereas the β2 isoform is the predominant isoform of nonmuscle tissues (Casella et al. 1987). In a landmark study, CP was one of the proteins found to be required for the reconstitution of Listeria actin-based motility *in vivo* with pure proteins (Loisel et al. 1999).

1.2.4 Severing proteins

Rapid actin disassembly is required for cells to dynamically reorganize their cytoskeletons in response to different signals and to maintain a pool of actin monomers available for new assembly. Actin-depolymerizing factor ADF/cofilin and gelsolin are the two major classes of actin-regulatory proteins that accelerate filament disassembly (Southwick 2000).

- **Gelsolin**

Gelsolin belongs to a family of seven related proteins: gelsolin, adseverin, villin, capG, advillin, supervillin, and flightless I (Silacci et al. 2004). Gelsolin is a ~80 kDa protein consisting of six homologous domains, numbered G1–G6, with G1 and G4 containing the primary gelsolin actin-binding domains. The ability of gelsolin to sever an actin filament depends on the presence of Ca^{2+}. However, some reports support that gelsolin acts more as a capping protein than severing protein, thereby preventing the reannealing of actin fragments (Gremm & Wegner 2000).

- **ADF/cofilin**

One of the most well-defined factors in actin turnover is ADF/cofilin. ADF/cofilin was first discovered and purified in 1980 from embryonic chick brain extracts by Bamburg et al. ADF/cofilin is small actin binding protein that is composed entirely of one ADF-H-domain and bind both actin monomers and filaments. Mammals have three cofilin isoforms: non-muscle cofilin (CFL1) is expressed in most tissues of embryonic and adult cells, muscle-specific cofilin (CFL2) is expressed in muscle cells, and ADF (destrin) is limited to epithelial and endothelial cells (Ono et al. 1994; Vartiainen et al. 2002). Genetic studies have distinct physiological roles. Cofilin-1 knockout mice exhibit an embryonic lethal phenotype at E9 (Gurniak et al. 2005) whereas ADF knockout leads to corneal thickening and blindness, and cofilin-2 knockout mice die by postnatal day 8(Agrawal et al. 2012). Aside from the cofilin role in actin cytoskeleton, it has also been postulated to modulate the intracellular Ca2+ signaling (Nusco et al. 2006).

All three ADF/cofilins have the ability to bind both G- and F-actin with similar affinities (Chin et al. 2016). Their binding to actin is regulated, at least in part, by the state of the nucleotide bound to actin. ADF/cofilin binds ADP-actin with about two orders of magnitude greater affinity than to ATP-actin or ADP-Pi –actin and inhibits the nucleotide exchange (Nishida 1985; Bamburg 1999). ADF/cofilin promoted actin depolymerization globally and this view is supported by the observation that is found only in the proximal portion of the cortex of motile cells and not at the distal edge where polymerization occurs. However, it has been further showed that the level of ADF/cofilin can influence the actin dynamics with a biphasic manner; lower concentration of ADF/cofilin exhibits higher binding affinity to ADP-bound actin in the filaments promoting depolymerization from the pointed end of actin filaments, while higher level of ADF/cofilin can stabilize the actin filaments and even initiate the nucleation of G-actins, which lead to elongation

and branched by profilin or Arp2/3 complex, respectively (Van Troys et al. 2008; Blanchoin et al. 2000; Bamburg & Bernstein 2008).

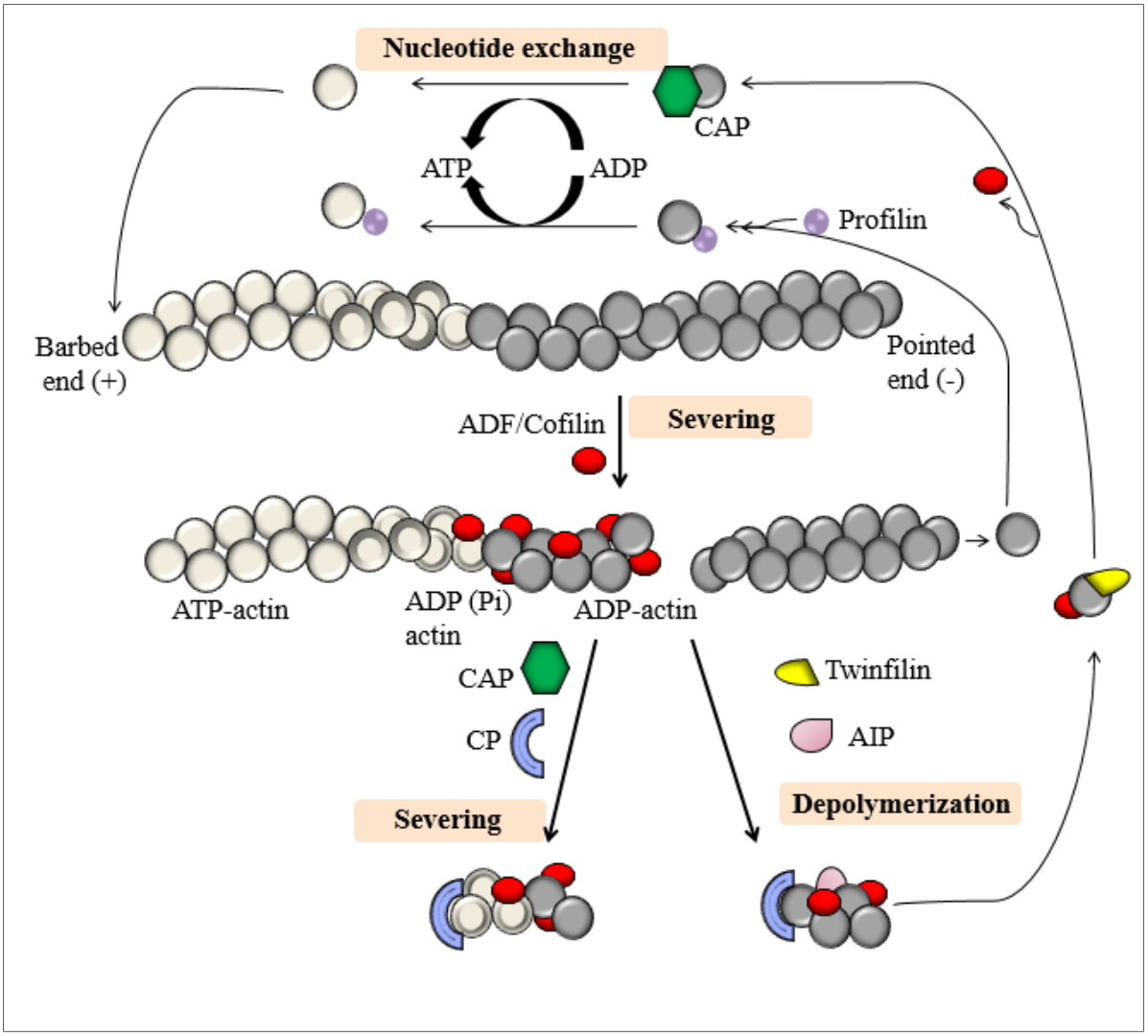

Figure 14: Regulation of actin filament dynamics by ADF/cofilin, profilin, CAP, twinfilin and AIP1. CAP accelerates the ADF/cofilin-bound actin severing and promotes nucleotide exchange of the G-actin. Profilin also promotes the nucleotide exchange at G-actin and, therefore, enhances actin turnover. AIP promotes the depolymerization of the ADF-cofilin-bound actin fragments whereas twinfilin inhibits nucleotide exchange of ADP-actin monomers and prevents their assembly into actin filaments

Cofilin promotes actin depolymerization by two distinct mechanisms. Numerous lines of evidence suggested that 1) cofilin depolymerizes actin due to a severing activity (Maciver et al. 1991) and 2) accelerates the treadmilling via the enhancement of the off-rate at the barbed end of the filament leading to actin filament destabilization (Carlier & Pantaloni 1997). A combination of both

mechanisms has also been suggested (Theriot 1997). Severing and pointed-end depolymerization are separate activities because they can be uncoupled by point mutations(Jiang et al. 1997; Pope et al. 2000; Moriyama & Yahara 1999; Ono et al. 2001). Importantly, recent findings showed that ADF and cofilin-2 display higher severing activity compared to cofilin-1 (Chin et al. 2016).

There are enormous numbers of computational, structural and biochemical and studies on the binding of cofilin to actin filaments. Electron microscopy first revealed that cofilin binds actin filaments cooperatively by bridging two longitudinally associated actin subunits (McGough et al. 1997; Ressad et al. 1998). This interaction alters the filament subunit tilt (Galkin et al. 2002), increases of the helical twist by ~5^{o} per subunit allosterically (McGough et al. 1997), weakens the lateral (between the two strands of actin filament) contacts (McGough & Chiu 1999; Bobkov et al. 2004), disrupts the longitudinal (between protomers within one helical strand of actin filament) contacts (Galkin et al. 2003; Bobkov et al. 2006) and inhibits the exchange of actin-bound nucleotides (Blanchoin & Pollard 1998; Nishida 1985; Hayden et al. 1993; Hawkins et al. 1993). Similarly, dense binding of cofilin shortens the helical pitch of actin filaments by 25% (McGough et al. 1997; Galkin et al. 2001) Time-resolved phosphorescence anisotropy (Prochniewicz et al. 2005) and differential scanning calorimetry (Bobkov et al. 2006) showed that one molecule of bound cofilin changes the structure of ~100 actin subunits. Essentially, studies using high-speed atomic force microscopy (HS-AFM) showed that conformational changes induced by cofilin binding are propagated unidirectionally to the pointed ends of the filaments (Umeki et al. 2016; Ngo et al. 2015). As a result, filament adopts a thermodynamically unstable conformation that spontaneously breaks at the junction between bare and cofilin-decorated regions (Suarez et al. 2011) and monomers released from the pointed-end.

Regulation of ADF/cofilin

The activity of ADF/cofilin can be regulated by several mechanisms including intracellular pH (Bernstein et al., 2000; Yonezawa et al., 1985), salt concentration, phosphorylation (Morgan et al. 1993; Agnew et al. 1995; Moriyama et al. 1996), association with PI(4,5)P2 (phosphatidylinositol *4*,*5*-bisphosphate) (Yonezawa et al. 1990) and binding of cortactin (Oser et al. 2009). In addition, ADF/cofilin competes with several other actin-binding proteins for binding to actin. Tropomyosin competes with ADF/cofilin for F-actin binding and inhibits

depolymerization and severing (Ono & Ono 2002; Bernstein & Bamburg 1982; Nishida et al. 1984). Profilin and cyclase-associated protein (CAP) also compete with ADF/cofilin for G-actin binding and synergistically increase actin filament turnover (Blanchoin & Pollard 1998; Didry et al. 1998; Wolven et al. 2000; Balcer et al. 2003; Skwarek-Maruszewska et al. 2009; Bertling et al. 2004).

The best characterized regulatory mechanism of ADF/cofilin is phosphorylation by two kinase families: the LIM (Lin-11/Isl-1/Mec-3) kinases and the TES (testis-specicific) kinases (**Figure 15**) (J Toshima et al. 2001; Jiro Toshima et al. 2001; Bernard 2007). LIM kinase is activated by phosphorylation by upstream kinases (Pak1, ROCK, protein kinase C) upon activation of small G-proteins (Rho, Rac, Cdc42) or diaglycerol (DAG) and subsequently catalyzes phosphorylation of ADF/cofilin at serine 3 residue. When ADF/cofilin is phosphorylated, it has low affinity for actin. ADP exchange in G-actin is strongly inhibited by cofilin, but once cofilin is phosphorylated, the released ADP-actin monomer can exchange with cytoplasmic ATP, and is re-incorporated at the barbed end of a growing filament. Suppression of cofilin activity by LIMK overexpression abolishes lamellipodium formation and polarized cell migration, which implicates cofilin in cell polarity and migration (Dawe et al. 2003). TESK activation is dependent on integrin engagement upon cell attachment and phosphorylates cofilin at the same residue (J Toshima et al. 2001). NCK-interacting kinase (NIK)-related kinase (NRK) also phosphorylates cofilin at Ser-3 (Nakano et al. 2003). A more recent report shows that the protein stability of ADF/cofilin is also dependent on the phosphorylation of tyrosine 68 residue by v-src protooncogene (Yoo et al. 2010). It has also been described that Aurora A kinase phosphorylates ADF/cofilin on Ser3, Ser8 and Thr25 (Ritchey & Chakrabarti 2014). On the other hand, ADF/ cofilin dephosphorylation and therefore activation is mediated by slingshot (SSH) phosphatase, chronophin (CIN) phosphatase, and protein phosphatase 1 and 2A (PP1 and PP2A) (Takuma et al. 1996; Gohla et al. 2005). Moreover, a 14–3-3 protein binds to phosphorylated ADF/cofilin and protects it from dephosphorylation (Gohla & Bokoch 2002), thereby providing an additional level of phosphoregulation of ADF/cofilin.

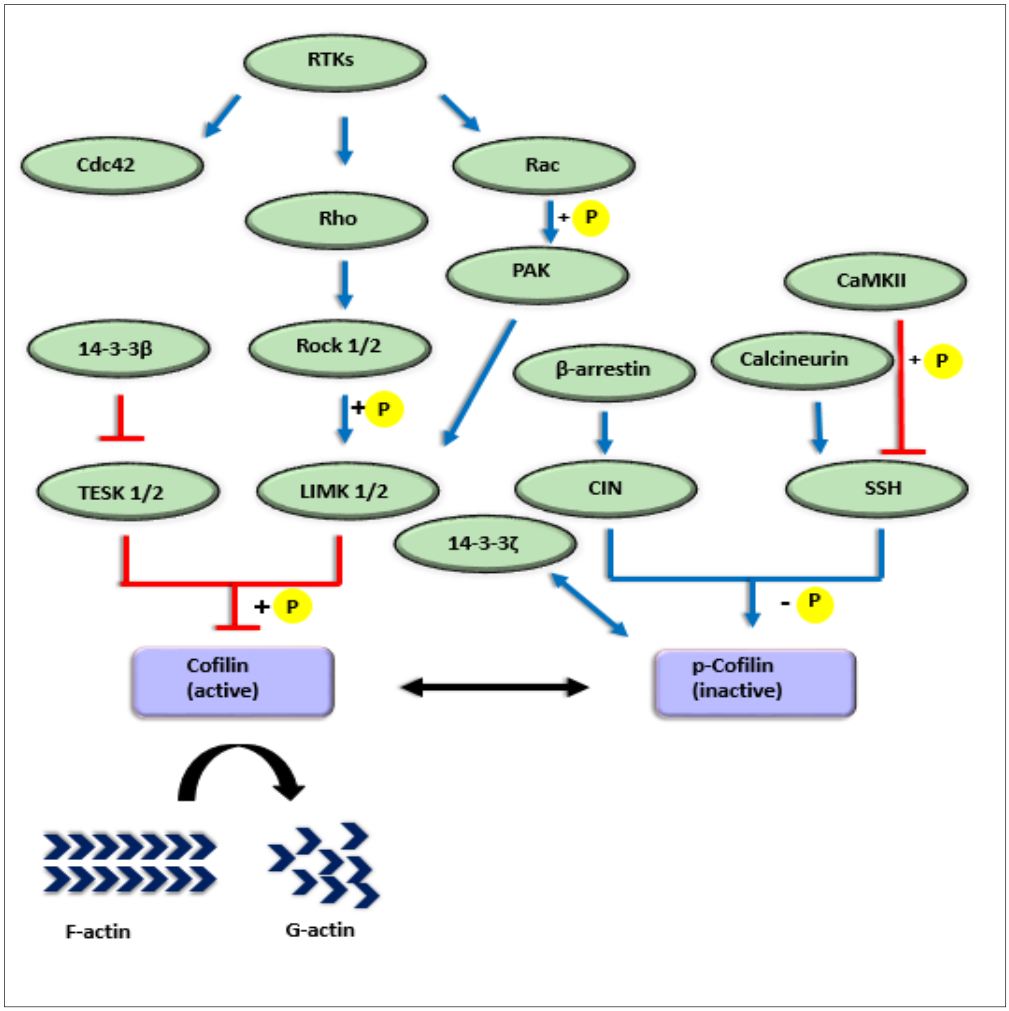

Figure 15: Schematic overview of the ADF/cofilin kinases and phosphatases and their upstream regulators. Blue double-arrow lines point an interaction which leads to stabilization. Red lines point an inhibition whereas blue lines point an activation. Rock; RHO-associated coiled-coil-containing protein kinase, CaMKII; Ca^{2+}/calmodulin-dependent protein kinase II, Cdc42; cell division cycle 42, PAK; p21-activated kinase, CIN; chronophin, TESK; testis-specific kinase, LIMK; LIM-kinase, SSH; slingshot-protein phosphatase

A growing body of evidence shows that, alteration of cofilin activity or expression participates in the pathogenesis of various diseases including cancer, neurodegenerative disorders (Alzheimer disease, corticobasal degeneration, William's syndrome, fragile X syndrome and spinal muscular dystrophy), immune deficiencies, skeletal muscle myopathies (nemaline myopathy) and dilated cardiomyopathy (Agrawal et al. 2007; Agrawal et al. 2012; Subramanian et al. 2015).

The severing activity of purified cofilin alone in vitro is not particularly efficient as visualized in real time by TIRF (total internal reflection fluorescence microscope) microscopy

(Andrianantoandro & Pollard 2006). This suggests that the high rates of filament turnover observed *in vivo* are further regulated and enhanced by additional factors. Mounting genetic and biochemical evidence has implicated three proteins, Srv2/CAP (cyclase-associated protein), AIP1 and Coronin, in functioning with cofilin to promote actin disassemble *in vivo*. Brieher and colleagues isolated Cofilin, AIP1 and Coronin as factors that induce the disassembly of *Listeria* actin comet-tails (Brieher et al. 2006). Recently, it has been demonstrated that these three proteins work in temporally ordered manner to rapidly disassemble F-actin with first coronin binding to ADP-actin subunits of older actin filaments, which in turn facilitates cofilin recruitment to filaments sides (Jansen et al. 2015; Brieher et al. 2006). Last comes AIP1, which promotes cofilin-mediated actin turnover by enhancing its severing activity and by capping the barbed ends of cofilin-severed actin filaments to block reannealing (Okada et al. 2002; Ono et al. 2004). Twinfilin is another actin-binding protein sequesters actin monomers and caps the plus ends of actin filaments preventing nucleotide exchange (Helfer et al. 2006) (**Figure 14**).

1.2.5 Actin filaments in muscle

Striated muscle cells are characterized by morphological and functional units called sarcomeres. Sarcomeres are composed of highly ordered arrangements of actin thin filaments and myosin thick filaments and a large number of proteins involved in the production of contractile forces (**Figure 16**). During muscle contraction, sarcomeres undergo a reversible shortening driven by central, bipolar myosin filaments pulling on actin filaments that are attached at the sarcomere boundaries, thus contracting the sarcomeres. Myosin-based thick filaments have myosin heads in bipolar orientations and registered in the middle of sarcomeres called M-line. The sarcomere can be further subdivided into a broad central A-band occupied by thick filaments and centered on the M-line, and I-bands that mark thick filament-free zones.

The barbed end of the thin filaments is anchored in the Z disc and the pointed end stretches to the middle of the sarcomere toward the M-band region (**Figure 16**). The actin filaments in sarcomeres are uniform in length and are positioned and stabilized by means of specific actin-binding proteins. Therefore, polymerization and depolymerization of actin must be precisely regulated during assembly and maintenance of striated myofibrils. Although both ends of sarcomeric actin filaments are capped, studies have provided evidence that actin is still dynamically polymerized and depolymerized at these ends in mature myofibrils (Imanaka-Yoshida et al. 1993; Komiyama et al.

1993; Littlefield et al. 2001; Gokhin & Fowler 2013). Nevertheless, FRAP (fluorescence-recovery after photobleaching) analyses suggested that also the entire thin filaments undergo turnover (Suzuki et al. 1998; Hasebe-kishi & Shimada 2001; Wang et al. 2005). Further studies showed that a subset of mature sarcomeric actin filaments undergo constant turnover as a result of myosin contractility (Skwarek-Maruszewska et al. 2009).

The dynamic remodeling of these sarcomeric filament arrays is consistent with the need for proteins that play role in actin nucleation, regulation of filament assembly and disassembly, nucleotide exchange and regulation of thin filaments length. A number of mutations in genes coding for thin filament structural and regulatory proteins, including cofilin, tropomyosin and nebulin, cause human hereditary myopathies, indicating that correct regulation of sarcomeric actin organization is vital for normal function of striated muscle (Laing 2007).

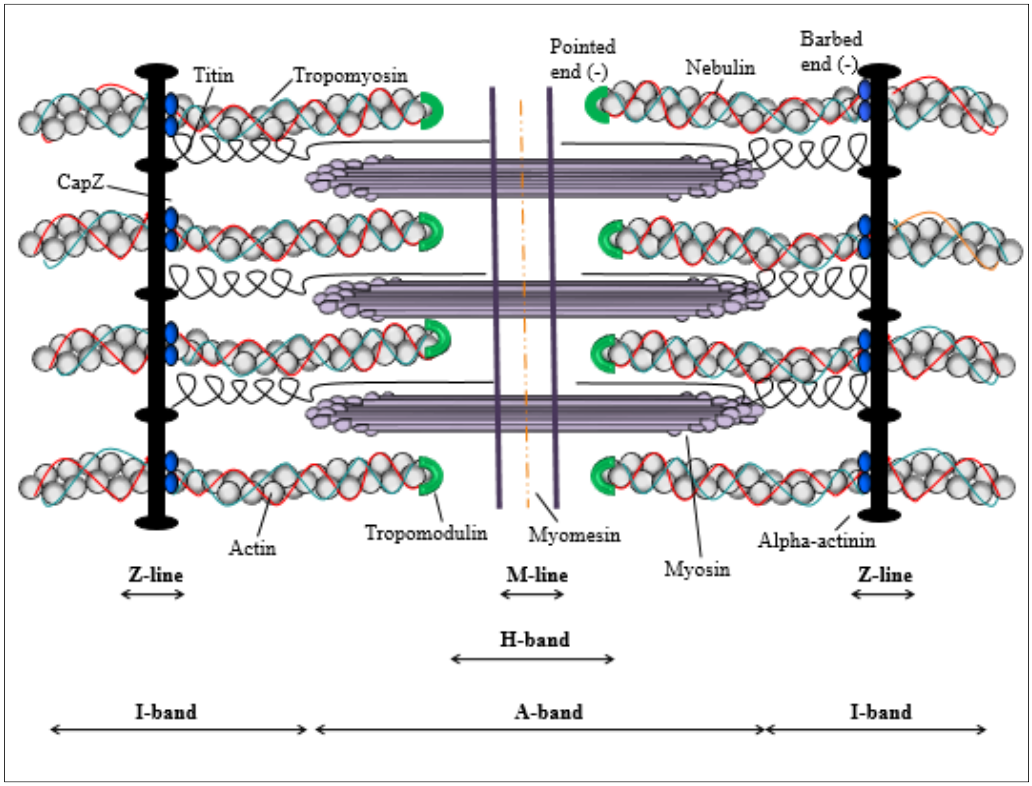

Figure 16: Schematic drawing of sarcomere structure and sarcomere-binding proteins. The sarcomere is a repeated structure along a myofibril. Two of the important proteins are myosin, which forms the thick filament, and actin, which forms the thin filament. Actin molecules are bound to the Z line, which forms the borders of the sarcomere.

1.2.6 Regulation of sarcomeric actin filaments

The specific mechanisms for actin nucleation and maintenance in sarcomeres are poorly understood (Ono 2010). Premyofibrils are believed to assemble through a similar mechanism to that of stress fibers in non-muscle cells (Sparrow & Schöck 2009). Thus, it is possible that formins and Arp2/3 complex are implicated in sarcomeric actin filaments assembly. Although the Arp2/3 complex is involved in myoblast fusion, there is no evidence indicates its involvement in the nucleation of sarcomeric filaments.(Richardson et al. 2008; Rochlin et al. 2010)

Formins, which nucleate unbranched linear actin structures are attractive candidates for the formation of sarcomeric thin filaments (Mattila & Lappalainen 2008; Schirenbeck et al. 2005). A study demonstrated that seven formins (DAAM1, mDia2, FMNL1, FMNL2, INF2, FHOD3, FMN1) localize to sarcomeres of cardiomyocytes (Rosado et al. 2014). Among these, DAAM1, mDia2 FMNL1 and FMNL2 are required for the myofibrillogenesis with the two latter playing role in myofibril repair. FHOD3 is required for normal sarcomere assembly in rat and mouse cardiomyocytes (Taniguchi et al. 2009; Kan-O et al. 2012; Kanaya et al. 2005; Iskratsch et al. 2013) and a knockout mice emphasizes its important in myofibrillogenesis during heart development (Kan-O et al. 2012). Further findings indicated that formin mutant sarcomeres contain thin filaments but have deformed thin filament-anchoring Z-line structures (Mi-Mi & Pruyne 2015).

Vertebrates have isoforms of profilin that are highly expressed in striated muscle, suggesting that they have important functions in muscle. Profilin-1, the isoform expressed in cardiac tissue, localizes to the Z-line of myofibrils and promotes actin polymerization by catalyzing ADP to ATP exchange on G-actin and through transient interactions of the profilin-ATP-actin complex with the barbed end of F-actin. In left ventricles of spontaneously hypertensive rats (SHRs), profilin-1 is highly expressed and promotes cardiac hypertrophy by modulating actin polymerization (Zhao et al. 2013). Profilin-1 knockdown attenuated cardiac hypertrophy, while overexpression promoted it. Although profilin-null mutant worms display only minor muscle defects, recent data in Drosophila demonstrated that elevated profilin levels resulted in elongated thin filaments and sarcomeres as well as myofibrillar disorganization, which correlated with impaired muscle function as evidenced by increased myocyte size and gene expression (Polet et al. 2006; Kooij et

al. 2016). These findings imply a role of profilin in cardiomyocyte hypertrophy and this is mediated through activation of the ERK1/2 signaling cascade (Kooij et al. 2016).

The barbed and pointed ends of actin filaments in sarcomeres are capped by two different capping proteins. CP purified from skeletal muscle is called CapZ (Casella et al. 1987). CapZ is a barbed end capping protein, that binds transiently to the Z-disc of thin filaments and regulates their polymerization and depolymerization (Pollard & Borisy 2003; Yamashita et al. 2003). Because, actin monomers are continually dissociating from the pointed filament ends, the net result of barbed end capping is shortening of the filament. Transgenic expression of a capping-deficient CapZ in the heart of mouse causes myofibril disorganization and cardiac defects (Hart & Cooper 1999).

Tropomodulins (Tmod) are pointed end binding proteins which interact with pointed actin filament ends via their C-terminal and with the tropomyosin, an F-actin binding protein, via their N-terminal (Krieger et al. 2002). The domains of Tmod are depicted on **Figure 17**. Tmod is associated with the filaments more dynamically than CapZ, therefore, exchange in sarcomeres is faster at pointed ends (Littlefield et al. 2001). Vertebrates contain two sarcomeric Tmod isoforms: Tmod1, expressed in striated muscles, erythrocytes, neurons and ocular lens fiber cells; and Tmod4 which is expressed in skeletal muscle cells. Tmods control thin filaments lengths by regulating actin subunit association/dissociation (Littlefield et al. 2001; Mardahl-Dumesnil & Fowler 2001). Gene knockout of tropomodulin 1 (Tmod1) in mice displays impaired assembly of myofibrils in the heart resulting in embryonic lethality at day (E) 9.5-10.5 (Chu et al. 2003; Fritz-Six et al. 2003) and to abnormally long thin filaments (Gregorio et al. 1995; Sussman 1998; Mudry et al. 2003; Gokhin et al. 2015). Consistently, overexpression of Tmod1 in primary cardiomyocyte cultures results in a shortening of actin filaments (Sussman et al. 1998; Littlefield et al. 2001) and heart-specific overexpression of Tmod in transgenic mice results in dilated cardiomyopathy in mouse hearts (Sussman et al. 1998).

Leiomodin (Lmod) belongs to the Tmod family and was discovered as a potential nucleation factor of muscle actin that stabilizes tropomyosin-decorated filaments (Chereau et al. 2008). However, studies showed that Lmod is not an early-stage actin-filament nucleator during premyofibril maturation but plays an important role in the mature sarcomeres of contractile cardiomyocytes (Skwarek-Maruszewska et al. 2010). Vertebrates have three Lmod isoforms: smooth muscle

(Lmod1), cardiac/skeletal (Lmod2) and fetal/skeletal (Lmod3). Lmod2 knockdown affects sarcomere maintenance in cardiomyocytes, whereas deletions or instability of Lmod3 results in thin filament disorganization and nemaline myopathy (Yuen et al. 2014; Garg et al. 2014). Lmod2 antagonizes Tmod1 for the binding to the pointed-end actin filaments. The structural diferences between striated forms of Tmod and Lmod families are illustrated in **Figure 17**.

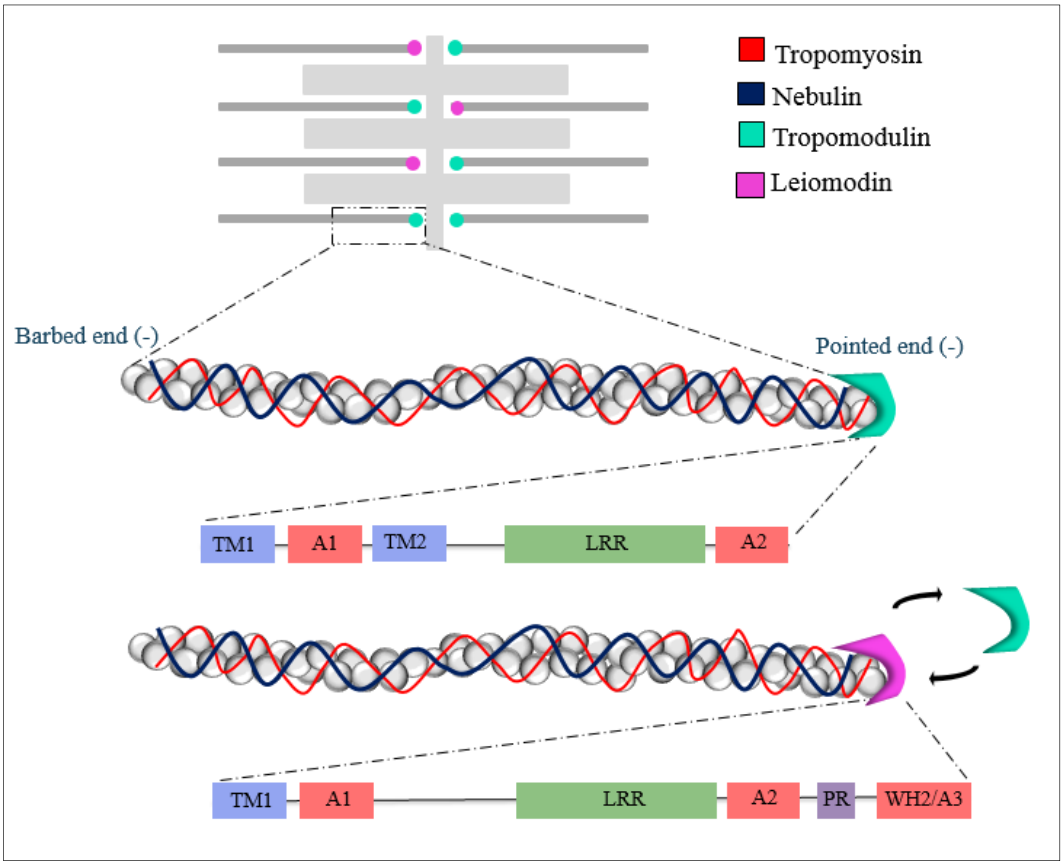

Figure 17: Domains of tropomodulin (Tmod) and leiomodin (Lmod) proteins. Tmod and Lmod share tropomyosin-binding (TM), actin-capping (A1, A2) and leucine-rich repeat (LRR) domains. The second TM2 is unique to Tmod while Lmod2 contains a C-terminal extension, which includes proline-rich (PR) and actin-binding Wiskott–Aldrich syndrome protein homology 2 (WH2) domain. Lmod binds the pointed ends of the thin filaments and competes for binding with Tmod.

Actin filament dynamics are further regulated by members of the actin-depolymerizing factor homology (ADF-H) family. ADF/cofilin has been characterized as a critical regulator of myofibril assembly (Ono et al. 2003). The body wall muscle of *C. elegans* is obliquely striated and has been used as a model to study assembly and function of striated muscle (Moerman & Fire 1997). Null or point mutations in the unc-60 (ADF/cofilin) gene result in slow moving or paralyzed nematodes

with large accumulations of thin filaments (Ono et al. 1999; Ono et al. 2003). Thus, unc-60 is required for proper positioning and the correct number of thin filaments in nematode striated muscle.

In mammalian muscle cells, cofilin-2 is upregulated during differentiation, whereas cofilin-1 is downregulated (Ono et al. 1994; Thirion et al. 2001; Vartiainen et al. 2002). Importantly, microinjection of high cofilin concentrations into skeletal myotubes induces disruption of sarcomeres and formation of cytoplasmic actin-cofilin rods (Nagaoka et al. 1995). Functional significance of cofilin in muscle has been further emphasized by the finding that a mutation in the human cofilin-2 gene causes nemaline myopathy (Agrawal et al. 2007). The mutation converts Ala-35 into Thr and significantly reduces the cofilin-2 protein level in skeletal muscle. Furthermore, deletion of cofilin from cardiac myocytes by RNA interference causes disorganization of sarcomeric actin (Skwarek-Maruszewska et al. 2009). These observations suggest that cofilin is important for maintenance of sarcomeric actin organization. Recently, muscle LIM protein (MLP) has been shown to interact with cofilin 2 and modulate the F-actin dynamics by accelerating the actin depolymerization process in cardiac and skeletal muscle and thus playing role in the pathogenesis of dilated cardiomyopathy (Papalouka et al. 2009). MLP is a critical regulator of myocyte cytoarchitecture, which localizes in the nucleus in differentiating striated muscle cells, promoting myogenic differentiation, while in adult muscle, it translocates to the cytoplasm. Genetic defects in MLP have been shown to lead to actin cytoskeleton disorganization and impaired cardiac myofibrillar structure.

1.3 TGF-β (Transforming growth factor-β) superfamily signaling

The TGF-β superfamily of secreted growth factors comprises 33 ligands including the anti-Müllerian hormone/Müllerian inhibiting substance (AMH/MIS) family, the nodal family, the activin/inhibin family, the bone morphogenetic protein (BMP) family, growth and differentiation factors family (GDFs) and the TGF-β family (Bartram & Speer 2004). Five distinct members of TGF-β family have been identified in vertebrates; three TGF-β isoforms are expressed in mammals (TGF- 1, 2, and 3) that are encoded by distinct genes in a tissue-specific and developmentally-regulated manner.

All TGF-βs and most BMPs are synthesized as pro-peptides and are biologically inactive containing an N-terminal prodomain termed latency-associated peptide (LAP), which prevents TGF-β from interacting with its receptors. Following proteolytic cleavage and separation of LAP, both TGF- β and BMPs become activated. A variety of other molecules have been described as TGF-β activators such as plasmin, thrombospondin (TSP)-1, matrix metalloproteinase (MMP)-2 and MMP-9 (Annes et al. 2003; Rifkin et al. 1999; Murphy-Ullrich & Poczatek 2000). Activated TGF-β and BMPs bind to their cell surface receptors and form tetrameric complexes of type I and type II receptors. There are a total of seven type I receptors and five type II receptors for the TGF-β superfamily (**Table 3**) (Horbelt et al. 2012).

Upon ligand binding, the type II receptor transphosphorylates the type I receptor at the conserved Gly-Ser-rich juxta-positioned domain (GS domain) (Wu et al. 2000). TGF-β signals through binding to type II (TβRII) receptor, which recruits and phosphorylates serine residues of the TGF-β type I (TβR1) receptor (Massagué 2008a) (**Figure 19**). Activation of TβRI by TβRII in the absence of ligand is prevented by FKBP1A (FKBP12), a peptidyl-prolyl cis-trans isomerase. FKBP1A forms a complex with inactive TβRI and dissociates from it only after TGF-β1 is recruited by TGF-β1-bound TβRII. The activated TβRI-TβRII complex is internalized by clathrin-mediated endocytosis into early endosomes (Le Roy et al. 2005).

Following receptor activation, the signal is propagated downstream through the intracellular mediators, the Smads which bind to the type I receptor (Kretzschmar & Massagué 1998; Massagué 1998) (**Figure 19**), with the assistance of adapter molecules like SARA (Smad anchor for receptor

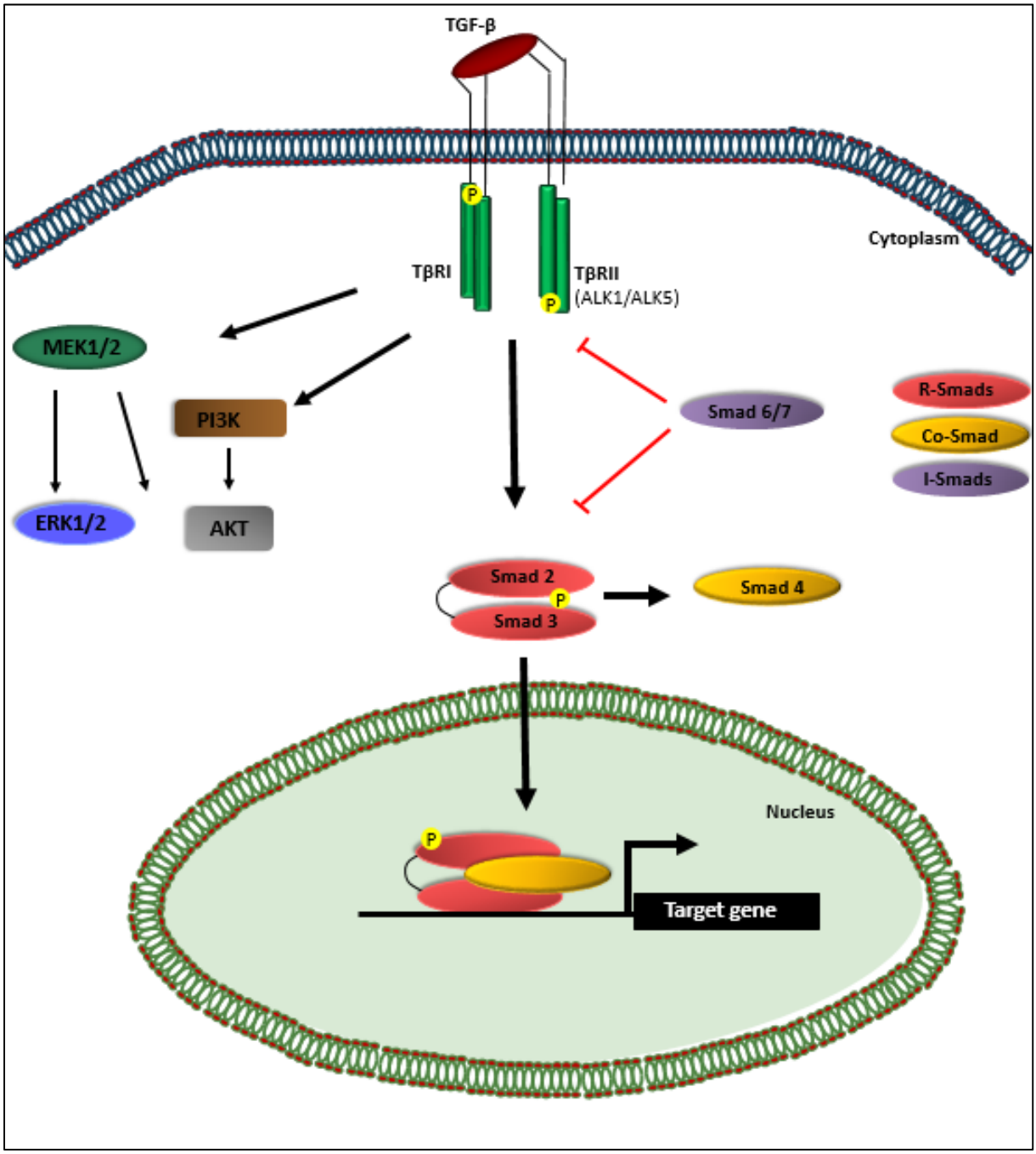

Figure 18: Schematic representation of the transforming growth factor-β (TGF-β) signaling pathway. Upon binding of active TGF-β to the TGF-β type I (ALK5) and type II (TβRII) receptor, ALK5 is phosphorylated and activated by TβRII. ALK5, in turn, phosphorylates and activates the R-Smads, Smad2 and Smad3, which form a complex with Smad4. This complex translocates to the nucleus, binds to DNA-binding transcription factors, co-activators and co-repressors regulating TGF-β/Smad target gene expression. In addition, TGF-β is also known to regulate non-Smad pathways, including ERK MAPK and PI3K-Akt.

activation) and HGS (Hepatocyte growth factor-regulated tyrosine kinase substrate) (Heldin et al. 1997; Shi & Massagué 2003). Smads can be divided into three major groups: the receptor-regulated Smads (R-Smads: Smad 1, Smad 2, Smad 3, Smad 5, and Smad 8), common-mediator Smad (Co- Smad: Smad 4), and inhibitory Smad (I-Smads: Smad 6 and Smad 7) (Massagué 1998). R-Smads are comprised of 400–500 amino acid residues and consist of an N-terminal Mad-homology domain (MH1) and a C-terminal MH2 domain, which are connected by a flexible linker whereas I-Smads lack the MH1 domain (Shi & Massagué 2003) (**Figure 20**). MH1 domain in R-Smads and Co-Smads contains N-terminus nuclear localization signals (NLS) and Smad4 has nuclear export signals (NES) in this domain. MH2 domain is rich in potential serine/threonine phosphorylation sites. The linker region can be phosphorylated by various kinases (e.g. MAPK, GSK, CDKs, CamKII) which determine cellular distribution or protein stability of Smads (Kretzschmar et al. 1997; Scherer & Graff 2000; Alarcon et al. 2009; Fuentealba et al. 2007).

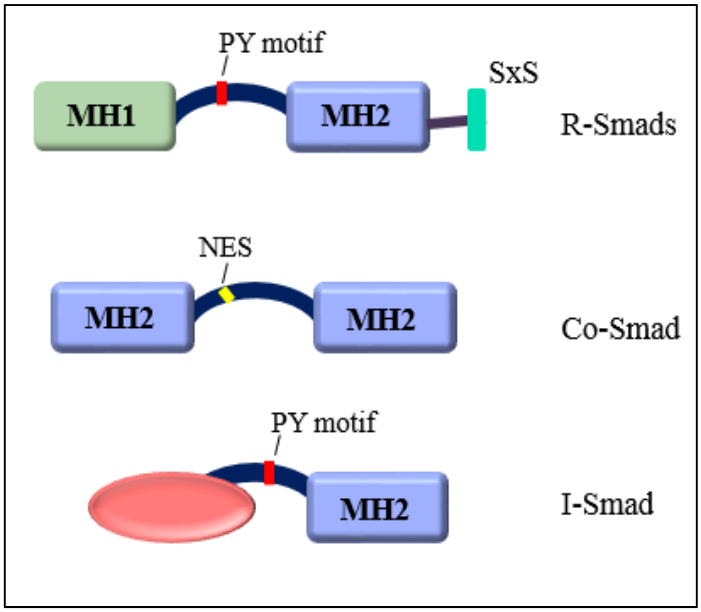

Figure 19: Functional domains of the three Smads subfamilies. Mad Homology domains MH1 and MH2 mediate interactions with cytoplasmic or nuclear proteins. The linker region between MH1 and MH2 contains a transcriptional activation motif and phosphorylation sites for different kinases. The inhibitory Smads lack MH1 domain. The PY motif mediates interaction with the ubiquitin ligases targeting Smads to degradation. R-Smads contain a SxS motif at which they become serine phosphorylated after ligand binding to their specific receptors

Smad2 and Smad3 (TGF-β signaling) are activated through phosphorylation by TβRI (ALK5), whereas Smad1, Smad5 and Smad8 (BMP signaling) are activated by ALK1 (ten Dijke & Hill 2004). The R-Smad phosphorylation changes the conformation of MH2 domain, promoting its dissociation from the adapter protein and TβRI (Souchelnytskyi et al. 2001). The phosphorylated

Table 3: TGF-β superfamily ligands and their receptor

	Receptors	Ligands
Type I	ALK1	Activin A, BMP-9, TGF-β1, TGF-β3
	ALK2	Activin A, BMP-6, BMP-7, MIS, TGF-β1, TGF-β2, TGF-β3
	ALK3 (BMPRIa)	BMP-2, BMP-4, BMP-6, BMP-7
	ALK4	Activin A, GDF-1, GDF-11, Nodal
	ALK5 (TβRI)	TGF-β1, TGF-β2, TGF-β3
	ALK6 (BMPRIb)	BMP-2, BMP-4, BMP-6, BMP-7, GDF-5, GDF-6, GDF-9b, MIS
	ALK7	Nodal
Type II	ActRII	Activin A, BMP-2, BMP-6, BMP-7, GDF-1, GDF-5, GDF-8, GDF-9b, GDF-11, Inhibin A, Inhibin B
	ActRIIb	Activin A, BMP-2, BMP-6, BMP-7, GDF-5, GDF-8, GDF-11, Inhibin A, Inhibin B, Nodal
	BMPRII	BMP-2, BMP-4, BMP-6, BMP-7, GDF-5, GDF-6, GDF-9b
	MISRII	MIS
	TβRII	TGF-β1, TGF-β2, TGF-β3

R-Smads have a high affinity for the Co-Smad, Smad4, and form a heteromeric Smad complex. The R-Smad-Co-Smad complex translocates and accumulates to the nucleus where, either alone or in association with transcription factors (i.e., AP-1) or chromatin remodelling factors (e.g. the SWI/SNF), binds to specific promoter elements and initiates the transcription of extracellular matrix proteins, such as fibronectin, collagen, c-Jun and endothelin-1 (Ross & Hill 2008; Euler-Taimor & Heger 2006; Zhang et al. 1998). These genes have important functions in ventricular remodeling or in vascular angiogenesis. AP-1/Smads signaling is also involved in the induction of fibrosis and apoptosis in the heart (Cheng & Grande 2002).

Smad 6 and Smad 7 function as inhibitors of TGF-β signaling by binding to type 1 receptors and interfering with the phosphorylation of R-Smads, or recruit the HECT-type E3-ubiquitin ligases Smurf1 (Smad ubiquitination-related factor 1) and Smurf2 (Smad ubiquitination-related factor 2) to induce proteasomal degradation of the receptor complexes (Massague & Chen 2000; Javelaud & Mauviel 2004). The expression of I-Smads 6 and 7 is induced by both activin/TGF-β and BMP signaling as part of a negative feedback loop. Typically, Smad 6 inhibits the BMP signaling and Smad 7 inhibits the TGF-β signaling.

1.3.1 Non-Smad signaling pathways

In parallel to canonical Smad pathways TGF-β signaling was shown to activate a number of Smad-independent pathways like extracellular receptor-linked kinase (ERK) (Mucsi et al. 1996; Hartsough & Mulder 1995), c-Jun N terminal kinase (JNK) (Hocevar et al. 1999), p38 mitogen-activated protein kinase (MAPK) (Hanafusa et al. 1999; Wang et al. 2002), phosphatidylinositol-3-kinase/Akt (Wilkes et al. 2005; Bakin et al. 2000), GTP-binding proteins (e.g., Ras, Rho, and Rac1), integrins (Zhang et al. 2015) and focal adhesion kinase (FAK) (Hong et al. 2011).

The precise mechanism by which TGF-β superfamily ligands activate these pathways has not been fully established. However the TGF-β- activated ERK pathway has been studied extensively. Activated TβRI recruits and phosphorylates the adaptor protein ShcA (Src homology domain 2 containing) on tyrosine and serine residues, thus promoting the formation of a ShcA/Grb2 (growth factor receptor binding protein 2)/Sos complex (Lee et al. 2007). Then, the ShcA/Grb2/Sos complex activates Ras at the plasma membrane, leading to sequential activation of c-Raf, MEK, and ERK. The target genes of TGF-β-activated ERK pathway are invlolved in cell motility, cell-matrix interaction and endocytosis (Zavadil et al. 2001). In addition, ERK activation is important for epithelial to mesenchymal transition (EMT), which is one of the major biological functions of TGF-β associated with tumor metastasis and fibrosis (Thiery 2003). Moreover, ERK can also phosphorylate Smads proteins, including Smad1, Smad2 and Smad3, and regulate their activities (Kretzschmar et al. 1997; Kretzschmar et al. 1999; Funaba et al. 2002).

Several non-canonical, Smad-independent signaling pathways for BMPs have been identified. BMP4, for example, was found to activate TAK-1, a serine/ethreonine kinase of the MAPKKK family (Derynck & Zhang 2003). In addition to the MAPK pathway, BMP signaling has been found to affect PI3K/Akt, P/kc, Rho-GTPases, and others (Zhang 2009). LIM kinase 1 (LIMK1), a downstream effector of the PAK network, can also associate with the BMP receptor type II (BMPRII) and this interaction synergizes with Cdc42 to activate the catalytic activity of LIMK1, thus increasing the phospho-cofilin(Ser3) level and causing changes in the actin cytoskeleton (Foletta et al. 2003; Lee-Hoeflich et al. 2004).

1.3.2 Role of TGF-β in diseases

The TGF-β superfamily members play fundamental role in the regulation of a plethora cellular processes, including cell growth, differentiation, tissue homeostasis, extracellular matrix (ECM) synthesis, embryonic development and apoptosis (Wrighton et al. 2009; Yue & Mulder 2001; Matsuura et al. 2010; Rahimi & Leof 2007). The best established role of TGF-β in the adult organism is its function as a tumor suppressor (Massagué 2008b). In normal epithelial cells TGF-β signaling prevents uncontrolled growth by inducing cell cycle arrest through expression of cyclin dependent kinases (CDKs) inhibitors p15INK4B and p21Cip1. Concomitantly, TGF-β has been also shown to induce apoptosis, as evident by the downregulation of Bcl-XL (Saltzman et al. 1998; Chipuk et al. 2001) and activation of caspase 3 and 8 (Shima et al. 1999; Chen & Chang 1997). However, TGF-β can also promote tumor growth, as underlined by its ability to induce changes in transcriptional activities that transit epithelial cells into mesenchymal cells, thereby facilitating tumor metastasis and invasion (Thiery 2003). Sporadic mutations in components of TGF-β signaling pathways were found in cancer (e.g. breast, colorectal, pancreatic, lung) (Gordon & Blobe 2008; Wang et al. 2007; Lin et al. 2006; Xu & Pasche 2007; Chen et al. 1998; Riggins et al. 1997). Antibody or antisense strategies are in clinical trials for the treatment of such tumors (Olivares et al. 2011; Nemunaitis et al. 2009; Fakhrai et al. 2006; Senzer et al. 2012; Morris et al. 2014).

Several lines of evidence link the development of a variety of human pathologies to aberrant regulation of TGF-β signaling. Dysregulation of TGF-β signaling was identified as the basis for cardiovascular, and skeletal diseases (Gordon & Blobe 2008). The importance of the TGF-β superfamily in cardiovascular development is evident from the phenotype of the TGFβ1-knockout mice, which are embryonic lethal due to an excessive systemic inflammatory response with massive infiltration of macrophages and lymphocytes into the heart and lungs. Notably, enhanced levels of TGF-β1 are found in patients with dilated, hypertrophic, and restrictive cardiomyopathy. ACE (angiotensin-converting enzyme) inhibitors and angiotensin I receptor antagonists, which are used therapeutically for cardiomyopathy patients (Pedersen et al. 1999; Madrid et al. 2002), might function by decreasing TGF-β1 expression and subsequent ECM production (El-Agroudy et al. 2003; Agarwal et al. 2002).

Many neurological disorders are also associated to an altered Smad signaling, comprising Smad nuclear reduction and stimulating pro-apoptotic signaling, therefore disturbing biological processes, which are essential for the development of nervous system and homeostasis. These include Alzheimer's disease, Parkinson's disease, spinal muscular atrophy and amyotrophic lateral sclerosis (ALS) (Mogi et al. 1995; van der Wal et al. 1993; Katsuno et al. 2010; Iłzecka et al. 2002).

1.3.3 TGF-β in fibrosis

TGF-β1 is a key regulator of fibroblast phenotype and function. Upon TGF-β stimulation, fibroblasts are activated and undergo transdifferentiation into myofibroblasts, the key effector cells in fibrotic states (**Figure 21**). Myofibroblasts share the phenotypic features of both fibroblasts (vimentin- and fibronectin-expressing cells) and smooth muscle cells (α-SMA-expressing cells) (Huard 2002). An earlier study showed that myofibroblasts can produce up to twice as much collagen as their fibroblast precursors (Lijnen & Petrov, 2002). This fibrotic transition is required for tissue repair and homeostasis in healing wounds; however, in pathologic conditions activated myofibroblasts lead to the development of fibrosis.

TGF-β overexpression induces marked fibrotic changes implying that TGF-β is a crucial mediator of tissue remodeling and fibrosis as a consequence of ECM accumulation in pathologic states (Ruiz-Ortega et al. 2007; Leask & Abraham 2004). This is based on the fact that TGF-β is known to induce the expression of ECM proteins (Hocevar & Howe 2000; Rudnicka et al. 1994; Xu et al. 1991; Peltonen et al. 1990), to increase the expression of integrins in a manner that increases cellular adhesions to the matrix (Warstat et al. 2010) and to stimulate the production of protease inhibitors that prevent enzymatic breakdown of the ECM (Massagué 1990). *In vitro*, TGF-β1 does not only initiate the production of fibrosis-related proteins but also downregulates the expression of myogenic proteins and prevents the myofibroblasts apoptosis, thereby promoting their survival (Li et al. 2004; Zhang & Phan 1999).

TGF-β1 contributes in the fibrotic pathogenesis of the kidney, liver, and lung (Epstein et al. 1994; Dooley & Ten Dijke 2012; Tatler & Jenkins 2012; Khalil & Greenberg 1991; Lan 2011) as well

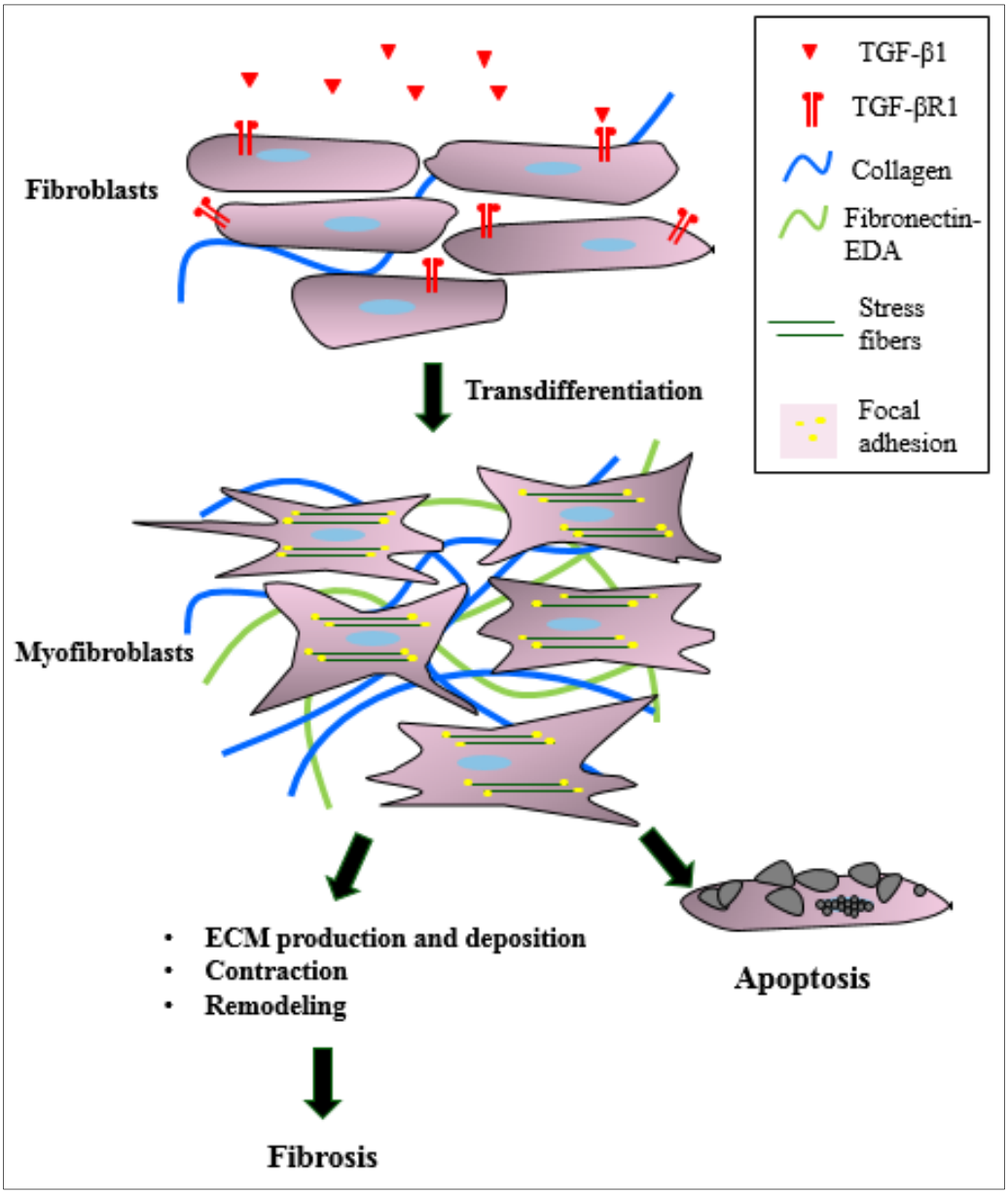

Figure 20: Schematic illustration of myofibroblast transdifferentiation. The myofibroblastic modulation of fibroblastic cells begins with of the TGF-β signaling pathway leading to the appearance of the differentiated myofibroblast, the most common variant of this cell, with stress fibers attached to focal adhesions. The presence of collagen and a splice variant form of fibronectin (ED-A fibronectin) in the microenvironment adjacent to the myofibroblast appears to be required for their differentiation. Myofibroblasts are implicated in many fibrotic and scarring diseases. However, in normal physiological situations, myofibroblasts undergo apoptosis.

as skeletal and heart muscle (Waltenberger et al. 1993; Khan & Sheppard 2006; Lijnen et al. 2000; Bernasconi et al. 1995). Some animal studies have shown that inhibiting the actions of TGF-β1 may attenuate fibrosis. In particular, TGF-β inhibition attenuated renal, hepatic, and cardiac fibrosis, highlighting the role of TGF-β in a wide range of fibrotic conditions (Kuwahara et al. 2002; Yanagita 2012; Chung et al. 2013; Border & Noble 1997; Liu et al. 2006). As TGF-β1 appears to be also involved in different cardio-pathological processes, it is an apt target for future therapy (Dobaczewski et al. 2011).

Extensive evidence suggests that the canonical ALK5/Smad3 pathway is critically involved in the pathogenesis of fibrosis in many tissues. In accordance, mice lacking Smad3 display attenuated fibrosis in a wide range of experimental models. The TGF-β1–Smad pathway appears to be involved in the activation of collagen-gene promoter sites, primarily enhancing the expression of collagen type I (COL1A1) (Tsuchida et al. 2003). In another study, the authors supported that the TGF-β1–Smad pathway in dermal fibroblasts caused the Smad3-dependent induction of *COL1A1*, along with *COL1A2, COL3A1, COL6A1* and *COL6A3* (Verrecchia et al. 2001).

Additionally, TGF-β1 can induce collagen synthesis and accumulation in cultured myoblasts through the p38 mitogen-activated protein kinase (MAPK) pathway via TGF-β-activated kinase (TAK1) (Rodríguez-Barbero et al. 2002) (**Figure 22**). TAK1 is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family and is thought to be activated by TGF-β1 (Zhang et al. 2000). Studies showed that the activation of TAK1 is mediated by the recruitment of the ubiquitin ligase (E3) TRAF6 to the TβRI which is required for the autophosphorylation of TRAF6 and activation of TAK1 (Sorrentino et al. 2008). In turn, activated TAK1 mediates p38 phosphorylation, which promotes phosphorylation of activated transcription factor-2 (ATF-2) (Hanafusa et al. 1999). Subsequently, the Smad complex combines with ATF-2 resulting in augmented protein synthesis (Sano et al. 1999). Alternative pathways for TGF-β1-induced fibrosis involving JNK1 and MKK4, also seem to be involved in TGF-β regulation of fibronectin synthesis (Hocevar et al. 1999). Moreover, many TGF-β responses involve AP-1- dependent genes such as human collagenase types 1 and 3 and the the type I collagen gene. In particular, TGF-β1 induces bFGF (basic fibroblast growth factor) protein synthesis using an unidentified mechanism and bFGF then activates the ERK/MEK-1 pathway through receptor tyrosine kinase (RTK) activation to induce AP-1 binding (Finlay et al. 2000).

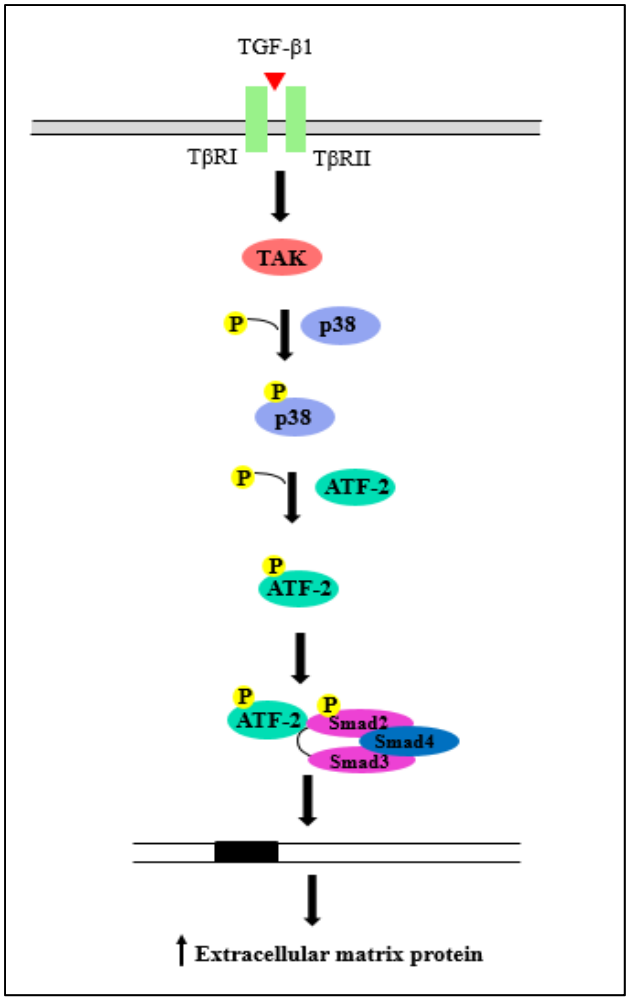

Figure 21: The TGF–TAK1 pathway. Following binding of the ligand TGF-β1 to the dimerized TGF-β receptor I (TβRI)–TGF-β receptor II (TBRII) complex, TAK1 (TGF-β activated kinase) is activated. Subsequently, TAK1 mediates p38 MAPK phosphorylation, which in turn promotes phosphorylation of ATF-2 (activated-transcription factor). Finally the Smad complex (Smad2-Smad3-Smad4) combines with ATF-2 resulting in enhanced protein synthesis.

1.3.4 TGF-β-induction of connective tissue growth factor

Some of the fibrotic actions of TGF-β signaling are mediated through upregulation of its downstream autocrine effector Connective Tissue Growth Factor (CTGF). Accordingly, transgenic mice expressing constitutively active TGF-β receptor I in fibroblasts, recapitulate features of the skin fibrosis and display high levels of CTGF expression, suggesting that both TGF-β and CTGF may have an important role in a sustained chronic fibrotic outcome (Sonnylal et al. 2007). CTGF is a secreted cysteine-rich protein that is implicated in a variety of cellular processes including fibroblast proliferation, chondrogenesis, angiogenesis, wound healing and ECM (i.e., fibronectin, procollagen) production. Mice lacking CTGF die shortly after birth, primarily due to skeletal defects which lead to respiratory failure, implying that CTGF plays a crucial role in the development of cartilage and ECM production. CTGF induces formation of myofibroblasts

through transdifferentiation of other cells, including epithelial cells, fibroblast and fibrocytes. There is growing amount of evidence that CTGF overproduction is associated with fibrosis in skin and in major internal organs. Based on this observation, it is considered a reliable marker for the severity of fibrosis (Dziadzio et al. 2005; Kovalenko et al. 2009; Pan et al. 2001; Paradis et al. 1999).

TGF-β directly induces CTGF synthesis in different fibroblasts subtypes for the stimulation of ECM synthesis (Grotendorst et al. 1996; Leask et al. 2003). This is supported by the observation that subcutaneous injection of TGF-β into neonatal mice resulted in an increase in CTGF mRNA, associated with excessive ECM (Frazier et al. 1996). Similarly, CTGF neutralizing antibodies are capable of reducing TGF-β-induced collagen synthesis in fibroblasts (Duncan et al. 1999). Importantly, CTGF alone is capable to induce fibrosis, as evidenced by the fact that injection of CTGF produced a similar effect to that induced by TGF-β (Frazier et al. 1996). A similar conclusion was reached from the analysis of transgenic mice specifically expressing high levels of CTGF in fibroblastic cells (Sonnylal et al. 2010). On the other hand, other studies showed that CTGF binds directly to TGF-β, and enhances its activity resulting in increased binding to TβRI and TβRII and thus, enhanced TGF-β-mediated fibrogenic actions.

Notably, TGF-β1 and CTGF levels were found to be overexpressed in biopsies from muscular dystrophy patients (Sun et al. 2008), in accordance with the high degree of fibrosis that characterize these degenerative diseases. It has been postulated that excessive deposition of fibrotic tissue in the heart results in cardiac pathology. Both TGF-β and CTGF/CCN2 mRNA are upregulated in many fibrotic disorders in rat model with post-infarction heart failure and in patients with cardiac ischemia (Kucich et al. 2001). Studies from Ohnishi and colleagues demonstrated an increase in CTGF/CCN2 mRNA in cardiac myocytes and mesenchymal cells in the infarct zone of rat hearts following myocardial infarction (Ohnishi et al. 1998). In human, CTGF/CCN2 mRNA was significantly increased in patients diagnosed with ischemic heart disease (Chen et al. 2000). TGF-β specifically mediates CTGF/CCN2 expression in cardiac fibroblast and cardiac myocytes, linked with a concomitant increased ECM synthesis. The TGF-β-induced CTGF/CCN2 induction is inhibited by cAMP and enhanced by MAPK inhibitors (Chen et al. 2000).

The regulation of CTGF expression by TGF-β appears to be regulated primarily at the level of gene transcription. In particular, TGF-β induces CTGF/CCN2 gene activation most probably by

acting on Smad-response elements which reside within the CTGF/CCN2 promoter, as evidenced by the enhanced-CTGF expression following co-transfection of Smad3 and 4 (Holmes et al. 2001). Of note, TGF-β-mediated induction of CTGF did not occur in embryonic fibroblasts cultured from Smad 3 knockout mice, suggesting that CTGF expression occurs in a Smad 2/3-independent manner (Flanders et al. 2003).

Chapter 2 Manuscripts

ERK1/2 directly acts on CTGF/CCN2 expression to mediate myocardial fibrosis in cardiomyopathy caused by mutations in the lamin A/C gene

Maria Chatzifrangkeskou, Caroline Le Dour, Wei Wu, John P. Morrow, Leroy C. Joseph, Maud Beuvin, Fusako Sera, Shunichi Homma, Nicolas Vignier, Nathalie Mougenot, Gisèle Bonne, Kenneth E. Lipson, Howard J. Worman and Antoine Muchir

ADF/cofilin-1 phosphorylation catalyzed by ERK1/2 alters cardiac actin in dilated cardiomyopathy caused by lamin A/C gene mutation

Maria Chatzifrangkeskou, David Yadin, Yannick Tanguy, Thibault Marais, Solenne Chardonnet, Nathalie Mougenot, Petra Knaus, Howard J. Worman, Wolfgang H. Goldmann, Gisèle Bonne, and Antoine Muchir

Manuscript in preparation.

ERK1/2 directly acts on CTGF/CCN2 expression to mediate myocardial fibrosis in cardiomyopathy caused by mutations in the lamin A/C gene

(*Human Molecular Genetics, 2016, DOI link*: https://doi.org/10.1093/hmg/ddw090)

Introduction

Mutations in *LMNA*, which encodes proteins of the inner nuclear membrane, have been associated with dilated cardiomyopathy (e.g., *LMNA* cardiomyopathy) (Fatkin et al. 1999; Bonne et al. 1999). *LMNA*-cardiomyopathy is characterized by increased myocardial fibrosis. There is currently no treatment for *LMNA* cardiomyopathy and heart transplantation is often the last therapeutic option. Previous studies demonstrated that ERK1/2 signaling improves the myocardial fibrosis and plays an important role in the development of this cardiac disease (Muchir et al. 2007; Wu et al. 2011; Muchir et al. 2012; Wu et al. 2014). Notwithstanding, the molecular mechanism(s) by which ERK1/2 activation promotes cardiac dysfunction remained unknown, as yet.

Results

Using a mouse model of *LMNA* cardiomyopathy and human heart tissue of patients with mutation in lamin A/C gene, we discover that the myocardial fibrosis that impairs the left ventricular function is caused by aberrant TGF-β signaling via canonical (Smad 2/3) and non-canonical (ERK1/2) pathways. Activated ERK1/2 contributes to myocardial fibrosis by increasing expression of CTGF/CCN2. Pharmacological blockade of CTGF/CCN2 slows down the onset of myocardial fibrosis and improves cardiac function, demonstrating the contribution of this factor in *LMNA* cardiomyopathy.

Impact

We here provide mechanistic insight into the role played by ERK1/2 signaling on the cardiac dysfunction in *LMNA* cardiomyopathy. Our findings indicate that therapeutic strategies targeting TGF-β-Smad signaling or CTGF/CCN2 may potentially be applied to patients with *LMNA* cardiomyopathy.

ADF/cofilin-1 phosphorylation catalyzed by ERK1/2 alters cardiac actin in dilated cardiomyopathy caused by lamin A/C gene mutation

(Manuscript *In preparation)*

Introduction

Dilated cardiomyopathy is characterized by an increase in both left ventricular dilatation and systolic dysfunction. In 1999, mutations in nuclear A-type lamins were reported to cause dilated cardiomyopathy (i.e., *LMNA* cardiomyopathy). Previous studies demonstrated an aberrant increased activity in mechanical-activated extracellular signal-regulated kinase (ERK1/2) signaling in hearts from mice and patients with *LMNA*-cardiomyopathy. This led to the conclusion that abnormal activation of ERK1/2 signaling is involved is the pathophysiology of left-ventricular contractile dysfunction in *LMNA* cardiomyopathy. However, we are lacking insight in the molecular and cellular mechanisms bridging ERK1/2 modulation and decreased systolic function.

Results

In this study, we identified for the first time that hyperactivated ERK1/2 signaling in heart i/ binds to ADF/cofilin-1, an actin binding protein, on a novel phospho-site on Threonine 25; ii/ activates the actin depolymerization action of ADF/cofilin-1 and, iii) alters the filamentous/globular (F/G) actin ratio. These events participate in the development of onset of overt cardiac dysfunction by disorganizing sarcomeric actin. Expressing a mutant form of ADF/cofilin-1 (T25A) attenuates the interaction between ADF/cofilin-1 and activated ERK1/2 and restores the actin dynamics in *LMNA*-cardiomyopathy. Inhibition of ERK1/2 signaling suppressed phospho-ADF/cofilin-1 and attenuated cardiac dysfunction. Our work clearly demonstrated some of the downstream alterations by which ERK1/2 signaling promotes cardiac dysfunction.

Impact

These findings unravel a role played by ERK1/2 signaling on actin dynamics that provide insight into the disease etiology for the cardiac phenotype in *LMNA*-cardiomyopathy and lay the groundwork for new therapeutic strategies.The mechanism by which *LMNA* mutation leads to ERK1/2 activation remains to be elucidated. However, our study clearly demonstrated some of the downstream alterations by which ERK1/2 signaling promotes cardiac dysfunction.

ADF/cofilin-1 phosphorylation catalyzed by ERK1/2 alters cardiac actin in dilated cardiomyopathy caused by lamin A/C gene mutation

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ABSTRACT

Hyper-activation of extracellular signal-regulated kinase (ERK) 1/2 signaling participates in the cardiac dysfunction when the *LMNA* gene encoding nuclear A-type lamins is mutated. Studies of pharmacological inhibition of ERK1/2 activity in mice have suggested it could be a promising therapy for future development in patients with cardiomyopathy caused by lamin A/C gene mutations. However, the mechanism of how hyper-activation of ERK1/2 signaling affects cardiac function is unknown. We show that active phosphorylated ERK1/2 directly binds to and catalyzes the phosphorylation of the actin depolymerizing factor (ADF) cofilin-1 on Thr25. ADF/cofilin-1 becomes active and disassembles actin filaments in cardiomyocytes. This activity leads to depolymerization of sarcomeric F-actin, ultimately altering cardiac function. Expressing a mutant form of ADF/cofilin-1 (T25A) blunted these events. These results highlight a novel role for ADF/cofilin-1 on actin dynamics in sarcomeres and provide a rationale as to why increased ERK1/2 signaling leads to dilated cardiomyopathy caused by lamin A/C gene mutations.

Keywords: ERK1/2, cofilin-1, *LMNA*, A-type lamins, dilated cardiomyopathy

INTRODUCTION

Dilated cardiomyopathy is characterized by left ventricular dilatation and systolic dysfunction and accounts for up to 30%-40% of all heart failures (Towbin and Bowles, 2002). Despite current efforts to manage dilated cardiomyopathy, the disorder remains a common cause of heart failure and a prevalent diagnosis in individuals requiring cardiac transplantation. Genetic mutations have been identified in 25%-35% of patients with dilated cardiomyopathy. In 1999, mutations in the lamin A/C gene (*LMNA*) were reported to cause an autosomal dominant inherited form of dilated cardiomyopathy (herein referred to as *LMNA* cardiomyopathy) (Fatkin et al., 1999; Bonne et al., 1999). *LMNA* encodes the A-type nuclear lamins, which arise from alternative splicing (Fisher et al., 1986; McKeon et al., 1986; Lin and Worman 1993) and are the main constituents of nuclear lamina. Despite our gaps in understanding many of their fundamental functions, much of the current research on the A-type lamins is focused on how mutations leading to alterations in these proteins cause dilated cardiomyopathy. We previously demonstrated that the mechanically activated extracellular signal-regulated kinase (ERK) 1/2 is hyper-activated in *LMNA* cardiomyopathy (Muchir et al., 2007). However, insights in the molecular mechanisms bridging ERK1/2 modulation and depressed cardiac function are lacking.

Alterations in cardiomyocyte mechanotransduction are likely to underlie molecular mechanisms of dilated cardiomyopathy and heart failure progression. Actin is one of the major cytoskeletal proteins in eukaryotic cells and plays an essential role in a number of cellular processes including mechano-resistance and contractile force generation. The contractile units of cardiomyocytes (called sarcomeres) generate force by the sliding of actin and myosin filaments. The actin filaments in sarcomeres are uniform in length and precisely oriented with their barbed-ends (+) facing the Z-disc, which are capped by CapZ (Casella et al., 1987) and their pointed-ends (-) directed toward the M-band, which are associated with tropomodulin. Actin filaments are additionally decorated along their length by tropomyosin and a large number of actin-binding proteins, which contribute to maintaining sarcomere structure and organization (Labeit and Kolmerer, 1995; Agarkova and Perriard, 2005; Chereau et al., 2008; Lange et al., 2006; Pappas et al., 2010; Sanger and Sanger, 2008). While it has been thought that actin filaments in sarcomeres are very stable, studies have provided evidence that sarcomeric actin filaments undergo a dynamic turnover. A number of actin-binding proteins show activities by enhancing actin filament turnover, promoting polymerization, depolymerization, or filament severing (Paavilainen et al., 2004;

Nicholson-Dykstra et al., 2005; Ono, 2007).

Defects in the regulation of the length or the organization of actin filaments in sarcomeres, due to mutations or de-regulated expression of cytoskeletal proteins, are a hallmark of many heart and skeletal muscle disorders, including several myopathies (Kho et al., 2012). Among the regulators of actin, ADF/cofilins play an essential role in the dynamics of filaments, including those in sarcomeres. ADF/cofilin enhances actin filament turnover by severing and promoting dissociation of actin monomers from the pointed ends (Van Troys et al., 2008). We here show in cultured cells, mouse tissue, and human tissue that phosphorylated ERK1/2 (pERK1/2) binds to and activates ADF/cofilin-1 in *LMNA* cardiomyopathy. The disassembly of actin occurs in sarcomeres of the heart from a mouse model of *LMNA* cardiomyopathy.

RESULTS

pERK1/2 alters F-actin dynamics in *LMNA* cardiomyopathy

We set out to unravel the consequences of abnormal ERK1/2 signaling in the heart of $Lmna^{H222P/H222P}$ mice, a model for dilated cardiomyopathy caused by mutation in *LMNA* (Figure S1A; Muchir et al., 2007; Muchir et al., 2012a). We observed an increase in cytoplasmic phosphorylated ERK1/2 (pERK1/2) as assessed by immunoblotting subcellular fractions (Figure S1B) and immunofluorescence microscopy (Figure 1A) in stably transfected C2C12 cells expressing H222P lamin A (C2-H222P) compared to those expressing wild type lamin A (C2-WT) (Choi et al., 2012a; Muchir et al., 2013). This pERK1/2 cytoplasmic accumulation, along with an increased nuclear staining, was previously observed in transiently transfected C2C12 cells expressing the mutated H222P lamin A compared to those expression wild type lamin A (Muchir et al., 2007). Although many ERK1/2 substrates are localized in the nucleus, several cytoskeletal proteins are targets (Chatzifrangkeskou et al., 2015). We reasoned that activation of pERK1/2 in cells with mutation in lamin A/C gene could be linked to alterations of cytosolic targets. Given that lamin A/C modulates cytosolic actin polymerization (Ho et al., 2013), we focused our attention on the modulators of actin dynamics. The ratio of filamentous (F) over globular (G) actin was significantly lower in C2-H222P cells (0.5) compared to C2-WT cells (0.75) ($p<0.005$) (Figure 1B, Figure S2A). The F/G actin ratio reflected the status of cellular actin network. When treating C2-H222P cells with cytochalasin D (inhibits actin polymerization) lowered the ratio (0.36) while

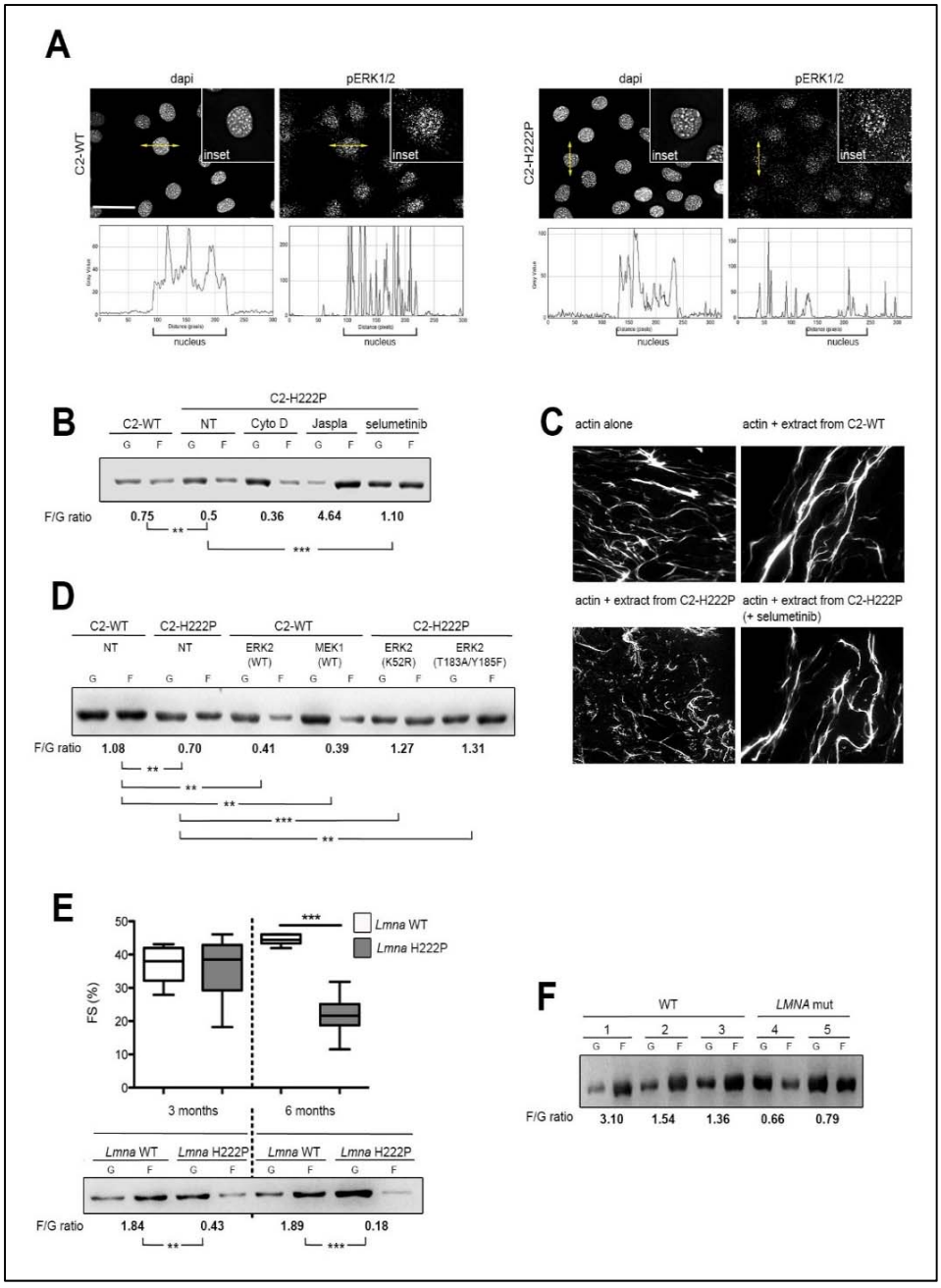

Figure 1. Altered F-actin dynamics in *LMNA*-cardiomyopathy

(A) Representative immuofluorescence microscopy images of pERK1/2 staining of C2-WT and C2-H222P cells. Nuclei counter-stained with 4',6 diamidino-2 phenylindole (dapi) are also shown. Scale bar, 25 µm. Scan lines graphs represent the intensity of pERK1/2 and dapi staining along the yellow arrow lines.

(B) Immunoblot illustrating the effect of A-type lamin H222P point mutation on the amounts of G-actin and F-actin and the calculated F/G actin ratios. Representative of three independent experiments each performed in triplicates. ***P*<0.005. Inhibiting ERK1/2 phosphorylation in C2-H222P cells using selumetinib reversed the F/G actin ratio. ****P*<0.0005. The use of cytochalasin D (cyto D) and jasplakinolide (Jaspla) validated the method. NT indicates not treated.

(C) Microscopic analyses of F-actin depolymerization and/or severing. Micrographs show *in vitro* F-actin filaments alone or incubated in presence of protein extracts from C2-WT or C2-H222P cells with or without selumetinib.

(D) Immunoblot illustrating the effects of ERK and MEK constructs on the amounts of G-actin and F-actin and the calculated F/G actin ratio. Representative of three independent experiments each performed in triplicates. NT indicates not transfected. ***P*<0.005, ****P*<0.0005.

(E) Graph showing means ± standard errors of means of left ventricular fractional shortening (FS) in 3 month-old and 6 month-old male *Lmna*$^{H222P/H222P}$ mice (*Lmna* H222P) (n=10) compared to *Lmna*$^{+/+}$ mice (*Lmna* WT) (n=19). ****P*<0.0005. Immunoblot illustrating the effect of A-type lamin H222P point mutation on the amounts of G-actin and F-actin and the calculated F/G actin ratio in mice at 3 months and 6 months of age. Representative of three independent experiments each performed in triplicates. ***P*<0.005, ****P*<0.0005.

(F) Immunoblot illustrating the effect of *LMNA* point mutations on the cardiac amounts of G-actin and F-actin and the calculated F/G actin ratio in patients compared to individuals without *LMNA* mutation.

treating with jasplakinolide, which promotes actin polymerization and stabilization, increased the ratio (4.64) compared to untreated C2-H222P (Figure 1B). These data suggest an abnormal depolymerization of F-actin in the presence of H222P lamin A in cells. Treating C2-H222P cells with inhibitors of MEK1/2 (the kinases that specifically phosphorylate ERK1/2) leads to the activation of F-actin polymerization (Figure 1B, Figure S2A,B,C). When the MEK1/2 inhibitor selumetinib was washed out from the media, in which C2-H222P cells were cultured, the F/G actin ratio progressively returned to a similar level of untreated cells (Figure S2D). This was also observed by immunofluorescence microscopy (Figure S2E). Treating both C2-WT and C2-H222P cells with cytochalasin D (or latrunculin B), brought about depolymerization of the actin network (Figure S2E). Washing out these drugs led to progressive polymerization of F-actin (Figure S2E). The polymerization of actin was, however, delayed in C2-H222P cells compared to C2-WT cells (30 minutes after washout for C2-H222P cells versus 15 minutes for C2-WT cells) (Figure S2E). This delay was diminished when we added selumetinib (Figure S2E). We next compared the effects of protein extracts from C2-WT or C2-H222P cells on the length of F-actin filaments by microscopic analysis of Alexa Fluor 488-labeled actin. When actin was polymerized in the presence of extracts from C2-H222P cells, the length of F-actin filaments was shortened compared to when in the presence of extracts from C2-WT cells (Figure 1C). This effect on the F-actin dynamics was blunted when C2-H222P cells were treated with selumetinib (Figure 1C). We then asked the question of whether ERK1/2 signaling contributed directly to F-actin cycling alteration. Transient transfection of C2-WT cells with either wild type ERK2 (ERK2-WT) or MEK1 (MEK1-WT) constructs bring about a decrease in the F/G actin ratio compared to untransfected cells (Figure 1D). Conversely, C2-H222P cells transfected with plasmids encoding ERK2 K52R (kinase dead) or ERK2-T183A/Y185F (dominant negative) had an increased F/G actin ratio compared to untransfected C2-H222P cells (Figure 1D). The level of F-actin in transfected C2-H222P cells is similar to C2-WT cells (Figure 1D). Thus, our data suggest that ERK1/2 signaling triggers depolymerization of actin in C2-H222P cells. Besides confirming that A-type lamins affect F-actin dynamics, we additionally found that ERK1/2 signaling is involved in this process.

To identify whether other *LMNA* mutations have the same effect on actin dynamics, we performed transient transfection with plasmid encoding A-type lamin variants E358K, L271P and N456I in C2C12 cells (Figure S3A). These mutations have been previously identified causing *LMNA* cardiomyopathy in humans. Transfecting cells with A-type lamin variants showed increased pERK1/2 activation (Figure S3A) and altered actin dynamics (Figure S3B) in C2C12 cells. These findings suggest that altered F-actin dynamics arises from *LMNA* mutations as a result of abnormal ERK1/2 activation.

Given that male *Lmna*$^{H222P/H222P}$ mice have enhanced activation of ERK1/2 signaling in the heart starting at 4 weeks of age (Muchir et al., 2007; Choi et al., 2012b), we investigated whether this could affect the actin cytoskeleton *in vivo*. F-actin dynamics were altered in the heart from *Lmna*$^{H222P/H222P}$ mice compared to *Lmna*$^{+/+}$ mice at 3 months of age (when left ventricular fractional shortening is normal) and more severely at 6 months of age (when the mice have impaired systolic function) (Figure 1E). This altered F/G actin ratio in the heart was reversed *in vivo* when *Lmna*$^{H222P/H222P}$ mice were crossed with *Erk1* knockout mice, in which the cardiac function was partially restored (Wu et al., 2014) (Figure S4). We obtained samples from heart tissues from two humans with *LMNA* cardiomyopathy. The F/G actin ratio was also significantly lower in the heart of humans carrying *LMNA* mutations compared to controls (Figure 1F). These data imply a central role of pERK1/2 in the regulation of F-actin dynamics in *LMNA* cardiomyopathy.

pERK1/2 binds to ADF/cofilin-1 on threonine 25 and alters its activity

We reasoned that pERK1/2 binds to ADF and/or NPF to alter F-actin cycling that leads to cardiac muscle disease. To test our hypothesis, we performed immunoprecipitations using extracts of C2-H222P cells and antibodies against ADF/cofilin-1, N-Wasp, ARP2, or profilin-1. We found that pERK1/2 interacted only with ADF/cofilin-1 (Figure 2A). The interaction between pERK1/2 and ADF/cofilin-1 was apparently enhanced in C2-H222P cells in comparison to C2-WT cells (Figure 2B), confirming that the higher level of pERK1/2 in the cytoplasm of C2-H222P cells points to a greater interaction with ADF/cofilin-1. The un-phosphorylated form of ERK1/2 (inactive) did not interact with ADF/cofilin-1 (Figure S5A). Similar interaction between pERK1/2 and ADF/cofilin-1 was found in the heart of 3 and 6 months old *Lmna*$^{H222P/H222P}$ mice, while it was not detected in *Lmna*$^{+/+}$ mice (Figure S5B). These results show that pERK1/2 binds to ADF/cofilin-1 in cells

expressing lamin A H222P. pERK1/2 catalyzes the phosphorylation of targets on serine or threonine residues (Wortzel and Seger, 2011). We observed that ADF/cofilin-1 is abnormally phosphorylated on threonine residues in C2-H222P cells compared to C2-WT cells, while phosphorylation on serine residues remains the same (Figure 2C). This threonine phosphorylation on ADF/cofilin-1 was blunted when C2-H222P cells were treated with selumetinib (data no shown). Threonine phosphorylation in ADF/cofilin-1 occurred in C2-WT cells transfected with a plasmid encoding the ERK2-WT (data not shown). ERK1/2 are "proline-directed" protein kinases. They phosphorylate serine or threonine residues, with Pro-Xaa-Ser/Thr-Pro being the most common consensus sequence for substrate recognition, although Ser/Thr-Pro can also serve as a substrate (Gonzalez et al., 1991). ADF/cofilin-1 contains 8 threonine residues but only Thr25 is in proximity of a proline, which could potentially be the target sequence for phosphorylation by pERK1/2 (Figure 2D). We therefore created plasmids encoding ADF/cofilin-1 mutants (T25A and T148A), which expressed a correctly localized ADF/cofilin-1 protein in C2C12 cells (Figure 2D). The interaction between pERK1/2 and mutant ADF/cofilin-1 T25A was attenuated, while the interaction with the ADF/cofilin-1 T148A construct was not altered (Figure 2E). This suggested that Thr25 is involved in the interaction with pERK1/2. ADF/Cofilin-2, the skeletal muscle isoform, does not have the Thr-Pro consensus site for phosphorylation catalyzed by ERK1/2 and therefore does not bind to it (Figure S5C). We next asked whether ADF/cofilin-1 T25A could alter actin dynamics. C2-H222P cells transfected with plasmids that expressed ADF/cofilin-1-WT or ADF/cofilin-1-T148A had a decreased F/G actin ratio (0.2 and 0.17, respectively) compared to non-transfected C2-H222P cells (Figure 2F). Expression of ADF/cofilin-1-T25A partially rescued the F/G actin ratio of C2-H222P cells (0.95 versus 0.53 for non-transfected cells) (Figure 2F). A phosphomimetic ADF/cofilin-1-T25D mutation blunted the rescue of actin depolymerization when expressed in C2-H222P cells (Figure S5D).

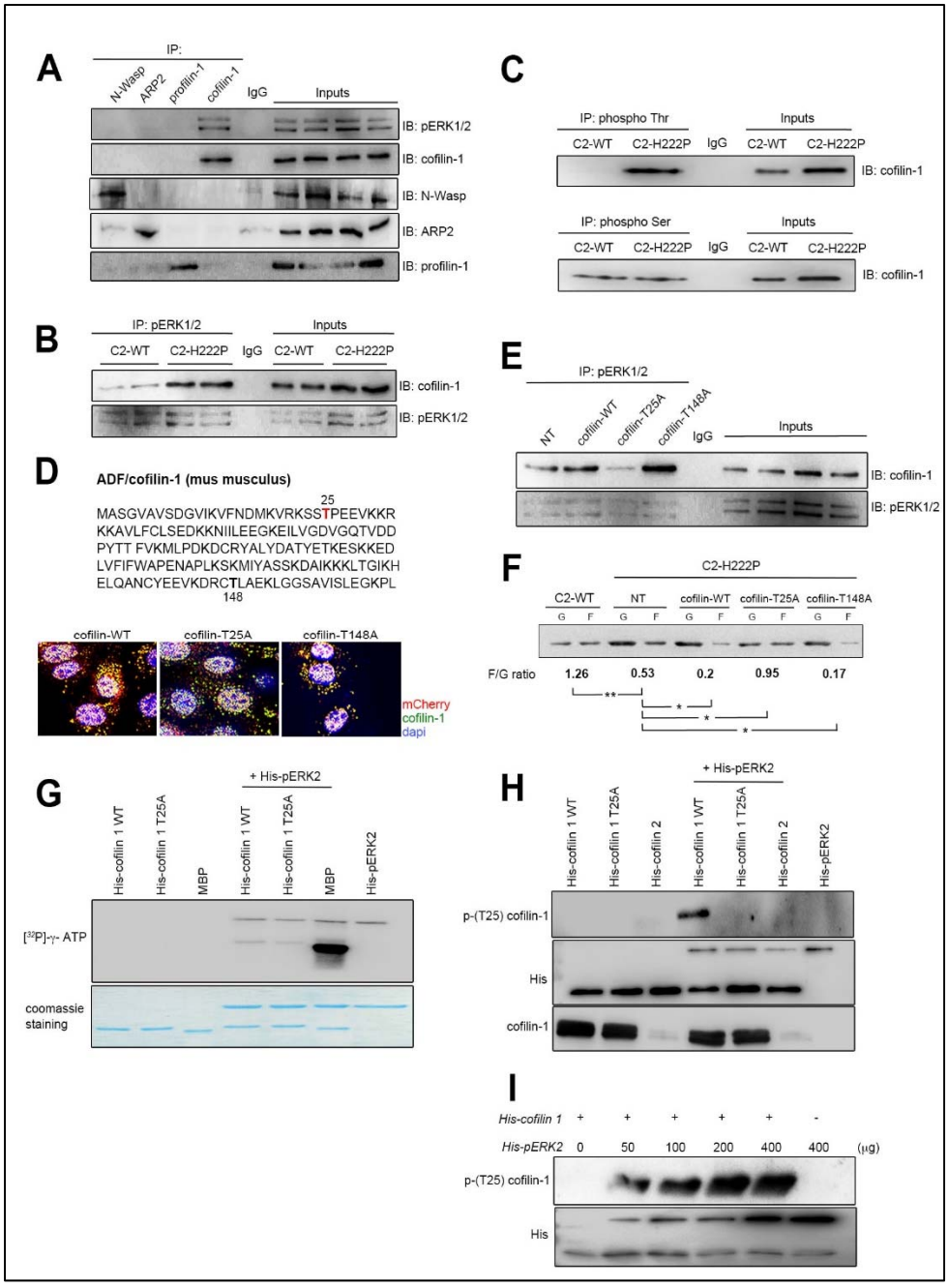

Figure 2. Interaction between ADF/cofilin-1 and pERK1/2

(A) Proteins extracted from C2C12 cells were subjected to immunoprecipitation using antibodies against cofilin-1, N-Wasp, ARP2 or profilin-1. The immunoprecipitates were separated by SDS-PAGE and immunoblotted using antibodies against pERK1/2, cofilin-1, N-Wasp, ARP2 or profilin-1. The immunoprecipitation was carried out with IgG as negative control.

(B) Proteins extracted from C2-WT and C2-H222P cells were subjected to immunoprecipitation using antibodies against pERK1/2. The immunoprecipitates were separated by SDS-PAGE and immunoblotted using antibodies against pERK1/2 and cofilin-1. The immunoprecipitation was carried out with IgG as negative control.

(C) Proteins extracted from C2-WT and C2-H222P cells were subjected to immunoprecipitation using antibodies specific to phospho-Thr or phospho-Ser. The immunoprecipitates were separated by SDS-PAGE and immunoblotted using antibody against cofilin-1. The immunoprecipitation was carried out with IgG as negative control.

(D) Amino acid sequence of murine ADF/cofilin-1 with highlighted Threonine 25 (red) and Threonine 148 (bold). Proteins extracted from C2-H222P transfected or not transfected (NT) with plasmids encoding cofilin-WT, cofilin-T25A or cofilin-T148A.

(E) C2-H222P cells transfected or not transfected were subjected to immunoprecipitation using antibodies againt pERK1/2. The immunoprecipitates were separated by SDS-PAGE and immunoblotted using antibodies against cofilin-1 or pERK1/2. The immunoprecipitation was carried out with IgG as negative control.

(F) Immunoblot illustrating the effect of different cofilin constructs on the amounts of G-actin and F-actin and the calculated F/G actin ratio. Representative of three independent experiments each performed in triplicates. *P<0.05, **P<0.005.

(G) *In vitro* kinase assay using recombinant cofilin 1-WT and cofilin 1-T25A, without or with the addition of recombinant pERK2, with MBP-positive control. [^{32}P]-γ-ATP indicates recombinant protein phosphorylation.
(H) *In vitro* kinase assay using recombinant cofilin 1-WT, cofilin 1-T25A and cofilin 2, without or with the addition of recombinant pERK2. Phosphorylated (Thr25)ADF/cofilin-1 was detected using specific antibody.
(I) *In vitro* kinase assay using recombinant cofilin 1-WT without or with the addition of recombinant pERK2 at ramping doses. Phosphorylated (Thr25)ADF/cofilin-1 was detected using specific antibody.

We next sought to show that pERK1/2 directly phosphorylated cofilin-1 on Thr25. In a radioactive *in vitro* kinase assay, incubation of His-pERK2 with His-cofilin-1 WT and [^{32}P]-γ-ATP resulted in the phosphorylation of ADF/cofilin-1 (Figure 2G). Our *in vitro* assay showed that incubation of His-pERK2 with [^{32}P]-γ-ATP resulted in phosphorylation of ERK2, which confirm the autophosphorylation of ERK2 (Lorenz et al. 2008). To further validate our data, we generated an antibody recognizing the region of phosphorylated (Thr25)ADF/cofilin-1 (between residues 21 and 35) (Figure S6A). To ensure that the identified phosphorylated (Thr25)ADF/cofilin-1 protein was truly overexpressed in C2-H222P cells, protein lysates were subjected to SDS-PAGE and blotted with antibodies against phosphorylated (Thr25)ADF/cofilin-1. As expected, the expression of phospho-(Thr25)ADF/cofilin-1 was significantly increased in C2-H222P cells compared to C2-WT cells (Figure S6B). Phospho-(Thr25)ADF/cofilin-1 is also found in the cytoplasm of C2-H222P cells (Figure S6C), similarly to pERK1/2 (Figure 1A), while it is mostly in the nucleus of C2-WT cells. We then tested pERK2 for its ability to phosphorylate ADF/cofilin-1 on Thr25. Phosphorylated ERK2 was incubated with His-cofilin-1 WT, His-cofilin-1 T25A or His-cofilin-2 WT and we evaluated the level of phosphorylation of ADF/cofilin-1 using our newly generated antibody. pERK2 substantially phosphorylated cofilin-1 WT on Thr25, but not cofilin-2 (Figure 2H). The phosphorylation of ADF/cofilin-1 correlates with amount of His-pERK2 (Figure 2I), indicating that the phosphorylation of ADF/cofilin-1 is attributable to ERK2 activity. This phosphorylation of cofilin-1 by pERK2 was blunted in the presence of mutant His-cofilin-1, in which the Thr25 amino acid are replaced by alanine reducing its ability to be phosphorylated (Figure 2H). Overall, these results demonstrated that ADF/cofilin-1 is phosphorylated on Thr25 by pERK1/2 in cells expressing lamin A H222P, promoting altered F-actin dynamics.

We next set out to detect endogenous ADF/cofilin-1 phosphorylated on Thr25 in cells. We extracted proteins from C2-WT, C2-H222P, and C2-H222P cells treated with selumetinib, separated the proteins on 2-dimensional gels and stained these with Cy2 dye (Figure 3A). Blotting

with antibodies against ADF/cofilin-1 identified a specific pattern of expression in C2-H222P cells compared to C2-WT cells and C2-H222P cells treated with selumetinib (Figure 3B). Each protein spot revealed by the antibody against ADF/cofilin-1 was excised and subjected to in-gel digestion and Orbitrap hybrid mass spectrometry analysis (Figure S7). We confirmed that ADF/cofilin-1 was present in each spot, and we identified a total of 13 phosphopeptides corresponding to ADF/cofilin-1 (Table S1). A phosphopeptide containing Thr25 phosphorylation site (SSTPEEVKK) was observed in a spot (spot D) from extracted proteins only from C2-H222P cells (Table 1, Table S2).

RhoA and Rho kinase (ROCK) regulate cofilin-mediated F-actin disassembly through LIMK-catalyzed phosphorylation of ADF/cofilin-1 (Bamburg, 1999). LIMK catalyzes the phosphorylation of ADF/cofilin-1 on Ser3 and inactivates its actin-severing activity (Agnew et al., 1995). We showed that the level of phosphorylated (Ser3)ADF/cofilin-1 was not different between C2-WT and C2-H222P cells (Figure S8A). Phosphorylated (Ser3)ADF/cofilin-1 was detected by Orbitrap hybrid mass spectrometry analysis (Table 1, Table S2). Treatment with Y27632, a selective inhibitor of ROCK, blocks the phosphorylation of LIMK and in turn the LIMK-catalyzed phosphorylation of ADF/cofilin-1, which leads to its activation (Maekawa et al., 1999). We showed that adding Y27632 to C2-WT and C2-H222P cells decreased phosphorylation on Ser3 of ADF/cofilin-1, without affecting the level of phosphorylation on Thr25 (Figure S8A). Adding selumetinib to C2-WT and C2-H222P cells did not alter a significant inactivation of the phospho-(Ser3)ADF/cofilin-1 (Figure S8A). Treating C2-WT cells with Y27632 led to a decreased F/G actin ratio compared to DMSO treatment (0.47 and 0.9, respectively), suggesting an activation of ADF/cofilin-1 severing activity (Figure S8B). However, similar treatment of C2-H222P cells did not alter the ADF/cofilin-1 severing activity (Figure S8B). These results suggest that ERK1/2 blocks the inhibitory activity of the ROCK pathway by catalyzing the phosphorylation on Thr25 and activation ADF/cofilin-1 in C2-H222P cells (Figure S8C).

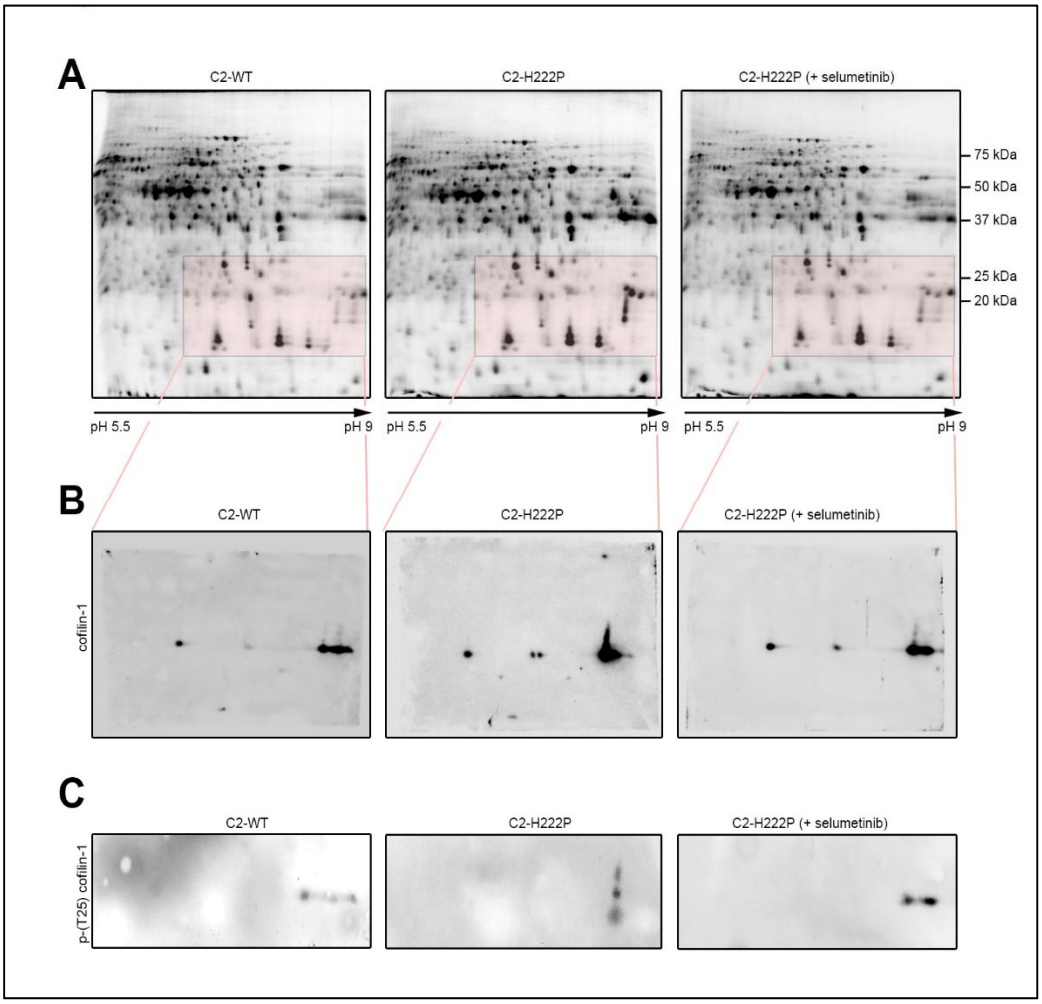

Figure 3. Detection of phosphorylated T25-ADF/cofilin-1
(A) Photographs of Cy2-stained 2-dimensional gels of protein extracts from C2-WT, C2-H222P and C2-H222P cells treated with selumetinib. Migrations of molecular mass standards are indicated at right and pH gradient at the bottom of each gel.
(B) Immunoblots using antibody against ADF/cofilin-1 to probe portions of the 2-dimensional gels (indicated by pink rectangles in Panel A) of C2-WT, C2-H222P and C2-H222P cells treated with selumetinib.
(C) Phosphorylated (Thr25)ADF/cofilin-1 detection in portion of 2-dimensional gels (indicated by pink rectangles in Panel A)) of C2-WT, C2-H222P and C2-H222P cells treated with selumetinib.

Phosphorylated Thr25/cofilin-1 alters sarcomere organization leading to cardiac dysfunction

Increasing the activity of ADF/cofilin-1 could have a detrimental effect on cardiac muscle. We therefore hypothesized that phosphorylation of Thr25 on ADF/cofilin-1 identified in the heart from *Lmna*$^{H222P/H222P}$ mice could have consequences on the cardiac muscle. Immunostaining of heart sections from *Lmna*$^{H222P/H222P}$ mice with phalloidin and antibodies against ADF/cofilin-1 showed

F-actin accumulations that co-localized with ADF/cofilin-1 (Figure 4A). These were indicative of F-actin depolymerization. Given that sarcomeres are composed of myosin (thick filaments) and actin (thin filaments), we next asked whether phospho-(Thr25)ADF/cofilin-1 could impact the dynamics of sarcomeric actin. Immunostaining with phalloidin and α-actinin antibodies showed disruption of sarcomeres organization (Figure 4B). This is followed by a reduced expression of sarcomeric actin and α-actinin (Figure 4C).

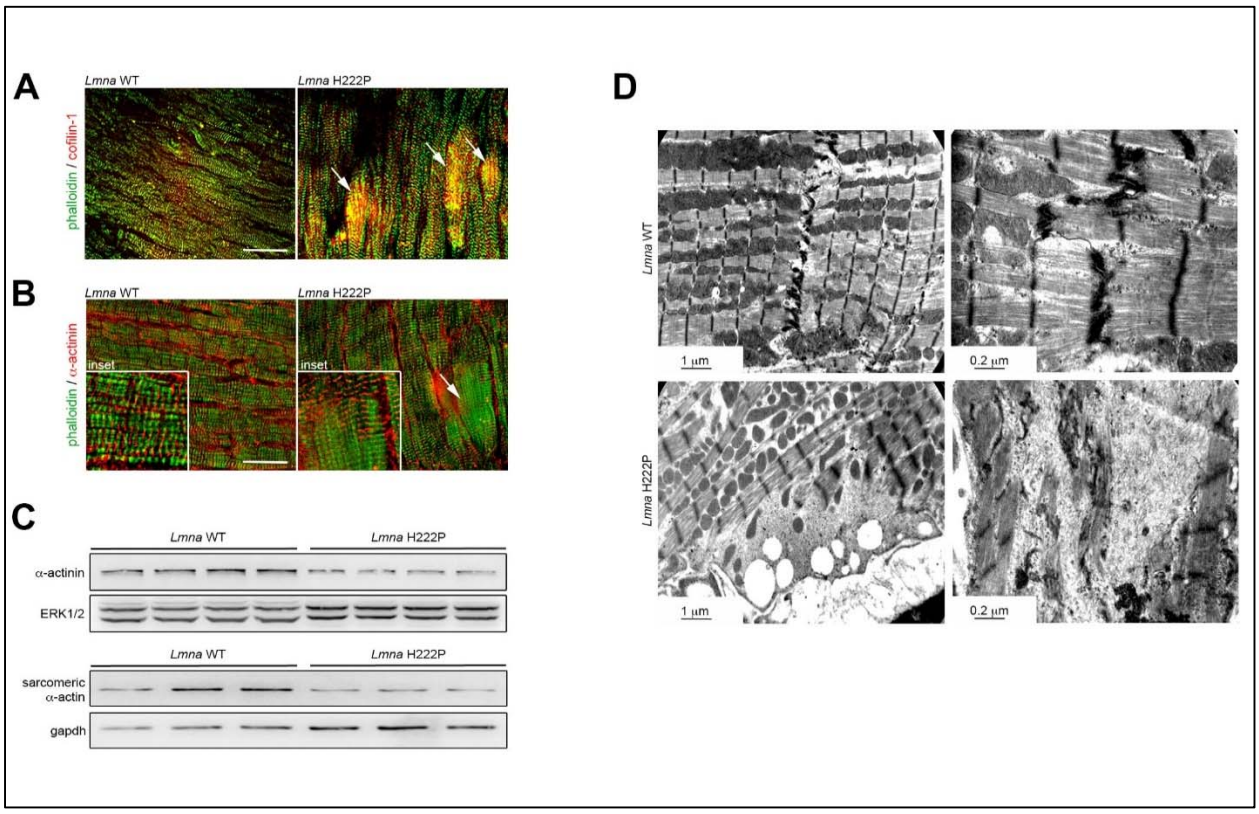

Figure 4. Alteration of sarcomere organization in *LMNA* cardiomyopathy

(A) Fluorescence micrographs showing phalloidin and cofilin-1 labeling of cross sections of hearts from 6 month-old male *Lmna*$^{+/+}$ (*Lmna* WT) mice and *Lmna*$^{H222P/H222P}$ (*Lmna* H222P) mice. Arrows indicate accumulation of ADF/cofilin-1 that co-localized with altered F-actin accumulations. Scale bar, 100 μm.

(B) Micrographs showing phalloidin and α-actinin of cross sections of hearts from 6 month-old male *Lmna*$^{+/+}$ (*Lmna* WT) mice and *Lmna*$^{H222P/H222P}$ (*Lmna* H222P) mice. Arrows indicate altered F-actin organization, which matches areas of disorganized sarcomeres (inset). Scale bar, 100 μm.

(C) Immunoblots showing α-actinin and sarcomeric α-actin protein levels in hearts from 6 month-old male *Lmna*$^{+/+}$ (*Lmna* WT) mice and *Lmna*$^{H222P/H222P}$ (*Lmna* H222P) mice. Representative of three independent experiments each performed in triplicates.

(D) Electron micrographs showing disruption of sarcomeric organization in hearts from 6 month-old male *Lmna*$^{+/+}$ (*Lmna* WT) mice and *Lmna*$^{H222P/H222P}$ (*Lmna* H222P) mice. In *Lmna* H222P mice, disorganized sarcomeres are seen.

To determine the ultrastructure of the heart from *Lmna*$^{H222P/H222P}$ mice in more detail, electron microscopy was performed. Left ventricular tissue from these mice exhibited severe disruption of myofibrillar structure including areas of sarcomere disorganization alongside of normal-looking fibers (Figure 4D). The expression of mRNAs such as *Lmod-3*, *Fmnl-1* and *Fhod-3* encoding sarcomeric actin nucleators (Chereau et al., 2008; Rosado et al., 2014; Taniguchi et al., 2009; Kan-O et al., 2012; Iskratsch et al., 2010) was also decreased and correlated with decreased, age-related cardiac function (Figure S9A). Furthermore, mRNAs encoding Srv2/Cyclase-associated protein and Twinfilin-1B (Johnston et al., 2015) were increased in the heart from *Lmna*$^{H222P/H222P}$ mice (Figure S9B), suggesting an accelerated shortening of F-actin.

We next tested the hypothesis that ADF/cofilin-1 influences cardiac function in *LMNA* cardiomyopathy. We injected a scAAVrh10 vector expressing wild type ADF/cofilin-1 into the orbital vein of 3 months-old *Lmna*$^{+/+}$ mice. Given that the ADF/cofilin-1 T25A phospho-dead mutation is able to partially restore the F-actin dynamics in the cellular model, we addressed the role played by this variant on left ventricular function to determine if it could blunt the cardiomyopathy. We injected a scAAVrh10 vector expressing either the wild type ADF/cofilin-1 or the ADF/cofilin-1 T25A mutant into the orbital veins of 3 month-old *Lmna*$^{H222P/H222P}$ mice. Three months after injection, all injected mice had increased ADF/cofilin-1 expression in the heart (Figure 5A). This was followed by depolymerization of F-actin in both *Lmna*$^{+/+}$ mice and *Lmna*$^{H222P/H222P}$ mice that received wild type ADF/cofilin-1 (Figure 5B). As expected, we observed a rescue on actin polymerization in *Lmna*$^{H222P/H222P}$ mice that received the AAVrh10-ADF/cofilin-1 T25A mutant (Figure 5B). Left ventricular end-systolic diameter and fractional shortening were significantly altered in *Lmna*$^{+/+}$ mice that received AAVrh10-cofilin-1 WT compared to non-treated animals (Figure 5C, Table 2), indicating the detrimental role of actin depolymerization on cardiac function. Left ventricular fractional shortening was significantly improved in *Lmna*$^{H222P/H222P}$ mice that received the AAVrh10-ADF/cofilin-1 T25A compared to the animals that received the wild type construct (Figure 5C, Table 2). Compared with *Lmna*$^{+/+}$ mice, *Lmna*$^{+/+}$ mice that received AAVrh10-cofilin-WT had significantly increased expression of *Col1a2*, which encoded collagen, *Myh7*, which encodes myosin heavy chain, and *Nppa and NppB*, which encodes the atrial natriuretic peptides (Figure S10A). Similarly, compared with *Lmna*$^{H222P/H222P}$ mice, mutated mice that received AAVrh10-cofilin-1 T25A had significantly increased expression of these genes (Figure S10A). Expression of these genes in *Lmna*$^{H222P/H222P}$ mice that received the

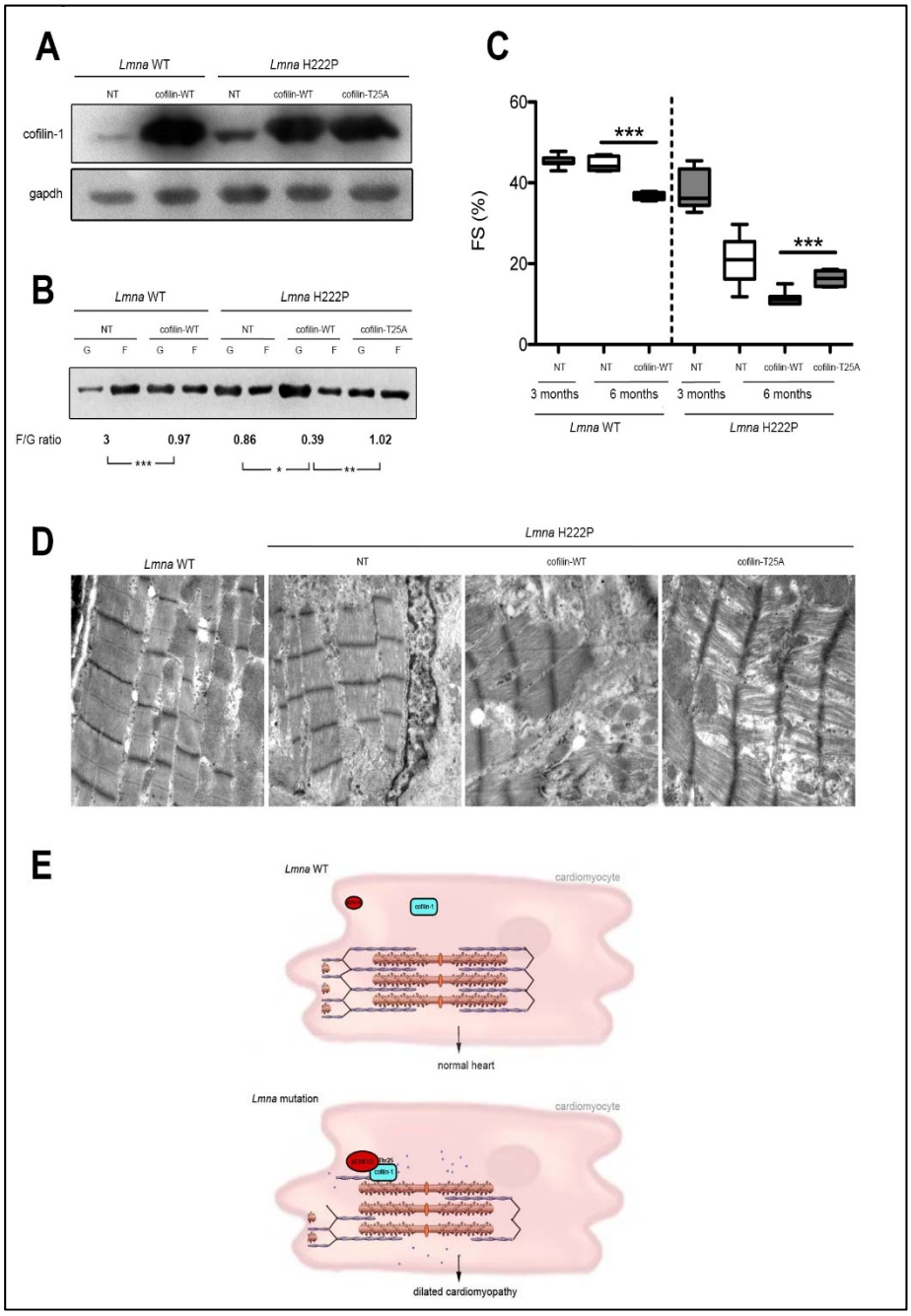

Figure 5. Phosphorylated (T25)ADF/cofilin-1 leads to cardiac dysfunction in *LMNA* cardiomyopathy

(A) Cofilin-1 protein level in hearts from *Lmna* WT or *Lmna* H222P transduced mice.

(B) Immunoblot illustrating the effect of AAVrh10 expressing cofilin-WT and cofilin-T25A constructs on the amounts of G-actin and F-actin and the calculated F/G actin ratio in hearts from *Lmna* WT or *Lmna* H222P transduced mice. Representative of three independent experiments each performed in triplicates. **P*<0.05, ***P*<0.005, ****P*<0.0005.

(C) Graph showing mean fraction shortening (FS) in 3-month-old (prior injection of AAV) and 6 month-old male transduced *Lmna* H222P mice and *Lmna* WT mice (see Table S3). **P*<0.05, ****P*<0.0005.

(D) Electron micrographs showing sarcomeric organization in hearts from 6 month-old male $Lmna^{+/+}$ (*Lmna* WT) mice and $Lmna^{H222P/H222P}$ (*Lmna* H222P) mice transduced with AAVrh10 expressing cofilin-WT and cofilin-T25A constructs. In *Lmna* H222P mice transduced with AAVrh10 expressing cofilin-T25A constructs, organized sarcomeres are seen compared to *Lmna* H222P mice transduced with AAVrh10 expressing cofilin-WT constructs or non-transduced.

(E) Proposed mechanism of ADF/cofilin-1 phosphorylation by ERK1/2 in heart from *Lmna* H222P mice and consequences on sarcomeric actin depolymerization.

AAVrh10-ADF/cofilin-1 T25A mutant was similar to that in non-treated *Lmna*$^{H222P/H222P}$ mice (Figure S10B). While left ventricular tissue from *Lmna*$^{H222P/H222P}$ mice that received AAVrh10-cofilin-1 WT exhibited severe disruption of myofibrillar structure (Figure 5D), this is partially restored in *Lmna*$^{H222P/H222P}$ mice that received AAVrh10-cofilin-1 T25A (Figure 5D). In summary, our findings identify a novel phosphorylation site on ADF/cofilin-1, which when phosphorylated by ERK1/2 leads to activation of its depolymerizing action on F-actin and alteration of cardiac function (Figure 5E). This is a novel mechanism in the development of *LMNA* cardiomyopathy.

DISCUSSION

Our data suggest a novel mechanism for A-type lamins in the regulation of actin dynamics. We show that phosphorylated ERK1/2 in *LMNA* cardiomyopathy binds to ADF/cofilin-1, an F-actin binding protein, phosphorylated it on Thr25 and that it activates the F-actin depolymerization function of ADF/cofilin-1. These events participate in the development of cardiac dysfunction. Inhibition of ERK1/2 signaling suppresses phospho-(Thr25)ADF/cofilin-1 and improves left ventricular dysfunction. The mechanism by which *LMNA* mutation leads to ERK1/2 activation remains to be elucidated. However, our study demonstrates some of the downstream alterations by which ERK1/2 signaling promotes cardiac dysfunction caused by *LMNA* mutations.

Although a role in actin depolymerization is known for ADF/cofilin-1, its function in the sarcomeric actin dynamics and the development of cardiac defect remains unclear. In muscle cells, actin is arranged, together with a large number of scaffolding and regulatory proteins into regular contractile sarcomeres (Ono, 2010). ADF/cofilins (e.g., destrin, cofilin-1 and cofilin-2) are required to disassemble sarcomeric F-actin filaments in cardiac cells (Skwarek-Maruszewska et al., 2009). Both ADF/cofilin-1 and ADF/cofilin-2 are expressed in cardiac muscle (Skwarek-Maruszewska et al., 2009). However, it has been thought that ADF/cofilin-2 was mostly involved in F-actin depolymerization in heart (Subramanian et al., 2015). We showed that ADF/cofilin-2 is not phosphorylated and activated by ERK1/2 signaling in *LMNA* cardiomyopathy, as it does not have the consensus Ser/Thr-Pro amino-acids sequences by pERK1/2. Functionally, ADF/cofilin regulation of F-actin dynamics is controlled by reversible phosphorylation. Several mechanisms regulate tightly the activation of ADF/cofilins, including phosphorylation at serine (Ser3) (Arber et al., 1998; Yang et al., 1998) by LIMK or testicular protein kinase 1. The LIMK-induced

phosphorylation of ADF/cofilin on Ser3 inactivates cofilin, leading to the accumulation of polymerized F-actin filaments (Sumi et al., 1999) and impaired contractility. It has also been described ADF/cofilins phosphorylations at Tyr68 by v-Src, which do not change cofilin activity, but induce increased ubiquitination of cofilin and its degradation through the proteasome pathway (Yoo et al., 2010). A study showed that Aurora A kinase catalyzes the phosphorylation ADF/cofilin on Ser3, Ser8 and Thr25 (Ritchey & Chakrabarti, 2014), during early mitotic stages to modulate its function. Our work is the first, to the best of our knowledge that identifies a phosphorylated residue within ADF/cofilin-1 which activate the depolymerizing role of ADF/cofilin-1.

We showed that active phosphorylated (Thr25)ADF/cofilin-1 is a *bona fide* effector of dilated cardiomyopathy. Functional significance of ADF/cofilins in muscle is also suggested by the finding that a mutation in the human gene causes nemaline myopathy (Agrawal et al. 2007). This myopathy is characterized by the presence of aggregates containing actin and other myofibrillar proteins (Clarkson et al., 2004). Patients with nemaline myopathy often develop a cardiomyopathy (Gatayama et al., 2013; Mir et al., 2012; D'Amico et al., 2006; Skyllouriotis et al., 1999; Van Antwerpen et al., 1988; Rosenson et al., 1986). In the patient muscle, actin is abnormally accumulated in aggregates. In the heart, ADF/cofilin-2 plays a pathological role in idiopathic dilated cardiomyopathy (Subramanian et al., 2015). Inactive phosphorylated ADF/cofilin-2 sequestered in aggregates is unable to maintain F-actin filament homeostasis and affects cardiomyocyte contractility. Sarcomeric actin filaments undergo dynamic turnover at both filaments barbed-end and pointed-ends (Littlefields et al., 2001; Bai et al., 2007), similar to cytoskeleton actin, and even in the entire thin filaments in myofibrils (Suzuki et al., 1998; Wang et al., 2005). Despite the predicted slow turnover rates, actin-dynamics enhancers and actin-filament stabilizers such as tropomodulin often antagonistically regulate the length of F-actin filaments in sarcomeres. Proteins that bind to the side of F-actin filaments are integral components of sarcomeric thin filaments and commonly stabilize F-actin filaments. It has been suggested a model in which ADF/cofilin promote sarcomeric actin filament severing to maintain thin filaments length in cooperation with the inhibitory effect of tropomodulin on actin elongation (Yamashiro et al., 2008). Failure in this machinery may therefore lead to loss of integrity and stability of the sarcomere and contribute to the resultant cardiomyopathy.

Our results further suggest that other members of the actin dynamics machinery (Ono, 2010) are altered in *LMNA* cardiomyopathy. *Lmod2* knockdown severely compromises sarcomere organization and assemble in muscle cells (Chereau et al., 2008). *Lmod2* null mice develop dilated cardiomyopathy (Pappas et al., 2015). Leiomodin-2 is an antagonist of tropomodulin-1 at the pointed end of the thin filaments in cardiac muscle (Tsukada et al., 2010), promoting actin filament elongation in sarcomere. A recent study revealed that mutation in *Lmod3* resulting in disorganization of F-actin filaments cause nemaline myopathy in human (Yuen et al., 2014). Mutations in *Fhod3* lead to alteration of sarcomere organization and caused dilated cardiomyopathy (Arimura et al., 2013). Profilin is another actin-binding protein that has been shown as implicated in the dynamic turnover of sarcomeric actin (Yamashiro et al., 2008). A study from Cammarato' group recently showed that profilin modulates sarcomeric organization and mediated cardiomyocyte hypertrophy in Drosophila (Kooij et al., 2016). Recent work demonstrated that knocking down *Wdr1* (an auxiliary factor for ADF/cofilin) in heart from mouse, leads to disruption of myofibrils and accumulation of ADF/cofilin (Yuan et al., 2014). The mice then develop a cardiomyopathy. Cyclase-associated protein 2 (CAP2) has been described as an actin regulatory protein that cooperates with ADF/cofilin to regulate the dynamics of F-actin filaments. CAP2 is expressed at high levels in heart where it localizes to the M-lines (Peche et al., 2007). *Cap2*-null mice die earlier than wild type mice and show a phenotype that is typically associated with dilated cardiomyopathy (Peche et al., 2013). Loss of Cap2 in mice leads to sudden cardiac death due to heart block (Field et al., 2015) and phenocopies many of the same features of *Lmna* $^{H222P/H222P}$ (Arimura et al., 2005) and *Lmna*$^{N195K/N195K}$ (Mounkes et al., 2005) mice as well as patients with *LMNA* cardiomyopathy. Our findings that sarcomere disorganization occurs in *Lmna*$^{H222P/H222P}$ mice suggest that F-actin depolymerization may be a pathway common to inherited dilated cardiomyopathies.

Our finding that *LMNA* mutations lead to alterations of the actin network is in line with work from others (Ho et al., 2013; Folker et al., 2011). It has been hypothesized that mutations associated with *LMNA* cardiomyopathy render cells more sensitive to mechanical stress (Lee et al., 2007; Zwerger et al., 2013) and are therefore defective in mechanotransduction (Lammerding et al., 2004). This could partially explain the aberrant activation of stress-activated ERK1/2 signaling that we reported in heart from *Lmna* mutant mice (Muchir et al., 2007). A-type lamins are connected to the actin cytoskeleton through the linker-of-nucleoskeleton-and-cytoskeleton (LINC)

complex (Crisp et al., 2006; Starr & Fridolfsson, 2010). The LINC complex comprises the nesprins and the SUNs proteins. The nesprins form a network surrounding the nucleus and anchor it to the cytoskeleton. This physical connection could function as a buffer against mechanical forces (Hale et al., 2008). An uncoupling between the nucleus and cytoskeleton has been described as playing a key role in *LMNA* cardiomyopathy pathogenesis (Nikolova-Krstevski et al., 2011). Mutations in *SYNE1* (encoding nesprin-1) have been found in patients with dilated cardiomyopathy (Zhang et al., 2007; Puckelwartz et al., 2010) and loss of nesprin-1 in mice leads to a cardiac phenotype similar to patients (Puckelwartz et al., 2009; Puckelwartz et al., 2010). Recent work has shown that a decoupling of the nucleus and cytoskeleton by knocking-down nesprin-2 and SUN1 or ADF/cofilin-1 are responsible for promoting nuclear deformation, likely by inverting increased contractile force to the nuclear envelope via its attachments to the LINC complex (Kanellos et al., 2015). This implies that dynamic regulation of actin by ADF/cofilin-1 is important for the actin-LINC complex to control nuclear shape that is lost in *LMNA* cardiomyopathy. Actin nucleation around the nucleus enables cells to deform their nucleus despite the presence of a stiff A-type lamins meshwork (Thiam et al., 2016).

Our work demonstrates a mechanism linking aberrant ERK1/2 signaling activation and F-actin defects, leading to the development of cardiomyopathy. Treatment of mice with ERK1/2 signaling inhibitors can improve cardiac phenotype, which may be attributed at least in part to the effect on ADF/cofilin-1 activity. These findings encourage further approaches to correct impaired actin dynamics to ameliorate the devastating *LMNA* cardiomyopathy.

MATERIAL AND METHODS

Cell culture and reagents

Generation of stable C2C12 cells expressing wild type (C2-WT) and H222P (C2-H222P) lamin A has been described previously (Choi et al., 2012a). C2-WT and C2-H222P cells were transiently transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. GFP-ERK2, RFP-MEK1, GFP-ERK2 K52R, and GFP-ERK2 T183A/Y185F plasmids were obtained from P. Stork (Oregon Health and Science University). GFP-lamin A, GFP-lamin A E358K, GFP-Lamin A L271P, and GFP-LaminA N456I were made available by HJ Worman (Columbia University). Working concentrations of 100 nM cytochalasin D, 100 nM

latrunculin B, 200 nM jasplakinolide, 10 µM Y27632, 10 µM MG132, and 50 µM selumetinib were prepared from stocks diluted in dimethylsulfoxyde. Cells were incubated with cytochalasin D for 45 min, jasplakinolide for 40 min, and selumetinib for 15 h.

Animals

Lmna$^{H222P/H222P}$ mice (Arimura et al., 2005) were fed chow and housed in a disease-free barrier facility at 12h/12h light/dark cycles. All animal experiments were carried out according to the French Ministry of Health at the Center for Research in Myology for the Care and Use of Experimental Animals.

Human heart tissue

Sections of explanted hearts from human subjects with *LMNA* mutations were obtained without identifiers from Myobank-AFM de l'lnstitut de Myologie. Myobank-AFM received approval from the French Ministry of Health and from the Committee for Protection of Patients to share tissues and cells of human origin for scientific purposes. Control human heart samples were obtained from the National Disease Research Interchange; information regarding donor confidentiality and consent can be found at http://www.ndriresource.org.

RNA isolation and real-time PCR

Total RNA was extracted from cells or mice hearts using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. 1 µg of total RNA was subjected to cDNA synthesis using the First-Strand cDNA Synthesis Kit (Life Technologies). Mouse primer sequences used for transcriptional analyses were as follows: *mCofilin-1* 5'- CGCAAGTCTTCAACACCAGA-3', 5'-TGAACACCAGGTCCTCCTTC-3'; *mLmod3* 5'-ACAGCAGCAGCAGCAACTTA-3', 5'-AAAGGGGACTTCCTGAGCAT-3'; *mFmnl1* 5'-CTGCTGAGCCAGTATGACAATG-3', 5'-CGGTATCCAGGTAGCTCTTCA-3'; *mFhod3* 5'-ACGACACCGATCCTTTCAAC-3', 5'-TTCTAGCTCATCCCGCTGTT-3'; *mTwfi2B* 5'-GCCCTTGAATCTGTGGTGTT-3, 5'-TCGCCGATCTCAATCTTCTT-3'. Real-time quantitative PCR reactions were performed on a LightCycler 480 (Roche) using the SYBR Green PCR Master Mix (Applied Biosystems). PCR products were subjected to melting curve analysis to exclude the synthesis of non-specific

products. Cycle threshold (C_t) values were quantified using a standard curve for the specific gene and relatively quantified using *RplR0* as an internal reference control. The Ct values were then normalized to the average expression levels of samples, calculated according to the $\Delta\Delta C_t$ method (Ponchel et al., 2003) and are presented as fold change over WT controls. All experiments were performed in triplicate.

Antibodies

Primary antibodies used were: anti-cofilin1 (Cell signaling), anti-phosphorylated (Ser3) cofilin1 (Cell signaling), anti-phosphorylated (Thr25) cofilin1 (GenScript), anti-cofilin2 (Thermo Scientific), anti-profilin1 (Cell signaling), anti-N-wasp (Cell signaling), anti-Arp2 (Cell signaling), anti-pERK1/2 antibody (Cell signaling), anti-ERK1/2 (Santa Cruz Biotechnology), anti-emerin (Novocastra laboratories), anti-lamin A/C (Santa Cruz Biotechnology) anti-gapdh (Santa Cruz Biotechnology) and anti-α-actinin (Abcam). Secondary antibodies for immunofluorescence were Alexa Fluor-488 conjugated goat anti-rabbit IgG, Alexa Fluor 568-conjugated goat anti-mouse IgG and Alexa-Fluor-488-conjugated donkey anti-goat IgG (Life Technologies). Secondary antibodies for immunoblotting were HRP-conjugated rabbit anti-mouse, goat-anti rabbit IgG (Jackson ImmunoResearch).

Protein extraction and immunoblotting

Total proteins were prepared by resuspending mouse heart tissue or cultured cells in extraction buffer (Cell Signaling) with the addition of protease inhibitors (25 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM 4-[2-aminoethyl]- benzene sulfonylfluoride hydrochloride and 2 mM Na_3VO_4). The lysates were sonicated (3 pulses of 10s at 30% amplitude) to allow dissociation of protein from chromatin and solubilization. Cytosolic and nuclear fractions were prepared using the NE-PER Nuclear and Cytosolic Extraction Reagents (Thermo Scientific) according to the manufacturer's instructions. Sample protein content was determined by the BiCinchoninic acid Assay protein assay (Thermo Scientific). Extracts were analyzed by SDS-PAGE using a 10% gel and transferred onto nitrocellulose membranes (Invitrogen). Subsequent to being washed with Tris-buffered saline containing 1% Tween 20 (TBS-T), the membranes were blocked in 5% bovine

serum albumin (BSA) in TBS-T for 1 h at room temperature, then incubated with the appropriate antibody overnight at 4°C. Subsequent to being washed with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies for 1h at room temperature. After washing with TBS-T, the signal was revealed using Immobilon Western Chemiluminescent HorseRadish Peroxidase (HRP) Substrate (Millipore) on a G-Box system with GeneSnap software (Ozyme).

Measurement of F-actin/G-actin ratio

The ratio of filamentous (F-) to globular (G-) actin was determined using the G-actin/F-actin in vivo assay kit (Cytoskeleton) according to manufacturer's instructions. Briefly, 2 mg of protein from cells or frozen heart tissues were homogenized in a lysis and F-actin stabilization buffer (LAS2) and centrifuged at 2,000 rpm for 5min to remove unbroken cells. F-actin was separated from G-actin by centrifugation at 100,000 g for 60 min at 37°C. The F-actin-containing pellet was resuspended in F-actin depolymerizing buffer at a volume equivalent to the G-actin-containing supernatant volume. The resuspended F-actin pellet was kept on ice for 60 min and was gently mixed every 15 min to dissociate F-actin. Equivalent volumes (10 µl) of supernatant and pellet were resolved by SDS-PAGE and subjected to immunoblot analysis, using an anti-pan actin antibody supplied in the kit. F/G actin ratio was quantified using ImageJ software.

Immunofluorescence microscopy

For immunofluorescence microscopy, frozen tissues were cut in 8 µm-thick sections. Cryosections were fixed (15 min, 4% paraformaldehyde in phosphate-buffered saline [PBS] at room temperature), permeabilized (10 min, 0.5% Triton X-100 in PBS) and blocked (1 h, PBS with 0.3% Triton X-100, 5% BSA). Sections were incubated with primary antibodies (overnight, 4°C, in PBS with 0.1% Triton X-100 and 1% BSA) and washed in PBS. The sections were then incubated for 1 h with secondary antibodies. Sections were washed with PBS and slides were mounted in vectashield mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories).

C2C12 cells were grown on coverslips, washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Cells were permeabilized with 0.2% Triton X-100 diluted in PBS for 7 min and non-specific signals were blocked with 0.2% Triton X-100, 5% BSA for 30 min. The samples were

then incubated with primary antibody for 1 h in PBS with 0.1% Triton X-100 and 1% BSA at room temperature. Cells were washed with PBS and incubated for 1 h with secondary antibodies. F-actin was stained with Alexa Fluor 568-phalloidin and G-actin with Alexa Fluor 488-deoxyribonuclease I for 1 h at room temperature. Cells and slides were then mounted in Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories). Immunofluorescence microscopy was performed using an Axiophot microscope (Carl Zeiss). All the images were digitally deconvolved using Autodeblur v9.1 (Autoquant) deconvolution software and were processed using Adobe Photoshop 6.0 (Adobe Systems).

Direct observation of F-actin severing

The F-actin–severing assay was performed as previously described (Ono *et al.* 2004). Purified muscle actin (Cytoskeleton) monomers were polymerized in 500 mM KCL, 10 nM ATP at room temperature for 1 h. Actin filaments were allowed to adhere on glass coverslips overnight at 4ºC, washed with buffer (5 mM Tris-HCl pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP and 0.5mM DTT) and incubated with protein extracts (10 μg/μl) for 30 min at 4ºC. Actin filaments were thereafter incubated with phalloidin Alexa Fluor 555 (ThermoFisher Scientific) and mounted with Vectashield mounting media (Vector Laboratories). Labelled F-actin filaments were viewed using a Zeiss Axiovert 200 fluorescent microscope (Zeiss).

Electron microscopy

Freshly harvested left ventricle apex was cut into small pieces and immediately fixed by immersion in 2.5% glutaraldehyde diluted in PBS for 1 h at room temperature. After washing in PBS, samples were post-fixed with 2% OsO_4, dehydrated in a graded series of acetone including 2% uranyl acetate in 70% acetone step and embedded in an epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, examined using a transmission electron microscope (Philips) and photographed with a digital SIS Morada camera (EMIS), using iTEM software.

Immunoprecipitation

Cells and cardiac tissues were lysed in 0.5ml of lysis buffer (50mM Tris-HCl, pH 7.5, 0.15M NaCl, 1mM EDTA, 1% NP-40). Mouse cardiac muscle tissue lysate was diluted to 2 mg/ml in lysis

buffer, pre-cleared with 20 µl washed protein G–Sepharose 4 fast-flow (GE Healthcare) and incubated with 20 µg of specific antibody overnight at 4°C. Next, 30 µl of washed beads was added and incubated for 2 h at 4°C. Pelleted beads were collected in sample buffer (0.25M Tris-HCl, 8% SDS, 40% glycerol, 20% β-mercaptoethanol) and subjected to SDS-PAGE and immunoblotting. For control reactions, we used rabbit immunoglobulin G1**.**

Construction of plasmids encoding wild type and ADF/cofilin-1 mutants

Mutagenesis was carried out using QuikChange II Site-Directed Mutagenesis Kit (Agilent technologies) according to the manufacturer's instructions. Cofilin-pmCherryC1 (Addgene) was used as a template for substituting threonine for alanine at position 25 and 148. Primers used for the introduction of single mutations are: mCofilin1-T25A 5'-CTTCACTTCTTCTGGTGCTGAAGACTTGCGAACCT-3', 5'-AGGTTCGCAAGTCTTCAGCACCAGAAGAAGTGAAG-3'; mCofilin1-T148A 5'-TTCTCTGCCAGGGCGCAGCGGTCCTTG-3', 5'-CAAGGACCGCTGCGCCCTGGCAGAGAA-3', mCofilin1-T25D 5'-CGTTTCTTCACTTCTTCTGGATCTGAAGACTTGCGAACCTTCATGTCA-3', 5'-TGACATGAAGGTTCGCAAGTCTTCAGATCCAGAAGAAGTGAAGAAACG-3'. The final plasmids were transformed in XL1- Blue super competent cells. Mutations were verified by DNA sequencing using an appropriate set of oligonucleotides to cover full length of the *cofilin1* gene.

Construction and injection of AAVs encoding ADF/cofilin-1

AAV vectors of serotype rh10, carrying wild type or mutant ADF/cofilin-1 under the control of the cytomegalovirus immediate/early (CMV) promoter were prepared by the triple transfection method in HEK293T cells as previously described (Dominguez et al., 2011). Briefly, cells were transfected with (i) the adenovirus helper plasmid, (ii) the AAV packaging plasmid encoding the rep2 and cap-rh10 genes and (iii) the AAV2 plasmid expressing CMV-cofilin-1. Seventy-two hours after transfection, cells were harvested and AAV vectors were purified by ultracentrifugation through an iodixanol gradient and concentrated with Ultra–Ultra cell 100K filter units (Amicon) in 0.1 M PBS, 1 mM $MgCl_2$ and 2.5 mM KCl. Aliquots were stored at -80°C until use. Vector titers were determined by real-time PCR. Three month-old mice (n=6) were injected with

scAAVrh10-ADF/cofilin wild type or AAVrh10-ADF/cofilin-1 T25A into the retro-orbital vein ($5x10^{13}$ viral genomes/kg in 100 µl) with an insulin syringe.

Protein expression and purification

Cofilin-1 WT, cofilin-1 T25A and cofilin-2 cDNAs were cloned in pNIC-CTHF vector expressing a cleavable C-terminal His_6 and FLAG tag. The plasmid encoding constitutively MEK1 and His-ERK2 kinase was purchased from Addgene (#39212). His-cofilin-1 and His-cofilin-2 recombinant proteins were expressed in Rosetta(DE3) *E.coli* and His-MEK1-ERK2 was expressed in BL21(DE3) *E.coli*. Bacteria were grown in 2 x YT medium until they reached an optical density (OD) of ~0.8 and recombinant protein expression was induced by the addition of 0.5 mM IPTG (isopropyl-thiogalactopyranoside) for 16-24 h at 18ºC for BL21(DE3) or 30oC for Rosetta(DE3) before harvesting. Cell pellets were stored at −20°C until needed. Cells were sonicated in lysis buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 5mM imidazole 0.5 mM TCEP with complete EDTA-free Protease Inhibitor Cocktail, Roche). After centrifugation at 40 000 g for 30 min at 4ºC, the soluble fraction was filtered through a 0.8 µm filter and subjected to metal affinity Ni^{2+}-nitrilotriacetic resin chromatography. Recombinant His_6-tagged proteins eluted with elution buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol and 250 mM imidazole). His-tagged cofilin proteins applied to gel fractionation on a HiLoad 16/60 Superdex 75 preparative-grade column. His-tagged active ERK2 was purified as described previously (Khokhlatchev et al., 1997). Briefly, eluted His-pERK2 was dialyzed overnight in 50mM HEPES pH 7.5, 50mM NaCl, 1mM DTT, 20% glycerol, then applied to a MonoQ GL 5/50 column and eluted on a gradient from 50 mM to 1 M NaCl.

In vitro kinase assay

Recombinant His-tagged cofilin-1 WT, cofilin-1 T25A and cofilin-2 (5 µg) were incubated alone or with 0.5 µg recombinant active His-ERK2, in kinase buffer (50 mM HEPES pH 7.5, 150 M NaCl, 5 mM MgCl2, 0.5 mM TCEP) in a total volume of 100 µl containing 1 mM ATP. Reaction mixtures were incubated at 37°C for 1 h and were terminated by the addition of 100 µl of 2 x SDS sample buffer. Proteins were resolved by SDS-PAGE. Blots were incubated with anti-His (Sigma-Aldrich H1029) and phosphorylated (Thr25)ADF/cofilin-1 antibodies.

Radioactive in vitro kinase assay

Cofilin-1, cofilin-1 T25A or dephosphorylated myelin basic protein (MBP) (Merck-Millipore) were incubated alone or in the presence of purified pERK2 in buffer comprising 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM TCEP and 0.5 mM ATP. 10 pmol of protein was used and 0.5 µCi [^{32}P]-γ-ATP was added per sample (total volume 25 µl). The samples were incubated for 30 min at 37°C and the reaction was terminated by the addition of 5 µl 6 x SDS-PAGE sample buffer. Proteins were then separated by SDS-PAGE and blotted onto nitrocellulose. The nitrocellulose membrane was then used to expose a phosphor screen (typically for 24 hours), which was then imaged using a Typhoon FLA 7000 system.

Two-dimensional electrophoresis

Proteins were extracted from cells in a buffer composed of 7 M urea, 2 M thiourea, 1% CHAPS, 10% isopropanol, 10% isobutanol, 0.5% Triton X-100 and 0.5% SB3-10. Proteins were precipitated using the Perfect Focus kit (G-Biosciences) and pellets were resuspended in the same buffer supplemented with 25 mM Tris-HCl (pH 8.8). Protein content was assessed with the Quick Start™ Bradford protein assay (BioRad) using BSA as standard. For each sample, 50 µg of proteins were labeled with 400 pmol of Cy2 (Fluoprobes), incubated 30 min on ice then quenched with 0.35 mM lysine for 10 min. Then, 700 µg of unlabeled proteins were added to the 50 µg of Cy2-labeled proteins.

Proteins were separated using 24 cm gels with pH range 3-10 using commercial strips (GE Healthcare). Strips were passively rehydrated overnight directly with the samples supplemented with 40 mM dithiothreitol and 0.5% ampholites. Isoelectric focusing migration was set as follows: 3 h at 50 V, 3 h at 200 V, 2 h gradient from 200 V to 1,000 V, 2 h at 1,000 V, 2 H gradient from 1000 V to 10000 V, 8h at 10000 V. A second step of isoelectric focusing was performed using the following parameters: 30 min at 200 V, 1 h gradient from 200 V to 10,000 V, 4 h at 10,000 V. The area of the strips corresponding to pH range from 5.5 to 9 was excised and frozen at -20°C until use for the second dimension. Strips were incubated for 15 min in equilibration buffer (6M urea, 75 mM Tris-HCl [pH 8.8], 26% and 2% SDS) supplemented with 65 mM dithiothreitol then for 20 min in equilibration buffer supplemented with 135 mM iodoacetamide. The second dimension was run in 12% acrylamide gels at 25 V for the first hour then 150 V and 12 mA per gel in a Tris-

glycine buffer. Gel images were acquired on a Ettan DIGE Imager (GE Healthcare). Each gel was run twice using the same conditions: first gel was used for immunoblotting and the second for protein identification. Gels used for protein identification were stained with ProQ-Diamond (Thermo Fisher) following the manufacturer's instructions except for a 3x dilution of the Pro-Q Diamond stain in ultra-pure water.

In-gel digestion and mass spectrometry for protein identification

Gels were stained with silver nitrate for 1 min sensitizing in 0.02% sodium thiosulfate, rinsed twice in ultra-pure water, incubated for 30 min in 0.212% silver nitrate, rinsed twice in ultra-pure water and developed in 3% sodium carbonate, 0.00125% sodium thiosulfate, 0.03% formalin. Staining was stopped by soaking the gels in 5% acetic acid. Stained protein spots of interest were manually excised, sliced into 1 mm cubes, destained in 50 mM sodium thiosulfate, 15 mM potassium ferricyanure and washed several times alternatively in water and acetonitrile. Tryptic digestion was performed overnight at 37°C, using 200 ng of mass spectrometry grade trypsin (G-Biosciences) in 50 mM ammonium bicarbonate, 5 % acetonitrile. Supernatants were collected and gel pieces washed twice for 15 min in an ultrasonic bath with 50 µl of 0.1% trifluoroacetic acid, 60% acetonitrile. Peptides solutions were dried using a vacuum centrifuge and resuspended in 12 µl of 5% acetonitrile, 0.1% formic acid.

Identification of phosphopeptide by mass spectrometry

3µl of each sample were analyzed in LC-MS-MS using an Ultimate 3000 Rapid Separation liquid chromatographic system coupled to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific). Peptides were loaded on a C18 reverse phase resin (2 µm particle size, 100 A pore size, 75 µm i.d.15 cm length) with a 50 min "effective" gradient from 99% A (0.1% formic acid and 100% H2O) to 40% B (80% ACN, 0.085% formic acid and 20% H2O). The Obitrap Fusion mass spectrometer acquired data throughout the elution process and operated in a data dependent scheme with full MS scans acquired with the Orbitrap, followed by stepped HCD MS/MS (top speed in 3 sec maximum) on the most abundant ions detected in the MS scan. Mass spectrometer settings were: full MS (AGC: 4E5, resolution: 1.2E5, m/z range 350-1500, maximum ion injection time: 60 ms); MS/MS (Normalized Collision Energy: 30, resolution: 17500, intensity threshold: 1E4, isolation window: 1.6 m/z, dynamic exclusion time setting: 15 s, AGC Target: 1E4 and

maximum injection time: 100 ms). The fragmentation was permitted of precursor with a charge state of 2 to 4. The software used to generate .mgf files is Proteome discoverer 1.4.

Statistics

Statistical analyses were performed using GraphPad Prism software. Statistical significance between groups of animals analyzed by echocardiography was analyzed with a corrected parametric test (Welch's *t* test), with a value of $P<0.05$ being considered significant. To validate results, we performed a non-parametric test (Wilcoxon-Mann-Whitney test). For all other experiments, a two-tailed Student's t test was used with a value of $P<0.05$ considered significant. Values with errors bars shown in the figures are means ± SEM. Sample sizes are indicated in the figure legends.

AUTHOR CONTRIBUTIONS

M.C. and A.M. designed the research. D,Y., T.M., J.R.L., Y.T., S.C., and N.M. helped with experiments. H.J.W., W.H.G. and G.B. provided input on the experimental design and took part in thoughtful discussions. M.C. and A.M. wrote the manuscript. M.C., A.M., and H.J.W edited the manuscript.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Alain Schmitt (Cochin microscopy facility) for electron microscopy. This work was financially supported by the Institut National de la Santé et de la Recherche Médicale, the Sorbonne Universités-Université Pierre et Marie Curie Paris 06, the Centre National de la Recherche Scientifique, the Association Française contre les Myopathies, Cure CMD, Fundación Andrés Marcio to A.M., and collaborative grant (ANR) between W.H.G., H.J.W. and A.M. H.J.W. was supported by NIH grant AR048997.

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Tables

Table 1. Orbitrap hybrid mass spectrometry analysis of phosphopeptides

Phospho-residue	S3			S23			S94			S156		
Genotype	C2-WT	C2-H222P	C2-H222P (selumetinib)	C2-WT	C2-H222P	C2-H222P (selumetinib)	C2-WT	C2-H222P	C2-H222P (selumetinib)	C2-WT	C2-H222P	C2-H222P (selumetinib)
Spot A	x	x	x	x						x	x	x
Spot B	x	x	x									
Spot C	x	x								x		
Spot D	x	x	x			x			x	x	x	x

Phospho-residue	T25			T63			T88		
Genotype	C2-WT	C2-H222P	C2-H222P (selumetinib)	C2-WT	C2-H222P	C2-H222P (selumetinib)	C2-WT	C2-H222P	C2-H222P (selumetinib)
Spot A				x	x	x			
Spot B									
Spot C									
Spot D		x		x	x	x	x	x	

Phospho-residue	Y82			Y89		
Genotype	C2-WT	C2-H222P	C2-H222P (selumetinib)	C2-WT	C2-H222P	C2-H222P (selumetinib)
Spot A	x	x	x			
Spot B	x	x	x			
Spot C	x	x	x			
Spot D	x	x	x			x

Table S1. Phosphopetides identified by Orbitrap for ADF/cofilin-1

Phosphopeptide ID	Sequence	Motifs	Number of phosphosites
cofilin-1 a1a5	ESKKEDLVFIFWAPENAPLK	79.96633148	1
cofilin-1 a1b14	YALYDATYETK	79.96633148	1
cofilin-1 a1b15	YALYDATYETKESK	79.96633148	1
cofilin-1 a1b16	YALYDATYETKESK	159.932663	2
cofilin-1 a2a1	ASGVAVSDGVIK	121.976902	1
cofilin-1 a2b11	SGVAVSDGVIK	79.96633148	1
cofilin-1 a2b12	SGVAVSDGVIK	121.976902	1
cofilin-1 a3a3	EILVGDVGQTVDDPYTTFVK	61.9557724	1
cofilin-1 a3a4	EILVGDVGQTVDDPYTTFVK	79.96633148	1
cofilin-1 a4a8	LGGSAVISLEGK	79.96633148	1
cofilin-1 a4a9	LGGSAVILIEGKPL	79.96633148	1
cofilin-1 a5a6	HELQANCYEEVK	136.987793	1
cofilin-1 a5a7	HELQANCYEEVKDR	136.987793	1
cofilin-1 a6b10	KSSTPEEVKK	79.96633148	1
cofilin-1 a6b13	SSTPEEVKK	79.96633148	1
cofilin-1 a7a2	AVLFCLSEDKK	136.987793	1

Table S2. Phosphopetides identified by Orbitrap for ADF/cofilin-1 in C2-WT, C2-H222P and C2-H222P cells treated with selumetinib

Phosphopeptide ID	Spot name and cell type	Sequence (top)	Phosphopeptide ID	Spot name and cell type	Sequence (top)	Phosphopeptide ID	Spot name and cell type	Sequence (top)
cofilin-1 a1b15	D- C2-H222P	YALYDATYETKESK	cofilin-1 a2a1	B- C2-H222P (selumetinib)	ASGVAVSDGVIK	cofilin-1 a3a4	D- C2-H222P	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	B- C2-H222P (selumetinib)	YALYDATYETKESK	cofilin-1 a2a1	I- C2-H222P	ASGVAVSDGVIK	cofilin-1 a3a4	D- C2-WT	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	A- C2-H222P (selumetinib)	YALYDATYETKESK	cofilin-1 a2a1	B- C2-WT	ASGVAVSDGVIK	cofilin-1 a3a4	D- C2-H222P (selumetinib)	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	D- C2-H222P	YALYDATYETKESK	cofilin-1 a2a1	A- C2-WT	ASGVAVSDGVIK	cofilin-1 a3a4	D- C2-H222P	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	D- C2-H222P (selumetinib)	YALYDATYETKESK	cofilin-1 a2a1	I- C2-H222P	ASGVAVSDGVIK	cofilin-1 a3a4	D- C2-H222P	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	F- C2-H222P	YALYDATYETKESK	cofilin-1 a2a1	E- C2-WT	ASGVAVSDGVIK	cofilin-1 a3a4	D- C2-WT	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	A- C2-WT	YALYDATYETKESK	cofilin-1 a2a1	D- C2-WT	ASGVAVSDGVIK	cofilin-1 a3a4	A- C2-H222P	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	D- C2-H222P (selumetinib)	YALYDATYETKESK	cofilin-1 a2a1	I- C2-H222P	ASGVAVSDGVIK	cofilin-1 a3a4	I- C2-H222P	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	B- C2-WT	YALYDATYETKESK	cofilin-1 a2a1	C- C2-H222P	ASGVAVSDGVIK	cofilin-1 a3a4	D- C2-H222P	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	E- C2-WT	YALYDATYETKESK	cofilin-1 a2a1	A- C2-H222P (selumetinib)	ASGVAVSDGVIK	cofilin-1 a3a4	F- C2-H222P	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	F- C2-H222P (selumetinib)	YALYDATYETKESK	cofilin-1 a2a1	A- C2-H222P (selumetinib)	ASGVAVSDGVIK	cofilin-1 a3a4	F- C2-H222P	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	D- C2-WT	YALYDATYETKESK	cofilin-1 a2a1	A- C2-WT	ASGVAVSDGVIK	cofilin-1 a3a4	A- C2-H222P	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	B- C2-H222P	YALYDATYETKESK	cofilin-1 a2a1	B- C2-H222P (selumetinib)	ASGVAVSDGVIK	cofilin-1 a3a4	D- C2-WT	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	C- C2-H222P	YALYDATYETKESK	cofilin-1 a2a1	A- C2-H222P (selumetinib)	ASGVAVSDGVIK	cofilin-1 a3a4	D- C2-WT	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	C- C2-WT	YALYDATYETKESK	cofilin-1 a2a1	A- C2-H222P	ASGVAVSDGVIK	cofilin-1 a3a3	D- C2-H222P (selumetinib)	EILVGDVGQTVDDPYTTFVK
cofilin-1 a1b15	I- C2-WT	YALYDATYETKESK	cofilin-1 a2a1	A- C2-H222P	ASGVAVSDGVIK	cofilin-1 a4a9	D- C2-H222P (selumetinib)	LGGSAVISLEGKPL

cofilin-1 a1b14	B- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a2a1	A- C2-H222P (selumetinib)	ASGVAVSDGVIK	cofilin-1 a4a9	D- C2-H222P	LGGSAVISLEGKPL
cofilin-1 a1b14	D- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a2a1	B- C2-H222P	ASGVAVSDGVIK	cofilin-1 a4a9	D- C2-H222P (selumetinib)	LGGSAVISLEGKPL
cofilin-1 a1b14	A- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a2a1	D- C2-WT	ASGVAVSDGVIK	cofilin-1 a4a9	D- C2-WT	LGGSAVISLEGKPL
cofilin-1 a1b14	E- C2-WT	YALYDATYETK	cofilin-1 a2a1	F- C2-H222P	ASGVAVSDGVIK	cofilin-1 a4a9	F- C2-H222P	LGGSAVISLEGKPL
cofilin-1 a1b14	C- C2-H222P	YALYDATYETK	cofilin-1 a2a1	F- C2-H222P (selumetinib)	ASGVAVSDGVIK	cofilin-1 a4a9	C- C2-WT	LGGSAVISLEGKPL
cofilin-1 a1b14	C- C2-WT	YALYDATYETK	cofilin-1 a2a1	D- C2-WT	ASGVAVSDGVIK	cofilin-1 a4a9	E- C2-WT	LGGSAVISLEGKPL
cofilin-1 a1b14	D- C2-WT	YALYDATYETK	cofilin-1 a2a1	I- C2-WT	ASGVAVSDGVIK	cofilin-1 a4a9	D- C2-H222P	LGGSAVISLEGKPL
cofilin-1 a1b14	D- C2-H222P	YALYDATYETK	cofilin-1 a2a1	D- C2-H222P	ASGVAVSDGVIK	cofilin-1 a4a9	A- C2-H222P (selumetinib)	LGGSAVISLEGKPL
cofilin-1 a1b14	B- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a2a1	C- C2-WT	ASGVAVSDGVIK	cofilin-1 a4a9	D- C2-H222P	LGGSAVISLEGKPL
cofilin-1 a1b14	D- C2-H222P	YALYDATYETK	cofilin-1 a2a1	D- C2-H222P	ASGVAVSDGVIK	cofilin-1 a4a9	I- C2-H222P	LGGSAVISLEGKPL
cofilin-1 a1b14	D- C2-WT	YALYDATYETK	cofilin-1 a2a1	I- C2-H222P	ASGVAVSDGVIK	cofilin-1 a4a9	A- C2-H222P	LGGSAVISLEGKPL
cofilin-1 a1b14	D- C2-H222P	YALYDATYETK	cofilin-1 a2a1	A- C2-WT	ASGVAVSDGVIK	cofilin-1 a4a9	I- C2-H222P	LGGSAVISLEGKPL
cofilin-1 a1b14	D- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a2a1	D- C2-H222P (selumetinib)	ASGVAVSDGVIK	cofilin-1 a4a9	D- C2-H222P	LGGSAVISLEGKPL
cofilin-1 a1b14	F- C2-H222P	YALYDATYETK	cofilin-1 a2a1	A- C2-H222P (selumetinib)	ASGVAVSDGVIK	cofilin-1 a4a9	D- C2-H222P (selumetinib)	LGGSAVISLEGKPL
cofilin-1 a1b14	A- C2-WT	YALYDATYETK	cofilin-1 a2a1	A- C2-H222P (selumetinib)	ASGVAVSDGVIK	cofilin-1 a4a9	A- C2-WT	LGGSAVISLEGKPL
cofilin-1 a1b14	A- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a2a1	I- C2-WT	ASGVAVSDGVIK	cofilin-1 a4a9	D- C2-H222P (selumetinib)	LGGSAVISLEGKPL
cofilin-1 a1b14	F- C2-H222P	YALYDATYETK	cofilin-1 a2a1	E- C2-WT	ASGVAVSDGVIK	cofilin-1 a4a9	E- C2-WT	LGGSAVISLEGKPL

cofilin-1 a1b14	D- C2-H222P	YALYDATYETK	cofilin-1 a2a1	D- C2-H222P (selumetinib)	ASGVAVSDGVIK	cofilin-1 a4a8	D- C2-H222P	LGGSAVISLEGK
cofilin-1 a1b14	D- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a2b11	A- C2-H222P (selumetinib)	SGVAVSDGVIK	cofilin-1 a5a7	D- C2-H222P	HELQANCYEEVKDR
cofilin-1 a1b14	I- C2-H222P	YALYDATYETK	cofilin-1 a2b11	A- C2-H222P	SGVAVSDGVIK	cofilin-1 a5a7	I- C2-H222P	HELQANCYEEVKDR
cofilin-1 a1b14	C- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a2b12	A- C2-H222P (selumetinib)	SGVAVSDGVIK	cofilin-1 a5a7	D- C2-H222P	HELQANCYEEVKDR
cofilin-1 a1b14	C- C2-H222P	YALYDATYETK	cofilin-1 a3a4	D- C2-H222P	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	D- C2-H222P (selumetinib)	HELQANCYEEVKDR
cofilin-1 a1b14	A- C2-H222P	YALYDATYETK	cofilin-1 a3a4	A- C2-WT	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	B- C2-WT	HELQANCYEEVKDR
cofilin-1 a1b14	D- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a3a4	A- C2-WT	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	D- C2-WT	HELQANCYEEVKDR
cofilin-1 a1b14	A- C2-H222P	YALYDATYETK	cofilin-1 a3a4	A- C2-H222P (selumetinib)	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	D- C2-H222P	HELQANCYEEVKDR
cofilin-1 a1b14	B- C2-WT	YALYDATYETK	cofilin-1 a3a4	E- C2-WT	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	A- C2-WT	HELQANCYEEVKDR
cofilin-1 a1b14	F- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a3a4	A- C2-H222P	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	F- C2-H222P (selumetinib)	HELQANCYEEVKDR
cofilin-1 a1b14	I- C2-H222P	YALYDATYETK	cofilin-1 a3a4	D- C2-WT	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	A- C2-H222P (selumetinib)	HELQANCYEEVKDR
cofilin-1 a1b14	E- C2-WT	YALYDATYETK	cofilin-1 a3a4	I- C2-H222P	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	D- C2-WT	HELQANCYEEVKDR
cofilin-1 a1b14	C- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a3a4	E- C2-WT	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	A- C2-H222P	HELQANCYEEVKDR
cofilin-1 a1b14	D- C2-H222P	YALYDATYETK	cofilin-1 a3a4	A- C2-H222P (selumetinib)	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	F- C2-H222P	HELQANCYEEVKDR
cofilin-1 a1b14	E- C2-WT	YALYDATYETK	cofilin-1 a3a4	D- C2-H222P	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	C- C2-H222P	HELQANCYEEVKDR
cofilin-1 a1b14	A- C2-WT	YALYDATYETK	cofilin-1 a3a4	D- C2-H222P	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	D- C2-H222P (selumetinib)	HELQANCYEEVKDR
cofilin-1 a1b14	D- C2-WT	YALYDATYETK	cofilin-1 a3a4	D- C2-H222P (selumetinib)	EILVGDVGQTVDDPYTTFVK	cofilin-1 a5a7	A- C2-H222P	HELQANCYEEVKDR
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cofilin-1 a1b14	D- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a3a4	D- C2-H222P (selumetinib)	EILVGDVGQTV DDPYTTFVK	cofilin-1 a5a6	A- C2-H222P (selumetinib)	HELQANCYEEVK
cofilin-1 a1b14	D- C2-WT	YALYDATYETK	cofilin-1 a3a4	F- C2-H222P	EILVGDVGQTV DDPYTTFVK	cofilin-1 a6b10	A- C2-WT	KSSTPEEVKK
cofilin-1 a1b14	C- C2-WT	YALYDATYETK	cofilin-1 a3a4	A- C2-H222P (selumetinib)	EILVGDVGQTV DDPYTTFVK	cofilin-1 a6b13	D- C2-H222P	SSTPEEVKK
cofilin-1 a1b14	B- C2-H222P	YALYDATYETK	cofilin-1 a3a4	D- C2-WT	EILVGDVGQTV DDPYTTFVK	cofilin-1 a6b13	E- C2-WT	SSTPEEVKK
cofilin-1 a1b14	D- C2-H222P (selumetinib)	YALYDATYETK	cofilin-1 a3a4	D- C2-H222P (selumetinib)	EILVGDVGQTV DDPYTTFVK	cofilin-1 a6b13	D- C2-H222P (selumetinib)	SSTPEEVKK
cofilin-1 a1b16	D- C2-WT	YALYDATYETKE SK	cofilin-1 a3a4	D- C2-H222P (selumetinib)	EILVGDVGQTV DDPYTTFVK	cofilin-1 a6b13	A- C2-WT	SSTPEEVKK
cofilin-1 a1a5	D- C2-H222P (selumetinib)	ESKKEDLVFIFWA PENAPLK	cofilin-1 a3a4	D- C2-WT	EILVGDVGQTV DDPYTTFVK	cofilin-1 a7a2	D- C2-H222P	AVLFCLSEDKK

Table S3. Quantification of left ventricular function in transduced *Lmna* WT and H222P

Treatment	n	Heart rate (beats/min)	LVEDD (mm)	LVESD (mm)	FS (%)
***Lmna* WT**					
NT	5	374.8 ± 17.6	3.7 ± 0.1	2.1 ± 0.1	44.7 ± 1.8
AAV-cofilin WT	5	338.6 ± 41.7	3.8 ± 0.3	2.4 ± 0.1 **	36.8 ± 0.9 ***
***Lmna* H222P**					
NT	3	231.7 ± 64.7	4.8 ± 0.7	3.9 ± 0.9	17.9 ± 5.2
AAV-cofilin WT	4	219.5 ± 21.1	4.9 ± 0.4	4.2 ± 0.4	12.2 ± 1.9
AAV-cofilin T25A	5	238.7 ± 49.1	4.4 ± 0.5	3.7 ± 0.5	16.6 ± 0.7 #

*P<0.05, **P<0.005, ***P<0.0005 between non treated and AAVrh10-cofilinWT-treated Lmna WT mice.

#P<0.05 between AAVrh10-cofilinWT-treated and AAVrh10-cofilinT25A-treated Lmna H222P mice.

SUPPLEMENTAL FIGURES

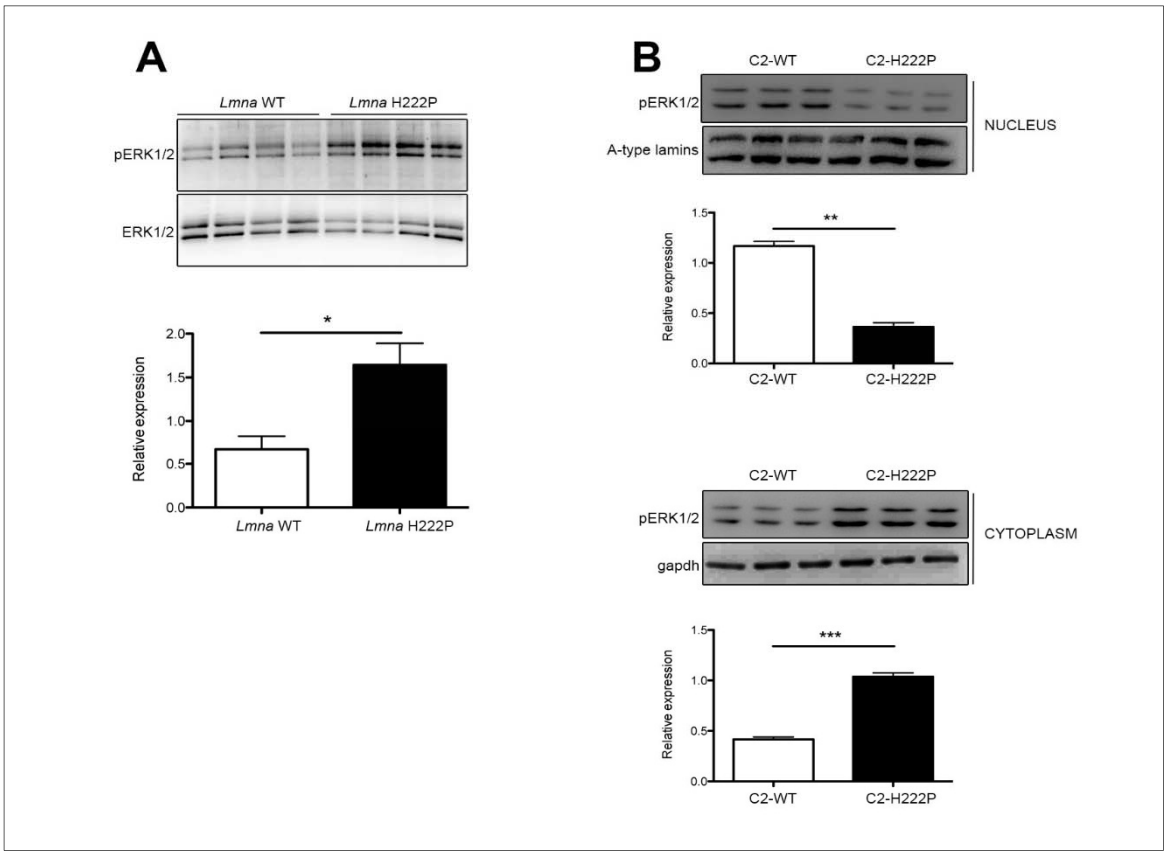

Figure S1. Aberrant ERK1/2 activation in cytoplasm of C2-H222P cells
(A) Immunoblots illustrating increased pERK1/2 protein in hearts from *Lmna*$^{H222P/H222P}$ mice. Representative of three independent experiments each performed in triplicates.*P<0.005.
(B) Immunoblots showing pERK1/2 and total ERK1/2 in extracts of nucleus and cytoplasm from C2-WT and C2-H222P cells. **P<0.005, ***P<0.0005.

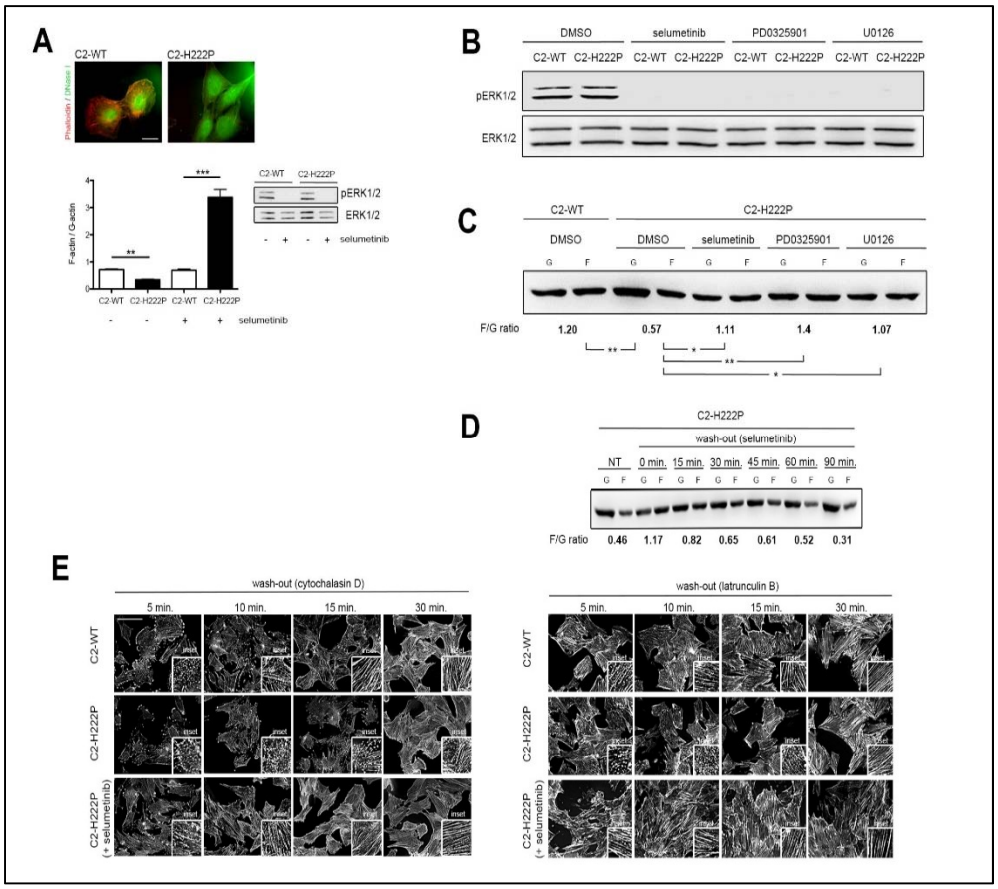

Figure S2. ERK1/2 signaling modulates F-actin dynamics

(A) Representative immunofluorescence micrographs showing phalloidin (F-actin) and DNase I (G-actin) staining of C2-WT and C2-H222P cells, with the resulting F/G actin ratio quantification shown below. Scale bar, 10 μm. The inhibition of ERK1/2 signaling by selumetinib results in decreased pERK1/2 (immunoblot) and an increase of the F/G actin ratio in C2-H222P cells. $^{**}P<0.005$, $^{***}P<0.0005$.

(B) Immunoblots illustrating the effect of several inhibitors of ERK1/2 signaling on the pERK1/2 level. DMSO: dimethylsulfoxide. Representative of three independent experiments each performed in triplicates.

(C) Immunoblot illustrating the effects of inhibitors of ERK1/2 signaling on the amounts of G-actin and F-actin and the calculated F/G actin ratio. Representative of three independent experiments each performed in triplicates. $^{*}P<0.05$, $^{**}P<0.005$.

(D) Representative immunoblot illustrating the effect of wash-out of selumetinib on the amounts of G-actin and F-actin and the calculated F/G actin ratio on C2-H222P cells.

(E) Micrographs of C2-WT and C2-H222P cells during cytochalasin D or latrunculin B washout experiments showing a delay of F-actin recovery in C2-H222P cells, which is blunted by treating cells with selumetinib (insets). Micrographs were take immediately after wash-out (0 min) and 5 min, 10 min, 15 min and 30 min later. Scale bars, 10 μm.

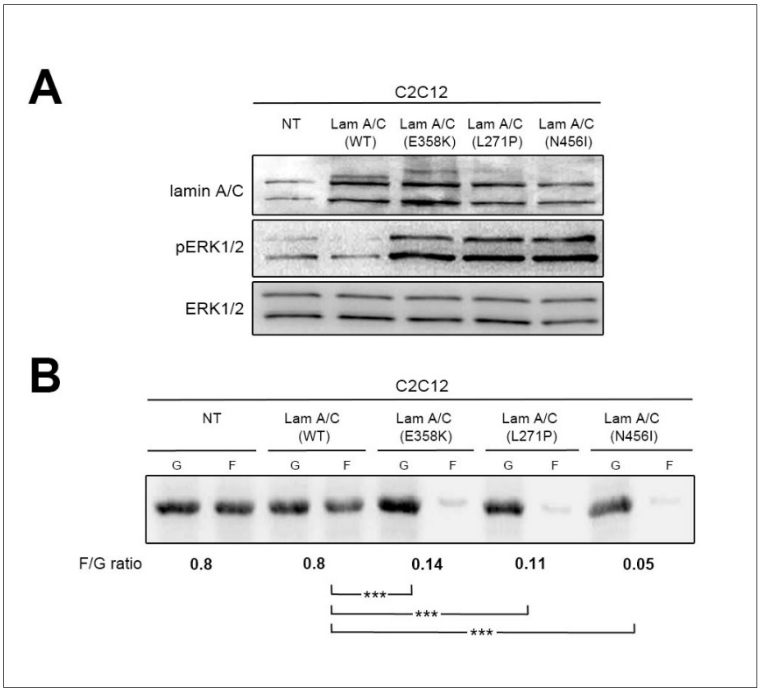

Figure S3. Lamin A variants modulate F-actin dynamics

(A) Immunoblots illustrating the effects of lamin A variants on the pERK1/2 levels. Representative of three independent experiments each performed in triplicates.

(B) Immunoblots illustrating the effects of lamin A variants on the amounts of G-actin and F-actin and the calculated F/G actin ratio. Representative of three independent experiments each performed in triplicates. ***P<0.0005. NT indicates not transfected.

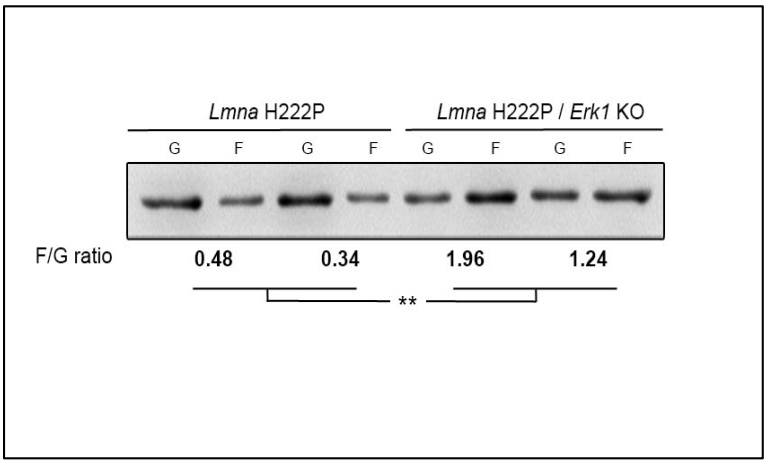

Figure S4. Depletion of *Erk1* in *Lmna*$^{H222P/H222P}$ mice improves the F-actin dynamics in heart

Immunoblot illustrating the effect of loss of *Erk1* on the amounts of G-actin and F-actin and the calculated F/G actin ratio in hearts of *Lmna*$^{H222P/H222P}$ mice. Representative of three independent experiments each performed in triplicates. **P<0.005.

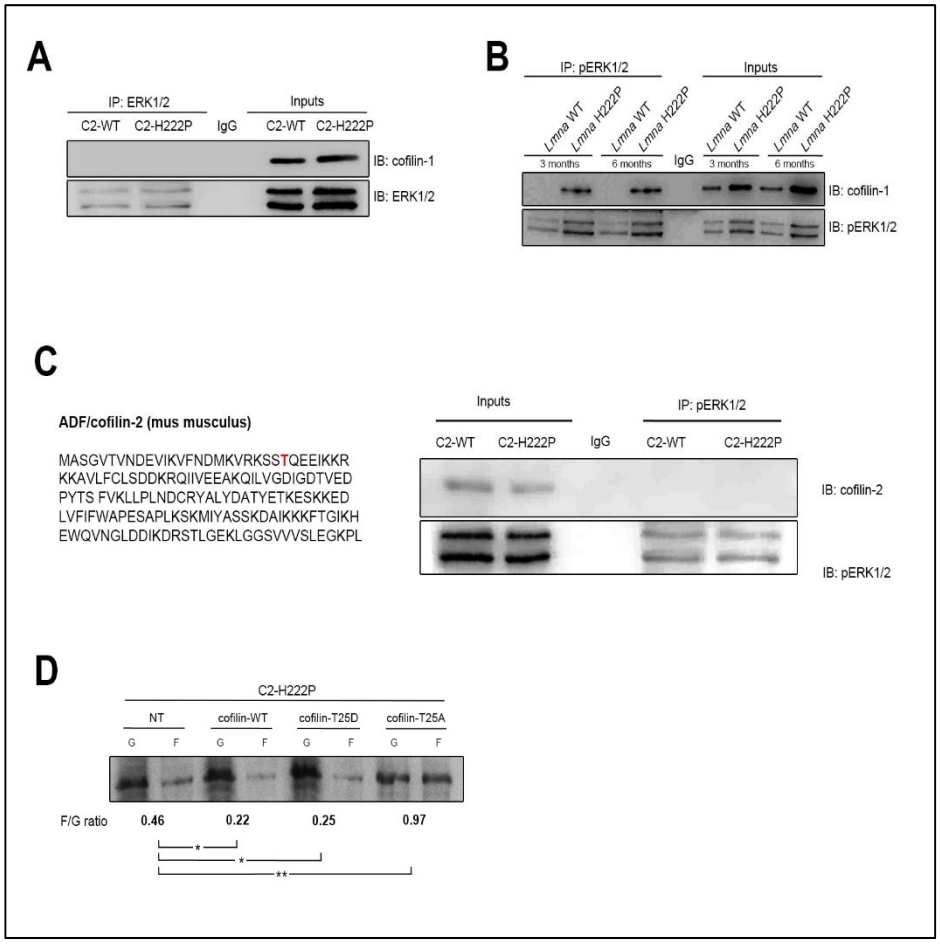

Figure S5. The phosphorylated form of ERK1/2 binds, phosphorylates cofilin-1 but not cofilin-2 on Thr25 to activate its actin depolymerizing activity

(A) Proteins extracted from C2C12 cells were subjected to immunoprecipitation using antibodies against ERK1/2. The immunoprecipitates were separated by SDS-PAGE and immunoblotted using antibodies against cofilin-1 and ERK1/2. The immunoprecipitation was carried out with IgG as negative control.

(B) Proteins extracted from *Lmna* WT and *Lmna* H222P (at 3 months and 6 months) cells were subjected to immunoprecipitation using antibodies against pERK1/2. The immunoprecipitates were separated by SDS-PAGE and immunoblotted using antibodies against cofilin-1 and ERK1/2. The immunoprecipitation was carried out with IgG as negative control.

(C) Amino acid sequence of murine ADF/cofilin-2 with highlighted Threonine 25 (red). Proteins extracted from C2C12 cells were subjected to immunoprecipitation using antibodies against pERK1/2. The immunoprecipitates were separated by SDS-PAGE and immunoblotted using antibodies against cofilin-1 or pERK1/2. The immunoprecipitation was carried out with IgG as negative control.

(D) Representative immunoblot illustrating the effect of different cofilin constructs on the amounts of G-actin and F-actin and the calculated F/G actin ratio. Representative of three independent experiments each performed in triplicates. **P*<0.05, ***P*<0.005.

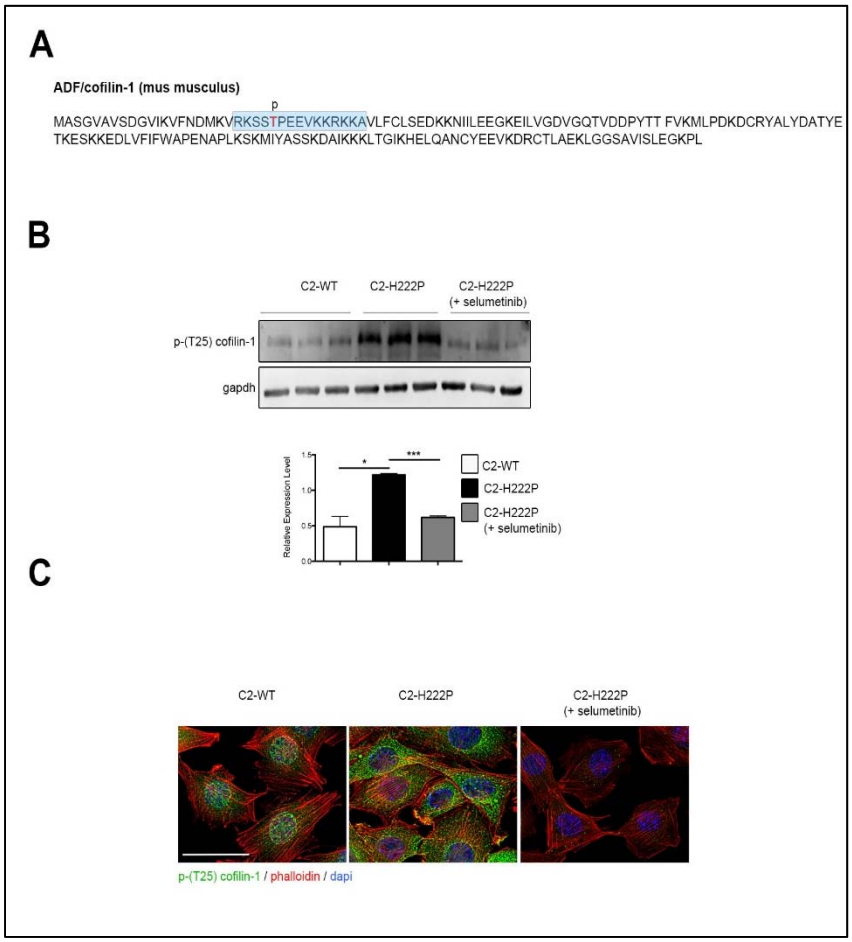

Figure S6. Characterization of newly generated antibody against phosphorylated (Thr25)ADF/cofilin-1

(A) Amino acid sequence of murine ADF/cofilin-1 and peptide recognized by the specific phosphorylated Thr25 antibody (highlighted in light blue with Thr25 in red). "p" indicates the phosphorylation of Thr25 residue.

(B) Immunoblots using antibodies phosphorylated (Thr25)ADF/cofilin-1 antibody to probe proteins extracted from C2-WT, C2-H222P and C2-H222P cells treated with selumetinib. Gapdh is the loading control. Representative of three independent experiments each performed in triplicates. The bar graph represents protein relative expression (means ± standard errors of means) of phosphorylated (Thr25)ADF/cofilin-1 (relative to gapdh). **P*<0.05, ****P*<0.0005.

(C)Representative immuofluorescence microscopy images of phosphorylated (Thr25)ADF/cofilin-1 (green) and phalloidin (red) staining of C2-WT, C2-H222P and C2-H222P cells treated with selumetinib. Nuclei counter-stained with 4',6-diamidino-2-phenylindole (dapi) are also shown. Scale bar, 25 □m.

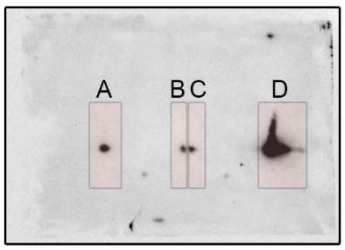

Figure S7. Protein spots revealed by the antibody against ADF/cofilin-1 that were excised and subjected to in-gel digestion and Orbitrap hybrid mass spectrometry analysis

Spots (indicated by pink rectangles A to D) detected by ADF/cofilin-1 antibody from C2-H222P that were excised for further proteomics analysis (see Table 1).

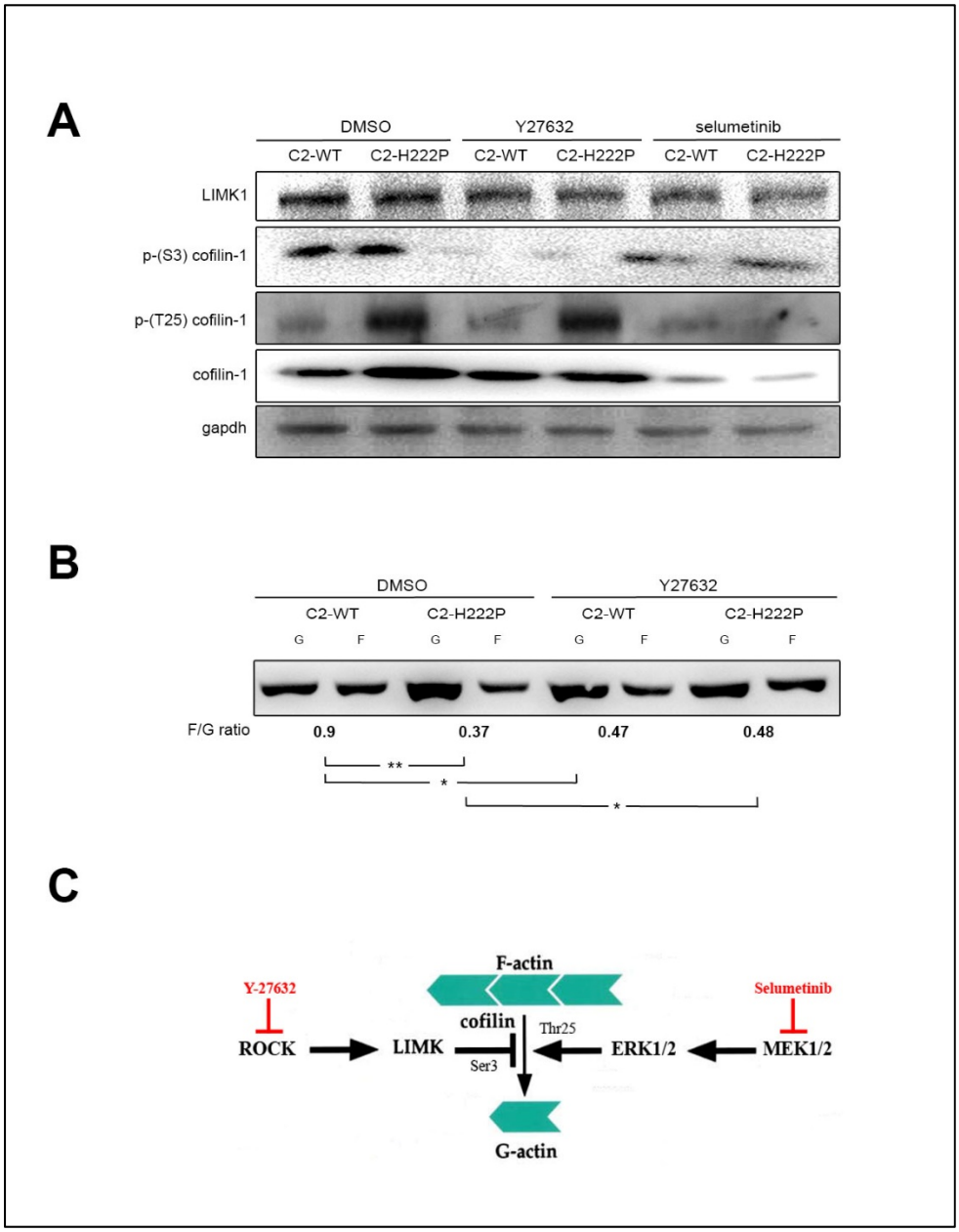

Figure S8. Phosphorylated (Ser3)ADF/cofilin-1 through ROCK signaling is not involved in the development of *LMNA* cardiomyopathy

(A) Representative immunoblots illustrating the effect of Y27632 and selumetinib on the amount of LIMK1, phosphorylated (Ser3)ADF/cofilin-1, phosphorylated (Thr25)ADF/cofilin-1 and cofilin-1 expression level in C2-WT and C2-H222P cells. Representative of three independent experiments each performed in triplicates.

(B) Representative immunoblot illustrating the effect of Y27632 the amounts of G-actin and F-actin and the calculated F/G actin ratio. Representative of three independent experiments each performed in triplicates. **P*<0.05, ***P*<0.005.

(C) Schematic representation of the phosphorylation of ADF/cofilin-1 by ROCK signaling on Ser3 and by ERK1/2 on Thr25 and the consequences on actin dynamics.

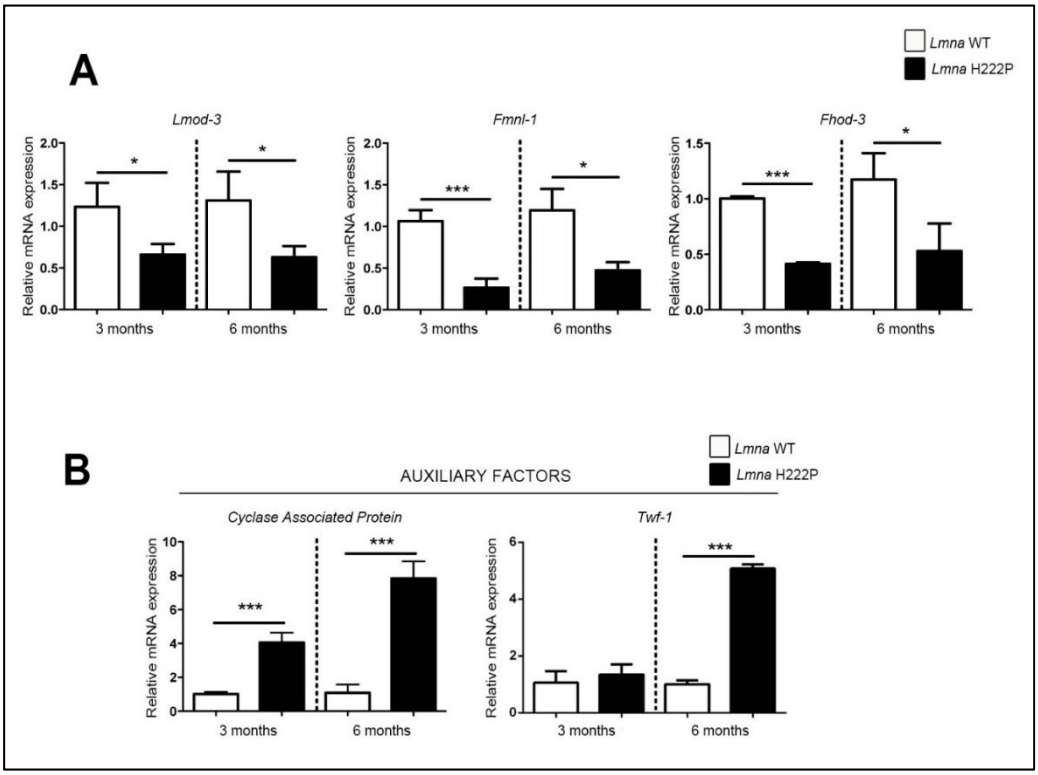

Figure S10. Altered expression of modulators of actin dynamics in *LMNA* cardiomyopathy

(A) Expression of *Lmod-3*, *Fmnl-1* and *Fhod-3* mRNA in hearts from 3 month-old and 6 month-old male *Lmna* WT and *Lmna* H222P mice Bars indicate means ± standard errors of means (n=9). **P*<0.05, ****P*<0.0005.
(A) Expression of *CAP2* and *Twf-1* mRNA in hearts from 3 month-old and 6 month-old male *Lmna* WT and *Lmna* H222P mice. Bars indicate means ± standard errors of means (n=9). ****P*<0.0005.

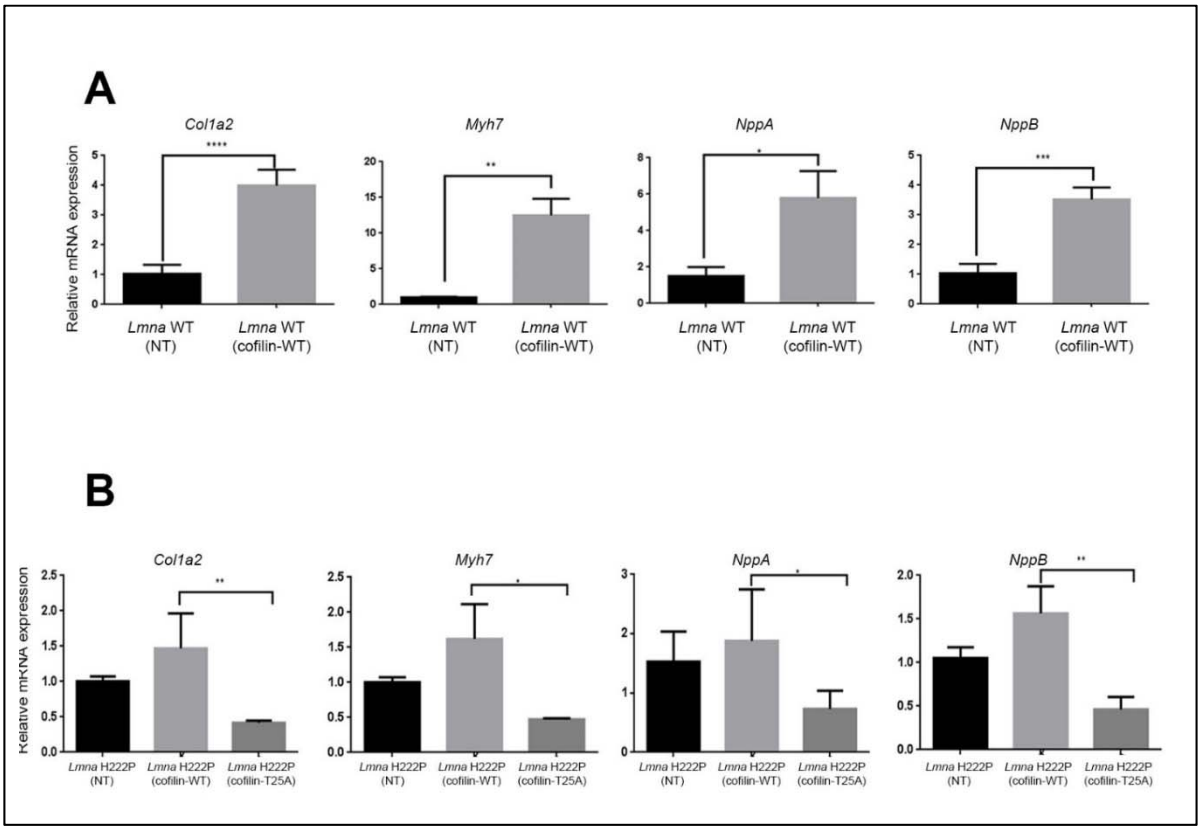

Figure S11. Phosphorylated (T25)ADF/cofilin-1 functions in cardiac remodelling in *LMNA* cardiomyopathy
(A) Expression of *Col1a1*, *Myh7*, *NppA* and *NppB* mRNA in hearts from 3 month-old and 6 month-old male $Lmna^{+/+}$ mice (*Lmna* WT) transduced with AAVrh10 expressing cofilin-WT construct. Bars indicate means ± standard errors of means (n=4). **P*<0.05, ***P*<0.005, ****P*<0.0005, *****P*<0.00005.
(B) Expression of *Col1a1*, *Myh7*, *NppA* and *NppB* mRNA in hearts from 3 month-old and 6 month-old male $Lmna^{+/+}$ mice (*Lmna* WT) transduced with AAVrh10 expressing cofilin-WT or AAVrh10 expressing cofilin-T25A constructs. Bars indicate means ± standard errors of means (n=3). **P*<0.05, ***P*<0.005.

Chapter 3 Discussion

LMNA-cardiomyopathy is caused by mutations in *LMNA* and is characterized by left ventricular dilatation and reduced systolic function accompanied by conduction system disease and/or arrhythmias. Mouse lines carrying mutations on *LMNA* gene have been a valuable resource in expanding our knowledge into novel functions of the nuclear lamina and are extremely helpful in exploring potential avenues that may offer therapies for the patients. Here, we used $Lmna^{H222P/H222P}$ knock-in mouse model of *Lmna*-cardiomyopathy. Previous work on the hearts of these mice showed that abnormal activation of ERK1/2 signaling is involved in the pathophysiology of left-ventricular contractile dysfunction and development of fibrosis. However, the intriguing question arises at this point is how impaired activated ERK1/2 signaling leads to cardiac pathogenesis. Our studies shed some light on this question. The present results showed that hyperactivation of cardiac ERK1/2 participates in TGF-β-mediated CTGF expression involved in the fibrosis development. Moreover, further data showed that ERK1/2 phosphorylates and activates ADF/cofilin-1 leading in to alteration of cardiac actin dynamics which might involved in the development of cardiac dysfunction.

ERK1/2 signaling plays role in the fibrosis

Myocardial fibrosis involves an increase in ECM deposition that adversely affects the function of the heart by disrupting electrical conductivity and mechanical performance. One of the cytokines of major importance in the pathogenesis of myocardial fibrosis is transforming growth factor (TGF-β). The role of TGF-b as a potent profibrotic cytokine has been demonstrated (Epstein et al. 1994) and up-regulation of TGF-β and its downstream Smad cascade is prevalent in many fibrotic diseases affecting the heart, lung, kidney, and liver (Epstein et al. 1994; Dooley & Ten Dijke 2012; Tatler & Jenkins 2012; Khalil & Greenberg 1991; Lan 2011; Khan & Sheppard 2006; Lijnen et al. 2000). In the context of the heart, fibrosis can cause defects of ventricular properties that causes both systolic and diastolic dysfunction (Klein et al. 2005). In addition, the excess of extracellular matrix proteins results in an alteration of ventricular properties (Klein et al. 2005) and in a non-homogeneous milieu for electrical propagation leading to arrhythmias (Verheule et al. 2004).

Here, I showed that cardiac tissue from *Lmna*$^{H222P/H222P}$ mice displayed elevated expression of TGF-β1 and TGF-β2 and activation of TGF-β/Smad signaling with increased p-Smad2/3 in the nucleus, as early as 12 weeks of age. Few other studies demonstrated that patients with dilated cardiomyopathy exhibited increased myocardial TGF-β1 and TGF-β2 expression (Pauschinger et al. 1999) and higher myocardial TGF-β1 levels were associated with absence of recovery in patients with dilated cardiomyopathy undergoing left ventricular assist device implantation (Felkin et al. 2009). However, the mechanism of the deregulated TGF-β signaling in the presence of mutated lamin A/C is still uncertain. It is known that A-type lamins can modulate TGF-β signaling through interaction with protein phosphatase 2A (PP2A) which can dephosphorylate R-Smads (Andrés & González 2009). In addition, Lin and colleagues indicated that MAN1, an integral protein of the inner nuclear membrane, binds to Smad 1 or Smad 2 and Smad 3 and inhibits BMP and TGF-β signaling, respectively (Raju et al. 2003; Osada 2003; Lin et al. 2005). MAN1 is predicted to be able to bind DNA and R-Smads simultaneously (Caputo et al. 2006). It has been also proposed that MAN1 is in complex with a phosphatase and the recruitment of R-Smads to MAN1 causes disassembly of the R-Smad–Smad4 complex, dephosphorylation of Smads and increased nuclear export (Pan et al. 2005). Given that MAN1 is associated with the nuclear lamina (Paulin-Levasseur et al. 1996; Bengtsson 2007), further investigation needs to be done. A hypothetical model is illustrated on **Figure 22**.

Several strategies to inhibit the TGF-β signaling pathway have been proposed (Pohlers et al. 2009); among which inhibitors of ALK-5/TβRI have been described (Inman et al. 2002). ALK5 phosphorylates the R-Smads (Smad 2 and Smad 3), downstream mediators of TGF-β signaling, which drive transcription of collagen genes, thereby triggering matrix deposition. SB-431542 has been characterized as a potent and selective inhibitor of ALK5/TβRI receptor that prevents TGFβ-induced Smad phosphorylation (Grygielko et al. 2005); inhibition of ALK-4 and ALK-7 was also noted (Callahan et al. 2002). To date, ALK5 inhibitors have been tested for therapeutic potentials including fibrosis (Hjelmeland et al. 2004; Laping 2002). It was therefore tempting to speculate that inhibition of TGF-β signaling via ALK5 inhibition may have a beneficial impact on cardiac fibrosis in *LMNA* cardiomyopathy. SB-421542-treated *Lmna*$^{H222P/H222P}$ have attenuated myocardial fibrosis and improved cardiac function. Hence, we showed that TGF-β signaling and downstream Smads play a mediator of *LMNA* cardiomyopathy.

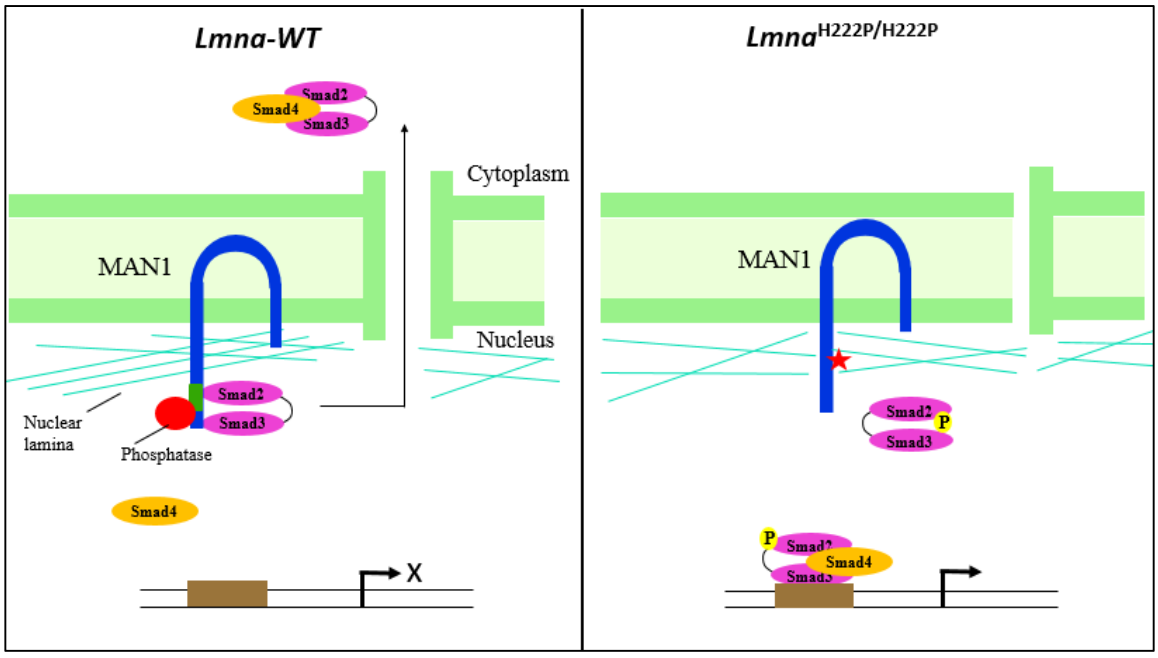

Figure 22: Hypothetical model of increased TGF-β signaling *Lmna*$^{H222P/H222P}$ mice. MAN1 disrupts R-Smad–Co-Smad complexes, induces dephosphorylation of R-Smads in order to attenuate the Smad-mediated signal and inhibitis the transcription of Smad-target genes. The *Lmna* mutation, we hypothesize that disrupts the interaction between MAN1 and R-Smads and abrogates the inhibitory effect of MAN1 on Smad signaling.

In addition to Smads, TGF-β has been shown to activate MAPK pathways by a Smad-independent manner (Mulder, 2000). TGF-β can activate distinct MAPKs including ERK1/2, c-Jun N-terminal kinase (JNK), and p38 in various types of cells. Previous studies reported that TGF-β1 may activate the ERK1/2 pathway through upstream factors, such as Ras/Raf, or directly through more downstream factors, such as MEK (Xie et al. 2004). Moreover, results from another study showed that TβRI directly phosphorylates ShcA to induce its association with Grb2/Sos, thus initiating the pathway known to link receptor tyrosine kinases to ERK1/2 kinases (Lee et al. 2007). In contrast, Hayashida et al. have raised the possibility of positive-crosstalk between the Smad and MAPK pathways for TGF-β-stimulated collagen gene expression (Hayashida et al. 1999). Further studies provided evidence that TGF-β-induced CTGF expression relies on an interplay between Smad3 and ERK1/2 (Leivonen et al. 2005). My work showed that abnormal overexpression of ERK1/2 occurs via TGF-β/Smad signaling since SB-431542 partially decreased the activated ERK1/2 cascade. Nevertheless, the cross-talk between TGF-β and MAPK pathways is not yet entirely elucidated. Studies described that activated ERK1/2 phosphorylates the linker region domains of

Smad2 and Smad3 and not the usual C-terminal SxS motif, which is phosphorylated by ALK5, resulting in the maintenance of Smad-mediated transcriptional activation (Blanchette et al. 2001). We next investigated the possible involvement of the ERK1/2 pathway in the activation of TGF-β signaling. Upon inhibition of ERK1/2 phosphorylation, the expression of p-Smad2/3, suggesting that a feedback control could exist between ERK1/2 and TGF-β.

CTGF (also known as CCN2) is a well-characterized downstream mediator of TGF-β action in connective tissue cells during the fibrotic response. Overexpression of CTGF has been also observed in fibrotic disorders of the liver, kidney, lung, skin and pancreas. In the heart, CTGF/CCN2 is secreted into the ECM by both fibroblasts and cardiomyocytes and has been shown to be an important mediator of TGF-β signaling. Similarly, CTGF is also increased in cardiac fibrosis and failing hearts (Ohnishi et al. 1998; Koshman et al. 2013). Cardiac tissue from *Lmna*$^{H222P/H222P}$ mice exhibited increased CTGF/CCN2 protein expression. There have been a number of studies examining the effects of overexpression of CTGF/CCN2 in cardiomyocytes. However, the data arising from these studies are contradictory. Studies using transgenic mice overexpressing CTGF/CCN2 pointed towards a cardio-protective effect of CTGF/CCN2, reporting similar amount of fibrosis after myocardial infarction or pressure overload (Gravning et al. 2012; Gravning et al. 2013; Panek et al. 2009). On the contrary, a recent study found that cardiac specific CTGF deletion in mice did not affect fibrosis upon pressure overload stimulation (Accornero et al. 2015). The exact reasons for these discrepancies remain unknown.

We further explored the effects of CTGF depletion by the usage of a small molecule CTGF inhibitor, namely FG-3019. Although it has been indicated that CTGF binds to TGF-β and enhances its activity, treatment with FG-3019 had no effect on Smad2/3 signaling in these mice. Furthermore, we investigated the effects of CTGF inhibition on cardiac function and showed a beneficial effect in *LMNA* cardiomyopathy. Our findings are in line with a recent study stating that pharmacologic inhibition of CTGF/CCN2 in a DCM mouse model significantly improved the cardiac function (Koshman et al. 2015). More interestingly, FG-3019 treatment of *mdx* mice decreases fibrosis and improves the strength of skeletal muscle (Morales et al. 2013). Hence, inhibiting CTGF/CCN2 could be beneficial for patients with *LMNA* cardiomyopathy by reducing cardiac fibrosis.

There is evidence that activation of ERK1/2 pathway is required for activation of CTGF promoter by TGF-β in fibroblasts (Leask et al. 2003). We therefore sought to determine whether ERK1/2 drives the expression of CTGF/CCN2 in the hearts of *Lmna*$^{H222P/H222P}$ mice. According to our results, inhibition of ERK1/2 phosphorylation lowers myocardial fibrosis by decreasing the expression of CTGF/CCN2 and the transcription from the CTGF/CCN2 promoter. Our findings are in agreement with a number of studies showing that CTGF-dependent fibrosis involves the ERK1/2 pathway (Ponticos et al. 2009; Sonnylal et al. 2010; Nakerakanti et al. 2011). Further reports by Hu and colleagues, indicated that pERK1/2 inhibition significantly inhibited CTGF-induced α-smooth muscle actin (α-SMA) and collagen I expression in human fibroblasts (Hu et al. 2013). Although the mechanism of ERK-induced CTGF activation is not fully investigated so far, it has been suggested that ERK1/2 might modulate transcription factors that act with Smad3 to activate the gene expression of CTGF. However, understanding the underlying mechanisms by which ERK1/2 signaling potentiates the CTGF expression, might be useful in developing novel anti-fibrotic therapeutics.

Collectively, the present findings revealed that inhibition of either TGF-β/Smad signaling, or ERK1/2 signaling or CTGF expression suppressed cardiac fibrosis and attenuated cardiac dysfunction, suggesting that these three components are part of a cascade which plays role in the progression of *LMNA*-cardiomyopathy by modulating the development of myocardial fibrosis.

ERK1/2 plays role in actin dynamics

pERK1/2 phosphorylates substrates (e.g. myosin light chain kinase (MCLK), focal-adhesion kinase (FAK), paxillin, the calpain protease, as well as nuclear proteins and transcription factors) on serine or threonine residues followed by proline. Here, I showed that pERK1/2 phosphorylates ADF/cofilin-1, at a new phosphorylation site and thus, plays a pivotal role in the regulation of actin dynamics. We further demonstrated that ADF/cofilin-1 expression and phosphorylation is increased in *LMNA* cardiomyopathy, leading to elevated G-actin pool.

Cofilin is a crucial regulator of actin that promotes F-actin filament severing and depolymerization and belongs to (actin-depolymerizing factor) ADF family. The severing activity of cofilin is inhibited by several mechanisms including phosphorylation at the residue Ser3 of cofilin. Four

kinases have been identified so far that block the activity of cofilin: LIM kinases 1 and 2 and TES kinases 1 and 2 (J Toshima et al. 2001; Jiro Toshima et al. 2001; Bernard 2007). Here, we describe a novel regulatory mechanism of ADF/cofilin-1 showing that phosphorylation at the residue Threonine 25 by pERK1/2 can activate its severing/depolymerizing activity. It is also unclear whether ADF/cofilin-1 could be regulated by other post-translational modifications and whether these may have a role in the regulation of ADF/cofilin-1 activity on actin dynamics. Interestingly, our mass spectrometry data encourage further approaches to advance our understanding of other phosphorylation sites of ADF/cofilin-1 occurring at the physiological and pathological stages and their role in the regulation of actin dynamics. For instance, phosphorylation on Ser 23 occurs in C2-WT but not in C2-H222P cells and this might be regulated also by pERK1/2 as shown in the C2-H222P-treated cells (page 123). In addition, our mass spectrometry analysis provide new phosphorylation sites of ADF/cofilin-1 such as Ser156, Thr63, Thr88 and Y82 and offer new directions for further research.

In muscle cells, actin-based thin filaments and myosin-based thick filaments are assembled into the repeated units of contractile apparatus known as sarcomeres. The actin filaments in sarcomeres are capped by CapZ at the barbed ends and by tropomodulin at the pointed ends; both of the capping proteins regulate the lengths of sarcomeric actin filaments (**Figure 17**). Although sarcomeric actin structures are believed to be relatively non-dynamic, many proteins promoting actin dynamics are also expressed in muscle cells. However, the exact mechanisms of the sarcomere turnover are largely unknown. Despite the fact that the role of ADF/cofilins in non-muscle actin dynamics is relatively well understood, their role in striated muscles remain unclear. It has been proposed that ADF/cofilins depolymerize the non-productive actin filaments in contraction and may also contribute to remodeling of non-sarcomeric cytoskeleton in muscle cells (Skwarek-Maruszewska et al. 2009). It is possible that the non-muscle cofilin-1 promotes actin dynamics differently from its muscle-specific counterpart.

Cofilin-2 is the isoform which is expressed in striated muscle (Ono et al. 1994; Vartiainen et al. 2002). There is a rapidly growing literature on the role of cofilin-2 on muscle actin dynamics. Mutation causes disruption of the sarcomeric architecture in mice (Agrawal et al. 2012) and nemaline myopathy in humans characterized by the presence of cytoplasmic actin-cofilin rods (Agrawal et al. 2007) indicating that correct regulation of sarcomeric actin filaments is crucial for normal function of striated muscle. It has been also demonstrated that cofilin-2 controls the length

of thin filaments in the contractile apparatus (Kremneva et al. 2014). While some researchers reported the binding of ADF/cofilin-1 on the sarcomere (Skwarek-Maruszewska et al. 2009), its function is still barely known. Accordingly, one group demonstrated severely disorganized sarcomeric actin filaments in ADF/cofilin-1 knockdown cardiomyocytes, implying that ADF/cofilin-1 plays also role in the sarcomere integrity and organization (Skwarek-Maruszewska et al. 2009). In our study we showed that ADF/cofilin-1 and not cofilin-2 is responsible for the cardiac sarcomere disorganization and is involved in the pathogenesis of *LMNA* cardiomyopathy.

Apart from ADF/cofilin-1, the transcript levels of different other factors of the actin dynamics machinery are also affected in *Lmna*$^{H222P/H222P}$ mice along the progression of cardiac dysfunction. In particular, down-regulation of the actin nucleators, *Lmod3*, *Fhod3* and *Fmnl1* along with an up-regulation of CAP2 and *Twf-2b* have been observed. Lmod3 promotes sarcomeric actin filament elongation and mutation in *Lmod3* results in disorganization of F-actin filaments leading to nemaline myopathy in human (Yuen et al. 2014). Fhod3 regulates actin assembly and sarcomere

IF; Immunofluorescence, MEFs; Mouse embryonic fibroblasts, AFM; Atomic Force Microscopy; L-CMD; *Lmna*- -related congenital muscular dystrophy. EDMD; Emery-Dreifuss Muscular Dystrophy

Observation	Cell type	Reference
Minor or no obvious differences of F-actin architecture	Lmna -/- MEFs	(Lammerding et al. 2004; Broers et al. 2004; J. S. H. Lee et al. 2007)
Reduction of stress fibers in the perinuclear region	Lmna -/- MEFs	(Houben et al. 2009)
Slightly disorganized actin network	Dermal fibroblasts from human EDMD and DCM	(Emerson et al. 2009)
Defective TAN lines anchoring	Lmna−/− MEFs	(Folker et al. 2011)
No actin cap	Lmna−/− MEFs	(Khatau et al. 2009)
Altered actin dynamics, Increased nuclear and cytoskeletal actin mobility	Cardiomyocytes from Lmna -/- and N196K mice	(Ho et al. 2013)
More pronounced stress fibers and increased F/G- actin ratio	Human myoblasts with *LMNA* mutations causen L-CMD	(Bertrand et al. 2014)
Decreased actin filament length and thickness	Neonatal rat ventricular cardiomyocytes expressing D192G *LMNA* mutation	(Lanzicher et al. 2015)

Table 4: Defects of actin cytoskeleton that have been found in a various models of striated muscle laminopathies

organization. Arimura and colleagues noted that mutations in *Fhod3* lead to alteration of sarcomere organization and cause dilated cardiomyopathy (Arimura et al. 2013). As described in the introduction, cyclase-associated protein 2 (CAP2) and Twinfilin-2b cooperate with ADF/cofilin in order to enhance the actin filaments turnover. *Cap2*-null mice die earlier than wild-type mice and show a phenotype that is typically associated with dilated cardiomyopathy (Peche et al. 2013). We reasoned that the changes observed are downstream effects of ADF/cofilin-1 upregulation but further studies should be done.

Despite several scientists have proposed alteration of actin network and dynamics in *LMNA* striated muscle diseases (**Table 4**), our study revealed the molecular mechanism. In conclusion, I provided evidence herein that aberrant phosphorylation form of ERK1/2 in DCM accumulates in the cytosol and actively disturbs actin organization by ADF/cofilin-1 phosphorylation. As noted earlier, treatment of mice with ERK1/2 signaling inhibitors can improve cardiac phenotype (Muchir et al. 2009). We reasoned that this improvement may be attributed at least in part to the effect on ADF/cofilin-1 activity. Investigating the phosphatase of Thr25 is another key target for future investigations. Bioinformatics analysis predicted that phosphatases SSH1 and CIN are strong candidates for this residue, but we need to experimentally clarify whether one or both are involved in the dephosphorylation of p-Thr25.

How alteration of actin dynamics could lead to the development of DCM?

We showed that there is a profound decrease of filamentous- to globular-actin (F/G) ratio in *LMNA* cardiomyopathy. I observed this alteration even prior any cardiac abnormality. F-actin in muscle cells serves as a rigid cytoskeleton platform for the binding of troponin-tropomyosin molecules and interacts with myosin for the myofilament activation and contractility. We thus can assume that any alteration of F/G actin ratio impacts the maintenance and correct organization of the sarcomeric apparatus in myofibrils. We believe that this aberrant actin dynamics leads to a sarcomeric disorganization as it has been shown by electron microscopy. Previous studies demonstrated that perturbation of the equilibrium between actin monomers and filamentous actin induces disassembly of premyofibrils and sarcomeric disorganization with a more visible M-band region (Wang et al. 2005; Skwarek-Maruszewska et al. 2009).

Further, actin dynamics also controls serum-response factor (SRF) activity by regulating the subcellular localization of the mechanosensitive transcription cofactor myocardin-related transcription factor A (MRTF-A). The G-actin pool is involved in the cytoplasmic sequestration of MRTF-A and thus, G-actin accumulation alters MRTF-A intracellular localization and represses activation of MRTF-A/SRF downstream genes (**Figure 23**). One major class of SRF targets is muscle-specific and comprises genes encoding sarcomeric proteins such as α-actin and myosin (Pipes et al. 2006). In accordance, one study indicated that disturbed nucleo-cytoplasmic shuttling of MRTF-A was caused by altered actin dynamics in *Lmna*$^{-/-}$ and N195K mutant cells (Ho et al. 2013). MRTF-SRF signaling induces actin expression in response to pathways that control cellular contractility by cooperating with the MEF2 transcription factors to regulate muscle-specific contractile protein genes required for muscle function. As MRTF-A/SRF axis plays crucial role in sarcomeric function, perturbation of this signaling in muscles is expected to disrupt sarcomeric integrity. In line with this statement, muscles lacking Lmod3 display accumulation of G-actin monomers and subsequent repression of MRTF-A expression, which in turn suppresses SRF-dependent target genes encoding cytoskeletal proteins and components of the contractile apparatus, leading to nemaline myopathy (Cenik et al. 2015).

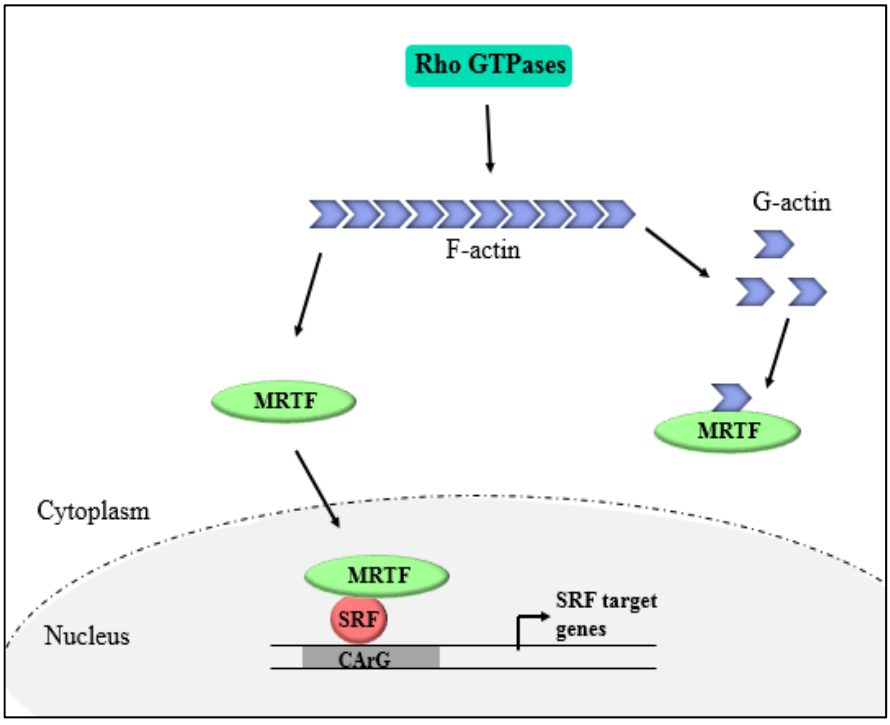

Figure 23: MRTF-SRF axis. MRTF, is a co-activator for serum response factor (SRF) Activation of Rho signaling promotes the assembly of F-actin from monomeric G-actin. Binding of G-actin to the MRTF prevents its nuclear accumulation and therefore repressing transcriptional activation by the MRTF– SRF complex

General discussion

Clearly we have come a considerable way in improving our understanding of increased pERK1/2 consequences on cardiac function. In conclusion, I have described a model in which hyperactivation of ERK1/2 signaling leads to increased fibrosis and abnormal actin dynamics in *LMNA* cardiomyopathy.

However, the question remains open: how mutations on *LMNA* gene leads to abnormal activation of ERK1/2 signaling? At this point, I could not pinpoint the molecular mechanism responsible for this impaired activation. However, one intriguing explanation could be provided by a study described that the coil 2 domain of A-type lamins bind the insert domain of active ERK1/2 (González et al. 2008). Further studies have found that lamin A protein serves as a scaffold for ERK1/2, anchoring it to the nucleus and presumably keeping it in close proximity with the transcription factors it normally activates (Rose et al. 2010,Gonzalez et al. 2008). For example, phosphorylation of c-Fos by ERK, both bound to lamins A/C, activates c-Fos/AP-1-driven transcription (González et al. 2008). Although no supporting data exist, we believe that mutations on lamin A/C directly abrogate its interaction with ERK which in turn becomes phosphorylated in the cytoplasm. Since ERK1/2 mediates the displacement of retinoblastoma protein (pRb) from lamins A/C (Rodríguez et al. 2010), another possibility is that in *LMNA* mutated cells, pRb or other proteins competitively inhibit the binding of ERK to lamins resulting in its nuclear and cytoplasmic release (**Figure 24**). Future studies will undoubtedly focus upon elucidating the precise mechanisms by which mutations on lamins result in hyperactivation of the ERK1/2 signaling.

Nevertheless, we still do not know why hyperactivation of pERK1/2 has been only observed in skeletal and cardiac muscle since lamins A/C are ubiquitously expressed. Understanding how ERK/12 is abnormally phosphorylated in the presence of *LMNA* mutations, opens up new avenues for a better understanding on how laminopathies arise and why they are tissue-specific.

It is challenging to explain the mechanisms by which lamins contribute to the tissue-selective pathology. While many investigators have suggested that *LMNA* mutations lead to alterations in gene expression (Perovanovic et al. 2016; Ho et al. 2013) that could have tissue-specific pathogenic consequences, other scientists have postulated that alterations in response to stress may underlie the development of striated muscle diseases (Lammerding et al. 2004; Broers et al. 2005; Bertrand et al. 2014). Since lamins have recently emerged as mechanosensors that respond to changes in structural and mechanical properties of cellular surroundings (Swift et al. 2013;

Isermann & Lammerding 2013), it is believed that mutations on lamins have a detrimental effect in tissues that experience high mechanical strains, like striated muscles.

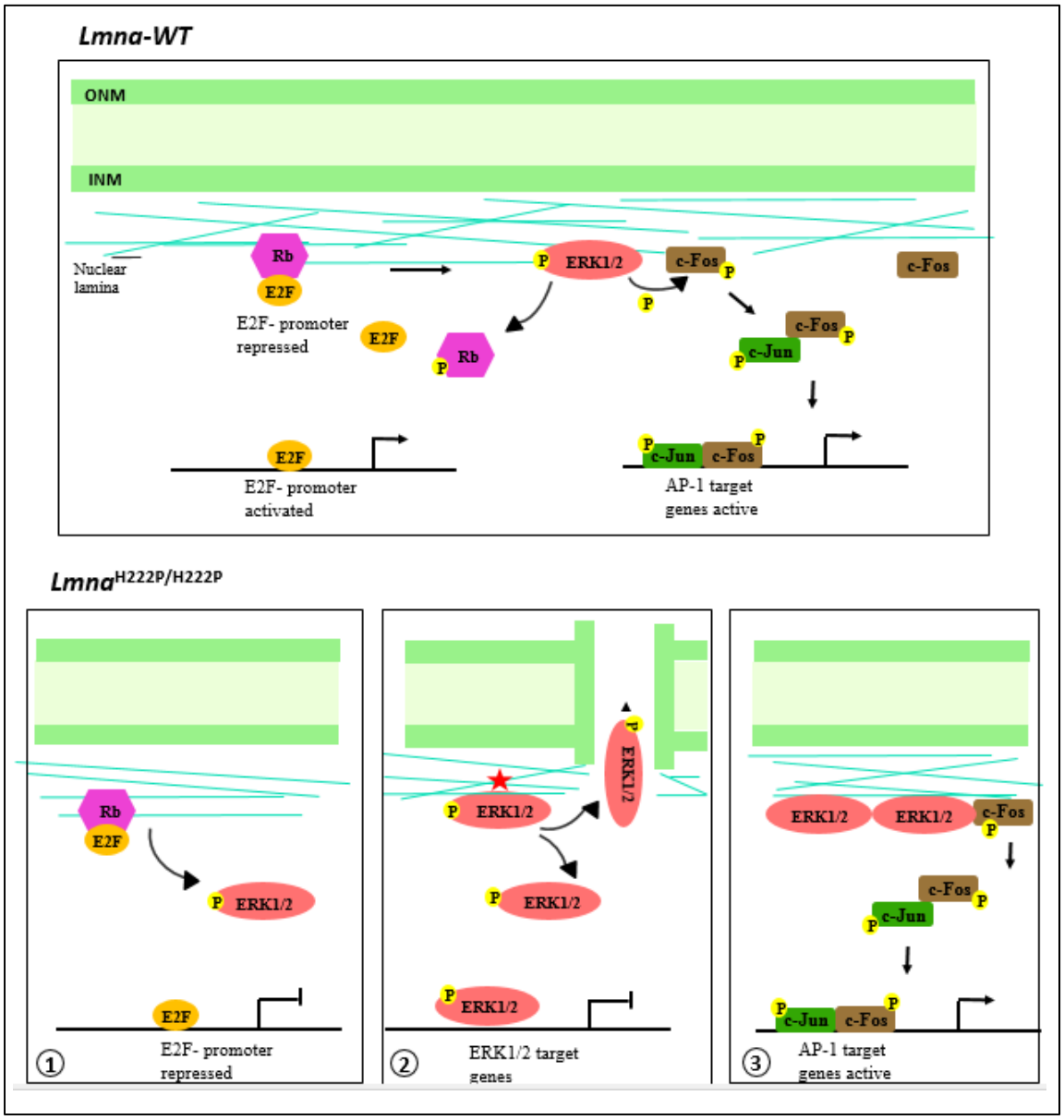

Figure 24: Models of how abnormalities of A-type lamins may lead to hyperactivation of ERK1/2. Phosphorylated ERK1/2 interact with A-type lamins and phosphorylate c-Fos, releasing it from the NE. c-Fos can THEN heterodimerize in the nucleoplasm with c-Jun, allowing the activation of AP-1 target genes. Rb is bound to lamin A and maintains in an active state bound to E2F. Phospho-ERK1/2 also disrupts Rb–lamin-A interaction. Rb is released to the nucleoplasm, where it will be phosphorylated and inactivated, liberating E2F. Here, there are three scenarios for the underlying mechanisms of ERK1/2 hyperactivation in *Lmna*$^{H222P/H222P}$: 1) Rb displaces ERK1/2 from nuclear lamina, 2) H222P aa substitution abrogates the interaction between lamina-pERK1/2. In both cases, pERK1/2 accumulates into the nucleoplasm and exported to the cytoplasm. 3) The mutation enhances the interaction between lamina-pERK1/2 which then reinforces the c-Jun phosphorylation and AP-1 target genes transcription creating a positive feedback on the activation of ERK1/2.

In such scenarios, it is hypothesized that muscle is unable to cope with the extreme loads and to exert the required force. In accordance, a number of studies suggested that mutations associated with *LMNA* cardiomyopathy have decreased mechanical stiffness (J. S. H. Lee et al. 2007; Zwerger et al. 2013; Lammerding et al. 2004; Broers et al. 2004) and exhibit impaired mechanotransduction (Lammerding et al. 2004). An interesting study supported that proteins of the Z-disc complex participate in mechanotransduction and these are defective in a subset of human dilated cardiomyopathy (Knöll et al. 2002). One of the major components of the Z disc is α-actinin, which crosslinks cardiac actin and titin molecules from neighboring sarcomeres. Accordingly, α-actinin staining in $Lmna^{H222P/H222P}$ cardiomyocytes is disrupted, which may affect cellular mechanics and therefore, mechanotransduction.

Could enhanced cofilin phosphorylation occur in other pathological or physiological conditions?

Besides the role of Thr25 phosphorylated cofilin in development of *LMNA* cardiomyopathy, it will be interesting to determine how such regulation is involved in normal physiological or other pathological processes. ERK has been implicated in the migration of numerous cell types. And inhibition of ERK represses cell migration (Lai et al. 2001). Cofilin is also a major regulator of actin dynamics in cell migration and manipulation of its activity has been shown to affect the cell migration process. It has a vital role in enhancing cell membrane protrusion at the leading edge of migrating cells by disassembling F-actin from the rear part of actin network to recycle G-actins to the front for further rounds of polymerization. Given the key functions of pERK/12 and ADF/cofilin in cell migration, it will be very interesting to determine whether the phosphorylation of Thr-25 is involved in ERK-mediated cell migration.

Since cell migration is a determining feature of cancer malignancy, the role of ADF/cofilins in cancer cell invasion is under intense investigation. Current data in cancer cell lines and rodent tumor models suggest that the ADF/cofilin expression is altered in correlation with invasive potential. In line with this argument, ADF/cofilins have been implicated in invadopodia formation (Yamaguchi et al. 2005). Invadopodia are actin-rich extensions of the plasma membrane that protrude into the matrix and are transiently formed by aggressive tumor cells. Accumulating evidence also suggests that components of the ERK1/2 pathway are frequently mutated in cancer

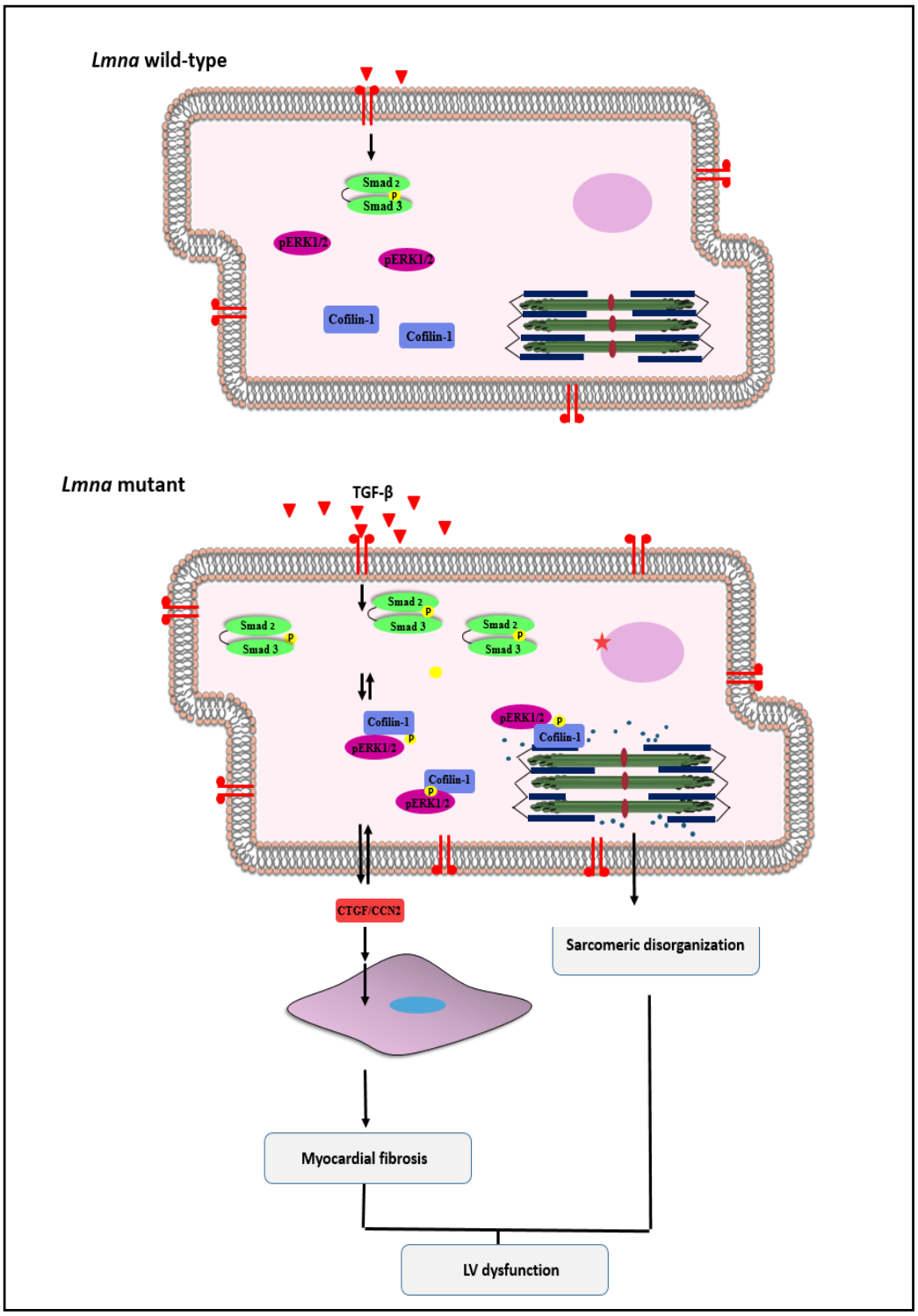

Figure 25: Schematic representation of the data obtained during this thesis. In the presence of *Lmna* mutation, cardiac tissue display enhanced activity of i) TGF-β and ii) ERK1/2 signaling pathways. Increased TGF-β mediates the induction of CTGF leading to the development of fibrosis. Enhanced pERK1/2 leads to phosphorylation and activation of ADF/cofilin-1. Phopshorylated ADF/cofilin-1 (pThr25) causes sarcomeric disorganization which might leads to the development of LV (left-ventrular) dysfunction

cells and this pathway is often upregulated in human tumors. Likewise, it would also be interesting to investigate the role of cofilin phosphorylation by activated ERK1/2 in tumor development and progression *in vivo* in future studies.

It has been previously reported that in human skeletal muscle, exercise (acute cycling) results in significant increase of ERK1/2 phosphorylation (Aronson et al. 1997). This exercise-induced ERK1/2 phosphorylation cannot be attributed to an increase in total ERK1/2 protein expression and is rapidly decreased upon cessation of exercise. Further studies are necessary to investigate whether cofilin-1 is phosphorylated by pERK1/2 during exercise.

Outlook

Since there is no curative treatment of *LMNA* cardiomyopathy so far, established management includes the use of angiotensin-converting enzyme (ACE) inhibitors or the combination of an ACE inhibitor with β-adrenergic receptor blockade. Even though ACE inhibitors are able to reduce the risk of heart failure, patients still experience left ventricular dysfunction. In most cases of cardiomyopathy caused by *LMNA* mutations, surgery is required for implantation of a cardioverter defibrillator to prevent malignant ventricular tachyarrhythmias (Meune et al. 2006). However, this invasive procedure carries a risk of infection, can be traumatic to patients, and is technically more demanding and liable to complication in young children and infants. This highlights the need for novel therapeutic options.

In summary, as depicted in **Figure 25** we propose the following model; in the presence of *LMNA* mutation, abnormally elevated TGF-β signaling leads to increase nuclear translocation and phosphorylation of Smad2/3 which is responsible in part for the hyperactivation of ERK1/2 signaling by an unknown mechanism. Enhanced phosphorylated ERK1/2 signaling has two main consequences: 1) induces the expression of CTGF which leads to the development of myocardial fibrosis 2) phosphorylates ADF/cofilin-1 at a novel residue, Thr 25, thus activating its severing activity. In turn, activation of ADF/cofilin-1 leads to an increase of the G-actin pool and therefore decrease of the F- to G-actin ratio, resulting in the disorganization of sarcomeric actin in the cardiac myofibrils.

Our findings of ADF/cofilin-1 phosphorylation by pERK1/2 in *LMNA*-cardiomyopathy raise the interesting question on whether this site could be a target in other types of cardiomyopathies such as dilated or hypertrophic cardiomyopathy resulting from other gene mutations. Clearly, future studies with a larger DCM mouse model pool (carrying other mutations) are required to confirm this finding as a general mechanism. An even more formidable task may be to perform CRISPR-based genome editing of the Thr25 into Ala in the hearts of the $Lmna^{H222P/H222P}$ mice at an early age. This may act to prevent rather than merely treat disease. However, more animal-based studies are required to understand the potential side-effects of such approach.

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Chapter 5 Appendices

Review 1: Extracellular signal-Regulated Kinases1/2 and their role in cardiac diseases

Maria Chatzifrangkeskou and Antoine Muchir

Science Proceedings, (2015); 2: e457

Review 2: Nuclear envelope and striated muscle diseases

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Current Opinion of Cell Biology, (2015) 32:1-6

5.1 Appendix I

Extracellular signal-Regulated Kinases1/2 and their role in cardiac diseases

This review provides an overview of the mitogen-activated protein kinase (MAPK) signaling pathways emphasizing on extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) branch. Alterations of ERK1/2 cascade in different cardiac diseases and current approaches for therapeutic development are also summarized.

Science Proceedings 2015; 2: e457. doi: 10.14800/sp.457; © 2015 by Maria Chatzifrangkeskou, *et al.*
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REVIEW

Extracellular signal-Regulated Kinases1/2 and their role in cardiac diseases

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Received: November 29, 2014
Published online: January 28, 2015

Mitogen-activated protein (MAP) kinases are members of a well-studied family of serine/threonine protein kinases involved in signal transduction pathways, which control multiple cellular processes. The extracellular signal-regulated kinase (ERK1/2) cascade is a MAP kinase pathway that transmits signals from the cell surface to substrates either in the nucleus or in the cytoplasm. The transmission of the signal through the ERK1/2 cascade is mediated by serial phosphorylations and activations of protein kinases. Abnormal regulation of the ERK1/2 signals has been linked to diseases and recent work clearly implicated ERK1/2 signaling in the development of cardiac pathologies. Understanding the underlying mechanism and the consequences of the aberrant modulation of ERK1/2 cascade will lead to the development of pharmacologic inhibitors for the treatment of these cardiac disorders.

Keywords: Mitogen-activated protein (MAP) kinases; Lamin; cardiomyopathy; Extracellular signal-regulated kinases 1/2

To cite this article: Maria Chatzifrangkeskou, *et al*. Extracellular signal-RegulatedKinases1/2 and their role in cardiac diseases. Sci Proc 2015; 2: e457. doi: 10.14800/nt.457.

Introduction

A proportion of inherited cardiomyopathies display deregulated MAP kinase signaling. As these diseases are a major cause of cardiac disease in human, it is therefore not surprising that the MAP kinase signaling continues to be the subject of intense researches for future pharmacological intervention. The development of molecules targeting this pathway has focused mostly on the development of small-molecule inhibitors. This review will gather information on MAP kinase signaling, focusing on the ERK1/2 branch and will discuss important research progresses in the field of inherited cardiomyopathies. The recent years provided some clues to explain the pathogenesis of such disease, involving ERK1/2, which might open novel and promising perspectives for future clinical trials.

MAP kinases

Protein kinases are important players to achieve the integrated function of cells. Protein kinases are able to transfer phosphoryl groups onto target proteins, altering their activity [1]. This mechanism participates in the transmission of intra- and extracellular signals throughout the cell and to the nucleus. Thus, protein kinases play a pivotal role in signaling pathways that could regulate cell growth, differentiation, development, and death [2]. Hence, any disruption of the phosphorylation could alter cell functions and may cause diseases [3].

The kinases are classified by the amino acids they phosphorylate [4]. The two main classes of kinases are tyrosine kinases, which phosphorylate tyrosine, and serine-threonine kinases, which phosphorylate serine or threonine [5]. Manning *et al*. have defined the human *«kinome space »* consisting of more than 500 protein kinase genes [6]. A growing number of diseases are caused by deregulation of the phosphorylation, and thus protein kinases are regarded as a promising therapeutic target for diseases.

There is a family of protein kinase cascades known as mitogen-activated protein (MAP) kinases [7, 8], which belongs to serine-threonine kinases. It comprises MAP kinases, MAP kinase kinases (MKKs) and MAP kinase kinase kinases (MKKKs) [9]. MKKKs phosphorylate and activate MKKs, which in turn phosphorylate and activate MAP kinases. In all currently known MAP kinase cascades, the kinase immediately upstream of the MAP kinase is a member of the MAP/ERK kinase (MEK) family [10-13], which are dual specificity enzymes that can phosphorylate serine/threonine and tyrosine residues. In spite of their ability to phosphorylate MAP kinases proteins, the substrate specificity of the known MEKs is very narrow: each MEK phosphorylates only one or a few of the MAP kinases. Much of the review highlights knowledge on extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), two of the known MAP kinases.

ERK1/2

ERK1 and ERK2 are ubiquitously expressed proteins of 44 and 42 kDa, which are nearly 85% identical in their amino-acid sequences [14-17]. Stimulation of different receptors can activate ERK1/2, e.g. receptors with intrinsic tyrosine kinase activity, cytokine receptors and G-protein-coupled receptors. Hence, ERK1/2 are stimulated by many extracellular ligands and cellular perturbations (e.g., mechanical stress, osmotic shock), with some cell type specificity [7]. ERK1 and ERK2 are activated by closely related MEKs, MEK1 and MEK2 [10, 18-21]. It has been assumed, from lack of evidence to the contrary, that MEK1/2 have no other substrates. This specificity of MEK1/2 has been used by several pharmacological companies to design potent inhibitors of ERK1/2 signaling [22]. A vast majority of the known MEK1/2 inhibitors are ATP non-competitive (i.e., they do not directly compete for the ATP binding site). These MEK1/2 inhibitors bind to a unique allosteric site adjacent to the ATP site, which explains their high specificity.

Of all the known MEKKs, Raf isoforms are the only ones known to date, which phosphorylate MEK1 and MEK2 [23-27]. Stimulation of extracellular receptors by ligands results in the activation of the protein Ras, which can interact with several effectors, including Raf isoforms. ERK1/2 signaling requires the spatial and temporal organization of three different kinases, Raf, MEK1/2 and ERK1/2. This is controlled by scaffold molecules [28], among which some have been identified in cardiac tissue (e.g., β-arrestin, FHL1, paxillin) [29]. These scaffold molecules have important functions: i/ they organize ERK1/2 for the efficient activation in the cascade; and ii/ they determine the output signal by localizing ERK1/2 to selected sites of action, (e.g., the transcription machinery, the actin cytoskeleton). Phosphorylation by ERK1/2 induces conformational change of the substrate, which thereby, either induces their activation, or regulates their association with other molecules [30]. To date, more than 150 substrates of ERK1/2 have been reported [31], which can be categorized into several categories including: i/ transcription factors, ii/ protein kinases and phosphatases, iii/ cytoskeletal proteins, iv/ receptors, and vi/ others. ERK1/2 can translocate to the nucleus where they are able to phosphorylate transcription factors to regulate their activities (e.g., c-Jun, c-Fos and ATF-2, Elk-1). Although many ERK1/2's substrates are localized in the nucleus and are phosphorylated after ERKs' nuclear translocation, the number of cytosolic ERK substrates is not much smaller. It has been described that several cytoskeletal element are targets for ERK1/2 (e.g., tubulin [32], vimentin [33] or cortactin [34]).

ERK1/2 and inherited cardiomyopathies

Studies point to ERK1/2 as a maladaptive signaling pathway in cardiomyopathy. Cardiomyopathy is an anatomic and pathologic condition associated with muscle and electrical dysfunction of the heart, which may be confined to the heart or may be part of a generalized systemic disorder, often leading to heart failure-related disability. A well-known negative effect of ERK1/2 signaling in human heart function is highlighted by the fact that different mutations able to increase ERK1/2 pathway activation lead to cardiac pathologies in patients with Noonan and related syndromes, such as Costello, LEOPARD and cardio-facio-cutaneous syndromes [35-38]. Knock-in mice expressing a Noonan syndrome-associated mutation exhibit enhanced ERK1/2 signaling and an accelerated transition toward heart failure in response to pressure overload. Interestingly, postnatal treatment with MEK1/2 inhibition normalizes cardiac defects [39]. Based on these results, Novartis recently launched a clinical trial on Noonan syndrome using a MEK1/2 inhibitor (ClinicalTrials.gov Identifier: NCT01556568).

A further example is represented by mutations in the A-type lamin gene and causing dilated cardiomyopathy. Since 1999, scientists have unraveled the role of the nuclear lamina in the development of cardiac disease [40]. *LMNA* encodes

nuclear A-type lamins via alternative splicing [41]. Lamins are intermediate filament proteins that polymerize to form the nuclear lamina, a fibrous meshwork underlining the inner nuclear membrane of most eukaryotic cells. We recently demonstrated an aberrant increase in ERK1/2 activity in hearts from a mouse model of the disease [42]. These results provide proof of principle for ERK1/2 inhibition as a therapeutic option to prevent or delay the onset of heart failure in *LMNA* cardiomyopathy. Pharmacological or genetic blockade of signaling in the ERK1/2 cascade in these mice improves left ventricular dilatation and deterioration in cardiac contractility [43-46].

Conclusion

Less than a decade ago the kinases constituting MAP kinase pathways were identified through intense efforts to understand the molecular events underlying cellular responses to extracellular signals. The kinases constituting ERK1/2 pathways appear to be key cellular signal transducers and thus attractive targets for drug development. These efforts are now beginning to bear fruit with the initiation of clinical trials in human cardiac diseases. Their positive outcome would be a triumph of translating basic scientific understanding of cellular function into successful human therapies.

Conflict of Interest

Dr. Muchir is inventor on a pending United States patent application on methods for treating and/or preventing cardiomyopathies by ERK inhibition filed by the Trustees of Columbia University in the City of New York.

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5.2 Appendix II

Review 2: Nuclear envelope and striated muscle diseases

This review discusses the two prevalent hypotheses behind the development of tissue specific effects observed in laminopathies, some of the animal models that have been used for the study of laminopathies affecting striated muscles and the signaling pathways that are proposed to contribute to the progression of the disease.

(Published in Human Molecular Genetics, DOI link: http://dx.doi.org/10.1016/j.ceb.2014.09.007*)*

Chapter 6 Acknowledgments

I would like to thank all the people who contributed in some way to the work described in this thesis. First and foremost, I thank the director of my thesis in Paris, Gisèle Bonne, for accepting me into her group. I am so deeply grateful for her help, professionalism, valuable guidance and support throughout this project and through my entire program of study.

Particular thanks go to my supervisor Antoine Muchir for his guidance, motivation and enthusiasm. You definitely provided me with the tools that I needed to choose the right direction and successfully complete my thesis.

Special thanks to the director of my thesis in Berlin Petra Knaus who has received me with open arms in her lab.

I gratefully acknowledge the reviewers of my thesis committee, Roland Foisner, Athanassia Sotiropoulos for the suggestions as well as Onnik Agbulut, Sigmar Stricker and Christien Hiepen for accepting being part of this. I would also like to thank the members of my 1st and 2nd year PhD committee: Eric Schirmer, Sophie Zinn and Vincent Mouly for their time and valuable feedback on a preliminary version of this thesis.

I thank a lot Stéphane Vassilopoulos for his advices during these three years and for his comments and suggestions on my thesis manuscript.

I would like to thank the members of the Bonne's group with whom I had the opportunity to work: Isabelle Nelson, Feriel Azibani, Anne Bertrand, Nicolas Vignier and Esma Ziat. They provided a friendly and cooperative atmosphere at work and also useful feedback and insightful comments on my work.

I would like also to thank all the members of Knaus lab, who did welcome me during the period I spent in Berlin. I would particularly like to thank David Yadin for his time, helpful comments and patience and Martina Fischer for helping in everything I needed during my time in Berlin. Vielen Dank für alles!

I thank the Myograd program for accepting me and more particularly Susanne Wissler who helps in the administrative work. In Paris side, I thank a lot Lidia Dolle for helping in everything we needed. Un gros merci pour tout ce que tu as fait pour nous, Lidia!

My time in Paris was made enjoyable in large part due to my colleagues and friends who became a part of my life. I am lucky to have met Coline Macquart here, and I thank her, not only because

is the only person who can spell my last name correctly but also for her friendhip and unyielding support. We started together this difficult journey and we become now besides colleagues, real friends. She is always beside me during the happy and hard moments. My time in Paris and in the lab would not have been the same without her. Of course, I could not forget to thank Matthew Thorley and Apostolos Malatras for all the great times and funny (until our stomachs hurt) chats that we have shared. You guys (aka breakthrough team) made my everyday work in the lab enjoyable. I am also thankful to Blanca Rodriguez Morales, Nada Essawy, Daniel Owens and Astrid Brull, who made my time here in Paris a lot more fun. Thank you all guys for the beautiful and unforgettable moments.

Finally, I would like to acknowledge my family. First and foremost I would like to thank my lovely parents, Vicky and Christos, for their constant love and unfailing support throughout my life and my studies. You are always there for me. I especially thank my beloved sister, Andrea, who is the most faithful person I have ever met. I would also like to thank my boyfriend, Marinos, whose faithful support and care, is so appreciated.