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„Chromatin association of LAP2alpha and A-type lamins regulates early myogenic differentiation“

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ABSTRACT

The organization and regulation of the genome are fundamental functions of eukaryotic nuclei and involve many proteins and nuclear structures. Among these, the nuclear lamina at the nuclear periphery plays an important role. Lamins, the main components of the nuclear lamina, are involved in a plethora of processes like chromatin anchorage to the nuclear periphery, gene regulation, DNA repair and replication, chromatin dynamics, and more. These functions are mediated by the direct binding of lamins to chromatin and indirectly through interaction of lamins with other chromatin associated factors. While B-type lamins are only confined to the nuclear periphery, A-type lamins also form a soluble, highly mobile pool that diffuses throughout the nucleus. This widespread nuclear localization makes A-type lamins particularly interesting for studying lamin functions in chromatin organization since they interact with both heterochromatic genomic regions at the periphery and with active chromatin in the nuclear interior. In concordance with their different localization, B-type lamins mainly associate with long, gene-poor, and repressed regions called lamina associated domains (cLADs), while A-type lamins also bind to active, gene-rich genomic regions. However, if and how A-type lamins regulate genes in active chromatin regions is unknow. A-type lamins interact with several factors in the nuclear interior, potentially increasing their range of functions. Among these interacting proteins, LAP2alpha is a prominent candidate for mediating genome regulation, since it directly and indirectly binds DNA via several domains, and it was reported to associate with active genomic regions. LAP2alpha also regulates the assembly state of nucleoplasmic lamins and the binding of A-type lamins and other epigenetic regulators to chromatin. Thus, studying the binding of A-type lamins and LAP2alpha to active chromatin opens the possibility to study their so far unexplored role in gene regulation in various cellular processes. In this thesis, I investigate association of A-type lamins and LAP2alpha to chromatin during myoblasts differentiation, in vitro. My data confirm the binding of these proteins to gene-rich genomic regions and additionally demonstrate a dynamic rearrangement of mainly LAP2alpha chromatin association, at early stages of myogenic differentiation. Unlike A-type lamins, LAP2alpha-bound genomic regions are enriched in differentially expressed genes during differentiation, although LAP2alpha is not enriched on the genes directly. Therefore, LAP2alpha may affect gene expression through local enrichment at chromatin regions adjacent to differentially expressed genes rather than through its direct association with genes. A-type lamins seem to be kept away from gene-rich regions and are strongly depleted at the gene transcription start site of expressed genes. Loss of LAP2alpha leads to the redistribution of A-type lamins to gene-rich genomic regions, particularly in proliferating myoblasts. In LAP2alpha-depleted myoblasts, A-type lamins enrich also in regions containing myogenic genes, a subgroup of which is deregulated in LAP2alpha knockout cells. My data indicate that the spreading of A-type lamins to genomic regions containing myoblast-relevant genes upon loss of LAP2alpha may impair their proper regulation, leading to differentiation defects, as previously shown. Finally, preliminary analyses show that chromatin accessibility may be changed genome-wide in LAP2alpha-depleted versus wild-type myoblasts. Overall, my data suggest that LAP2alpha facilitates correct gene regulation during early myogenesis by providing a chromatin environment around active myogenic genes, allowing their regulation by other chromatin regulatory proteins. In addition, LAP2alpha may inhibit spreading of A-type lamins to active chromatin by so far unknown mechanisms. Elucidating the mechanisms through which LAP2alpha and A-type lamins affect chromatin regulation is an important step towards understanding their role in lamin-linked diseases (laminopathies), possibly bringing us closer to the development of effective therapies.

ZUSAMMENFASSUNG

Die Organisation und Regulierung des Genoms sind grundlegende Funktionen der eukaryontischen Zellkerne. Viele Proteine und Kernstrukturen sind an diesen Prozessen beteiligt und für die korrekte Regulierung des Genoms notwendig. Unter diesen spielt die Kernlamina eine wichtige Rolle. Lamine, die Hauptkomponenten der Kernlamina, sind an einer Vielzahl von Prozessen beteiligt, wie der Verankerung des Chromatins an der Kernperipherie, der Regulierung der Genexpression, der DNA-Reparatur und -Replikation, der Regulierung der Loci-Dynamik und vielem mehr. Diese Funktionen können das Ergebnis einer direkten Bindung von Lamin an Chromatin oder indirekter Effekte sein, die auf die Interaktion von Laminen mit anderen Faktoren zurückzuführen sind. Während Lamine des B-Typs nur an der Kernperipherie vorkommen, bilden Lamine des A-Typs auch einen löslichen Pool, der sich im gesamten Zellkern verteilt. Diese weit verbreitete Lokalisierung macht die A-Typ-Lamine sehr interessant für die Untersuchung der Funktionen von Lamin in der Chromatinorganisation, da sie sowohl mit dem an der Peripherie befindlichen Teil des Genoms als auch mit dem Chromatin im Kerninneren interagieren können. In Übereinstimmung mit ihrer unterschiedlichen Lokalisierung wurde die Bindung von B-Typ-Laminen an Chromatin hauptsächlich in langen, genarmen und unterdrückten Regionen, den so genannten lamina-assoziierten Domänen (cLADs), beobachtet, während A-Typ-Lamine auch an aktive, genreiche Genomregionen binden. Die Untersuchung von A-Typ-Laminen kann daher dazu beitragen, Mechanismen zu verstehen, die für die Regulierung des aktiven Teils des Genoms und ihre Rolle bei der Genexpression relevant sind. Darüber hinaus können die A-Typ-Lamine mit weiteren Faktoren im Kerninneren interagieren, was ihr Funktionsspektrum möglicherweise erweitert. Unter diesen interagierenden Proteinen ist LAP2alpha ein interessanter Kandidat für die Genomregulation, da es direkt und indirekt an die DNA binden kann und mit aktiven Genomregionen assoziiert ist. LAP2alpha reguliert auch den Zustand des Zusammenbaus der nukleoplasmatischen Lamine und die Bindung von A-Typ-Laminen und anderen epigenetischen Regulatoren an Chromatin. Die Bindung von A-Typ-Laminen und LAP2alpha an aktives Chromatin eröffnet somit die Möglichkeit, ihre bisher unerforschte Rolle bei der Genregulation in verschiedenen zellulären Prozessen zu untersuchen. In dieser Arbeit untersuche ich die Bindung von A-Typ-Laminen und LAP2alpha an Chromatin während der Differenzierung von Myoblasten. Ich bestätige die Bindung dieser Proteine an genreiche genomische Regionen und zeige, dass sich diese Bindung im Verlauf der Differenzierung dynamisch umgestaltet. Im Gegensatz zu Lamin A/C, sind die an LAP2alpha gebundenen genomischen Regionen während der Differenzierung mit unterschiedlich exprimierten Genen angereichert, aber die Gene werden nur selten direkt von LAP2alpha und Lamin A/C gebunden. Lamin A/C und insbesondere LAP2alpha können also die Genexpression eher durch lokale Anreicherung in Chromatinregionen beeinflussen, die an unterschiedlich exprimierte Gene angrenzen, als durch ihre direkte Bindung an Gene. Dies stimmt mit meiner Beobachtung überein, dass die Abreicherung von Lamin A/C an der Startstelle der Gentranskription stark mit der Genexpression korreliert und bei nicht-exprimierten Genen fehlt. Darüber hinaus zeige ich, dass der Verlust von LAP2alpha zu einer Umverteilung von A-Typ-Laminen auf dem Chromatin führt, insbesondere in proliferierenden Myoblasten. In LAP2alpha-depletierten Myoblasten reichern sich die A-Typ-Lamine in Regionen an, die Gene enthalten, die an der Myoblastendifferenzierung beteiligt sind und von denen einige dereguliert werden. Daher könnte die Anreicherung von A-Typ-Laminen in Regionen, die für Myoblasten relevante Gene enthalten, nach dem Verlust von LAP2alpha deren ordnungsgemäße Regulierung beeinträchtigen, was zu Differenzierungsdefekten führt. Schließlich berichte ich über vorläufige Daten, die Veränderungen der Chromatinzugänglichkeit in LAP2alpha-depletierten Myoblasten im Vergleich zu ihren WT-Pendants zeigen. Ich stelle die Hypothese auf, dass Veränderungen in der Chromatinzugänglichkeit ein Mechanismus sein könnten, durch den der Verlust von LAP2alpha und folglich die Umverteilung von Lamin des Typs A auf dem Chromatin die

Genexpression beeinflusst. Die Aufklärung der Mechanismen, durch die LAP2alpha und Lamin A/C die Chromatinregulation beeinflussen, ist ein wichtiger Schritt, um ihre Rolle bei Krankheiten wie Laminopathien zu verstehen, und könnte uns möglicherweise der Entwicklung wirksamer Behandlungen näherbringen.

ABBREVIATIONS

ADLD = adult-onset autosomal dominant leukodystrophy

AP-1 = activator protein 1

ATAC-seq = assay for transposase-accessible chromatin - sequencing

BAF = barrier-to-autointegration factor

BioID = proximity-dependent Biotin identification

CDM1A = dilated cardiomyopathy

ChIP-seq = chromatin immunoprecipitation - sequencing

cLADs = constitutive LADs

CMD = congenital-type muscular dystrophy

CTCF = CCCTC-binding factor

DamID = DNA adenine methyltransferase identification

Des = desmin

DSBs = double-strand breaks

ER = endoplasmic reticuluum

ERK = extracellular signal-regulated kinase

fLADs = facultative LADs

FPLD2 = familial partial lipodystrophy type 2

GO = gene ontology

H3K27me3 = histone 3 lysine 27 trimethylation

H3K9me2/3 = histone 3 lysine 9 di-/tri-methylation

HDR = homology-directed repair

HGPS = Hutchinson-Gilford progeria syndrome

HMG5 = high mobility group (HMG) box-containing protein 5

IE = regions inside EDD peaks

IG-Fold = immunoglobulin fold

INM = inner nuclear membrane

JNK = c-Jun N-terminal kinase

KASH = Klarsicht/ANC-1/Syne-1 homology

LA = lamin A/C

LADs = lamina-associated domains

LAP = lamin-associated polypeptides

LBR = lamin B receptor

Lco1 = Lamin companion 1

LEM = LAP2-emerin- MAN1

LGMD1B = limb-girdle muscular dystrophy type 1B

LINC = linker of nucleoskeleton and cytoskeleton

MAD = mandibuloacral dysplasia

MAPK = mitogen-activated protein kinase

MARs = matrix association regions

mESCs = mouse embryonic stem cells

MRF s= myogenic regulatory factors

MyHC = myosin heavy chain

MyoD = myoblast determination protein 1

Myog = myogenin

Narf = nuclear prelamin A recognition factor

NE = nuclear envelope

NETs = nuclear envelope transmembrane proteins

NHEJ = non-homologous end joining

NL = nuclear lamins

NLS = nuclear loclization singnal

NPCs = nuclear pore complexes

NTRs = nuclear transport receptors

NUPs = nucleoporins

OE = regions outside EDD peaks

O-GlcNAcylation = O-linked-N-acetylglucosaminylation

ONM = outer nuclear membrane

PcG = polycomb-group proteins

PCNA = Proliferating Cell Nuclear Antigen

PLA = proximity ligation assay

PNS = perinuclear space

PP2A = Protein phosphatase 2

pRB = retinoblastoma protein

P-Ser22 = phospho-Serine 22

RFC = Replication Factor Complex

RNA-seq = RNA - sequencing

rRNA = ribosomal RNA

SMAD = Mothers against decapentaplegic (MAD)

SREBP1 = sterol regulatory element-binding transcription factor 1

SUN = Sad1p, UNC-84

TADs = topologically associating domains

TGF-β1 = Transforming growth factor beta 1

Tmpo = Thymopoietin gene

Note: Throughout this thesis, Lamin A/C and A-type lamins are used interchangeably.

CHAPTER 1: INTRODUCTION

The information necessary to produce every living organism is stored in their genetic material. It is therefore not surprising that the study of the genome, its regulation, preservation, and inheritance are such important fields in biology.

One of the main evolutionary differences among organisms is their cellular complexity (Baum & Baum, 2014; Mast et al., 2014), which is reflected also in the organization of their DNA (Talbert et al., 2019). Eukaryotic cells have a complex cellular organization that includes a high level of compartmentalization (Diekmann & Pereira-Leal, 2013; Gabaldón & Pittis, 2015). Cellular sub-compartmentalization is a very useful tool for keeping different biochemical environments separated from each other, it is achieved in different ways, and does not necessarily require the use of lipid membranes (Boeynaems et al., 2018; Wheeler & Hyman, 2018). Sub-compartments are enriched in specific proteins and factors, usually involved in the same biological processes. This allows these proteins to concentrate in specific areas and interact with each other efficiently, separating them from other signals that may interfere with their functions (Diekmann & Pereira-Leal, 2013; Harrington et al., 2013; Martin, 2010).

It is therefore not surprising that in complex organisms like eukaryotes the genetic material is isolated from the rest of the cell. In eukaryotic cells, the DNA is bound by proteins and spatially confined inside a double membrane organelle called the cell nucleus. The evolutionary importance of the nucleus is such that this compartment represents one of the main features that distinguishes Eukarya from the other two domains of life, Archaea and Bacteria (Simonson et al., 2005).

The nucleus creates an efficient environment to protect, organize and regulate the genome (Cho et al., 2019; D'Angelo et al., 2012; Galy et al., 2000; Guerreiro & Kind, 2019; Lim et al., 2016; Misteli, 2020; Palancade et al., 2007; Schreiner et al., 2015; Ulianov et al., 2019).

1.1 THE CELL NUCLEUS

The cell nucleus is a membranous organelle found in eukaryotic cells (Figure i1). It is separated from the cytosol by the nuclear envelope (NE), a structure consisting of two double-layered lipid membranes, divided by a perinuclear space (PNS). The external membrane of the NE, the outer nuclear membrane (ONM), is continuous with the endoplasmic reticulum (ER) (Lu et al., 2011). Nevertheless, the protein composition of the ONM and the ER membrane are very different, allowing the two compartments to carry on different functions (Hirano et al., 2020). The ONM contains proteins that interact with cytosolic components outside the nucleus. For example, a group of ONM proteins can bind to the cytoskeleton and regulate processes like the positioning of the nucleus inside the cell and the integration of mechanical signals from the cytoplasm to the nucleus (Lombardi et al., 2011; Starr & Fridolfsson, 2014). The membrane of the NE facing the inside of the nucleus is called inner nuclear membrane (INM). The INM contains proteins that are known to interact with chromatin and/or with other components inside the nucleus, like the nuclear lamina (NL) (Katta et al., 2014). The ONM and the INM are continuous in several points of the NE, surrounding large proteinaceous channels called nuclear pore complexes (NPCs) (Watson, 1955). The NPCs connect the cytoplasm with the nucleoplasm and allow a regulated exchange of proteins, RNA, and riboprotein complexes between the two compartments (Naim et al., 2007).

Inside the nucleus, underneath the INM, lies the NL. This is a proteinaceous mesh mainly composed of lamins and their interacting partners. The NL is fundamental for the mechanical properties of the nucleus. This structure is involved in maintaining the nuclear structural integrity, sensing the mechanical signals coming from the cytoskeleton, and affecting several cell-signaling pathways (Gruenbaum & Foisner, 2015; Lammerding et al., 2004; Lombardi et al., 2011). Lamins and some of their interacting proteins can also bind to chromatin either directly or indirectly, thereby contributing to the spatial organization and regulation of the genome (Kim et al., 2019; Pradhan et al., 2020; Ulianov et al., 2019).

The most important component of the nucleus, and the central structure for all its processes and functions, is the chromatin. Chromatin consists of DNA molecules wrapped around histone proteins and further bound by other factors to obtain a dynamic 3D conformation. The folding of the genetic material occurs in several structural layers, starting with the DNA helix twist, its wrapping around histones to form nucleosomes, and their further assembly in chromatin fibers and higher structures, up to the formation of mitotic chromosomes (Kornberg & Klug, 1981; Maeshima et al., 2019).

The chromatin structure must be dynamic and able to remodel in order to allow differential gene expression in various processes. The cell, in fact, needs to adapt its gene expression in processes like cell differentiation or in response to stimuli, like growth factors, inflammatory signals and many others. This regulation is achieved through epigenetic modifications (Karlić et al., 2010), binding of transcription factors (Iwafuchi-Doi & Zaret, 2014), chromatin remodelers (De la Serna et al., 2001; Lessard et al., 2007) and many other mechanisms (Ho & Crabtree, 2010; Wu, 1997).

The presence of sub-nuclear compartments further facilitates the organization of some DNA-related processes by creating microenvironments with specific protein composition and biochemical properties inside the nucleus. The better understood among these structures are the nucleoli. These are specialized areas forming around chromatin regions containing a high number of ribosomal RNA (rRNA) genes. The nucleoli contain all the factors needed for rRNA transcription and are the sites of ribosome subunit assembly. Other sub-nuclear compartments have been found inside the nucleus, but their functions and organization are less understood (Misteli & Dundr, 2001).

In conclusion, the cell nucleus is a very complex organelle, whose function is maintained by multiple layers of organization. Foremost, it has to integrate different mechanisms and structures to efficiently preserve and control the genetic material and its expression.

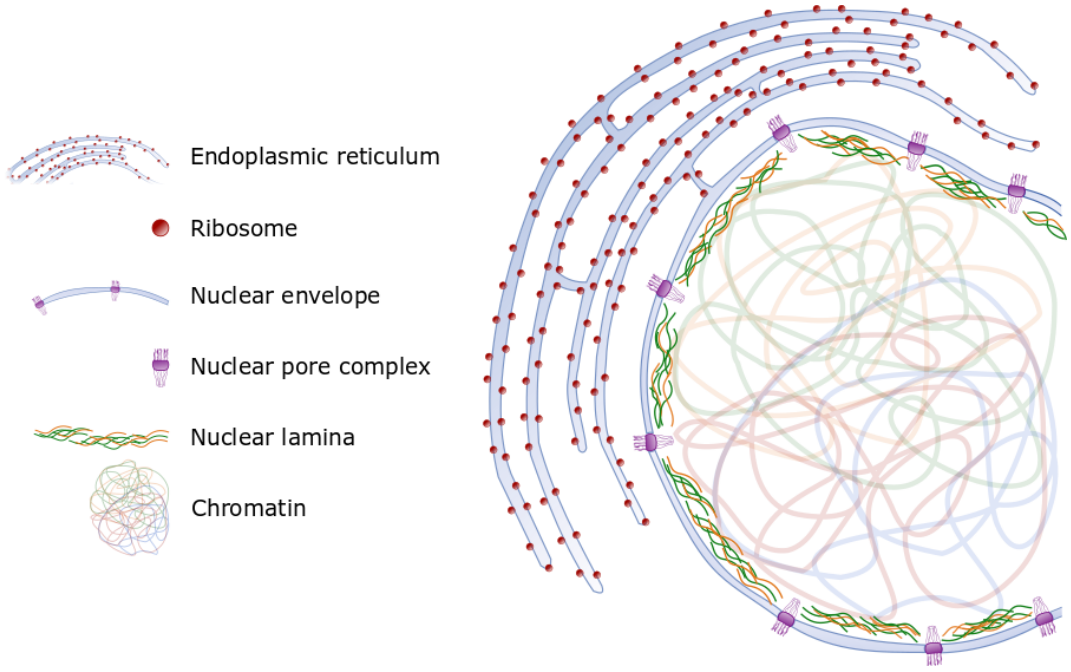


Figure i1: *Cartoon representing the main structures of the cell nucleus, including the rough endoplasmic reticulum and its ribosomes, the nuclear envelope, the nuclear pore complexes, the nuclear lamina, and the chromatin.*

1.2 THE GENOME AND ITS ORGANIZATION

As discussed above, the central component of the cell nucleus is the genomic material. A human cell nucleus contains around 2 m of DNA. To properly fit such an amount of DNA inside an organelle of a few µm in diameter, the DNA molecules need to be efficiently packed. Yet, the compaction has to allow proteins involved in processes like gene expression to efficiently interact with the DNA. The first level of organization of the genome consists in the formation of chromatin fibers. The basic unit of chromatin fibers is the nucleosome. Nucleosomes consist of 146 bp of DNA wrapped around an octamer of core histone proteins and connected to each other by approximately 60 bp of linker DNA. The chromatin fiber has a diameter of 5-24 nm and forms higher-order structures, such as chromatin loops and domains (Maeshima et al., 2019; Ou et al., 2017). Loops are usually formed by self-interactions inside one chromatin fiber and their length can range from Kilobases (Kb) to Megabases (Mb). Loops can mediate the interaction of regulatory elements, such as two or multiple enhancers, with promoter regions (Greenwald et al., 2019). Larger loops often serve in the formation and regulation of gene clusters and affect the 3D compaction of the genome (Misteli, 2020; Solovei & Mirny, 2021).

The chromatin is further organized in domains, which are regions of the genome preferentially interacting with each other rather than with the rest of the chromatin. These regions are also called topologically associated domains (TADs). TADs are formed by loop-extrusion through cohesin rings, and their boundaries are delimited by binding sites for the protein CTCF (Nanni et al., 2020). It is believed that TADs are important to restrict the effect of regulatory elements only to the genes inside a specific TAD. Although some studies point towards genes deregulation after disruption of TADs, others seem to suggest that on a genome-wide level, only a few genes are impaired by alteration of these domains (Dixon et al., 2016; Lupiáñez et al., 2016; Szabo et al., 2019). These data support the hypothesis that the overall function of genes depends on several layers of regulatory

mechanisms which facilitate their correct regulation. Impairment of only one of these mechanisms may not lead to drastic changes but only to an increased possibility of deregulation of the genes, especially those strongly relying on that specific mechanism for their regulation.

Chromatin domains further assemble into higher-order chromatin compartments. Based on their frequency of long-range interactions, each locus in the genome has been associated to one of two main chromatin compartments, called A and B. A and B compartments roughly correspond to euchromatin and heterochromatin, respectively (Lieberman-Aiden et al., 2009). Finally, the genomic material is divided in chromosomes, which exist in interphase as territories occupying a relatively compact region of the nucleus. Chromosome territories are roughly shaped as spherical structures that contain a complex network of channels, important to allow transcription factors and other proteins to readily access the DNA (Cremer & Cremer, 2001). Chromosome territories tend to locate in specific regions of the nucleus, and so do specific gene loci. For example, repressed or inactive genes are preferentially located at the nuclear periphery, while active genes tend to locate to the nuclear interior. The molecular processes that lead to these specific subnuclear localizations are still largely unknown (Misteli, 2020).

The study of the spatial organization of the genome is not enough to understand its regulation and functions. It is fundamental to also understand the relationship of chromatin with the various structures of the nucleus, and the functions that these structures carry on. A prominent nuclear structure, involved in the organization and regulation of chromatin, is the nuclear envelope, with its components.

1.3 THE NUCLEAR ENVELOPE

The NE is the outer barrier that separates the nucleus from the rest of the cell. This barrier protects the genome from mechanical damage and isolates the factors involved in genome regulation from the cytosol. Impairment of the structure or functions of the NE affects the viability of the cell as well as the stability of the genome (Denais et al., 2016; Raab et al., 2016).

As briefly described above, the NE is composed of two membrane layers: the inner- and outer-nuclear membrane (INM and ONM, respectively). These membranes are separated by a perinuclear space (PNS) but connected at sites of nuclear pore complexes (NPCs). The NPCs are large protein structures forming channels through the NE, thereby allowing the transport of selected molecules from the cytosol to the nucleus and *vice versa*. The main components of the NPCs are a class of proteins called nucleoporins (or Nups) (Hetzer et al., 2005). Although the INM and the ONM are connected, their protein composition is different and strictly regulated. The ONM contains a set of proteins responsible for the interaction with the cytoskeleton. These proteins, called Nesprins, are characterized by a common domain, the KASH domain. In the perinuclear space, the KASH domain mediates the interaction of Nesprins with the SUN-domain proteins, located in the INM (Sosa et al., 2012). The KASH- and SUN-proteins create a complex called “linker of nucleoskeleton to cytoskeleton” (LINC) complex. Together these proteins form a bridge through the NE that enables fundamental nuclear processes to take place. The LINC complex is involved in processes like the positioning of the nucleus inside the cell (Grady et al., 2005; Lei et al., 2009) and the sensing and transduction of mechanical and stress signals from the cytoskeleton to the nuclear interior (Méjat & Misteli, 2010). The INM also contains a group of transmembrane proteins (the “nuclear envelope transmembrane proteins” or NETs) that are able to interact with chromatin and are involved in processes like gene expression and chromatin organization (Zuleger et al., 2013). Some NETs remain

uncharacterized functionally, but others are known to interact with lamins in the NL (Clements et al., 2000; Q. Zhang et al., 2005) and with chromatin and chromatin regulating proteins (Czapiewski et al., 2016; de las Heras et al., 2017).

As discussed in this paragraph, the NE includes several specialized regions essential to carry-on different nuclear functions. Nevertheless, this structure undergoes extensive morphological changes during various cell processes, particularly during the cell cycle and mitosis. In interphase, for example, the surface of the NE grows. The formation of new lipids, which supports this process, is believed to happen at the NE/ER interface and to be regulated by a balance of enzymes involved in lipid synthesis, like torsin and lipin (Bahmanyar et al., 2014; Grillet et al., 2016). The newly synthesized lipids can then freely diffuse and contribute to the NE growth. During interphase, new membrane proteins also need to be incorporated into the expanding NE. The synthesis of these proteins happens in the ER and they afterwards diffuse within the connected membrane layers until they reach the proper place in the membrane. Most of the proteins inserted in the INM can freely diffuse and reach the inner layer through the membrane surrounding the NPCs, others require active transport (De Magistris & Antonin, 2018). The major NE rearrangement, however, is observed during mitosis. While in closed and semi-closed mitosis, observed in budding and fission yeast, at least part of the NE remains intact, higher eukaryotes undergo what is defined as open mitosis. During this process, the NE is completely dismantled, detached from the (also disassembled) lamina and from the chromosomes, and absorbed into the ER. After the end of mitosis, regions of the ER start making contacts with the chromatin. The flattening and expansion of these membranes is thought to give rise to the new NE. The contacts between the growing NE and chromatin require the presence of proteins of the INM, including lamin-binding proteins, nucleoporins and many more (Schooley et al., 2012).

1.4 THE NUCLEAR PORE COMPLEXES

The NPCs, as discussed above, are the communication channels between the cytosol and the nucleoplasm. They are large macromolecular assemblies, inserted into the NE at sites where the ONM and the INM are continuous (Fichtman et al., 2010). These channels are made up of around 30 different proteins, present in 8 to 64 copies each, and can reach molecular masses above 120 MDa, like in human cells (Cronshaw et al., 2002).

The NPC structure contains an inner pore ring, spanning through the NE and forming the central channel structure. The channel is anchored to a nucleoplasmic ring and a cytosolic ring. Peripheral elements emanate both from the nucleoplasmic and cytosolic ring: they form the nuclear basket and the cytoplasmic filaments, respectively (Bui et al., 2013; Maimon et al., 2012). The proteins involved in the formation of NPCs are called nucleoporins (NUPs) and are structurally conserved across eukaryotes. The NUPs can be divided into scaffold NUPs and FG-NUPs. Scaffold NUPs are mainly involved in the formation of the pore structure. The FG-NUPs contain FG (Phe-Gly) repeats, which are disordered domains spanning through the pore channel and constituting the permeability barrier (Yamada et al., 2010). Although small molecules (<30-40 kDa) can freely diffuse through the nuclear pores, molecules with higher molecular weight require active transport. This active transport is mediated by specific proteins, the nuclear transport receptors (NTRs). The FG-domains of the central NUPs form a disordered, hydrophobic hydrogel in which the FG-repeats interact to form a mesh. To pass through it, the cargo needs to be bound by transport receptors, which can interact with the FG-regions through transient and low-affinity interactions (Ghavami et al., 2016). Importin α and β are examples of import NTRs that bind to the nuclear localization signal (NLS) on cargo proteins in the

cytoplasm and facilitate their transport into the nucleus (Goldfarb et al., 2004; Lowe et al., 2015). Exportin 1, instead, works to facilitate the transport in the opposite direction (Seedorf et al., 1999; Wente & Rout, 2010).

1.5 THE NUCLEAR LAMINA

Underneath the INM of the NE, a class of proteins called lamins assembles into a mesh, to form, in association with lamin-binding proteins, a scaffold structure defined as nuclear lamina (NL). The NL is involved in many cellular functions. Initial studies showed its importance in maintaining the nuclear shape, the integrity of the NE, the mechanical properties of the nucleus, and in sensing and transducing mechanical stimuli coming from the cytosol (Dahl et al., 2008). Later, the NL has also been shown to take part in many other nuclear processes, like organization and spatial positioning of chromatin, gene expression, cell signaling, and more (Gruenbaum & Foisner, 2015; Isermann & Lammerding, 2013). To fulfill many of these functions, lamins need to interact with proteins inserted in the INM, bound to chromatin, or present in the nucleoplasm.

1.5.1 Structure of nuclear lamins

Lamins are type V intermediate filaments. Like other intermediate filaments, they are composed of an N-terminal (head) domain, a central (rod) domain and a C-terminal (tail) domain. The C-terminal domain includes a nuclear localization signal (NLS), an IG-fold motif, and the CaaX-box (Ahn et al., 2019). The CaaX-box is a particular amino acidic sequence, composed of a cysteine (C), followed by two aliphatic amino acids (aa), and a random amino acid (X). This sequence is the substrate of enzymatic modifications that regulate lamin localization and further processing (Barrowman et al., 2008; Gruenbaum & Medalia, 2015). Lamins can be classified into two major groups, based on their post-translational modifications and localization in the nucleus: A-type lamins (including lamin A and lamin C) and B-type lamins. Lamin B1 and B2, the main B-type lamins, are encoded by two different genes, *LMNB1* and *LMNB2* (Biamonti et al., 1992; Feng Lin & Worman, 1995). A-type lamins, on the other hand, are the product of differential splicing of the same gene, *LMNA* (F. Lin & Worman, 1993).

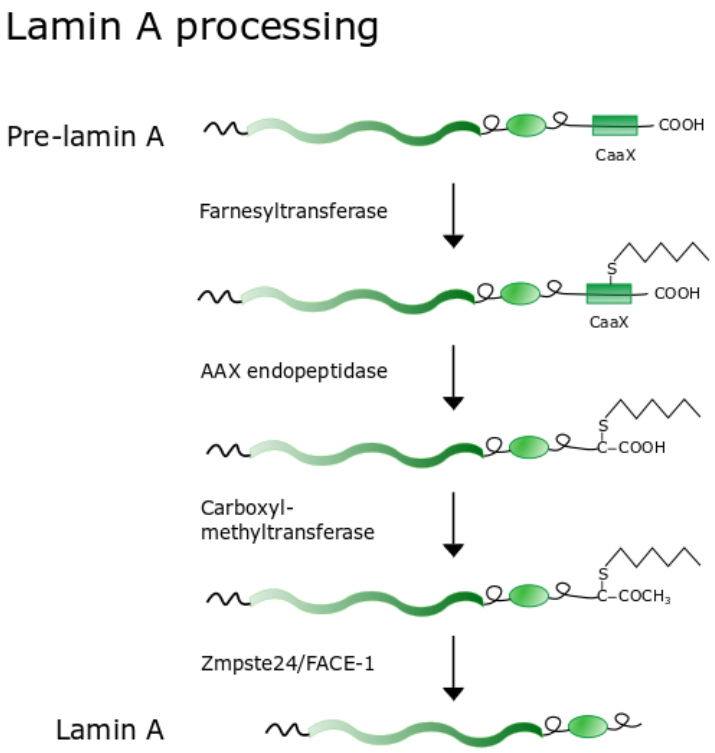


Figure i2*: Scheme depicting the various steps of Lamin A processing. First, the enzyme farnesyltransferase adds a farnesyl group to the cysteine in the C-terminal CaaX box. Then, an endopeptidase removes the -aaX terminal peptide and the remaining carboxy-terminal group is methylated. Finally, the Zinc metallopeptidase STE24 (Zmpste24; FACE-1 in human cells) removes 15 C-terminal amino acids, including the farnesyl group, producing the mature (and soluble) Lamin A.*

A- and B-type lamins undergo different post-translational processing, which influences their localization in the nucleus. Lamin C lacks the C-terminal domain containing the CaaX-box and is, therefore, not post-translationally processed (Al-Saaidi & Bross, 2015). B-type lamins and lamin A are initially farnesylated on the cysteine of their CaaX motif by a farnesyltransferase. The last three amino acids (aaX) are then removed by an AAX endopeptidase, and a methyl group is added to the remaining cysteine (Adam et al., 2013; Maske et al., 2003). In addition to these steps, lamin A, but not lamins B1 and B2, undergoes further modifications (Figure i2). The Zinc metallopeptidase ZMPSTE24 removes the last 15 amino acids of the lamin A C-terminus, eliminating the farnesyl group (Holtz et al., 1989; Pendás et al., 2002). Due to their permanent C-terminal modifications, B-type lamins localize mainly at the nuclear periphery, while A-type lamins are initially incorporated into the NL, but also partially released into the nucleoplasm as soluble pool after losing their farnesylation. The presence of the farnesyl group is essential, but not sufficient, for the localization of lamins at the nuclear membrane (Adam & Goldman, 2012; Davies et al., 2009).

Lamin assembly into filaments at the nuclear periphery is not completely understood. In vitro data suggest that lamins form dimers by interaction of their α-helical rod domains in a parallel fashion (Ahn et al., 2019). These dimers then assemble longitudinally in a head-to-tail manner to form proto-filaments, which interact laterally to form the mature filaments (Davidson & Lammerding, 2014) (Figure i3). Recent cryo-electron microscopy data show that the structure of lamin filaments is different from the one displayed by cytoplasmic intermediate filaments (IFs). Lamins, in fact, form

3,5 nm thick filaments, as opposed to the canonical 10 nm thick IFs (Ahn et al., 2019; Y. Turgay & Medalia, 2017; Yagmur Turgay et al., 2017), which is consistent with having two protofilaments associated laterally. In vitro studies showed that lamin A and B can interact and form mixed filaments, but in vivo photobleaching and immunofluorescence studies seem to point towards the formation of separate filaments which could either be connected or just positioned next to one another (Davidson & Lammerding, 2014). In contrast to the assembly state of lamin filaments at the periphery, little is known about the structure of A-type lamins in the nuclear interior (Naetar et al., 2021).

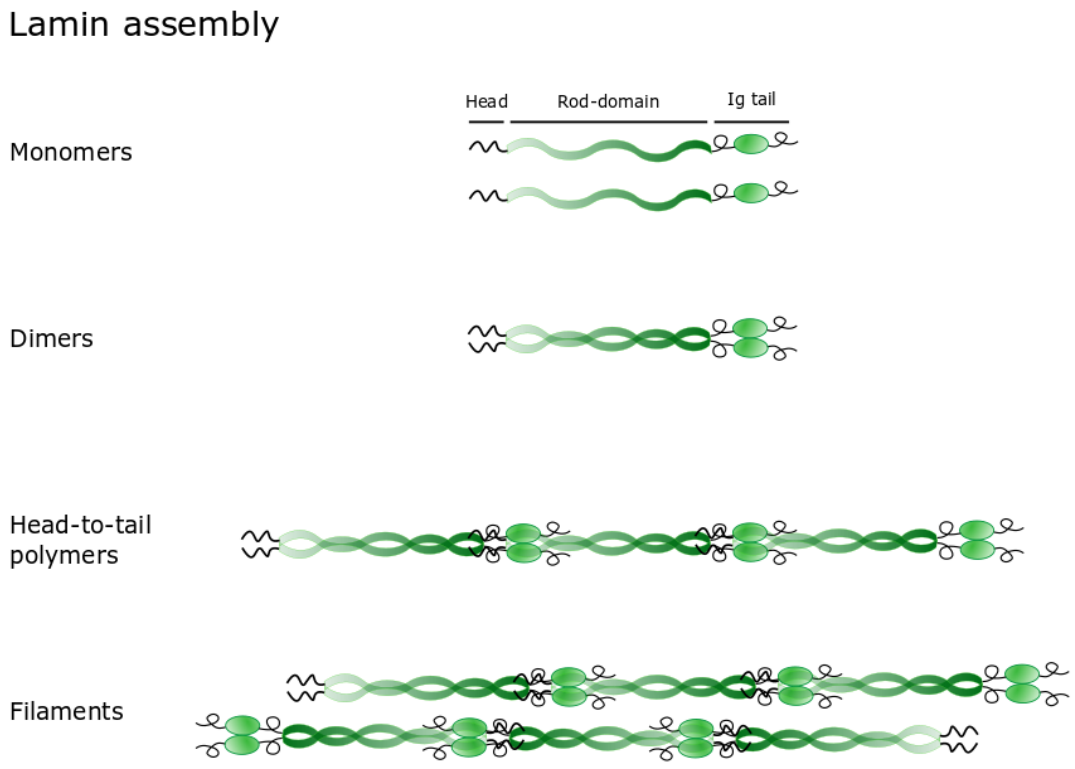


Figure i3: *Scheme depicting lamin filament assembly. Two lamin monomers interact in a parallel fashion, through their rod-domains, to form dimers. Then, dimers assemble head-to-tail to form polymers. Two polymers associate with each other laterally, likely in an anti-parallel direction, to form filaments.*

1.5.2 Lamin binding partners

Lamins interact with many proteins, which help them carry out their functions. The first lamin binding proteins discovered are transmembrane proteins of the NE and are referred to as lamin-associated polypeptides (LAPs) (Foisner & Gerace, 1993). Some examples are the LAP1 and the LAP2 protein families (Furukawa et al., 1998; Maison et al., 1997), each of them including multiple isoforms, and the lamin B receptor (LBR) (Ye & Worman, 1994). Further studies identified also soluble lamin binding partners. Among these soluble proteins are, for example, the alpha isoform of the LAP2 family (LAP2α) (T. Dechat et al., 2000), Narf (Barton & Worman, 1999), and Lco1 (Vlcek et al., 2004). Many other proteins have been suggested to interact with lamins, some stably and others, like Rb, PCNA, and other factors, more transiently. The transient interaction of lamins to some of these proteins is believed to have a regulatory effect and modulates the protein´s functions in specific cell

processes or in response to cell stimuli (Wilson & Foisner, 2010). In addition, proteomic studies revealed many additional inner nuclear membrane proteins, termed nuclear envelope transmembrane proteins (NETs), which are expressed in a tissue-specific manner (de las Heras et al., 2013, 2017), but most of them have been barely analyzed.

Thus, Lamin binding partners include a variety of proteins with very different structures and functions. Some of the better characterized lamin binding proteins are involved in structural and mechanical processes. I already introduced the LINC complex, which spans through the NE linking the cytoskeleton and the nucleus. In the nuclear interior of mammals, the INM proteins SUN1 and SUN2 interact via their N-terminal domain with nuclear lamins (Haque et al., 2010). In the lumen between INM and ONM, SUN proteins interact with KASH proteins, located in the ONM, and those, in turn, interact with the cytoskeleton, thereby providing a link between lamins and cytoskeletal filaments (Sosa et al., 2012). Furthermore, in human cells, two KASH proteins (nesprin 1α and 2) are known to also localize to the INM, directly interacting with lamins and with the LEM-protein emerin (Haque et al., 2010; Mislow et al., 2002; Yang et al., 2013).

Another important class of lamin binding proteins is the LEM (LAP2-emerin-MAN1) protein family (Brachner & Foisner, 2011). These factors share a common motif, the LEM-domain, which allows them to interact with a soluble protein, called barrier-to-autointegration factor (BAF) (Cai et al., 2001; Shumaker et al., 2001). BAF is highly conserved among metazoan cells and exists as a dimer in solution. Each monomer contains two DNA binding sites and studies suggest that its binding to DNA induces conformational changes in BAF, leading to the formation of oligomers of 6 molecules (Zheng et al., 2000). With the ability to bind DNA in a sequence-independent fashion and to interact with histones, BAF is a perfect bridge between the INM and chromatin. In addition to LEM-containing proteins, BAF can bind to other factors, including lamin A, therefore creating an additional link between chromatin and the NL (Montes De Oca et al., 2005). Some of the lamin binding partners also interact with chromatin independently of the LEM domain and of BAF. All the members of the LAP2 family, for example, share a LEM-like domain that has been proven to directly bind to DNA in vitro (Furukawa et al., 1997). MAN1 also contains an additional DNA binding motif (Caputo et al., 2006) and LAP2β can interact with HA95, which in turn binds to DNA (Martins et al., 2003). In addition, some of the NETs were found to interact with chromatin (Czapiewski et al., 2016; de las Heras et al., 2017), but only a few of them have been shown so far to interact with lamins. Lamins can therefore regulate chromatin indirectly by binding to other proteins which interact with DNA. Additionally, lamins have been shown to bind to chromatin directly through their C-terminal histone binding domain (Thomas Dechat et al., 2009; Taniura et al., 1995). The intricate network of interactions between the NL and chromatin puts lamins at the center of many nuclear processes, like chromatin organization, gene expression, and DNA replication.

1.5.3 LAP2alpha

As already mentioned, the LAP2 family of proteins is one of the main lamin interactors. These proteins are encoded by the gene *Tmpo*, and include 6 isoforms (α, β, γ, δ, ε, ζ; Figure i4) produced by alternative splicing (Berger et al., 1996). The LAP2 isoforms share a common N-terminal domain which includes the LEM-motif and the LEM-like domain. All the isoforms are, therefore, able to bind to DNA both directly, through the LEM-like domain, and indirectly, through the interaction of the LEM-domain with BAF (Cai et al., 2001). Most of the LAP2 isoforms are structurally very similar also in their C-terminal tail, which contains a single transmembrane domain anchoring them to the INM. LAP2ζ is the smallest isoform and has only a few amino acids after the N-terminal domain, therefore lacking the

transmembrane anchor. Its localization is mainly cytosolic, although a low amount is also found in the nucleus (Shaklai et al., 2008). The other isoform, which has a completely different C-terminus lacking a transmembrane domain, is LAP2α (or LAP2alpha). LAP2alpha is the longest LAP2 isoform and is distributed throughout the nucleus (Thomas Dechat et al., 1998). Its specific C-terminal domain contains binding sites for A-type lamins and other factors, like pRb and HMGN5 (T. Dechat et al., 2000; Markiewicz et al., 2002; S. Zhang et al., 2013). The LAP2alpha unique domain is therefore responsible for the specific functions of this protein. While the other LAP2 isoforms interact preferentially with B-type lamins, LAP2alpha interacts exclusively with A-type lamins in the nuclear interior and it has been shown to regulate the interaction of lamin A/C with chromatin (Gesson et al., 2016). This is particularly interesting since both LAP2alpha and the nucleoplasmic pool of A-type lamins are distributed throughout the nucleus and this localization allows them to bind not only to peripheral, repressed, and gene-poor chromatin regions at the NL, but also to more open and active areas in the nucleoplasm (T. Dechat et al., 2000; Gesson et al., 2016). It is therefore tempting to speculate that LAP2alpha-mediated direct or indirect chromatin regulation can affect the expression of genes required for specific cell functions. In support of this theory, LAP2alpha has been shown to interact with proteins involved in chromatin regulation. Its binding to the high mobility group protein N5 (HMGN5) affects the positioning of the latter on DNA, therefore regulating chromatin compaction in defined areas of the genome, as discussed in more detail later in this thesis (Furusawa et al., 2015; S. Zhang et al., 2013).

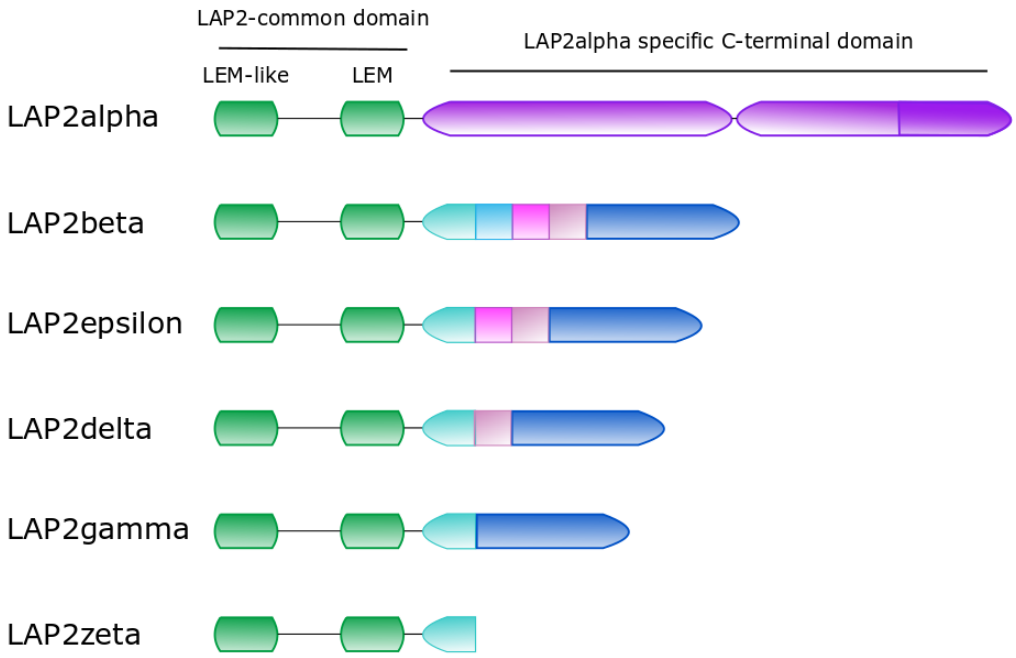


Figure i4: *Schematic representation of the 6 mammalian LAP2 isoforms. All isoforms contain LEM-like and LEM domains (depicted in green). Different C-terminal domains of the isoforms confer specific localization and functions.*

LAP2alpha is also important for certain functions of the tumor suppressor retinoblastoma protein (pRb). More specifically, together with A-type lamins LAP2alpha is required for correct localization and phosphorylation of pRb. Lack of LAP2alpha alters pRb regulation, therefore affecting the downstream signaling and cell cycle-dependent transcription (Markiewicz et al., 2002; Pekovic et al.,

2007). This is yet another indication that the unique structure and, consequently, the specific localization and functions of this LAP2 isoform may allow LAP2alpha to be involved in the regulation of active chromatin, aiding other factors like pRb to maintain proper transcriptional control.

LAP2alpha functions have also been investigated in vivo in complete and conditional LAP2alpha knockout mice. Mice lacking this protein show systolic dysfunction of the heart at a young age and sporadic cardiac fibrosis later in life (Gotic, Leschnik, et al., 2010). Moreover, LAP2alpha knockout mice display mild hyperplasia in highly proliferating tissues, such as skin and colon, associated with changes in A-type lamins localization and pRb dysfunction (Naetar et al., 2008). No obvious defects are visible in the skeletal muscle tissue. Nevertheless, a detailed analysis of LAP2alpha knockout primary myoblasts shows deregulation in their differentiation. This is accompanied by delayed upregulation of myogenic-specific markers and prolonged expression of stem cell factors (Gotic, Schmidt, et al., 2010).

1.5.4 Lamin functions

To properly analyze lamin functions in the cell nucleus, one has to distinguish between functions of B-type lamins and A-type lamins. Moreover, we should also distinguish between the contributions of the peripheral pool of A-type lamins, which is embedded in the NL, and the soluble pool, distributed throughout the nucleoplasm. However, these pools are not easy to evaluate separately, as knockouts of A-type lamins, for example, always affect both the nucleoplasmic and the peripheral lamin A/C pool. Therefore, the individual contribution of these pools of lamin A/C in carrying out lamin-dependent functions is often not clear. Nevertheless, many studies focused on A-type lamin-mediated mechanisms and, although the details are not fully understood yet, many advances have been made in elucidating lamin functions.

One of the main differences between A- and B-type lamins, which mirrors their different functions in the cell, is their expression pattern. Lamin B is ubiquitously expressed throughout development in all differentiation stages and cell types (Feng Lin & Worman, 1997; Stewart & Burke, 1987). On the contrary, A-type lamins are upregulated during differentiation and are thought to be more involved in tissue-specific functions (Eckersley-Maslin et al., 2013).

1.5.5 Functions of B-type lamins

Unravelling lamin B functions is a challenging task. While mutations in A-type lamins cause a very wide range of phenotypes and diseases, only one lamin B-linked disease has been described, so far: the adult-onset autosomal dominant leukodystrophy (ADLD). The main reason for this difference might be that B-type lamins are necessary for organogenesis and cell survival. Defects in their functions, in fact, are mainly incompatible with life (Vergnes et al., 2004).

Several mouse models have been generated with mutated forms or full depletions of B-type lamins, to better analyze their impact in whole organisms. Mice with a mutated form of lamin B1 did not survive after birth and displayed reduced size, organ and tissue abnormalities, and defects in genome stability in somatic cells (Vergnes et al., 2004). Lamin B2-deficient mice, on the other hand, were born alive and died shortly after birth, displaying mainly neuronal defects, probably related to impaired neuron migration (Coffinier et al., 2010). Depletion of both lamin B1 and B2 does not seem to affect the functions and viability of mouse embryonic stem cells (mESCs). Nevertheless, mice

generated from B-type lamin-depleted stem cells die immediately after birth because of their inability to breath (Kim et al., 2011). Although these mice show multiple organ/tissue defects and brain anomalies, not all organs are affected by the loss of B-type lamins. The reason why some organs may not be affected by this depletion could be a predominant role of A-type lamins in the homeostasis of their cells (Thomas Dechat et al., 2009; Vergnes et al., 2004).

In vitro studies in Xenopus extracts showed that depletion or mutations in B-type lamins lead to fragile nuclei, unable to replicate DNA and with normally formed but mis-localized NPCs. To date, it is not clear which step of DNA replication requires functional B-type lamins, although some data point towards either the initiation phase (Ellis et al., 1997; Izumi et al., 2000) or the elongation phase (Moir et al., 2000; Spann et al., 1997). Functional lamin B filaments have also been linked to normal DNA transcription (Spann et al., 2002). Other studies showed that B-type lamin depletion leads to cell apoptosis and the impaired assembly and maintenance of the mitotic spindle (Tsai et al., 2006). Additionally, more and more data point towards a role of lamin B1 in cellular senescence (Shimi et al., 2011). In some cell lines, in fact, its downregulation leads to p53-mediated cell senescence (Freund et al., 2012). Other studies seem to indicate that lamin-associated senescence depends on the ratio of B and A-type lamins, rather than loss of B-type lamins alone (Dreesen et al., 2013; C. J. Hutchison, 2014; Christopher J. Hutchison, 2012).

The correct ratio between A and B type lamins is also fundamental for the mechanical properties of the nucleus. Knockdown of lamin B1 has been associated to increased nuclear stiffness, in erythroid cells (Swift et al., 2013). Nevertheless, this effect relies also on A-type lamins expression: cells characterized by low levels of lamin A/C show increased nuclear stiffness upon lamin B1 depletion, while the effect seems negligible in the presence of high levels of A-type lamins (Stephens et al., 2017).

In addition to DNA replication and translation, positioning of the mitotic spindle, NPCs localization, nuclear mechanics and structure, and cell migration, B-type lamins have been implicated in chromatin organization, heterochromatin maintenance, and gene repression. The functions of lamins in chromatin regulation will be discussed in more detail in the next chapters.

1.5.6 Functions of A-type lamins

The functions of A-type lamins have been extensively investigated. Their mutations can cause a wide range of diseases, collectively named “laminopathies”, that can affect different tissues. The differential effect of A-type lamins in various tissues is partially due to their expression pattern. In contrast to B-type lamins, A-type lamins are expressed at very low levels in mESCs and upregulated later during differentiation (Eckersley-Maslin et al., 2013). This means that fully differentiated cells usually display higher A-type lamin levels than cell precursors but also that not all the tissues express these lamins or rely on them in the same way.

Mutations in A-type lamins have been linked to the deregulation of various cell signaling pathways. For example, upregulation of MAPK signaling through hyperactivation of ERK and JNK has been demonstrated both in patients affected by laminopathies and in cell lines (Muchir et al., 2007, 2009). Additionally, there is evidence of modulation of the TGF-β1 pathway by A-type lamins. Cell lines depleted of Lamin A/C show alterations in the downstream response to TGF- β1, with increased SMAD-dependent collagen production and a reduced interaction of the phosphatase PP2A with the cell cycle regulator Retinoblastoma protein (pRb). This correlates with the deregulation of pRb

phosphorylation and cell proliferation. Impairment of the TGF- β1 signal has been suggested as one of the mechanisms responsible for mesenchymal cell defects in laminopathies (Van Berlo et al., 2005). Cellular or phenotypical changes in other lamin-linked diseases also suggest A-type lamin modulation of the Notch signaling pathway, involved in stem cell differentiation and the determination of cell fate (Scaffidi & Misteli, 2008). The molecular details of this interplay have not been elucidated yet.

A-type lamins are also involved in the regulation of cell cycle-dependent transcription. In vitro studies show that A-type lamins bind to pRb and these two molecules co-localize in perinucleolar foci, in fibroblasts (Kennedy et al., 2000; Mancini et al., 1994; Van Berlo et al., 2005). As described above, their binding partner LAP2alpha has also been suggested to interact with pRb, inhibiting E2F-mediated transcription and delaying cell cycle entry from G0 (Dorner et al., 2006; Markiewicz et al., 2002). Several mechanisms have been proposed to explain A-type lamin-dependent regulation of pRb, including indirect effects through the modulation of the TGF-β1 pathway (as described above). A-type lamins also modulate pRb localization, its post-translational modification, and proteasomal degradation (Johnson et al., 2004; Van Berlo et al., 2005). In vivo data from patients and in vitro studies show that deregulation of pRb pathways is one of the cellular defects leading to alterations in skeletal muscle- and adipocyte-differentiation in laminopathies. These tissues are, in fact, frequently affected in patients with A-type lamins- (or lamin-binding partners-) related disorders. In muscular dystrophies and other muscular phenotypes, mutations in lamins are also associated with defective activation of the MyoD pathway and deregulation of other myogenic regulatory factors (MRFs) essential for skeletal muscle functions. Studies in cell lines show that A-type lamins can also interact with the transcription factor c-Fos, modulating its interaction with the AP-1 transcriptional complex and the downstream gene expression (Gonzàlez et al., 2008). Moreover, lamin A/C can influence the localization of the cholesterol response element binding protein SREBP1, in adipocytes, and interact with several other transcription factors involved in gene expression and cell differentiation (Graziano et al., 2018).

Laminopathies also exhibit defects in nuclear mechanics (Dahl et al., 2006; Lammerding et al., 2004). Lamin A/C expression has been linked to nuclear stiffness and viscosity (Buxboim et al., 2017; Swift et al., 2013). Nuclear stiffness limits 3D cell migration but is fundamental for nuclear integrity: knockdown of A-type lamins reduces the stiffness of the nuclei but increases their susceptibility to mechanical stress, leading to DNA damage and decreased viability (Harada et al., 2014). Similarly, mutations of lamin A/C, as well as loss of all lamins, have been linked to the formation of non-lethal nuclear ruptures and consequent DNA damage (Chen et al., 2018; De Vos et al., 2010).

A-type lamins have also been shown to be involved in several processes related to chromatin dynamics and DNA repair. They influence the dynamics of telomeres, centromeres, and other genomic loci by transiently interacting with these regions. Depletion of A-type lamins has been shown to change the diffusion of genomic loci from slow-anomalous to fast-normal, although the implications of these changes on genome regulation and stability are unclear (Bronshtein et al., 2015; De Vos et al., 2010). The binding of lamins to telomeres has also a role in the maintenance of these structures since LMNA knockout (KO) mice show both a decrease in telomere length and a high rate of telomere loss in chromosomes (Gonzalez-Suarez et al., 2009). Furthermore, mouse embryonic fibroblasts depleted of A-type lamins display an impaired double-strand break (DSBs) repair mechanism by both homology-directed repair (HDR) and non-homologous end joining (NHEJ) (Redwood et al., 2011). This leads to aneuploidies, chromosome and chromatid breaks, and increased levels of DNA damage markers. The effect on the DSB repair efficiency is probably due to changes in the expression and stability of proteins involved in these repair pathways (Gonzalez-

Suarez et al., 2011). Genomic instability has also been confirmed in vivo in some laminopathies, like the Hutchinson-Gilford progeria syndrome (HGPS) (Allsopp et al., 1992; Decker et al., 2009).

The functions of A-type lamins in DNA replication are much less clear. Studies in Xenopus laevis egg extracts suggest that a functional NL is fundamental for the correct localization of replication factors like the Proliferating Cell Nuclear Antigen (PCNA) and the Replication Factor Complex (RFC) (Spann et al., 1997). DNA replication defects have been found in HGPS cells and further studies proposed that A-type lamins may be required to properly restart stalled DNA replication forks (Singh et al., 2013; Tang et al., 2012). Nevertheless, the molecular mechanisms of their involvement in these processes have yet to be investigated.

All these studies show that A- and B-type lamins are involved in many different aspects of nuclear homeostasis and response to stimuli, as well as in DNA preservation and replication. One of the most fascinating and extensively studied topics is, however, their involvement in spatial chromatin organization and the regulation of gene expression.

1.5.7 Lamin functions in chromatin structure and regulation

As discussed above, lamins are fundamental for many nuclear functions and are involved in different aspects of chromosome dynamics, DNA repair, and replication. The ability of these proteins to directly bind to the chromatin makes them also the perfect candidates to be involved in chromatin regulation processes, as I will discuss in this paragraph.

The NL was initially shown to interact with chromatin in regions called matrix association regions (MARs) (Blasquez et al., 1989). To better characterize these interactions, several in vitro studies have been performed, leading to evidence of the direct binding of lamins to the DNA (Ludérus et al., 1992; Zhao et al., 1996). Lamins have also been reported to bind to histones, initially in Drosophila (Goldberg et al., 1999) and other organisms (Yuan et al., 1991) and later also in mammalian cells (Taniura et al., 1995). The establishment of whole-genome sequencing techniques allowed researchers to analyze these binding sites on a genome-wide scale. To do so, Lamin B1 interactions with chromatin were first detected by DamID. In this technique, the E.coli DNA adenine methyltransferase (Dam) is fused to lamin B1 or another protein of interest, and the DNA that comes in close proximity to this fusion protein becomes methylated. The type of methylation introduced by Dam is easy to identify because it does not naturally occur in most eukaryotes. The specific binding sites can therefore be isolated and sequenced (Greil et al., 2006; Van Steensel & Henikoff, 2000; Vogel et al., 2007). The Dam-ID genome-wide mapping of lamin B1 biding sites was initially performed in Drosophila and, afterwards, in mammalian genomes. The identified lamin B1-bound regions are called lamina-associated domains (LADs), their size ranges from 0,1 to 10 megabases (Mb), and they are characterized by a repressive chromatin environment with H3K9me2/3 and H3K27me3 enrichment and low gene density. More than 1000 LADs have been identified, covering about 30% of the genome and being distributed across all chromosomes. The borders of these interaction regions are often sharp and delimited by specific sequences like CTCF binding sites (Guelen et al., 2008; Peric-Hupkes et al., 2010).

Despite the fact that the gene density in LADs is low, most of the genes within these regions are repressed or expressed at very low levels. To understand whether the binding to the NL is causally responsible for gene repression, several laboratories artificially tethered reporter genes to the lamina. Although in some studies this tethering led to gene repression, one study did not show any

difference in the expression levels of the reporter gene after translocation to the NL (Finlan et al., 2008; Kumaran & Spector, 2008; Reddy et al., 2008). Therefore, the repression of the genes inside LADs may not be strictly linked to their confinement at the nuclear periphery. In agreement with this theory, single-cell analyses of LADs concluded that only 30% of these genomic lamin-binding regions are at the nuclear periphery in each cell and that the contacts with the NL are dynamic and not inheritable after mitosis. In fact, LADs are randomly re-shuffled in daughter cells after cell division (Kind et al., 2013). Most of the LADs are positioned in the nuclear interior and may be bound to nucleoplasmic A-type lamins. Many of these interactions (about 30% of the total LADs) occur in peri-nucleolar areas, suggesting a link between A-type lamins and DNA sequences positioned around the nucleoli (Kind & Steensel, 2014).

In general, lamins have a very prominent role in chromatin organization and spatial distribution. For example, lamin A has been linked to the correct tethering of heterochromatin at the nuclear periphery, together with the lamin B receptor (LBR). These two proteins are sequentially expressed during differentiation and their simultaneous loss leads to an inversion of chromatin architecture, with centrally distributed heterochromatin and peripheral euchromatin (Solovei et al., 2013).

Despite these findings, peripheral LAD localization does not seem to strictly require the presence of lamins. mESC depleted of both A- and B-type lamins maintain their overall LAD organization (Amendola & Steensel, 2015). This could be due to the redundant functions of lamins and lamin binding proteins, such as LBR and the LEM-proteins interacting with BAF. The binding of BAF to the genome is, in fact, largely overlapping with that of lamins. Knockdown of BAF increases the interactions of lamin B1 with LADs, supporting the idea of their competitive binding in these regions (Kind & Steensel, 2014).

Although the LAD organization seems to be mainly preserved among cells and species, differentiation-induced changes have been reported. Genomic maps of Lamin B1 interaction with chromatin were generated for different cell types during neuronal differentiation: mESC, committed neuronal precursors, and terminally differentiated astrocytes. Although the majority of the LADs are conserved during this lineage differentiation, some differences are detectable. The LADs preserved in different cell types are called constitutive LADs (cLADs), while the others are referred to as facultative LADs (fLADs) (Meuleman et al., 2013). Areas of differential lamin B1 binding often contain genes involved in the differentiation process. Moreover, increased gene binding to lamin B1 has been shown to correlate with gene repression (Sadaie et al., 2013).

In light of more recent findings, the conservative view of lamins interacting only with repressive and gene-poor chromatin has been more and more challenged. Different results depend partially on the type of technique employed and on technical differences in the treatment of the samples, which can lead to the enrichment or loss of active genomic regions. In particular, chromatin immunoprecipitation (ChIP) seems more efficient in detecting transient and dynamic interactions of lamins with the genome, therefore also highlighting their binding to active chromatin. DamID, instead, could mainly reveal the stable interactions that occur at the periphery with repressed chromatin. DNA sonication settings have also been shown to be crucial to enrich for specific types of chromatin in ChIP experiments (Gesson et al., 2016).

Analysis of adipocytes by ChIP followed by array hybridization revealed interactions of A-type lamins with promoter regions of genes inside and outside LADs. Depending on the specific sub-promoter region, the interaction with A-type lamins has different effects on the transcriptional activity of the gene (Lund et al., 2013). The effect of A-type lamin binding to a specific sub-promoter region also seems to be cell type-specific. In adipocytes and their progenitors, A-type lamins differentially bind to

adipogenic genes during differentiation, potentially contributing to their transcriptional regulation. In this process, lamin binding seems to be also influenced by the metabolic state of the cells. During adipocyte differentiation, the metabolic changes occurring in the cells are associated with GlcNAcylation of histone 2B (H2B) which has been shown to mark the sites of newly formed LADs (Rønningen et al., 2015).

Lamin A/C interaction with specific chromatin regions depends also on their phosphorylation state. Binding of the Ser22-phosphorylated (pSer22) A-type lamins, in fact, has been detected on active enhancers and has been found to be altered in Hutchinson-Gilford progeria syndrome (HGPS) cells, leading to deregulation of genes involved in the progeria phenotype (Ikegami et al., 2020).

The results obtained analyzing laminopathies and differentiation systems suggest that A-type lamin binding does not occur only in repressed and gene-poor regions. Similarly, another study showed that A-type lamins also bind to euchromatin and, in those regions, colocalize with their interacting protein LAP2alpha. The presence of LAP2alpha may be important for proper A-type lamin association with euchromatin. Cells lacking LAP2alpha display A-type lamin re-localization towards more repressed regions of the genome and these changes also correlate with changes in active and repressive histone marks (Gesson et al., 2016). Although it is tempting to speculate that A-type lamin and LAP2alpha binding may directly affect chromatin regulation, several studies showed that both proteins can interact with and affect the localization or activity of other factors involved in chromatin regulation.

LAP2alpha has been shown to interact with chromatin modifiers, like the high mobility group N 5 protein (HMGN5). HMNG5 can dynamically bind to nucleosomes in a sequence-independent manner and reduce chromatin compaction by destabilizing histone H1 chromatin interactions (Furusawa et al., 2015). HMNG5 has been shown to directly bind to LAP2alpha in living cells and these two proteins influence each other´s binding sites on chromatin. Loss of LAP2alpha leads to a significant redistribution of HMNG5 chromatin binding and to a reduction of its dynamics in cells, probably due to the more stable interaction of HMNG5 with chromatin. The regulation of these two proteins is reciprocal since downregulation of HMNG5 was also shown to change LAP2alpha binding sites on chromatin (S. Zhang et al., 2013).

Lamins can also affect chromatin regulation through the Polycomb group (PcG) of proteins. PcGs are protein complexes involved in epigenetic control of developmental and differentiation processes by repressing genes that are not needed in a particular cell type/stage of differentiation (Prezioso & Orlando, 2011). A-type lamins interact with PcGs and their presence is important for the correct assembly and localization of PcGs foci and, therefore, for correct gene repression (Cesarini et al., 2015). Analyses of these interactions by proximity ligation assay (PLA) and the use of specific algorithms for image analysis suggested a preferential distribution of PcGs foci in the nuclear interior and, therefore, a likely involvement of the soluble pool of A-type lamins in PcGs regulation, rather than the peripheral lamin filaments (Marullo et al., 2016).

1.5.8 Lamins in diseases

Given the broad range of functions carried out by lamins, it is not surprising that their mutations have been associated with several diseases. The term describing A-type lamin-related diseases is “laminopathies”. As already mentioned, most diseases are linked to mutations in A-type lamins (*LMNA* gene) because B-type lamins are required for viability. Diseases linked to B-type lamins or

lamin-binding proteins are often referred to as secondary laminopathies. More than 400 mutations in *LMNA* have been described, resulting in different phenotypes including skeletal muscle impairment, cardiomyopathies, lipodystrophies, neuronal and dermal defects, and phenotypes involving the whole organism, like the premature aging syndrome Hutchinson-Gilford progeria (HGPS) (J. L.V. Broers et al., 2006; Kang et al., 2018).

Since A-type lamins are expressed in most differentiated cells, it is surprising that the majority of lamin mutations do not affect all tissues. Two main hypotheses have been suggested to explain this incongruence: a structural model and one involving gene-expression regulation. In the first case, it is proposed that mutations in lamins induce structural/mechanical defects in the nucleus of all cells and that mainly the tissues subjected to high mechanical stress, like for example the skeletal muscle, develop a phenotype. The other hypothesis is based on the knowledge that A-type lamins are involved in differential gene regulation during differentiation and suggests that they regulate the development of mesodermal tissues, which are the most often affected in laminopathies. The two theories are not mutually exclusive. In fact, combined impairment of multiple lamin functions may be responsible for the different phenotypes (Osmanagic-Myers & Foisner, 2019). Another model proposes that the cell type-specific expression of lamin A-interacting NETs at the INM may be the reason why only specific tissues are affected in different laminopathies (Wong et al., 2014; Worman & Schirmer, 2015).

Among laminopathies, the Hutchinson-Gilford progeria syndrome (HGPS) has one of the most dramatic phenotypes. Within the first year of life patients begin to show symptoms like growth retardation, loss of subcutaneous fat, hair loss, skin defects, reduced bone density, and others. As the patients grow older, they develop severe osteoporosis, atherosclerosis, and cardiovascular disease. The latter is the main reason of death for HGPS patients, leading to heart failure at an average age of 14 (Piekarowicz et al., 2019). HGPS is caused by a single LMNA mutation leading to the activation of a cryptic splicing site, deletion of 50 amino acids in the C-terminal domain of lamin A and, ultimately, the production of a permanently farnesylated lamin A protein that is retained at the nuclear periphery and is referred to as progerin (Gonzalo et al., 2017). Expression of the HGPS-linked lamin A mutant (or progerin) has been shown to affect the nucleoplasmic pool of wildtype lamin A (expressed from the wildtype allele) (Vidak et al., 2015) and chromatin organization (Ikegami et al., 2020; McCord et al., 2013; Sebestyén et al., 2020). More interestingly, during the normal aging process, the cryptic site responsible for the production of progerin is also increasingly used, leading to the accumulation of this protein variant in normally aged cells (McClintock et al., 2007; Scaffidi & Misteli, 2008). The molecular mechanisms underlying the development of HGPS are still object of intense studies, although changes in nuclear integrity, chromatin binding, gene expression, DNA stability, and cell signaling pathways have been shown (Vidak & Foisner, 2016).

A-type lamin mutations can also lead to diseases involving preferentially a single tissue type, like in the case of lipodystrophies. Lipodystrophies are characterized by the loss of subcutaneous fat, which can lead to insulin resistance and diabetes. The main lipodystrophies associated with *LMNA* mutations are the Familial partial lipodystrophy type 2 (FPLD2) and the Mandibuloacral dysplasia (MAD) (Kang et al., 2018). In other cases, changes in A-type lamins lead to pathologies involving the heart tissue, like in the case of the dilated cardiomyopathy (CDM1A) (Pankuweit, 2018). These defects often appear early in life and with high penetrance. Less frequently, *LMNA* mutations can lead to the impairment of other tissues, like in the case of some neuropathies and dermopathies (Hasselberg et al., 2018). The most common defects associated with laminopathies, however, are skeletal muscle dysfunctions.

1.5.9 Skeletal muscle-related laminopathies

Laminopathies can affect the skeletal muscle with various degrees of severity. The muscle defects can represent the main phenotype, like in the case of muscle dystrophies, or be part of a wider phenotype, like in HGPS patients. The first muscular dystrophy associated with changes in A-type lamins was the Emery-Dreifuss muscular dystrophy (EDMD) (Bonne et al., 1999), which is characterized by joint contractures, muscle weakening, and cardiac abnormalities, often fatal. This muscular dystrophy can also be associated with mutations in the LEM-protein emerin (Bione et al., 1994). Other laminopathies affecting the skeletal muscle are the limb-girdle muscular dystrophy type 1B (LGMD1B) (Muchir et al., 2000) and one congenital-type muscular dystrophy (CMD) (Prigogine et al., 2010). All of them are associated with a high penetrance of cardiac defects.

The molecular mechanisms behind these pathologies have been extensively studied and show that multiple mechanisms may contribute to their phenotype.

First, reduced expression of A-type lamins or their binding partners, like LAP2alpha, can lead to the deregulation of factors required for muscle differentiation (Cohen et al., 2013; Frock et al., 2006), like the myogenic factors MyoD and desmin, the cell cycle regulator pRb, adhesion molecules like M-cadherin and also non-proteic factors, like micro RNAs (Chen et al., 2006; Sylvius et al., 2011).

NE proteins are also mis-localized in some in vitro models of muscle disease linked to A-type lamin mutations (Steele-Stallard et al., 2018). For example, some laminopathies have been associated with changes in MAN1 localization. The latter is a LEM protein serving as regulator of the TGF-β-induced cellular response by binding and sequestering downstream signaling molecules, the R-Smads, at the nuclear periphery (Bengtsson, 2007; Feng Lin et al., 2005; Moustakas et al., 2001). Changes in A-type lamins can therefore impair the TGF-β pathway, which has been shown to be involved in skeletal muscle regeneration (Delaney et al., 2017) and being altered in several other myopathies (Ismaeel et al., 2019). The downstream effect on Smad proteins could also depend on A-type lamin interaction with PP2A, a phosphatase involved in Smad dephosphorylation (Van Berlo et al., 2005). The proper activation of the TGF-β pathway is fundamental for muscle functions since has also been shown to regulate the activation of muscle related factors (MRFs), like MyoD, Myf5, myogenin and MRF4 (Cohen et al., 2013). Therefore, its deregulation in laminopathies can strongly affect the muscle tissue.

Furthermore, when expressed in cell cultures, many muscle-disease-causing lamin A mutants lead to alteration in the shape of the nucleus and to an increase in nuclear deformability, due to a reduction in stiffness (Jos L.V. Broers et al., 2004; Chatzifrangkeskou et al., 2020; Goldman et al., 2004). This has been shown to depend on alterations in lamin integration into the NL and on defective mechano-coupling of the lamina with the cytoskeleton (Zwerger et al., 2013).

The reduced stability of the nuclei leads also to more frequent ruptures in the NE and, consequently, DNA damage (Earle et al., 2020). It is therefore clear that *LMNA* mutations can affect different functions of these proteins, and the various impaired functionalities can contribute more or less extensively to the final muscle phenotype.

1.5.10 Role of LAP2alpha in the skeletal muscle

LAP2alpha has also been shown to have an important role in the skeletal muscle. The upregulation of LAP2alpha observed in *LMNA* knockout mice, for example, seems to exacerbate the defects in their skeletal muscle differentiation. In fact, loss of LAP2alpha in these mice leads to a partial rescue of the phenotype (Cohen et al., 2013). One of the reasons for this effect could be A-type lamins and LAP2alpha opposite influence on pRb activation in the satellite cells, the adult stem cells of the skeletal muscle. While satellite cells lacking A-type lamins show a reduced proliferation rate, loss of LAP2alpha has the opposite effect on these cells. The combined loss of both proteins, therefore, restores partially the proliferation defect in muscle progenitor cells. Similarly, depletion of LAP2alpha restores the expression levels of several muscle-specific factors affected by A-type lamins loss, like Myh1 and Myh16b (Cohen et al., 2013).

The generation of a LAP2alpha knockout mouse model gave additional insights into the role of this protein in various tissues. Loss of LAP2alpha seems to mainly affect highly proliferating tissues by interfering with the pRb pathway and leading to hyperproliferation of erythroid and epidermal progenitor cells. The overall development, regeneration, and function of the skeletal muscle are, on the contrary, unaffected (Naetar et al., 2008). Nevertheless, a more detailed analysis of muscle tissue and its cells showed a reduced ratio of slow to fast muscle fibers and an increased number of satellite cells in the absence of LAP2alpha. When cultivated and differentiated in vitro, LAP2alpha knockout myoblasts display a prolonged expression of stem cell markers and delayed differentiation, associated with delayed up-regulation of differentiation markers like myogenin and myosin heavy chain (Gotic, Schmidt, et al., 2010; Gotic & Foisner, 2010).

PROLOGUE

The main objective of this Doctorate project is to study the role of A-type lamins and their interacting protein LAP2alpha in chromatin organization during myogenic differentiation.

I aim to:

- Characterize Lamin A/C and LAP2alpha binding to chromatin in myoblasts on a genome-wide level
- Analyze the changes of this binding during myoblasts differentiation
- Investigate the correlation between Lamin A/C and LAP2alpha binding to chromatin and gene expression regulation
- Understand the consequences of LAP2alpha loss on chromatin and its effects on gene expression

I chose to investigate Lamin A/C and LAP2alpha functions in chromatin regulation in myoblasts because, as discussed in the introduction of this thesis, mutations in Lamin A/C have been linked to many diseases involving different degrees of skeletal muscle defects (Carboni et al., 2013; Dubinska-Magiera et al., 2013; Maggi et al., 2016). In addition, loss of LAP2alpha has also been shown to impact myoblast differentiation (Gotic, Schmidt, et al., 2010). The described skeletal muscle defects can be partially linked, at least for Lamin A/C mutations, to mechanical defects of the nucleus or altered interactions with other proteins (Maggi et al., 2016). Nevertheless, a comprehensive understanding of the mechanisms behind these phenotypes is still missing and newly emerging concepts also point to chromatin defects in these diseases. My hypothesis is that, in addition to the described mechanisms, impaired chromatin regulation plays an important role in the reported cellular defects. The ability of Lamin A/C and LAP2alpha to bind to chromatin both at the nuclear periphery and in the nucleoplasm allows them to interact also with euchromatic regions, containing active genes (Gesson et al., 2016). By binding to euchromatin, these proteins can potentially affect the regulation of genes relevant for specific cellular processes. Additionally, the choice of using myoblasts for this investigation offers the advantage of studying a dynamic process: the muscle differentiation. Myoblasts, in fact, can be induced to differentiate *in vitro*, and the various stages of this process can be analyzed to identify changes in the chromatin state over time.

Here I show that both Lamin A/C and LAP2alpha binding to chromatin changes during differentiation. In this process, LAP2alpha enriches in genomic regions containing genes relevant for myoblast differentiation, suggesting that this protein plays a role in the regulation of myoblast differentiation. I propose that the binding of LAP2alpha to certain genomic regions generates a specific chromatin environment facilitating efficient gene expression changes during differentiation.
Additionally, I generated LAP2alpha-deficient myoblasts that recapitulate the impairment of differentiation previously described (Gotic, Schmidt, et al., 2010). Myoblasts depleted of LAP2alpha display a relevant redistribution of Lamin A/C towards accessible genomic regions, which are enriched in expressed genes. In the newly Lamin A/C-bound areas I find a significant fraction of the genes that are deregulated upon LAP2alpha loss. This suggests that the differentiation impairment in LAP2alpha-depleted myoblasts is, at least partially, linked to a de-regulation of the binding of A-type lamins to chromatin. Additionally, I show that a consistent depletion of Lamin A/C can be found at the TSS of expressed genes, independently of their regulation during myoblast differentiation (e.g., up-, down-regulation). I hypothesize that absence of Lamin A/C binding at the TSS of active genes is important to allow their expression.

CHAPTER 2: RESULTS

For this study I used immortalized myoblasts derived from a p53 knockout mouse model (IM; p53KO - courtesy of G. Wiche). Additionally, I generated LAP2alpha-deficient myoblasts by editing the genome of IM; p53KO cells using the CRISPR-Cas technology. I transfected IM; p53KO cells with a vector encoding the Cas9 protein and an sgRNA targeting exon4 of the *Tmpo* gene (sgRNA3, Figure 1A). The exon 4 is specific for the alpha isoform of the *Tmpo* gene, therefore abolishing only the production of the LAP2alpha protein. I also generated a control cell line by treating IM; p53KO with a vector encoding the Cas9 protein, but without adding the specific sgRNA. In this case, the *Tmpo* locus is not modified. In both cases, transfected cells were enriched by FACS, performing a bulk sorting. As a result of the system limitations, the LAP2alpha-depleted cell line still contains around 10% of myoblasts whose *Tmpo* locus was not modified and therefore express wild-type LAP2alpha protein. The control line, treated with the Cas9 but no sgRNA, shows no significant signs of genome editing at the *Tmpo* locus (Suppl. Figure 1A). Throughout this study, the control cell line will be referred to as LAP2alpha WT and their counterpart, largely depleted of LAP2alpha, as LAP2alpha KO. The editing of the *Tmpo* locus allows to study the role of LAP2alpha in chromatin organization by analyzing the changes occurring in the absence of the protein in an otherwise isogenic background. Moreover, LAP2alpha-deficient myoblasts can give additional insights into the role and regulation of Lamin A/C binding to chromatin, as it has been previously shown that loss of LAP2alpha affects the distribution of A-type lamins on the chromatin, in fibroblasts (Gesson et al., 2016). The generation of a control cell line is important to ensure that the observed results are due to the selective depletion of LAP2alpha in these cells and not due to other unrelated changes occurring during cell manipulation.

2.1 DEPLETION OF LAP2alpha IN IMMORTALIZED MYOBLASTS RECAPITULATES THE DIFFERENTIATION DEFECTS PREVIOUSLY DESCRIBED

Following the generation of LAP2alpha KO cells and their controls, I checked the expression of LAP2alpha and Lamin A/C in these cells. Lamin A/C expression does not seem to be affected in LAP2alpha-deficient myoblasts both by Western blot (WB) analysis and by immunofluorescence (IF) staining (Figure 1B, D). LAP2alpha KO cultures display no visible LAP2alpha specific band by WB analysis (Figure 1B), but some nuclei (approximately 10%) remain positive by IF (Figure 1D). As expected, since the genome of LAP2alpha WT cells was not edited, their LAP2alpha expression is unaffected (Figure 1B, D). From these analyses, I can also conclude that LAP2alpha and Lamin A/C are normally expressed throughout all differentiation stages of myoblasts, allowing me to follow their function in any of the chosen differentiation stages.

After the generation of the cell lines, I compared the ability of LAP2alpha KO and WT cells to differentiate and, as previously observed (Gotic, Schmidt, et al., 2010), I found that LAP2alpha loss is associated with a reduced differentiation potential, as shown by light microscopy images (Figure 1C) and by the reduction of the protein expression of some myoblast-specific markers, like Myogenin (Myog) and Myosin Heavy Chain (MyHC) (Figure 1B).

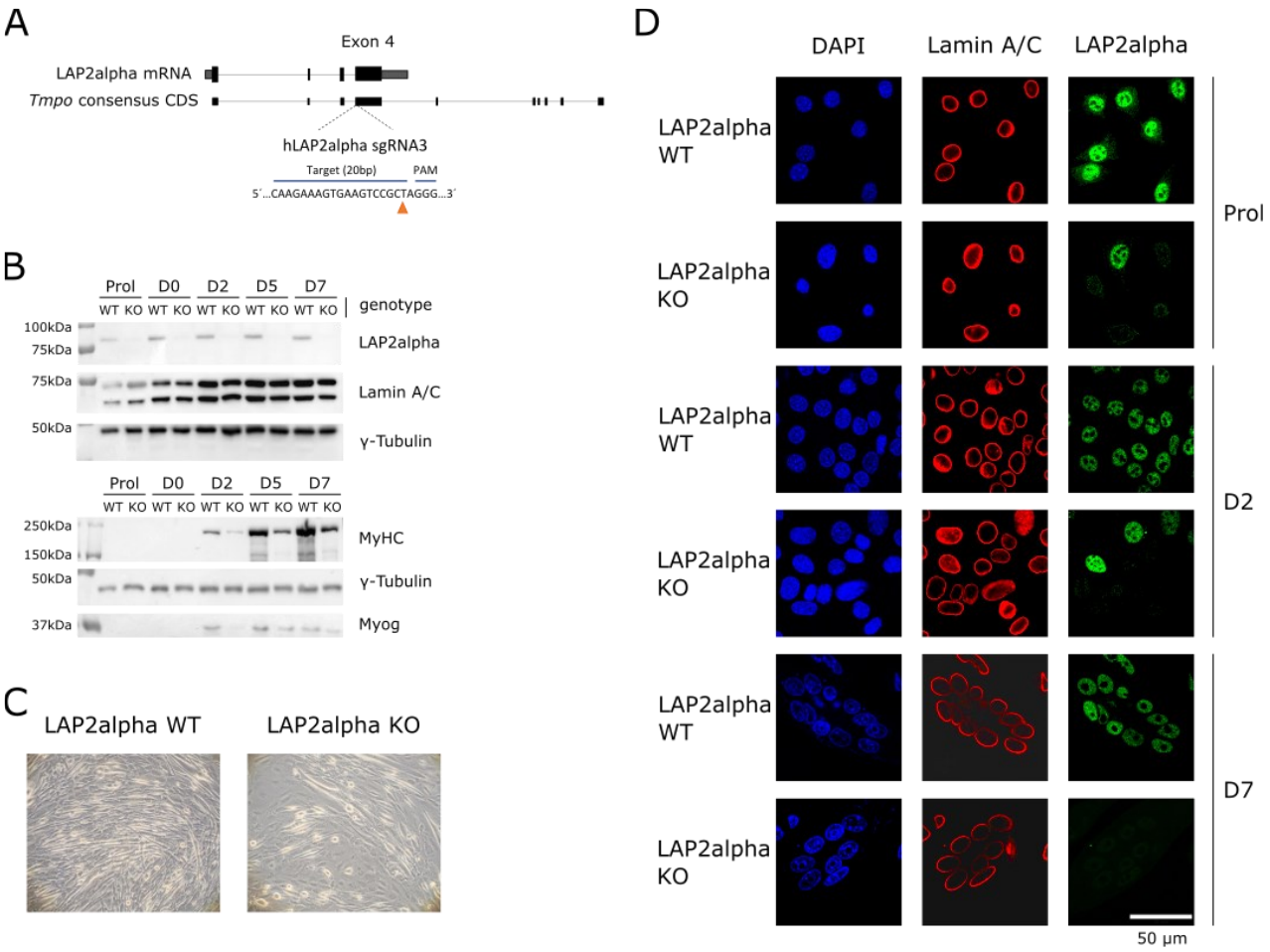


Figure 1. *(A) Schematic view of the sgRNA used to specifically target the alpha isoform of the LAP2 (Tmpo) gene. The targeted exon is viewed in relation to LAP2alpha mRNA and to the Tmpo consensus CDS. (B) Western blots of different stages of myoblast differentiation in LAP2alpha WT and KO cells. The upper blot shows LAP2alpha and Lamin A/C expression in these cells in the various stages analyzed. The lower blot shows the expression of myoblast differentiation markers (myosin heavy chain - MyHC and myogenin - Myog), in the same stages of differentiation. In both western blots γ-Tubulin expression was used as normalization marker. (C) Light microscopy pictures of LAP2alpha WT and KO myoblasts 7 days after the induction of the differentiation program. (D) Immunofluorescence showing Lamin A/C (red) and LAP2alpha (green) expression in LAP2alpha WT and KO myoblasts in various stages of differentiation. 4',6-diamidino-2-phenylindoleDAPI staining (blue) was used to identify all nuclei.*

2.2 LOSS OF LAP2alpha IS LINKED TO DEREGULATION OF GENE EXPRESSION ALREADY IN EARLY STAGES OF DIFFERENTIATION

To better characterize the differentiation defects in LAP2alpha-depleted myoblasts, I performed mRNA-seq on these cells, and then compared the changes in gene expression in KO cells to their LAP2alpha-expressing WT counterparts.

One of the aims of this project is to analyze potential correlations of chromatin changes with gene expression deregulation, in the absence of LAP2alpha. Since such changes are likely to happen early during myoblasts differentiation, I decided to focus on the initial stages of this process throughout the analyses. *In vitro*, myoblasts start their differentiation program when reaching high confluency and continue this process when kept in low-serum conditions. I analyzed proliferating myoblasts (Prol), which are still dividing and in which the differentiation program has not been activated yet, D0 myoblast, which have reached a high confluency and are initiating differentiation, and D2 myoblasts, which have been switched to a low-serum medium for two days after reaching high confluency and are therefore further progressed into the differentiation process.

The mRNA-seq analysis was conducted in collaboration with Celine Prakash, from the Center for Integrative Bioinformatics Vienna (CIBIV). Analysis of LAP2alpha-expressing WT myoblasts shows very little difference in gene expression between D0 and Prol myoblasts. At D0 only 56 genes are differentially expressed (DE) compared to the proliferating stage (Figure 2A, upper-left plot). As the differentiation proceeds, the changes in gene expression become more and more evident. At D2, while the cells do not show strong phenotypical changes (data not shown), the transcription program is already altered to the point that we could identify 4257 DE genes, compared to the Prol stage (Figure 2A, upper-right plot). The LAP2alpha KO cell line shows a similar trend, confirming that also the differentiation of LAP2alpha-deficient myoblast leads to expression changes in many genes. In this case, we find 35 genes differentially expressed at D0 and 4426 at D2 (Figure 2A, lower-left and -right plots, respectively). This similar trend in the changes in gene expression is expected since the loss of LAP2alpha, although reducing the differentiation potential of myoblasts, does not abolish differentiation completely. This observation suggests the deregulation of a specific subset of genes in LAP2alpha KO versus WT cells rather than a complete incapability to activate the differentiation program itself. When comparing LAP2alpha KO cells to their WT counterpart, we find that there are deregulated genes in each of the analyzed stages. Interestingly, myoblasts lacking LAP2alpha are already characterized by some degree of gene expression deregulation in their proliferating stage. We report 26 DE genes between these cell lines, mainly downregulated in absence of LAP2alpha (Figure 2B, left plot). This finding supports the idea that LAP2alpha loss may affect the basal chromatin state of myoblasts and that the differentiation defects are, at least partially, the result of a reduced ability of these cells to properly regulate gene expression in response to differentiation stimuli. The further the differentiation program proceeds, the more the differences between the two cell lines (LAP2alpha WT and KO) exacerbate. At D0, 35 genes are deregulated in LAP2alpha-depleted myoblasts (Figure 2B, middle plot), while at D2 stage of differentiation we observe 215 DE genes (Figure 2B, right plot). Among the latter, as expected, we find many genes involved in skeletal muscle differentiation. The majority of the deregulated myogenic genes is downregulated in the absence of LAP2alpha, in line with the observed phenotype of an impaired differentiation. Since the differences between the Prol and the D0 stages are not so pronounced, from this point on I decided to focus mainly on the analyses of proliferating myoblasts and their differentiation stage D2.

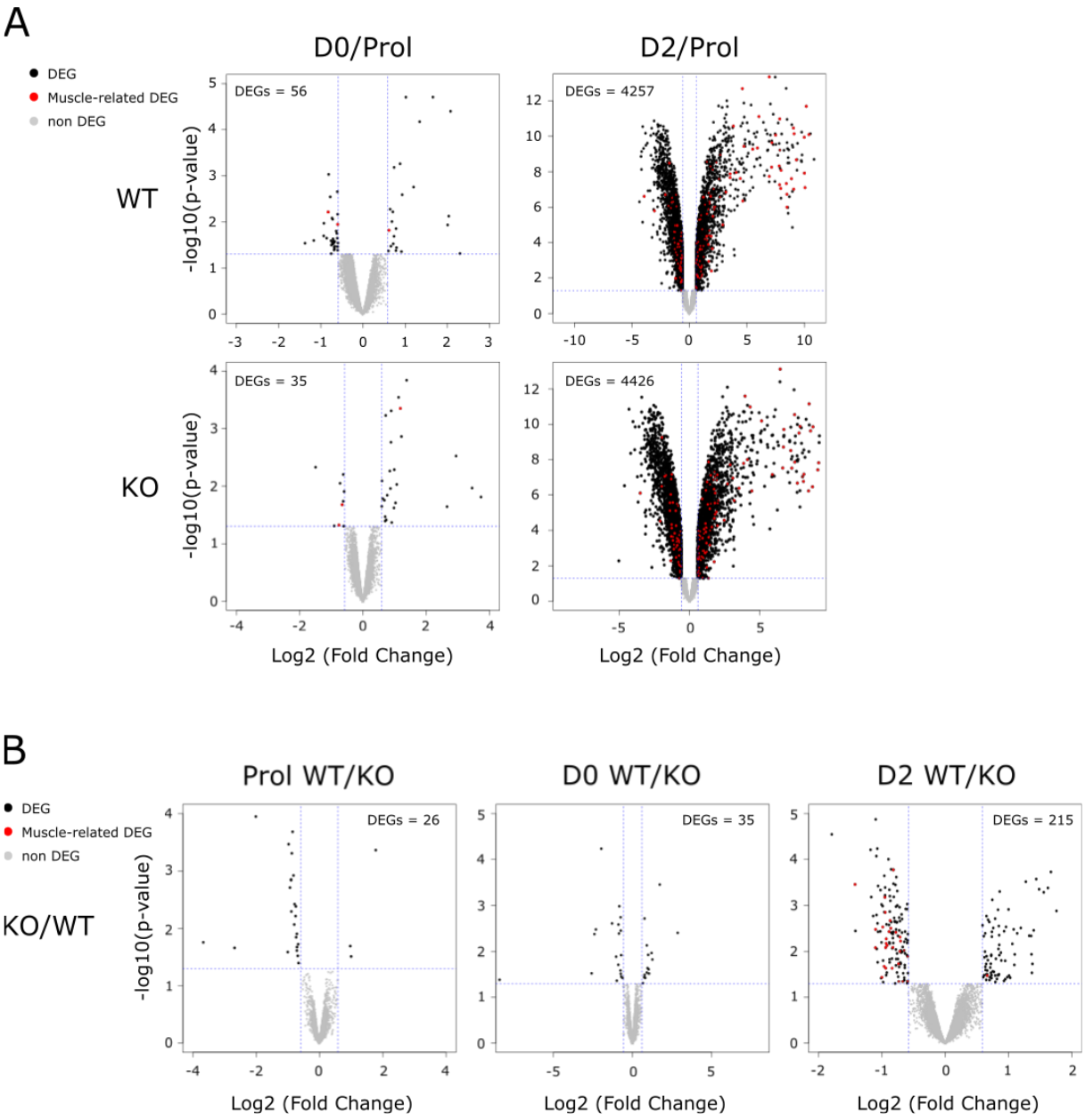


Figure 2. *(A) Volcano plots showing the genes differentially regulated during differentiation in LAP2alpha WT myoblasts (upper row) and LAP2alpha KO myoblasts (lower row). In both cases, the volcano plots on the left show the genes differentially regulated at day 0 (D0) of the differentiation program compared to proliferating myoblasts (Prol). The volcano plots on the right, instead, show the difference in gene expression between day 2 of differentiation (D2) and proliferating myoblasts (Prol). (B) Volcano plots showing the genes differentially expressed in LAP2alpha KO myoblasts, compared to LAP2alpha WT cells, in the differentiation stages analyzed (proliferating - Prol; differentiation day 0 - D0; and differentiation day 2 - D2). DEG (black) = differentially regulated gene; Muscle-related DEG (red) = differentially regulated genes related to muscle differentiation; non DEG (grey) = genes non differentially regulated.*

2.3 Lamin A/C AND LAP2alpha BIND TO REGIONS OUTSIDE THE REPORTED cLADs AND THEY REDISTRIBUTE ON CHROMATIN DURING MYOBLAST DIFFERENTIATION

I then performed a genome-wide analysis of LAP2alpha and Lamin A/C binding to chromatin with the aim of combining these data with the myoblast transcription profiles discussed above. More precisely, I wanted to test a potential correlation between these proteins´ binding to chromatin and changes in gene expression.

I performed chromatin immunoprecipitation followed by deep sequencing analysis (ChIP-seq analysis) of LAP2alpha WT cells and, for this, I used one antibody to detect LAP2alpha and two different antibodies to recognize different pools of Lamin A/C. The two lamin A/C antibodies used in this project, in fact, recognize different regions of Lamin A/C. The antibody referred to as "3A6" binds to the C-terminus of A-type lamins and the other antibody, "E1", to the N-terminus. Since LAP2alpha also binds to the C-terminus of A-type lamins, we believe that 3A6 is less able to recognize the pool of lamins interacting with LAP2alpha. By binding to the Lamin A/C N-terminus, the antibody E1 can most likely equally well recognize lamins in a complex with LAP2alpha and lamins not interacting with this protein, as revealed by co-immunoprecipitation analyses (Suppl. Figure 1B). ChIP-seq analyses provide genome-wide information on LAP2alpha and Lamin A/C binding to chromatin. Since my main objective is to correlate DNA binding and gene expression, I enriched for euchromatin (containing active genes) by using a low number of sonication cycles for chromatin preparation, as previously described in another work (Gesson et al., 2016). The downstream bioinformatic analyses were performed in collaboration with Celine Prakash (CIBIV) and Fatih Sarigöl (Max Perutz Labs). The mapped sequences were first analyzed with the Enriched Domain Detector (EDD) peak caller, specifically designed for detecting lamin-interacting chromatin regions (Lund et al., 2014). Both Lamin A/C and LAP2alpha do not recognize a specific DNA sequence (Stierlé et al., 2003), and, thus, their binding pattern is diffused over long stretches of the genome, rather than highly enriched in narrow windows, as usually found when looking at the interaction of transcription factors with chromatin. The EDD peak caller, therefore, identifies long regions with a high density of protein binding, despite a lower overall signal intensity.

When analyzing the regions identified by the EDD peak caller in LAP2alpha WT cells in the IGV browser view, it is evident that Lamin A/C- and LAP2apha-bound chromatin regions largely overlap (Figure 3A). Nevertheless, we also identify genomic areas which are uniquely bound by either of these proteins. At a first glance, the analysis of EDD peaks distribution confirmed that the experimental settings successfully enrich for euchromatic regions. Lamin A/C and LAP2alpha peaks, in fact, are largely located outside of the previously described repressive cLADs (Figure 3A, in orange), although some overlap is still present. The Lamin A/C peaks detected with the two different lamin antibodies are largely, but not completely, overlapping. This confirms their ability to detect different Lamin A/C pools.

On a genome-wide level, we show the comparisons between EDD peaks of different proteins as base pairs (bp) overlaps: we define the length of EDD peaks as the bp of DNA covered by that peak and compare how many bp overlap, for example, in two different samples or stages.

First, we determined the portion of the genome bound by each protein. LAP2alpha peaks cover between 11 and 16% of the genome, depending on the differentiation stage of the myoblasts (Suppl. Figure 2A). Lamin A/C peaks cover between 15 and 21% of the total genome sequence (Suppl. Figure 2A) and their overlap with cLADs is around 45-55% (Suppl. Figure 2B), independently of the

differentiation stage. Notably, LAP2alpha also displays around 62% overlap with cLADs in proliferating myoblasts, but only around 14% concordance with these regions in the D2 stage, suggesting that LAP2alpha redistributes away from these heterochromatic regions during differentiation (Suppl. Figure 2B).

We then analyzed the overlaps between the regions covered by A-type lamins, LAP2alpha and the previously reported cLADs. In the Prol stage the two antibodies recognizing Lamin A/C display a highly overlapping chromatin association, as shown by Venn diagrams (Figure 3B, left). LAP2alpha also overlaps largely with Lamin A/C peaks, as demonstrated previously (Gesson et al., 2016). As mentioned above, a large fraction of the LAP2alpha and Lamin A/C associated regions does not overlap with cLADs. Similar results were obtained in the D2 stage (Figure 3B, right).

We next compared Lamin A/C and LAP2alpha binding to chromatin in the proliferating and the D2 differentiation stages. During differentiation, Lamin A/C partially redistributes on the chromatin. The majority of the Lamin A/C EDD peaks overlaps in the two stages, but we also show some stage-specific binding in Prol and D2 myoblasts. This is similar when using either the 3A6 or the E1 antibody (Figure 3C, upper-left and -middle diagrams)

Notably, LAP2alpha has a much more pronounced redistribution on the chromatin during differentiation (Figure 3C, upper-right diagram). Its overlap with cLADs, as described above, is strongly reduced in D2 samples (Figure 3C, lower-right diagram), pointing towards LAP2alpha binding to more active regions of the genome during differentiation (as confirmed later in this thesis).

From these analyses I conclude that the two lamin antibodies detect a similar, but not identical Lamin A/C binding pattern on chromatin, reinforcing the idea that they recognize different pools of A-type lamins. I also show that LAP2alpha binding to chromatin is highly dynamic during myoblast differentiation and that this protein tends to move away from heterochromatic cLADs after the differentiation started. Lamin A/C binding is, instead, more stable, with only minor changes in chromatin binding during early stages of myogenic differentiation.

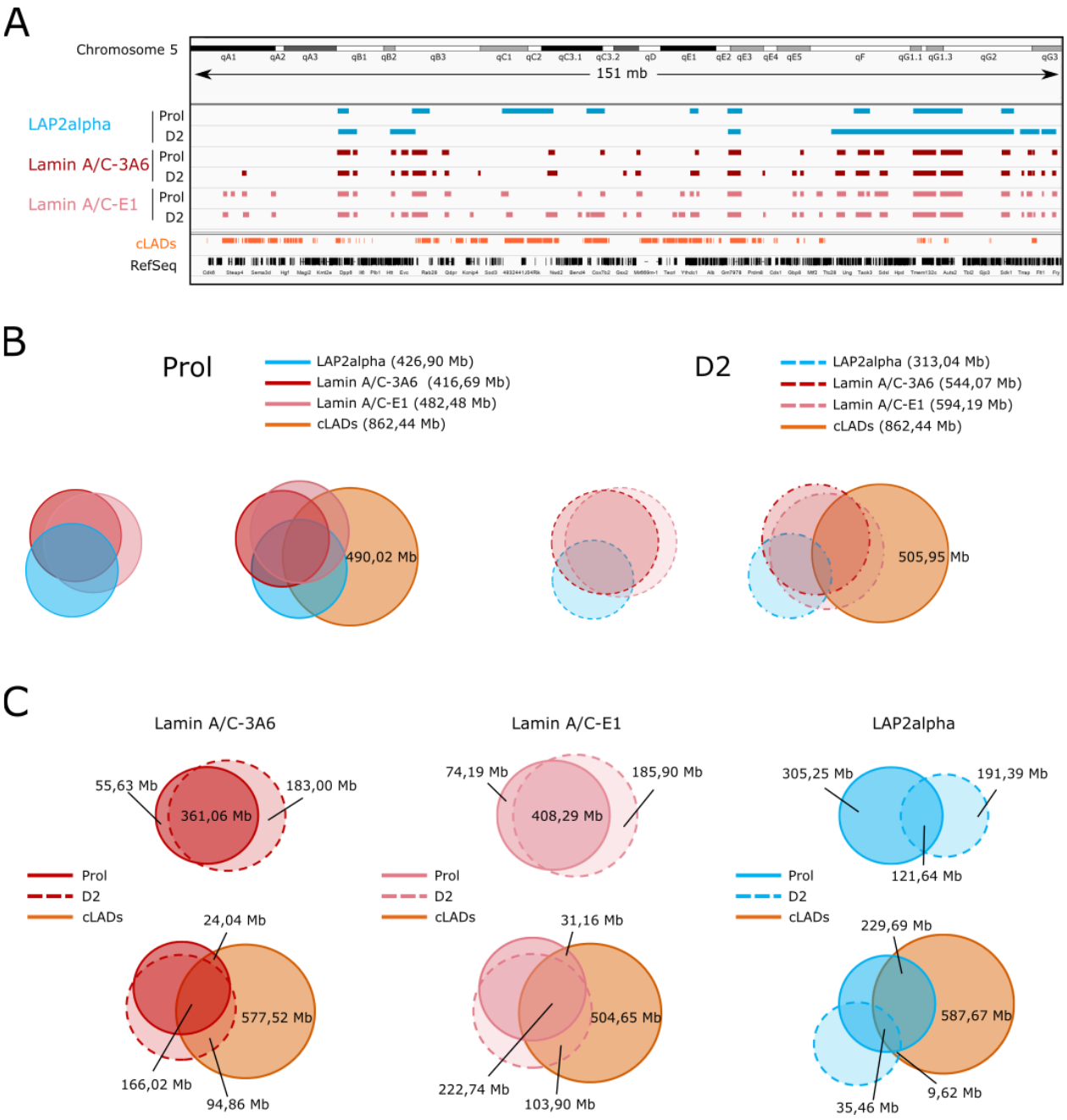


Figure 3. *(A) IGV browser view of the entire chromosome 5 showing the EDD peaks obtained analyzing LAP2alpha (light blue) and Lamin A/C binding to chromatin by ChIP-seq. Lamin A/C binding was detected both with the Lamin A/C-3A6 (red) and Lamin A/C-E1 antibodies (pink). The EDD peaks are shown for both stages of differentiation analyzed (Prol and D2). (B) Venn diagrams showing the overlap between LAP2alpha (light blue), Lamin A/C 3A6 (red) and Lamin A/C E1 (pink) binding to chromatin, alone and with the reported cLADs (orange). The overlap is calculated for the Prol stage (diagrams on the left) and for the D2 stage of differentiation (on the right). (C) Venn diagrams representing the overlaps between the Prol and D2 stages of differentiation of the EDD peaks detected for Lamin A/C-3A6 (red), Lamin A/C-E1 (pink), and LAP2alpha (light blue). In the lower row the same overlaps are represented in comparison with the reported cLADs. Numbers in Venn diagrams indicate total length of EDD peaks in Mb in the respective area.*

2.4 THE PORTION OF EDD PEAKS NOT OVERLAPPING WITH cLADs CONTAINS A HIGHER PROPORTION OF EXPRESSED GENES

The reduced overlap of Lamin A/C and LAP2alpha with cLADs, per se, does not necessarily imply that these proteins are binding to more active regions of the genome. To better understand the nature of the binding of these proteins to chromatin, we decided to investigate which specific genes were found inside EDD peaks in our samples, in the various conditions.

Generally, this analysis reveals that, for each of the antibodies used, the EDD peaks detected cover several thousands of genes (between ~7000 and ~9000 genes for each of the analyzed conditions). In most cases more than 40% of these genes lie in the regions overlapping with cLADs (Figure 4A, in dark- and light grey). The genes within LAP2alpha EDD peaks in the D2 stage of differentiation represent an exception, with only ~14% of them overlapping with cLADs. Regardless of these differences, most of the genes found in the EDD peaks overlapping with cLADs are not expressed in myoblasts (more than 96% of the total). If we consider, instead, the EDD peaks outside the cLADs regions, the proportion of expressed genes changes drastically, varying from ~11% up to ~37% (Suppl. Figure 2C).

These results show that, when selectively enriching for euchromatin in ChIP-seq analyses, we can detect Lamin A/C and LAP2alpha chromatin binding outside the reported cLADs both in proliferating and differentiating myoblasts, as observed previously in fibroblasts (Gesson et al., 2016). In these regions both proteins can bind to more active chromatin and therefore can potentially be involved in the regulation of gene expression. In addition, LAP2alpha-bound chromatin regions seem to contain a significantly higher number of expressed genes particularly at the D2 stage, compared to Lamin A-bound regions.

2.5 GENES INSIDE EDD PEAKS ARE NOT DIRECTLY BOUND BY Lamin A/C OR LAP2alpha

Another question arising from these data is whether the genes in LAP2alpha and Lamin A/C EDD peaks are directly bound by these proteins. As previously mentioned, the EDD peak caller defines as peaks regions of the genome with a high density of binding of the protein of interest. This means that a peak represents a long genomic region in which the binding of the protein of interest is significantly higher than in the areas surrounding that peak. However, EDD peaks also include regions not directly bound by the analyzed protein but located inside a long stretch of genome whose protein binding density is significantly higher than the background. Therefore, genes inside the EDD peaks are not necessarily directly bound by Lamin A/C or LAP2alpha.

For analyzing protein binding to genes, we define a gene as bound by Lamin A/C or LAP2alpha by calculating the mean log2ratio of the ChIP/INPUT signal on the gene-body, and selecting genes that pass a specific cutoff threshold, as described in the Material and Methods section.

Surprisingly, most of the genes inside Lamin A/C and LAP2alpha EDD peaks are not directly bound by these proteins using these criteria. This is true both for peaks overlapping with cLADs and peaks outside these regions, as depicted by the pie charts in Figure 4B. Particularly, also LAP2alpha bound regions at the D2 stage, which contain many expressed genes (Figure 4A) do not show a higher percentage of directly bound genes (Figure 4B). The genes directly bound by either Lamin A/C or

LAP2alpha within EDD peaks outside cLADs amount to ~10-13% of the total number of genes in these regions. Inside cLADs, 20-21% of the genes have direct Lamin A/C enrichment and around 15% of them are directly bound by LAP2alpha (Suppl. Figure 2D). The fact that there is a higher fraction of genes directly bound by these proteins inside the cLADs, which are enriched in non-expressed genes, points towards a negative correlation between direct Lamin A/C or LAP2alpha binding to gene-bodies and gene expression. Nevertheless, the positioning of genes inside LAP2alpha EDD peaks, particularly at the D2 stage of differentiation, seems to be concordant with their expression. This seems to indicate that, although its direct binding to the gene-body may repress gene expression, LAP2alpha binding to the gene´s neighboring chromatin may positively regulate this process.

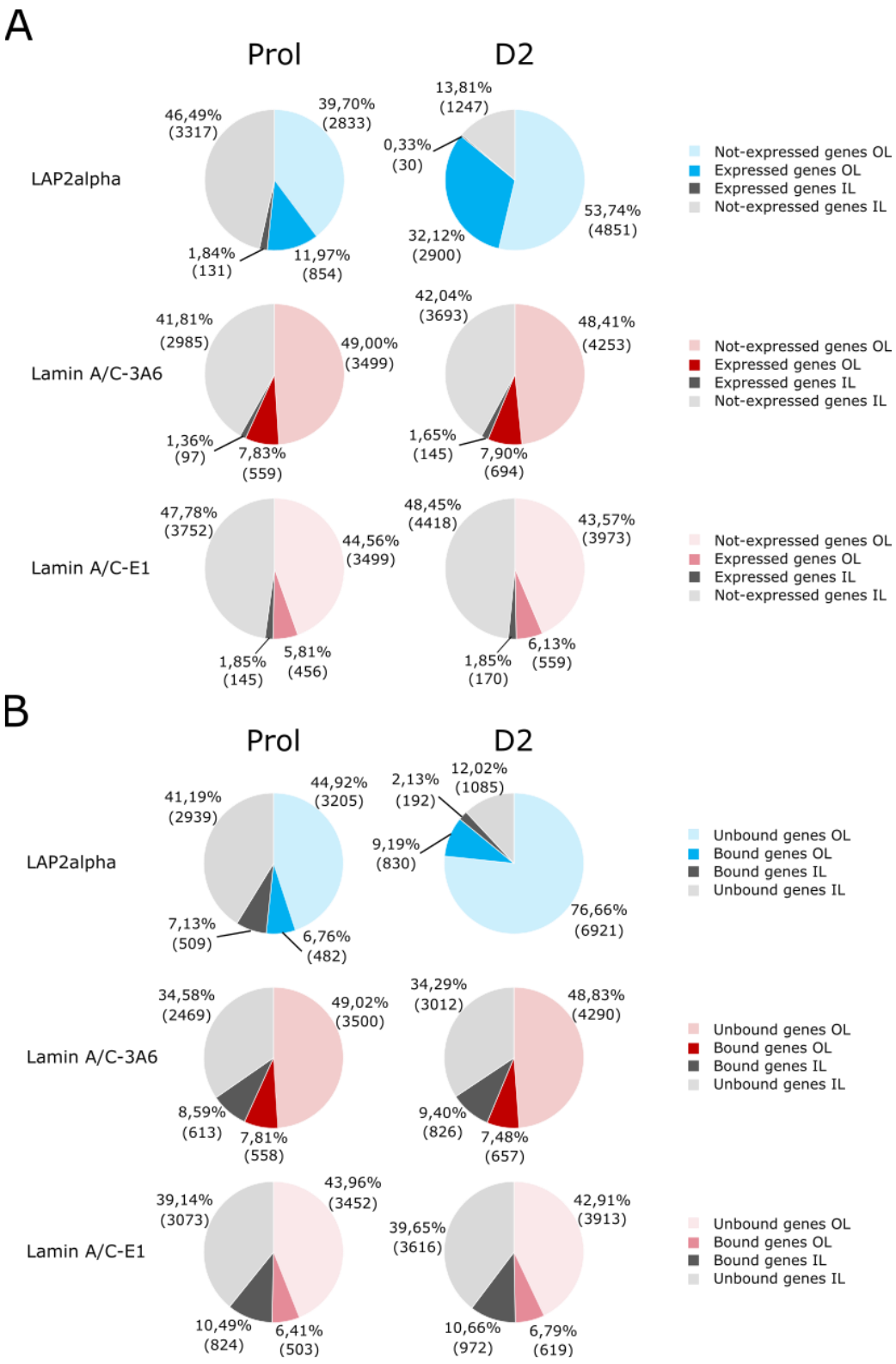


Figure 4. *(A) Pie charts representing the percentage of expressed and not-expressed genes located inside EDD peaks, for LAP2alpha, Lamin A/C-3A6 and amin A/C-E1 (upper, middle, and lower row, respectively). The genes were also divided in those found in regions overlapping with cLADs (IL) and outside cLADs (OL). Both the gene number and the percentage over the total are shown. (B) Pie charts representing the percentage of bound and unbound genes located inside EDD peaks, for LAP2alpha, Lamin A/C-3A6 and Lamin A/C-E1 (upper, middle, and*

lower row, respectively). The genes were also divided according to their location inside or outside cLADs (IL and OL, respectively). Number of genes and percentages over the total are shown.

2.6 EXPRESSED GENES IN LAP2alpha EDD PEAKS ARE INVOLVED IN CHROMATIN REGULATION

To better address the potential functional relevance of LAP2alpha and Lamin A/C binding to chromatin, I performed gene ontology (GO) analysis on the genes located inside their respective EDD peaks.

When analyzing all genes within LAP2alpha or Lamin A/C EDD peaks, I see no relevant enrichment for specific gene functions. Nevertheless, if one tests specifically the expressed genes outside cLADs, LAP2alpha EDD peaks in the proliferating stage are enriched in genes involved in the regulation of chromatin silencing, nucleosome positioning and other processes related to chromatin organization (see Suppl. data file1, Sheet1 and Suppl. Figure 3A). While the differentiation proceeds, although still containing genes involved in chromatin regulation, LAP2alpha EDD peaks extend to genes relevant for processes like mitochondrial-related functions, apoptosis, and protein synthesis (see Suppl. data file1, Sheet2 and Suppl. Figure 3B). Lamin A/C EDD peaks contain genes not enriched for specific cellular functions. Nevertheless, when selectively looking at expressed genes, I find a significant number of expressed genes involved in RNA transcription and regulation and in other metabolic processes. This is confirmed in both stages of differentiation and for both lamin antibodies used (see Suppl. data file1, Sheet3 to 6). This analysis indicates that LAP2alpha binding to chromatin during myoblast differentiation may also affect genes encoding chromatin-regulating proteins and might thus affect chromatin organization and accessibility globally, but this hypothesis will require further investigation.

2.7 LAP2alpha IS ENRICHED IN REGIONS OF THE GENOME CONTAINING GENES IMPORTANT FOR THE DIFFERENTIATION PROCESS

I next wondered whether Lamin A/C and LAP2alpha can influence the expression of genes involved in myoblast differentiation and if Lamin A/C has a role in the deregulation of gene expression observed after LAP2alpha loss. For answering these questions, I first checked whether genes involved in these processes are found in Lamin A/C and/or LAP2alpha EDD peaks and whether they are directly bound by these proteins.

As shown before, 4257 genes are differentially expressed in LAP2alpha WT cells 2 days after the induction of differentiation when compared to their proliferating counterparts (DEGs D2/Prol). Only a small portion (~5%) of those genes is found inside Lamin A/C EDD peaks (independently of the antibody used for the immunoprecipitation step of the ChIP experiments), and very few of them are directly bound by these proteins (Figure 5A, upper panel and 5C, left table). As expected, most of the DE genes inside EDD peaks are located outside cLAD regions (Figure 5B, left panel). This finding, in concordance with the previous analyses, points towards absence of direct A-type lamin enrichment on genes that need to be actively regulated in myogenic differentiation.

In contrast, the LAP2alpha EDD peaks contain much more DEGs (D2/Prol) than the Lamin A/C EDD peaks (Figure 5A upper row). In the Prol stage, LAP2alpha EDD peaks contain more than 200 DEGs, and, at D2, LAP2alpha redistributes on the chromatin to cover more than 800 of these genes (~20% of the total DEGs D2/Prol - Figure 5A, upper row). However, only about 10% of these were directly bound by LAP2alpha (Figure 5C, left table). This suggests that the enrichment of LAP2alpha on the chromatin surrounding the genes helps to control their regulation, either directly or indirectly. LAP2alpha binding could potentially change the chromatin state and make it more accessible for other regulatory factors. Alternatively, LAP2alpha could directly target transcription factors or chromatin remodelers to the areas where differentiation-relevant genes are located. To clarify the exact mechanism, more experiments are required.

Lamin A/C and LAP2alpha EDD peaks cover a large part of the genome, and they broadly redistribute on the chromatin during differentiation. It is therefore important to define whether the number of DEGs D2/Prol found inside EDD peaks is indeed statistically significantly enriched or whether it is the result of a random distribution of the EDD peaks on the genes in some regions of the genome. To address this problem, random permutation tests were performed (Figure 6) to determine how many DEGs D2/Prol one would expect to find by chance inside EDD peaks, given the number of genes in the mouse genome, the number of DEGs D2/Prol, the length of the EDD peaks and the total length of the genome itself.

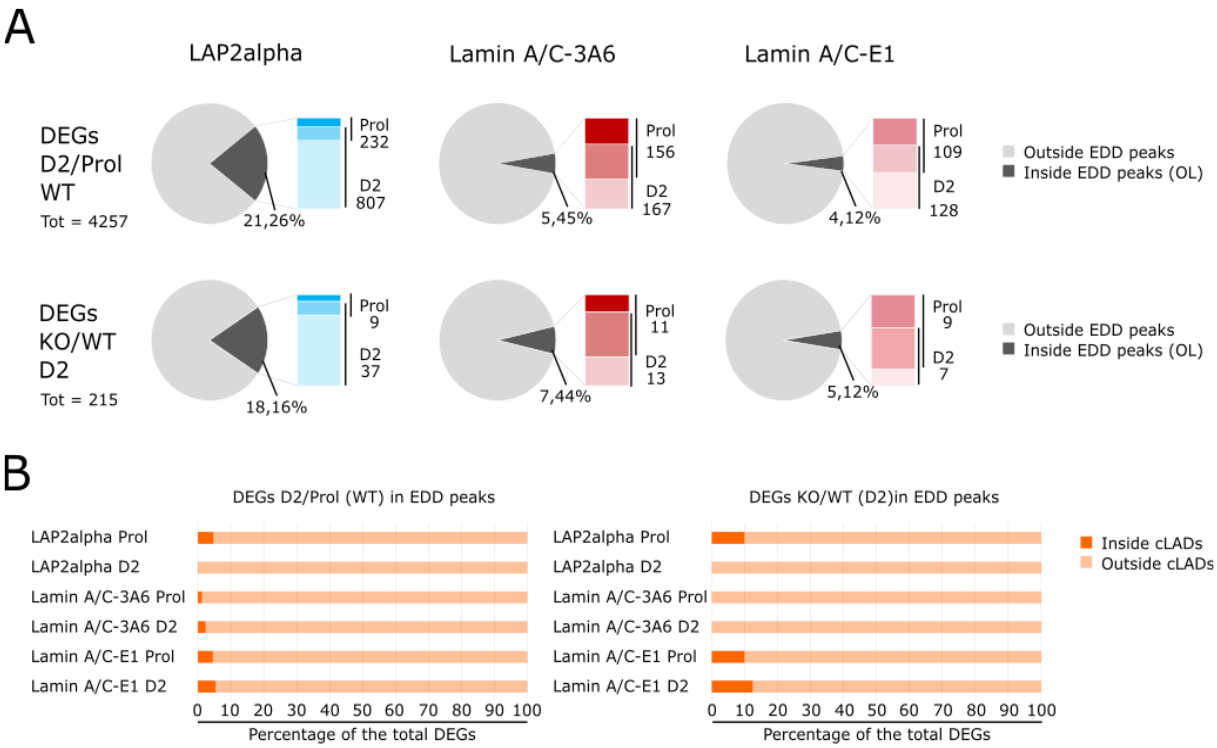
Random permutation analyses revealed that DEGs D2/Prol are not significantly enriched in Lamin A/C EDD peaks, independently of which lamin antibody was used (Figure 6A, middle and right plots). LAP2alpha EDD peaks, instead, contain significantly more differentially expressed genes than what is expected by chance (Figure 6A, left plots). Especially in the D2 stage, LAP2alpha redistributes to regions that contain around 10 times a higher number of differentially expressed genes than would be expected by chance. Interestingly, in the D2 stage, genes up-and downregulated during differentiation are equally significantly enriched in LAP2alpha EDD peaks (Figure 6B). We can therefore speculate that the localization of these genes within LAP2alpha EDD peaks may facilitate their expression regulation (up- and down-regulation) during differentiation. Furthermore, in the LAP2alpha KO cells, the absence of LAP2alpha enrichment in these genomic regions (see below) could contribute to their deregulation and to the impairment of the differentiation program. From these analyses I can also conclude that the potential LAP2alpha-mediated regulatory mechanism is not dependent on a direct binding of LAP2alpha to the gene bodies, but that it is rather the effect of the changes in the surrounding chromatin environment that can influence the expression of these analyzed genes.

2.8 LAP2alpha IS ENRICHED IN REGIONS OF THE CHROMATIN CONTAINING GENES DEREGULATED IN LAP2alpha-DEFICIENT MYOBLASTS

Based on the finding that LAP2alpha EDD peaks are enriched in differentially expressed genes during myoblast differentiation, EDD peaks were next checked for the presence of genes whose expression is affected by LAP2alpha depletion (DEGs KO/WT). I hypothesize that if these genes are affected by the Lamin A/C or LAP2alpha-enriched chromatin environment, the absence of LAP2alpha could affect their regulation either directly, or indirectly through changes in Lamin A/C binding.

DEGs KO/WT are not found significantly enriched in Lamin A/C EDD peaks, independently of the differentiation stage, nor in LAP2alpha EDD peaks, in the Prol stage. In fact, very few of these genes are located within EDD peaks in each of the above-mentioned conditions (Figure 5A, lower row). Random permutation tests confirm no significant enrichment of these DE genes in EDD peaks (Figure 6C). However, in differentiation stage D2, the LAP2alpha-bound regions in WT myoblasts contain ~15% of the DEGs KO/WT, deregulated upon knockout of LAP2alpha (Figure 5A, lower row, on the left). Random permutation tests show that this number is significantly higher than what would be expected by chance (Figure 6C, left-bottom graph). These data indicate that a significant number of genes located in LAP2alpha-bound regions in WT cells are deregulated in LAP2alpha KO cells.

In summary I conclude that LAP2alpha and Lamin A/C redistribute on the chromatin during myoblasts differentiation. Unlike Lamin A/C, LAP2alpha is enriched in regions containing a significantly higher number of genes involved in the differentiation process, compared to random distribution. In absence of LAP2alpha several of these genes fail to be properly regulated. In fact, of the 215 genes deregulated in LAP2alpha KO myoblasts, 163 are differentially regulated during differentiation in WT myoblasts (DEGs D2/Prol). Of these 163 genes, 150 are upregulated during differentiation, in WT cells. The majority of them fails to be properly upregulated in LAP2alpha KO myoblasts. This suggests a role for LAP2alpha in the regulation of myogenic gene expression, mediated by its enrichment on chromatin regions around relevant genes. The mechanism through which LAP2alpha influences gene expression is yet to be understood (see discussion below).

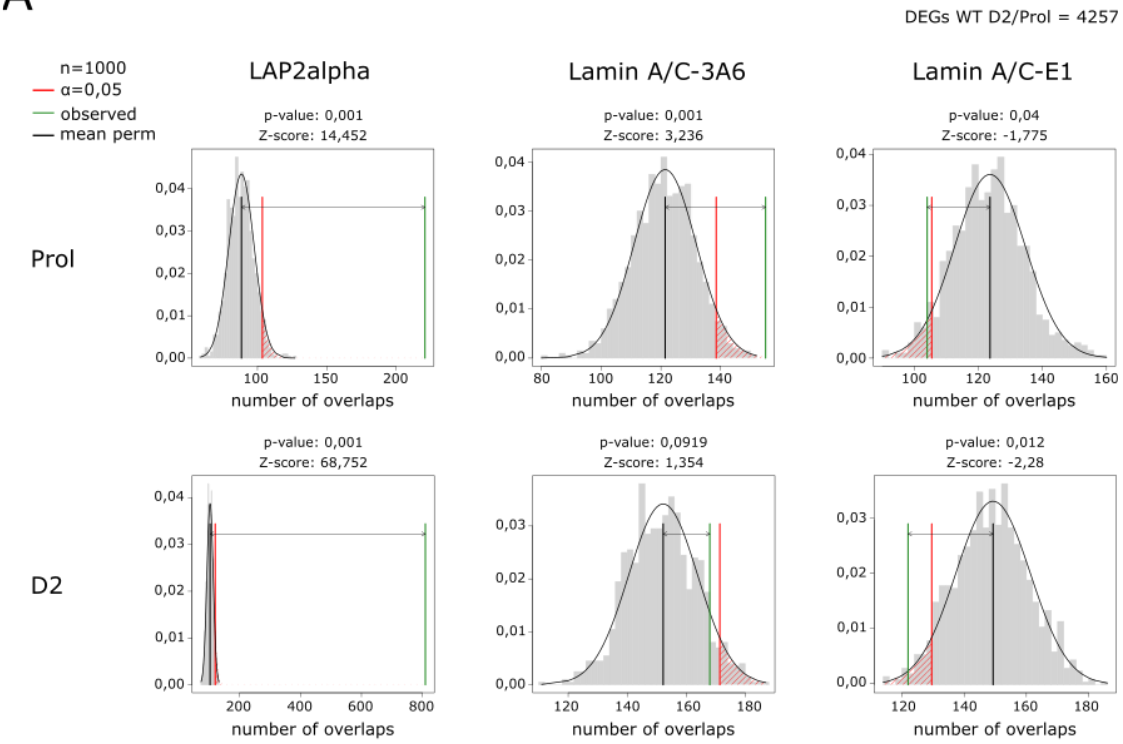


C

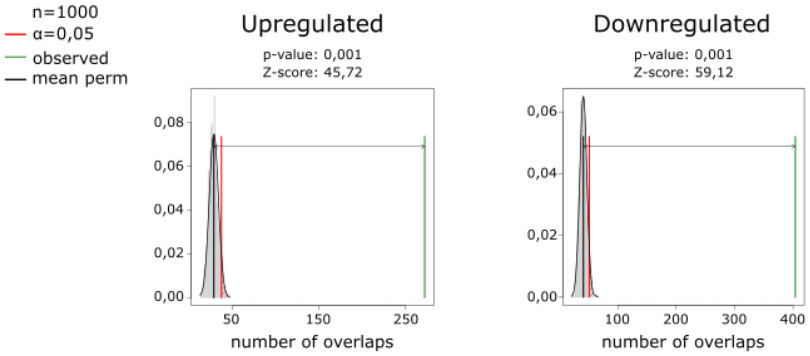
	DEGs D2/Prol (WT)				DEGs KO/WT (D2)			
	Outside cLADs		Inside cLADs		Outside cLADs		Inside cLADs	
	All	Bound	All	Bound	All	Bound	All	Bound
LAP2alpha Prol	221	33	11	1	9	1	1	0
LAP2alpha D2	806	32	1	0	37	1	0	0
Lamin A/C-3A6 Prol	154	0	2	0	11	0	0	0
Lamin A/C-3A6 D2	163	0	4	0	13	0	0	0
Lamin A/C-E1 Prol	104	1	5	3	9	0	1	0
Lamin A/C-E1 D2	121	2	7	0	7	0	1	0

Figure 5. *(A) Pie charts representing genes deregulated during myoblast differentiation (DEGs D2/Prol, WT - upper row) and in LAP2alpha KO myoblasts (DEGs KO/WT, D2 - lower row). For each of these groups of genes we show the percentage found inside LAP2alpha, Lamin A/C-3A6 and Lamin A/C-E1 EDD peaks, outside cLADs (respectively left, middle and right pie charts, in dark grey). The number of genes found inside EDD peaks is divided for each differentiation stage (bar on the side of the charts) and the number of these genes is reported on the side of the bar. (B) Bar graphs representing the genes differentially expressed during myoblasts differentiation (DEGs D2/Prol, WT - left) and deregulated in LAP2alpha KO myoblasts (DEGs KO/WT, D2 - right) that are located inside LAP2alpha and Lamin A/C (3A6 and E1) EDD peaks in both differentiation stages analyzed. The percentage of these genes found in regions overlapping cLADs is reported in dark orange, while the genes found in EDD peaks outside cLADs are in light orange. The percentage refers to the total number of DEGs located inside those specific EDD peaks and not to the total number of genes found deregulated. (C) Table representing an overview of genes differentially expressed during myoblast differentiation (DEGs D2/Prol WT) and in LAP2alpha KO cells (DEGs KO/WT D2) found inside EDD peaks. These genes are divided according to their location inside or outside cLADs. The number of genes that are directly bound by LAP2alpha and Lamin A/C is also reported.*

A


DEGs WT D2/Prol = 4257
n=1000
α=0,05
observed
mean perm
LAP2alpha
Lamin A/C-3A6
Lamin A/C-E1
Prol
D2
p-value: 0,001
Z-score: 14,452
p-value: 0,001
Z-score: 3,236
p-value: 0,04
Z-score: -1,775
p-value: 0,001
Z-score: 68,752
p-value: 0,0919
Z-score: 1,354
p-value: 0,012
Z-score: -2,28
number of overlaps

B


n=1000
α=0,05
observed
mean perm
Upregulated
Downregulated
p-value: 0,001
Z-score: 45,72
p-value: 0,001
Z-score: 59,12
number of overlaps

C

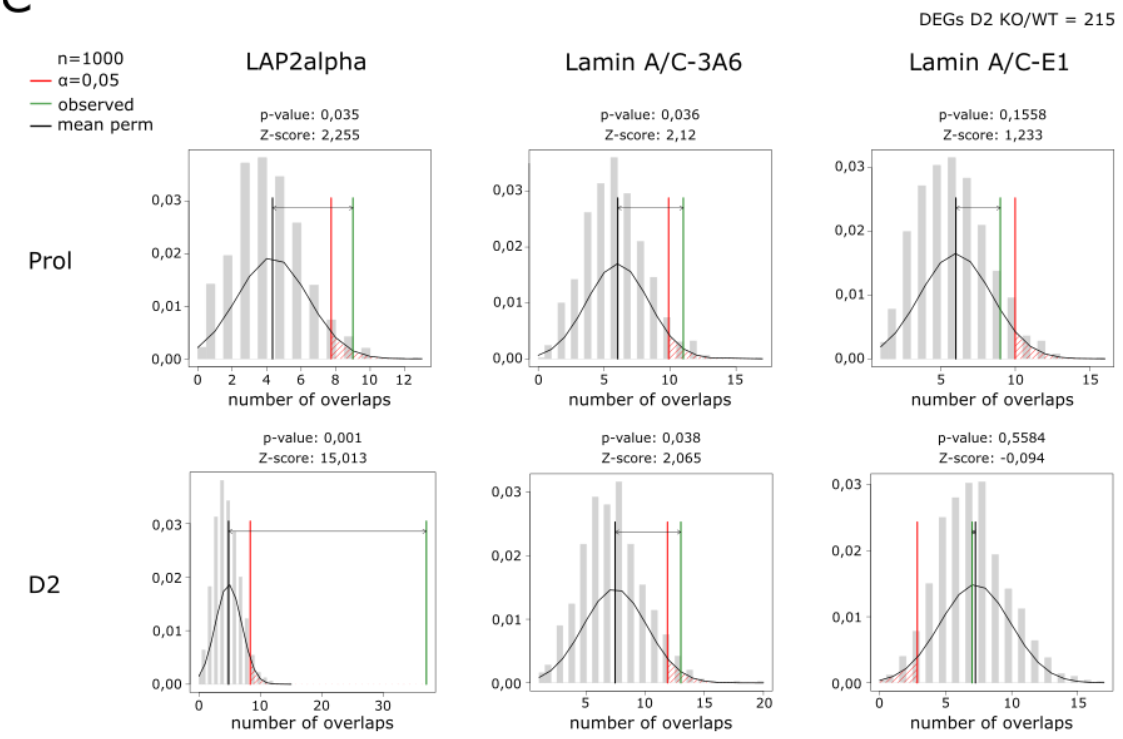

DEGs D2 KO/WT = 215
n=1000
α=0,05
observed
mean perm
LAP2alpha
Lamin A/C-3A6
Lamin A/C-E1
Prol
D2
p-value: 0,035
Z-score: 2,255
p-value: 0,036
Z-score: 2,12
p-value: 0,1558
Z-score: 1,233
p-value: 0,001
Z-score: 15,013
p-value: 0,038
Z-score: 2,065
p-value: 0,5584
Z-score: -0,094
number of overlaps

Figure 6. *(A) Plots of random permutation tests comparing the number of DEGs D2/Prol found inside EDD peaks (for each of the analyzed conditions) to the number of genes that one would expect to find by chance in genomic regions of the same size as the analyzed EDD peaks. The curve represents the distribution of the values found by running 1000 random permutation tests, the red line represents the cutoff at which the observed gene number can be considered significantly different from the expected distribution, for α=0,05. The green line represents the observed number of genes in the respective EDD peaks. Tests were run for LAP2alpha and Lamin A/C (with EDD peaks identified with both 3A6 and E1 antibodies), in proliferating myoblasts and at D2 stage of differentiation. (B) Random permutation tests, performed as described above, were conduct for DEGs D2/Prol found inside LAP2alpha-enriched EDD peaks, in myoblasts, at the D2 differentiation stage. The tests were run separately for DEGs D2/Prol upregulated and downregulated during myoblast differentiation. (C) Random permutation tests performed, as described in Figure 6A, but considering the DEGs KO/WT found inside the EDD peaks.*

2.9 LOSS OF LAP2alpha INDUCES REDISTRIBUTION OF LAMIN A/C ON CHROMATIN

To better understand LAP2alpha-mediated gene regulation and to characterize the changes occurring at the chromatin level in absence of LAP2alpha, I also performed ChIP-seq analysis of LAP2alpha KO myoblasts.

The bioinformatic analyses of the deep-sequencing data were again performed in collaboration with Celine Prakash (CIBIV) and Fatih Sarigöl (Max Perutz Labs). When looking at the EDD peaks distribution on the genome in LAP2alpha KO cells in the IGV genome browser, we again see a strong overlap between binding regions detected by the two Lamin A/C antibodies (Figure 7A). As the differentiation proceeds, we also detect some redistribution of lamin A/C on the genome. Interestingly, in the Prol stage, the C-terminal Lamin A/C 3A6 antibody recognizes a pool of A-type lamins that has a significantly different distribution on the chromatin when compared to the pool recognized by the N-terminal E1 antibody (Figure 7A, in dark red). This could be interpreted in different ways: for example, loss of LAP2alpha may induce changes in the assembly state of lamins, which, consequently, cannot be detected by the E1 antibody. Indeed, masking of the N-terminal epitope of Lamin A/C in the absence of LAP2alpha has been recently reported (Naetar et al., 2021). Alternatively, Lamin A/C, lacking its main nucleoplasmic interactor LAP2alpha, may be free to form complexes with other proteins, which prevents their recognition by E1. Further studies are required to elucidate the exact mechanism behind this difference.

On a genome-wide level, I can confirm that the pool of lamins recognized by 3A6 is enriched in different regions of the genome compared to the E1 Lamin A/C pool (Figure 7B). Interestingly, this difference is strongly reduced as the differentiation proceeds (D2 myoblasts), suggesting that the putative epitope-masking for E1 antibody is greatly reduced after the onset of differentiation. In LAP2alpha depleted cells Lamin A/C is still able to bind to large genomic regions outside cLADs. This is particularly evident for the 3A6 lamin A/C bound regions, in the proliferating stage. In fact, the identified EDD peaks even show a reduced overlap with the repressive cLAD regions when compared to their WT counterparts (Figure 7B, 8B). Thus, LAP2alpha loss seems to induce an increased binding of Lamin A/C to more active regions of the chromatin (see also below).

LAP2alpha KO cells also show genome-wide changes in Lamin A/C distribution on the chromatin, during differentiation. In case of the lamin pool recognized by E1, the redistribution occurs to a similar extent as seen in the control WT myoblasts (Figure 7C, right diagrams, compare with Figure 3B). The lamin pool recognized by 3A6, on the other hand, redistributes more extensively (Figure 7A and C, left diagrams) in the absence of LAP2alpha.

When comparing side by side the Lamin A/C binding in LAP2alpha WT vs KO cells, one can better appreciate the changes in Lamin A/C binding upon loss of LAP2alpha. As shown in Figure 8A in the IGV browser view, most of the lamin EDD peaks seem to localize in similar regions of the genome, supporting the specificity of Lamin A/C ChIP results using both Lamin A/C antibodies. Nevertheless, there are peculiar differences as well, reflecting the different Lamin A/C pools these antibodies detect, and highlighting differences in the behavior of these different lamin pools.

A genome-wide analysis of the Lamin A/C EDD peaks shows that in LAP2alpha-depleted myoblasts A-type lamins, although covering a similar proportion of the genome compared to LAP2alpha WT cells (Suppl. Figure 4A), have a clearly reduced overlap with cLADs (Figure 8B). This is seen for all conditions, but particularly in the Prol stage for the Lamin A/C pool recognized with the antibody 3A6 (Figure 8B, upper-right diagrams). The percentages of Lamin A/C EDD peaks overlapping with cLADs are listed in the Suppl. Figure 4B. Overall, these results indicate that Lamin A/C redistributes on chromatin upon loss of LAP2alpha, particularly in the proliferating stage. To test whether the Lamin A/C redistribution occurs towards regions formerly covered by LAP2alpha in WT cells, we checked the overlap of the Lamin A/C EDD peaks with the WT LAP2alpha EDD peaks, both in WT and KO myoblasts. The overlap of Lamin A/C 3A6 peaks with LAP2alpha EDD peaks in the proliferating stage was dramatically reduced from 55% in LAP2alpha WT cells to 15% in LAP2alpha KO cells (Figure 8C and Suppl. Figure 4C). A similar, although less pronounced, decrease in overlap was seen for the Lamin A/C EDD peaks detected with the antibody E1. In contrast, we observe a slight increase in the overlap between Lamin A/C EDD peaks, for both lamin antibodies, at D2 of the differentiation process and the genomic regions covered by LAP2alpha in WT cells at D2. Altogether, this indicates that loss of LAP2alpha leads to the redistribution of Lamin A/C to regions outside of the previously LAP2alpha-bound regions in proliferating cells. In contrast, Lamin A/C tends to relocate at least partially to regions normally covered by LAP2alpha in WT cells at differentiation day 2. This peculiar rearrangement of Lamin A/C on chromatin in the absence of LAP2alpa can potentially affect genes located in these regions. Thus, we checked for genes in the Lamin A/C bound regions in the LAP2alpha KO cells.

Figure 7. *(A) IGV browser view of the entire chromosome 5 showing the EDD peaks obtained analyzing Lamin A/C binding to chromatin by ChIP-seq, in LAP2alpha KO myoblasts. Lamin A/C binding was detected both with the Lamin A/C-3A6 (red) and Lamin A/C-E1 antibodies (pink). The EDD peaks are shown for both stages of differentiation analyzed (Prol and D2). (B) Venn diagrams showing the overlap between the Lamin A/C binding detected with the Lamin A/C 3A6 (red) and Lamin A/C E1 (pink) antibodies, alone and with the reported cLADs (orange). The overlap is shown for the Prol stage (diagrams on the left) and for the D2 stage of differentiation (on the right). (C) Venn diagrams representing the overlaps between the Prol and D2 stages of differentiation of the EDD peaks detected for Lamin A/C-3A6 (red) and Lamin A/C-E1 (pink). The same overlaps are represented also in comparison with the reported cLADs. Numbers in Venn diagrams indicate total length of EDD peaks in Mb in the respective area.*

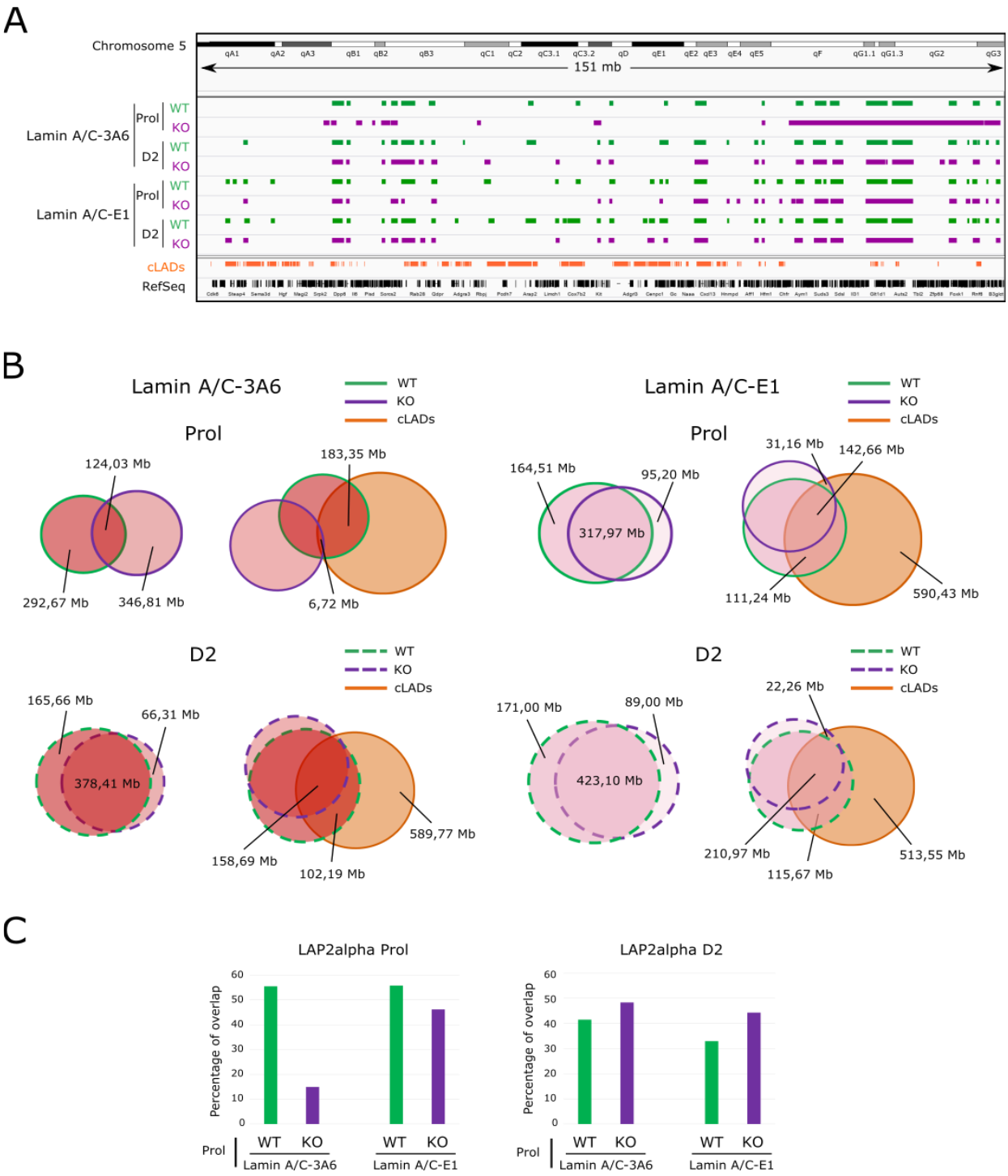


Figure 8. *(A) IGV browser view of the entire chromosome 5 showing the EDD peaks obtained analyzing Lamin A/C binding to chromatin by ChIP-seq, in LAP2alpha WT and KO myoblasts. The binding was detected both with the Lamin A/C-3A6 (red) and Lamin A/C-E1 (pink) antibodies. The EDD peaks are shown for both stages of differentiation analyzed (Prol and D2). For comparison, the reported cLADs are shown in orange. (B) Venn diagrams representing the overlap between the Lamin A/C binding detected in LAP2alpha WT and KO myoblasts, for the Lamin A/C 3A6 (red) and Lamin A/C E1 (pink) antibodies, alone and with the reported cLADs (orange). The overlap is shown for the Prol stage (upper rows) and for the D2 stage of differentiation (lower rows). The same overlaps are represented also in comparison to the reported cLADs. Numbers in Venn diagrams indicate total length of EDD peaks in Mb in the respective area. (C) Bar graphs representing the % of overlap between Lamin A/C-enriched EDD peaks in proliferating myoblasts and LAP2alpha-enriched EDD peaks either in proliferating*

myoblasts (Prol, left graph) or at D2 stage of differentiation (D2, graph on the right). The overlap is shown both for EDD peaks identified with the Lamin A/C-3A6 antibody and with the Lamin A/C-E1 antibody in LAP2alpha-expressing (WT) and -depleted (KO) cells.

2.10 IN ABSENCE OF LAP2alpha, LAMIN A/C BINDING INCREASES IN ACTIVE REGIONS OF THE GENOME BUT NOT DIRECTLY ON GENES

When looking at the genes located inside Lamin A/C EDD peaks in LAP2alpha KO myoblasts, we observed that, not surprisingly, the increased binding of Lamin A/C outside the cLADs in these cells correlates with a higher number of expressed genes found inside the Lamin A/C EDD peaks (Figure 9A). This is particularly evident for the Lamin A/C bound regions detected by 3A6 antibody in the proliferating stage (5601 expressed genes in LAP2alpha KO versus 656 genes in WT cells). Moreover, in the absence of LAP2alpha, a much higher percentage of expressed genes is detected in the regions of the EDD peaks not overlapping with cLADs. On the contrary, the proportion of expressed genes in EDD peaks overlapping with cLADs is mainly unchanged, compared to WT cells (Suppl. Figure 4D).

Altogether we conclude that, in the absence of LAP2alpha, Lamin A/C redistributes to active chromatin regions containing a higher number of expressed genes. Changes in the chromatin environment surrounding these active genes may interfere with their regulation, as we will discuss in more detail below.

We next tested direct binding of A-type lamins to genes within Lamin A/C EDD peaks in the absence of LAP2alpha. As observed in LAP2alpha WT myoblasts, only a small portion of these genes both inside and outside of cLADs was directly bound by Lamin A/C (Figure 9B). In most conditions, the proportion of Lamin A/C bound genes in EDD peaks in LAP2alpha KO myoblasts is similar to that observed in WT cells. While around 20% of the genes in the EDD peaks overlapping with cLADs are enriched in Lamin A/C, only around 11-13% of the genes outside cLADs are directly bound (Suppl. Figure 4E). This proportion of genes within cLADs is only different for the 3A6-detected pool of lamins in the Prol stage, which could, however, be related to the fact that only a very small portion of Lamin A/C EDD peaks detected by 3A6 overlaps with cLADs in these conditions.

Altogether I conclude that, in the absence of LAP2alpha, Lamin A/C redistributes to active chromatin regions containing a high number of expressed genes. Binding of Lamin A/C to the chromatin environment surrounding these genes, rather than binding to these genes directly, may affect their regulation.

To further characterize the changes happening in the absence of LAP2alpha, I performed a gene ontology (GO) analysis of the genes located inside Lamin A/C EDD peaks in LAP2alpha KO myoblasts. In the Prol stage, the 3A6 pool of lamins binds to regions enriched in genes relevant for RNA-related processes, in concordance with what was observed in WT cells. In addition, in LAP2alpha KO cells I also find enrichment of expressed genes involved in apoptosis, mitochondrial regulation, autophagy and histone acetylation (Suppl. Data file2, Sheet1). Similarly, the EDD peaks identified with the antibody E1 are also enriched for RNA-related genes and, additionally, genes involved in chromatin regulation and gene expression (Suppl. Data file2, Sheet3). As differentiation proceeds, both pools of A-type lamins bind to regions enriched in genes involved in several metabolic processes and RNA regulation (Suppl. Data file2, Sheet2 and 4).

This analysis shows that, in LAP2alpha-depleted cells, Lamin A/C EDD peaks contain expressed genes involved in specific cellular processes, that differ from those in LAP2alpha WT myoblasts. Altogether, in the absence of LAP2alpha, Lamin A/C redistributes to regions containing more expressed genes compared to LAP2alpha WT myoblasts, and a significant number of these genes is involved in differentiation-relevant processes like chromatin regulation and metabolic functions. The binding of lamins to these regions may therefore have an impact on their regulation.

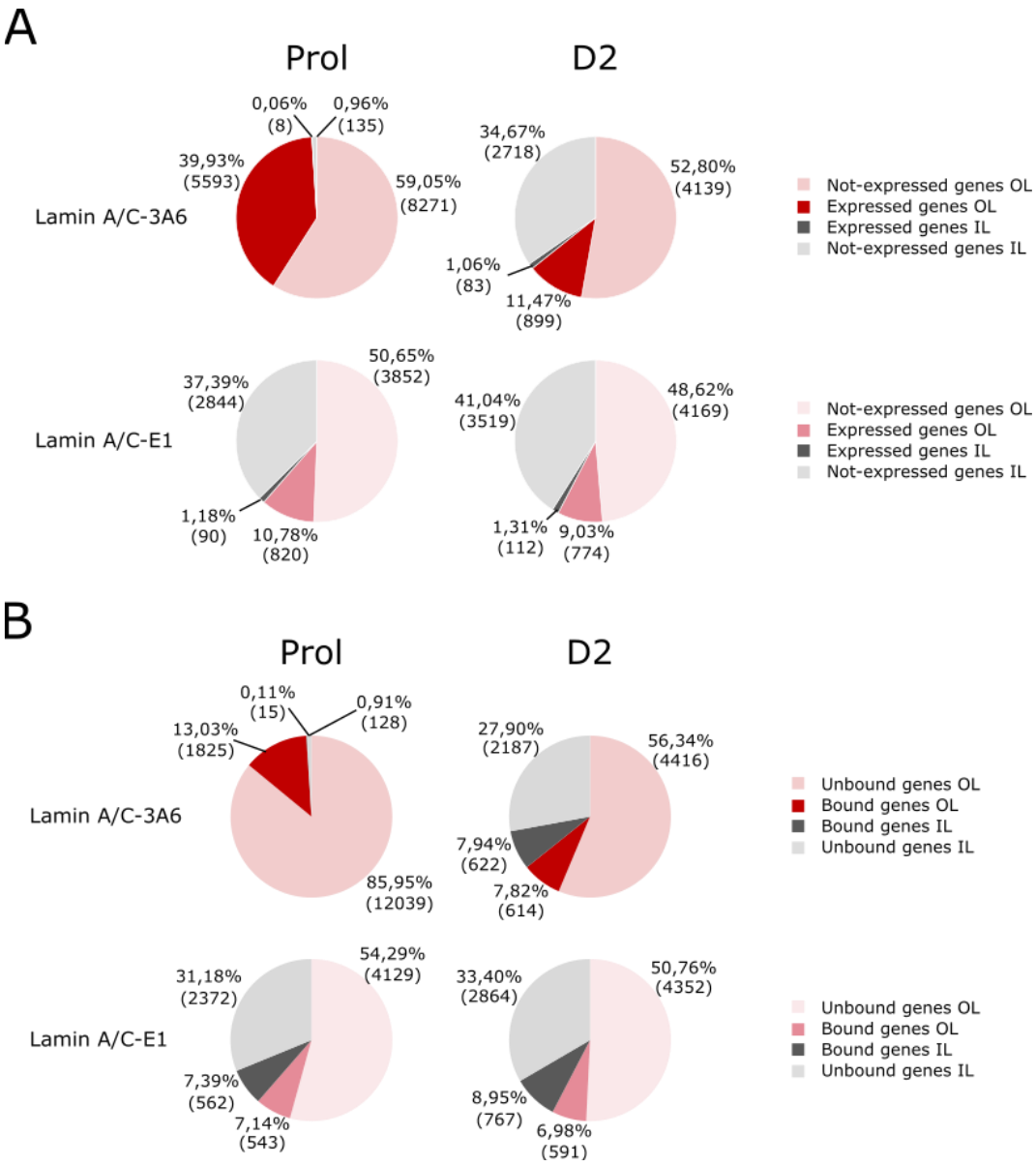


Figure 9. *(A) Pie charts representing the percentage of expressed and not-expressed genes located inside EDD peaks, for Lamin A/C-3A6 and amin A/C-E1 (upper and lower row, respectively). The genes were also divided in those found in regions overlapping with cLADs (IL) and outside cLADs (OL). Both the gene number and the respective percentages of the total are shown. (B) Pie charts representing the percentage of bound and unbound genes located inside EDD peaks, for Lamin A/C-3A6 and Lamin A/C-E1 (upper and lower row, respectively). The genes were divided according to their location inside or outside cLADs (IL and OL, respectively). Number of genes and percentages of the total are shown.*

2.11 IN THE ABSENCE OF LAP2alpha, LAMIN A/C REDISTRIBUTES TO REGIONS ENRICHED IN GENES RELEVANT FOR MYOBLAST DIFFERENTIATION

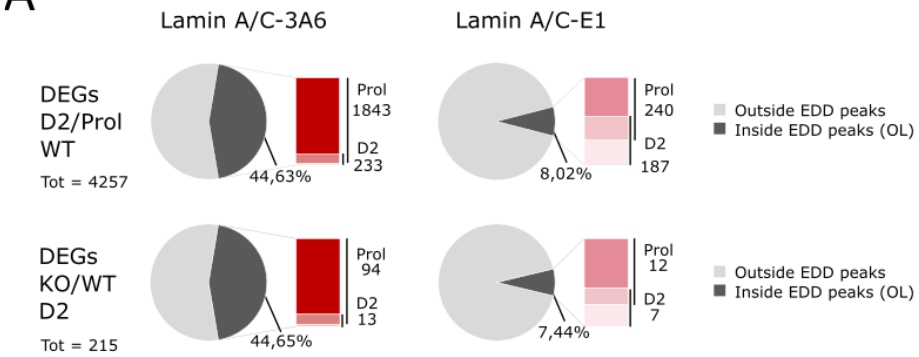
We next tested whether Lamin A/C relocalization on chromatin in the absence of LAP2alpha affects regions containing genes differentially expressed during myoblasts differentiation (DEGs D2/Prol) or genes that are de-regulated in LAP2alpha KO myoblasts (DEGs KO/WT). While in LAP2alpha-expressing myoblasts only 2-3% of these genes are found in chromatin regions enriched in lamin A/C in each stage of differentiation (Figure 5A), the percentage of DEGs D2/Prol in the EDD peaks identified with the 3A6 antibody increases strongly to around 44% in the proliferation stage upon LAP2alpha depletion (Figure 10A, upper panel). In the D2 stage and in E1 antibody-identified genomic regions, we also see some enrichment of genes involved in myoblast differentiation in lamin A/C EDD peaks, but this is much less pronounced (Figure 10C, columns on the left). Random permutation tests suggest that in proliferating and differentiating (D2) myoblasts both pools of lamins A/C bind to significantly more genes involved in myoblast differentiation (DEGs D2/Prol) than one would expect by chance (Figure 11A). Thus, in the absence of LAP2alpha, the deregulated pools of Lamins A/C, particularly that recognized by 3A6, bind to regions enriched in genes differentially expressed during myoblast differentiation (DEGs D2/Prol).

We also find that in proliferating LAP2alpha KO myoblasts, the 3A6-detected pool of lamins redistributes to regions containing more than 44% of the genes deregulated in the absence of LAP2alpha (DEGs KO/WT – Figure 10A, lower row). This is not true for the differentiation stage D2 or for the E1-detected pool of lamins in either stage (Figure 10C, columns on the right). Random permutation tests also endorse these conclusions (Figure 11B).

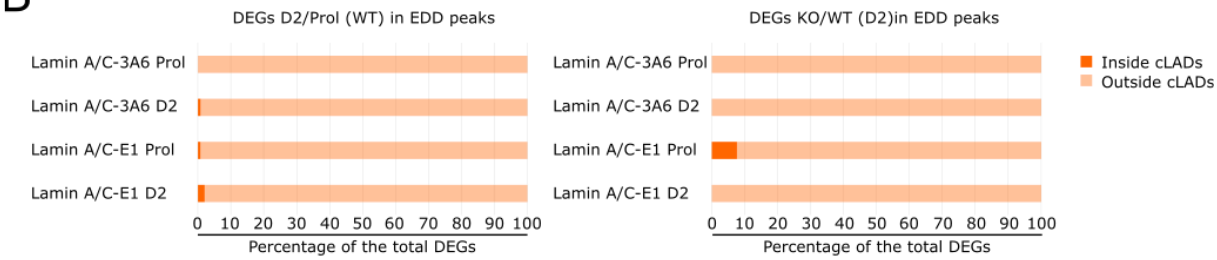
As expected, most of the differentially expressed genes (DEGs D2/Prol and DEGs KO/WT) are localized within regions of the Lamin A/C EDD peaks not overlapping with cLADs (Figure 10B). Moreover, barely any of the deregulated genes is directly bound by lamin A/C (Figure 10C), reinforcing the idea that enrichment of lamins on the gene-body is irrelevant for the regulation of expressed genes. Only 6% (116 out of 1843, Figure 10C, first two columns) of the DEGs D2/Prol is directly bound by lamin A/C in the Prol stage, which is less than the generally observed binding of lamin A/C to all genes inside EDD peaks (~11-13% - Suppl. Figure 4E).

In summary, the data obtained so far suggest that binding of LAP2alpha and Lamin A/C to long genomic regions may create a local environment that is able to influence the regulation of genes located in these regions. LAP2alpha enrichment around expressed genes suggests a role of this protein in facilitating the regulation of active chromatin. In accordance with this hypothesis, loss of LAP2alpha is associated with a de-regulation of many genes involved in myogenic functions. On the contrary, Lamin A/C enrichment seems less relevant for active genes. Instead, a pronounced abnormal Lamin A/C enrichment around genes in the absence of LAP2alpha could make their regulation less efficient, as suggested by the enrichment of Lamin A/C in areas around the deregulated genes.

A



B



C

	DEGs D2/Prol (WT)				DEGs KO/WT (D2)			
	Outside cLADs		Inside cLADs		Outside cLADs		Inside cLADs	
	All	Bound	All	Bound	All	Bound	All	Bound
Lamin A/C-3A6 Prol	1843	116	0	0	94	0	0	0
Lamin A/C-3A6 D2	233	0	2	0	13	0	0	0
Lamin A/C-E1 Prol	240	5	2	0	12	0	1	0
Lamin A/C-E1 D2	187	0	4	0	7	0	0	0

Figure 10. *(A) Pie charts representing genes differentially regulated during myoblast differentiation (DEGs D2/Prol, WT - upper row) and deregulated in LAP2alpha KO myoblasts (DEGs KO/WT, D2 - lower row). For each of these groups of genes we show the percentage found inside Lamin A/C-3A6 and Lamin A/C-E1 EDD peaks, outside cLADs in LAP2alpha KO myoblasts (respectively left and right pie charts, in dark grey). The number of genes found inside EDD peaks is divided for each differentiation stage (bar on the side of the charts) and the number of these genes is reported on the side of the bar. (B) Bar graphs representing the genes differentially expressed during myoblasts differentiation (DEGs D2/Prol, WT - left) and deregulated in LAP2alpha KO myoblasts (DEGs KO/WT, D2 - right) that are located inside Lamin A/C (3A6 and E1) EDD peaks, in LAP2alpha KO myoblasts, in both differentiation stages analyzed. The percentage of these genes found in regions overlapping cLADs is reported in dark orange, while the genes found in EDD peaks outside cLADs are represented in light orange. The percentage refers to the total number of DEGs located inside those specific EDD peaks and not to the total number of genes found deregulated. (C) Table representing an overview of genes differentially expressed during myoblast differentiation (DEGs D2/Prol WT) and in LAP2alpha KO cells (DEGs KO/WT D2) found inside EDD peaks in LAP2alpha KO myoblasts. These genes are divided according to their location inside or outside cLADs. The number of genes that are directly bound by Lamin A/C (for the two antibodies used for ChIP-seq) is also reported.*

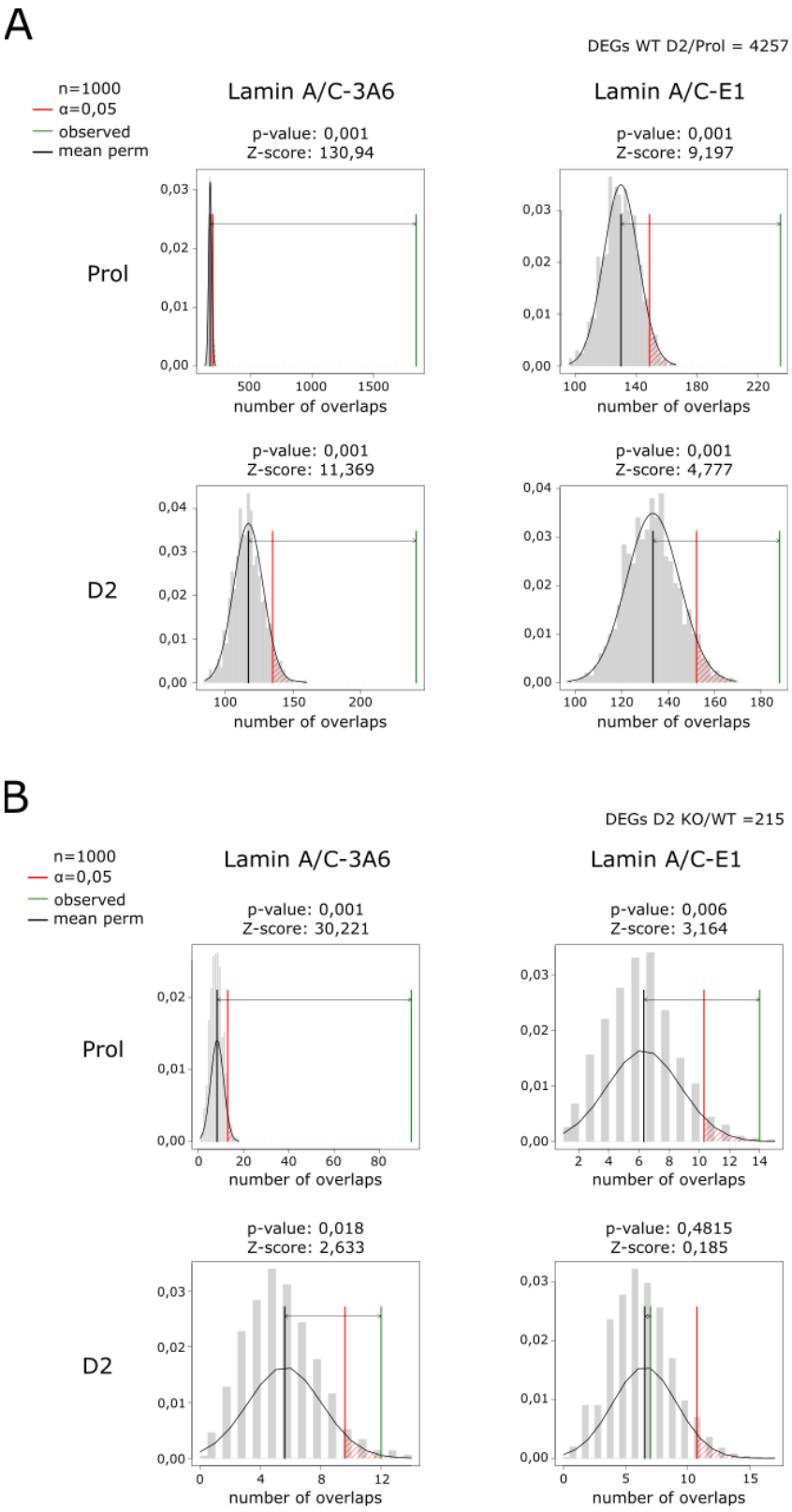


Figure 11. *(A) Plots of random permutation tests comparing the number of genes differentially regulated during myoblast differentiation (DEGs D2/Prol, WT) found inside EDD peaks in the analyzed conditions and the distribution of the number one would expect to find by chance in regions of equal length. Permutation test were performed for Lamin A/C-3A6 and E1 in both differentiation stages for EDD peaks detected in LAP2alpha KO*

myoblasts. (B) Plots of random permutation tests obtained with the same procedure (and in the same conditions) described in (A) but testing genes deregulated in LAP2lapha KO (DEGs KO/WT, Prol).

2.12 THE MAJORITY OF LAMIN A/C- AND LAP2alpha-BOUND GENES ARE LOCATED OUTSIDE EDD PEAKS

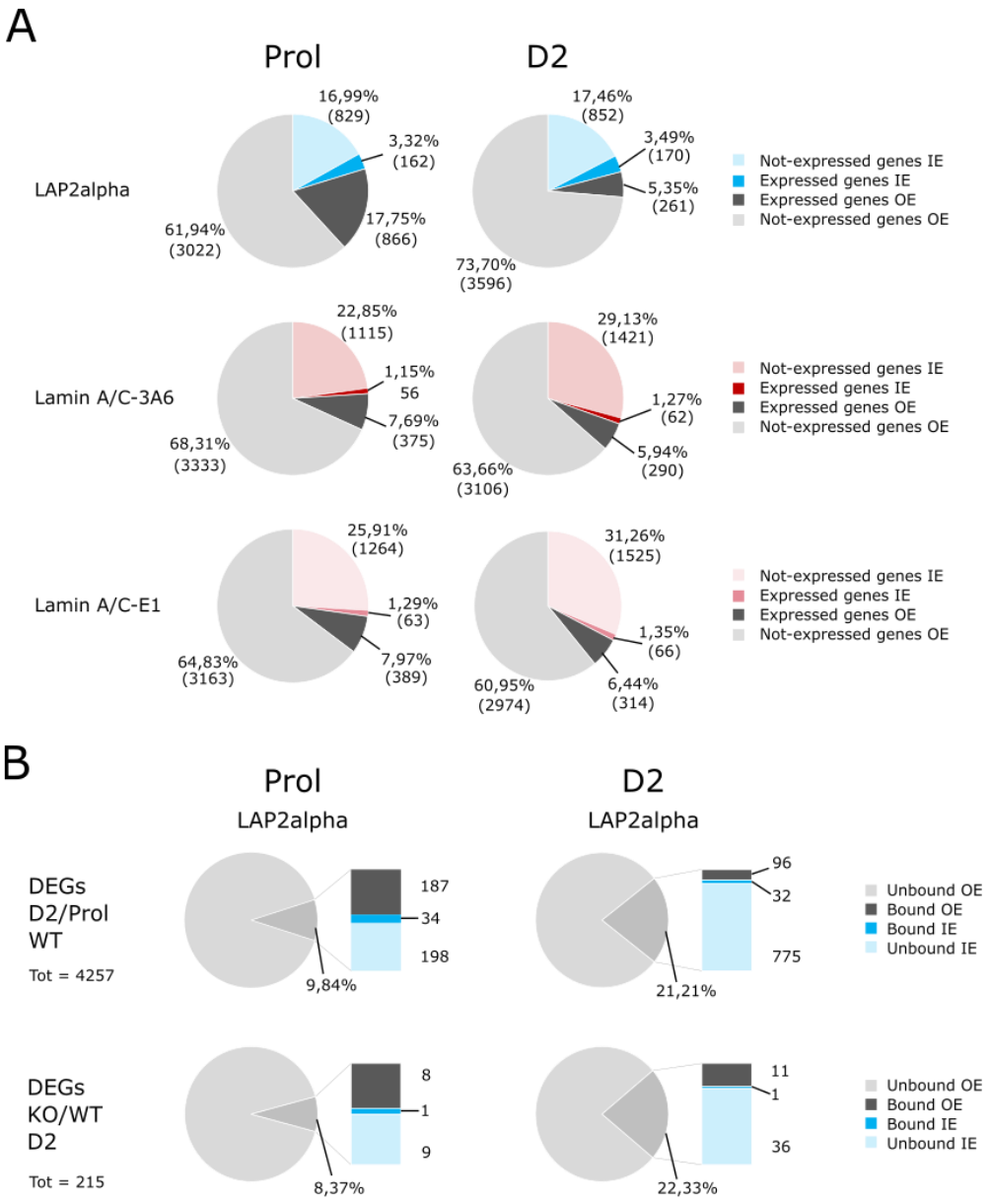
Inside EDD peaks, direct binding of Lamin A/C or LAP2alpha to genes seems to negatively correlate with their expression. This is supported by both the lack of direct binding of Lamin A/C and LAP2alpha to genes involved in myoblast differentiation and by the increased binding to genes in more repressed regions, like cLADs, that are mainly not expressed. Whether the increased A-type lamin binding to non-expressed genes is a mechanism to keep them inaccessible to factors involved in gene expression or a consequence of the putative lack of transcriptional regulators on these genes remains unknown.

To obtain more insights into the general effect of direct binding of LAP2alpha and Lamin A/C to genes, we extended our analysis to regions outside EDD peaks (OE). These OE regions lack the high binding density of LAP2alpha and Lamin A/C observed in EDD peaks and are therefore not detected by the EDD peak caller. Nevertheless, this does not exclude LAP2alpha and Lamin A/C binding to shorter genomic regions or even selectively to specific genes. Indeed, we find that around 70% of the genes that show direct Lamin A/C and/or LAP2alpha binding are located outside EDD peaks (Figure 12A, light and dark grey). In concordance with previous results, most of the bound genes are not expressed. However, LAP2alpha binds to a higher number of expressed genes in proliferating myoblasts. This is in line with the previous observation that LAP2alpha EDD peaks contain more expressed genes than Lamin A/C EDD peaks, especially in D2 (Figure 4A) and that LAP2alpha directly binds to more expressed genes within EDD peaks compared to A-type lamins (Figure 12A). Nevertheless, the strongest difference in the number of LAP2alpha and A-type lamins bound genes is seen in the regions outside the EDD peaks (OE regions): In these areas, more than 20% of the LAP2alpha-bound genes are expressed, compared to 5-7% expressed genes bound to either of the two Lamin A/C pools. This suggests a putative role for LAP2alpha in the direct regulation of active genes compared to A-type lamins.

Similarly, LAP2alpha may contribute to the efficient regulation of many DEGs during differentiation by two mechanisms: direct binding to genes and binding to the gene environment (in EDD peaks). Out of the 4257 DEGs D2/Prol, 9,84% were either directly bound by LAP2alpha or localized within EDD peaks in the Prol stage. Within this 9,84%, LAP2alpha directly binds to 187 DEGs D2/Prol outside EDD peaks and additional 232 DEGs D2/Prol are located inside LAP2alpha EDD peaks but only 34 of these are directly bound by LAP2alpha (Figure 12B, left). At D2, much more DEGs D2/Prol are found inside EDD peaks, although the number of DEGs directly bound by LAP2alpha in the EDD peaks does not increase. This is also true for the genes deregulated in the absence of LAP2alpha (DEGs KO/WT) (Figure 12B, lower row). Overall, these findings indicate a more important role of local enrichment of LAP2alpha on the chromatin than its direct binding on genes in the myogenic differentiation process.

Direct binding of Lamin A/C to DEGs D2/Prol both outside and inside EDD peaks is neglectable (Figure 12C, left columns). This is also true for the genes deregulated in the absence of LAP2alpha (Figure 12C, right columns).

In LAP2alpha-depeleted myoblasts, the extent of direct binding of Lamin A/C to genes is comparable to that observed in WT cells in most cases, except for the Lamin A/C pool recognized by the 3A6 antibody. In the Prol stage, this pool of lamins binds directly to an increased number of genes inside EDD peaks, compared to WT myoblasts (Figure 13A, left-upper chart), and around 1/3 of those genes are expressed. Outside EDD peaks we also detect an increased proportion of expressed genes bound by the 3A6 Lamin A/C pool compared to the LAP2alpha WT cells. The redistribution of this pool of lamins to active genes may impair gene regulation also by other factors. Even though in proliferating myoblasts the 3A6 lamin pool tends to bind to more expressed genes in the absence of LAP2alpha, neither the DEGs D2/Prol nor the DEGs KO/WT show a significant increase in direct Lamin A/C binding (Figure 13B, left and right chart, respectively). The lamin pool detected by E1 is not significantly bound to DEGs D2/Prol or DEGs KO/WT in any of the differentiation stages (Figure 13C). These data suggest that direct binding of Lamin A/C to the gene-body is most likely not a mechanism for the regulation of genes relevant for myoblast differentiation.

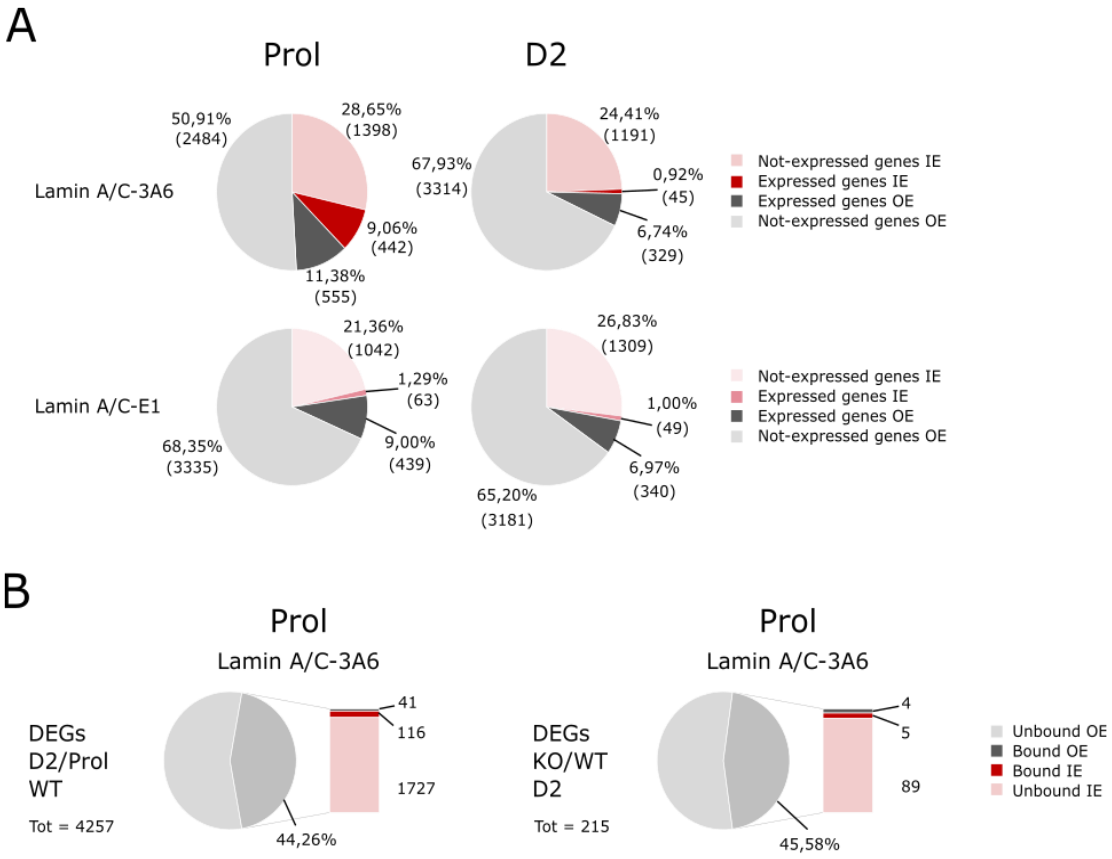


C

	DEGs D2/Prol (WT)				DEGs KO/WT (D2)			
	Unbound outside EDD peaks	Bound outside EDD peaks	Bound inside EDD peaks	Unbound inside EDD peaks	Unbound outside EDD peaks	Bound outside EDD peaks	Bound inside EDD peaks	Unbound inside EDD peaks
Lamin A/C-3A6 Prol	4083	18	0	156	202	2	0	11
Lamin A/C-3A6 D2	4073	17	1	166	202	0	0	13
Lamin A/C-E1 Prol	4134	14	4	105	205	0	0	10
Lamin A/C-E1 D2	4113	16	2	126	207	0	0	8

Figure 12. *(A) Pie charts representing the genes bound by LAP2alpha (upper row) in LAP2alpha WT myoblasts. The genes are divided for their localization inside LAP2alpha EDD peaks (IE) or outside these peaks (OE). A further distinction was made between expressed and not expressed genes. The same analysis was performed for genes bound by A-type Lamins and detected with the Lamin A/C-3A6 antibody (middle row) and with the Lamin A/C-E1 antibody (lower row) by ChIP-seq analysis. (B) Pie charts representing LAP2alpha binding to genes that are differentially expressed during myoblast differentiation (DEGs D2/Prol, WT - upper row) and found deregulated in the absence of LAP2alpha (DEGs KO/WT, Prol - lower row). For each pie chart, the percentage of genes enriched*

in LAP2alpha binding (as defined by either location of the gene within LAP2alpha EDD peaks or directly bound by LAP2alpha) is shown in darker grey and a bar on the side divides them in those bound outside EDD peaks (OE, dark grey), bound inside EDD peaks (IE, dark blue) and those found inside EDD peaks, but not directly enriched in LAP2alpha binding (Unbound IE, light blue). (C) Table of the genes differentially regulated during myoblast differentiation (left side) and deregulated in LAP2alpha KO cells (right side). The table shows how many of these genes are found inside or outside Lamin A/C EDD peaks in LAP2alpha WT myoblasts and how many have been shown to be directly bound. The analysis was performed with both the Lamin A/C 3A6 and E1 antibodies and for both differentiation stages (Prol and D2).



C

	DEGs D2/Prol (WT)				DEGs KO/WT (D2)			
	Unbound outside EDD peaks	Bound outside EDD peaks	Bound inside EDD peaks	Unbound inside EDD peaks	Unbound outside EDD peaks	Bound outside EDD peaks	Bound inside EDD peaks	Unbound inside EDD peaks
Lamin A/C-3A6 D2	4010	12	0	235	200	2	0	13
Lamin A/C-E1 Prol	3991	24	5	237	198	4	0	13
Lamin A/C-E1 D2	4048	18	0	191	205	3	0	7

Figure 13. *(A) Pie charts representing the genes bound by Lamin A/C in LAP2alpha KO myoblasts. The genes are divided for their localization inside Lamin A/C EDD peaks (IE) or outside these peaks (OE). A further distinction was made between expressed and not expressed genes. The analysis was performed for binding detected with the Lamin A/C-3A6 antibody (upper row) and with the Lamin A/C-E1 antibody (lower row) by ChIP-seq analysis. (B) Pie charts representing Lamin A/C -3A6 binding to genes that are differentially expressed during myoblast differentiation (DEGs D2/Prol, WT - left) and genes found deregulated in the absence of LAP2alpha (DEGs KO/WT,*

Prol - right). For each pie chart, the percentage of genes enriched in Lamin A/C binding (as defined by either location of the gene within Lamin A/C EDD peaks or directly bound by Lamin A/C) is shown in darker grey and a bar on the side divides them in those bound outside EDD peaks (OE, dark grey), bound inside EDD peaks (IE, dark red) and those found inside EDD peaks, but not directly enriched in Lamin A/C binding (Unbound IE, light red). (C) Table of the genes differentially regulated during myoblast differentiation (left side) and deregulated in LAP2alpha KO cells (right side). The table shows how many of these genes are found inside or outside Lamin A/C EDD peaks in LAP2alpha KO myoblasts and how many have been shown to be directly bound. The analysis was performed with both the Lamin A/C 3A6 and E1 antibodies and for both differentiation stages (Prol and D2). The results obtained for Lamin A/C-3A6 in the Prol stage are represented with pie charts in panel (B) of the figure.

2.13 LAMIN A/C DEPLETION AT THE TRANSCRIPTION START SITE IS A CONSISTENT FEATURE OF EXPRESSED GENES

The results described above indicate that direct binding, especially of Lamin A/C, to genes negatively correlates with their expression. These data were obtained considering the mean protein binding throughout the gene-body and do not exclude the possibility that Lamin A/C and LAP2alpha may bind specifically to shorter regions on active genes, such as the transcription start site (TSS), transcription termination site (TTS), intron/exon junctions, or gene promoters. To obtain a deeper insight into Lamin A/C and LAP2alpha binding patterns on genes and on their up-and downstream regions, we looked at log2ratios of ChIP/INPUT sequences along the genes. First, we randomly selected 500 genes upregulated, downregulated or non-differentially expressed (non DE) during myoblast differentiation, and 500 genes not expressed in any of the analyzed stages or genotypes. Then, we computed for LAP2alpha and each of the Lamin A/C sample profiles the mean and median of the log2ratios of the ChIP/INPUT signal over these 500 genes and the regions flanking the gene bodies. In this analysis one can expect a mean/median binding signal close to 0 for genes or regions of the genes that have no consistent enrichment or depletion of these proteins, since in each of the 500 genes we would have a different (positive or negative) and random enrichment over the gene body. On the contrary, if LAP2alpha or Lamin A/C binding is consistently enriched or depleted in a specific region of the gene (e.g., at the promoter), the mean/median would have a positive or negative signal, respectively.

In LAP2alpha WT myoblasts, non-expressed genes show a flat profile: Both mean and median are flat lines, pointing towards random LAP2alpha and Lamin A/C binding along these genes (Figure 14, left column). Lamin A/C profiles on genes in all other categories (upregulated, downregulated, not differentially expressed genes) present a consistent depletion on the TSS (Figure 14) independent of the specific antibody used. LAP2alpha binding patterns on these genes, in contrast, are less clear. Although LAP2alpha behaves similarly to A-type lamins on non-expressed genes, the signal at the TSS of expressed genes seems variable, sometimes even showing a slight increase on these regions (Figure 14). Overall, these results point towards a random binding of Lamin A/C and LAP2alpha on genes that are not expressed and thus not regulated during myoblast differentiation. In contrast, depletion of Lamin A/C seems to be a common feature at the TSS of genes whose expression still need to be regulated, in myoblasts.

The depletion of Lamin A/C binding at the TSS of expressed genes and the lack of depletion in non-expressed genes is detectable more clearly when plotting the unscaled biding profiles around the TSS (+/- 2kb up-and downstream) of the same genes analyzed before (Figure 15). Lamin A/C is clearly depleted at the TSS of genes downregulated and non-DE during myoblast differentiation. The

depletion on the upregulated genes is not as pronounced in the Prol stage, before their upregulation (the median profile looks flat, while the mean still shows signs of depletion). When the genes are upregulated during differentiation (their expression increases) the depletion at the TSS becomes more evident. This indicates that depletion of Lamin A/C at the TSS of genes correlates with their expression levels. As mentioned before LAP2alpha depletion is not detectable at the TSS of these genes, in fact there seems to be a slight increase in LAP2alpha binding around the TSS of some regulated genes.

A

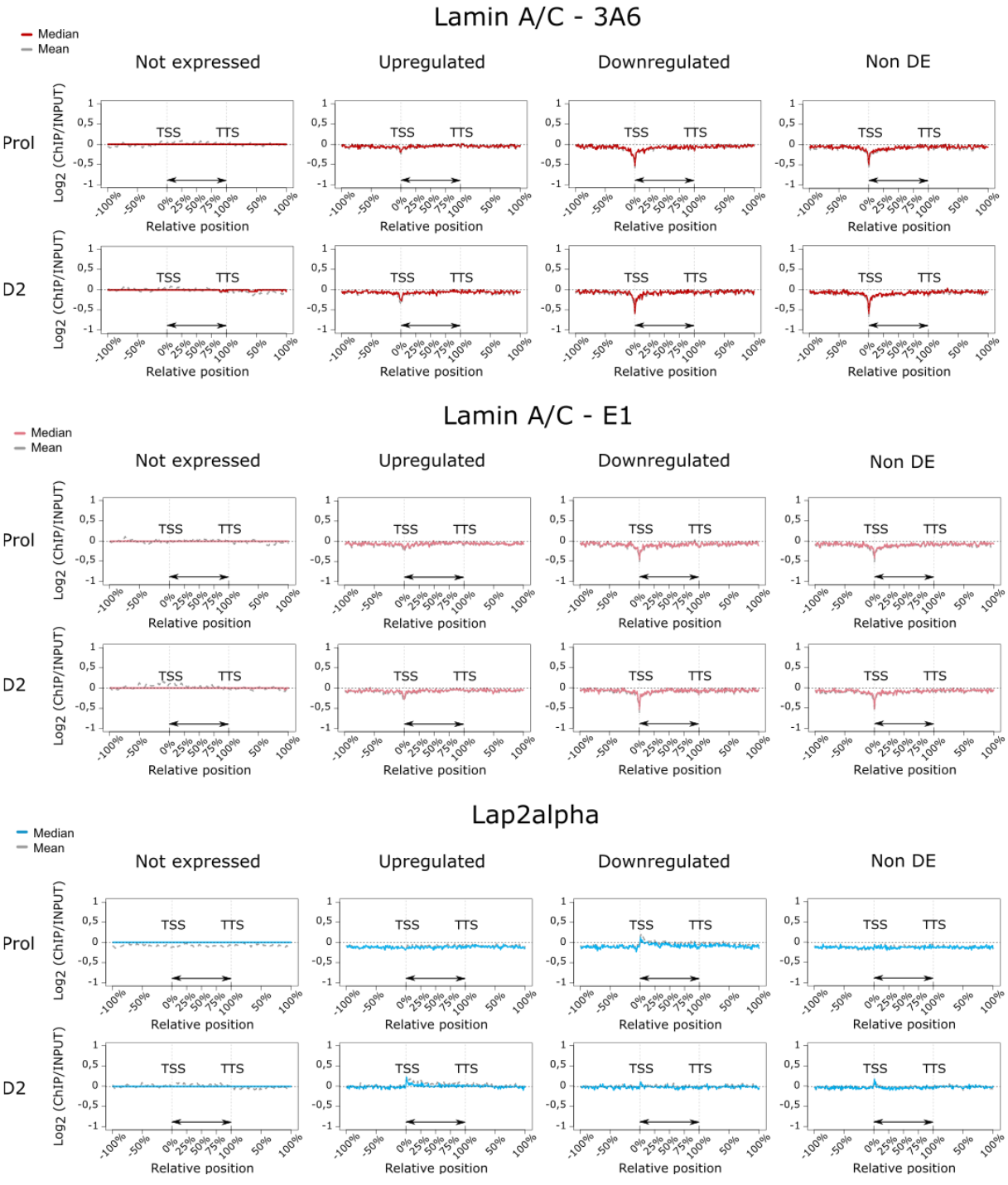


Figure 14. *(A) Scaled profiles of Lamin A/C and LAP2alpha binding to genes and their surrounding genomic sequences, obtained by ChIP-seq analysis. 500 genes were chosen for each of the analyzed categories: genes not expressed in all the analyzed stages of myoblast differentiation (not expressed) and genes upregulated, downregulated and whose expression was unchanged (non DE) during myoblast differentiation. The analysis was performed for both differentiation stages, in LAP2alpha WT myoblasts. Transcription start site (TSS) and transcription termination site (TTS) are highlighted. Y-axis shows log2ratios of ChIP/INPUT signals.*

A

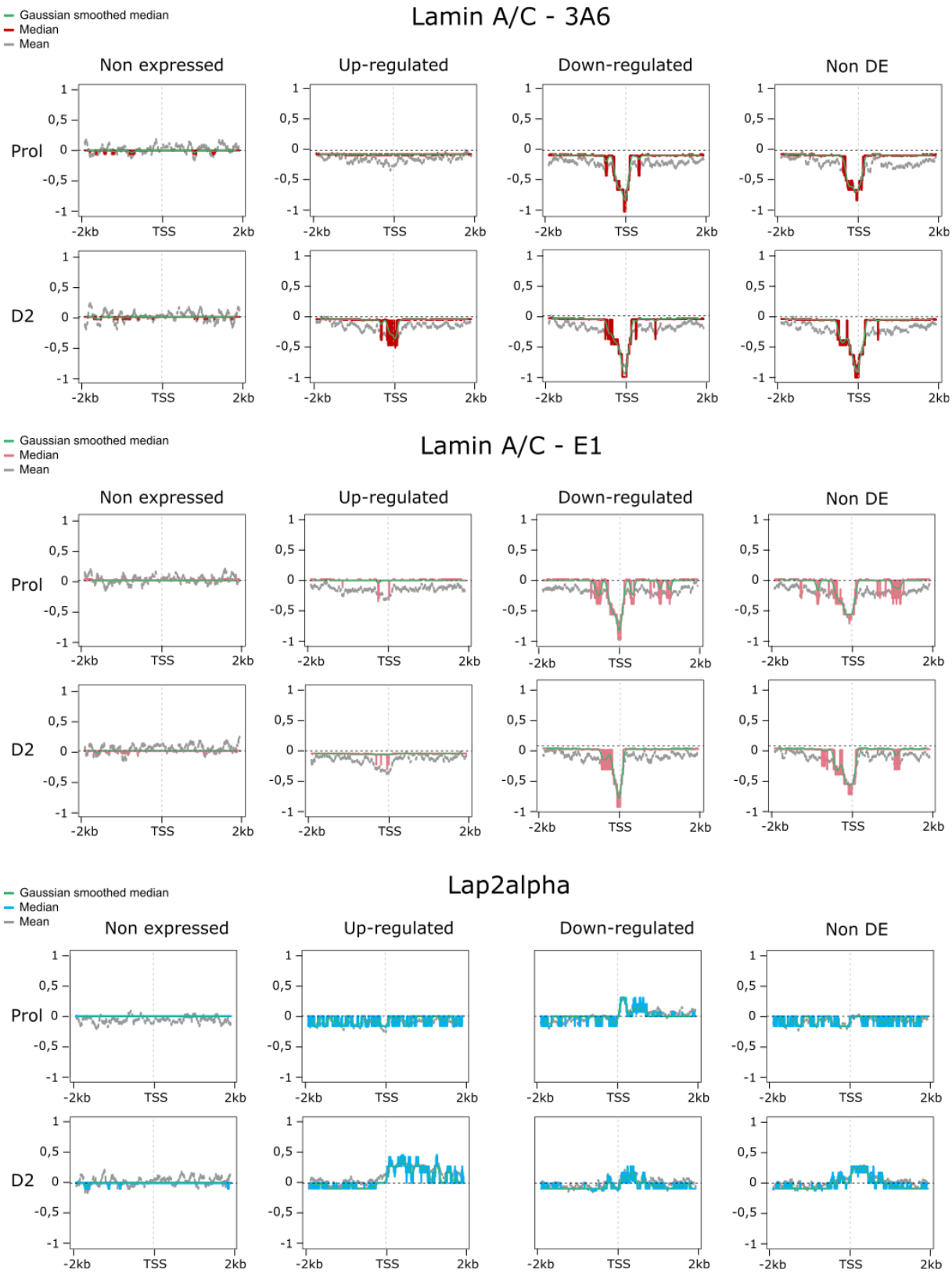


Figure 15. *(A) Unscaled profiles of Lamin A/C and LAP2alpha binding to the TSS of genes and their surrounding genomic sequences, obtained by ChIP-seq analysis with the Lamin A/C-3A6 and E1 antibodies, and the LAP2alpha-specific antibody, in LAP2alpha WT myoblasts. 500 genes were chosen for each of the analyzed categories: genes*

not expressed in all the analyzed stages of myoblast differentiation (not expressed) and genes upregulated, downregulated and whose expression was unchanged (non DE) during myoblast differentiation. The analysis was performed for both differentiation stages. Transcription start site (TSS) and transcription termination site (TTS) are highlighted. Y-axis shows log2ratios of ChIP/INPUT signals.

2.14 LOSS OF LAP2alpha AFFECTS LAMIN A/C BINDING PROFILE AT THE TSS OF EXPRESSED GENES

As shown above, loss of LAP2alpha in myoblasts is accompanied by a deregulation of chromatin-binding of A-type lamins on a genome-wide level. In particular, in proliferating cells, the 3A6-detected pool of Lamin A/C redistributes to genomic areas containing expressed genes, many of which involved in myoblast differentiation. We therefore analyzed Lamin A/C binding profiles on genes and their up- and down-stream regions in LAP2alpha KO myoblasts, to assess how loss of LAP2alpha influences Lamin A/C binding patterns on these genes. Non-expressed genes show no specific Lamin A/C binding pattern in all of the analyzed stages/genotypes, and in concordance with LAP2alpha WT myoblasts. Their mean/median binding profile is represented by a flat line around 0 (Figure 16, left column). Interestingly, in proliferating LAP2alpha KO myoblasts, the 3A6-derived pool of lamins shows a consistent binding around the TSS of expressed genes, independently of their specific expression changes during differentiation (Figure 16A, upper row). This is in stark contrast to what we observe in WT myoblasts (Figures 14 and 15), and it is in line with our hypothesis that changes in Lamin A/C binding may influence the ability of transcription factors and other proteins to properly access gene regulatory regions such as the TSS. Although the E1-derived pool of Lamin A/C does not show a clear binding to the TSS in the Prol stage of LAP2alpha KO cells, the depletion observed at the TSS is much less pronounced compared to WT (Figure 16B, upper row). In the D2 stage of differentiation, both the 3A6- and E1-derived pools of Lamin A/C are depleted around the TSS of down-regulated genes and genes non-DE, in concordance with the finding in LAP2alpha WT myoblasts (Figure 16A and 16B, lower rows), while the depletion at the TSS of upregulated genes is subtle (Figure 16A and 16B, second column). These patterns are more clearly visible when analyzing the unscaled lamin A/C binding profile around the TSS (Figure 17). These data, in addition to the analyses shown above, suggest that loss of LAP2alpha influences Lamin A/C binding to chromatin mainly in the Prol stage. These changes in Lamin A/C binding may affect the differentiation process by making gene regulation less efficient. This is, of course, not a black and white situation, since gene expression relies on multiple levels of regulation like chromatin accessibility, histone modifications, regulation of transcription factors and their modulators, and more (Venters & Pugh, 2009).

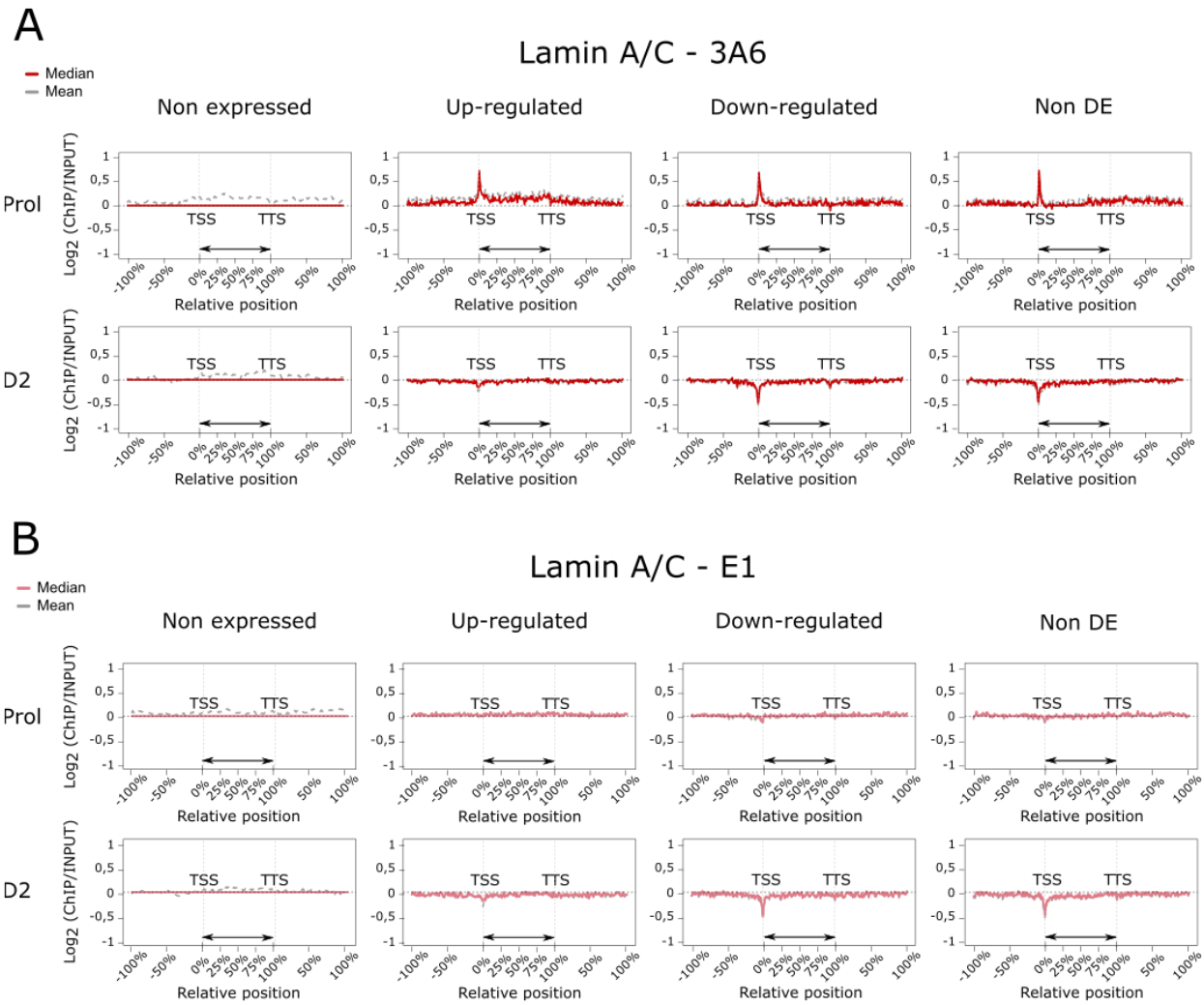


Figure 16. *(A) Scaled profiles of Lamin A/C binding to genes and their surrounding genomic sequences, obtained by ChIP-seq analysis with the Lamin A/C-3A6 and E1 antibodies, in LAP2alpha KO myoblasts. 500 genes were chosen for each of the analyzed categories: genes not expressed in all the analyzed stages of myoblast differentiation (not expressed) and genes upregulated, downregulated and whose expression was unchanged (non DE) during myoblast differentiation. The analysis was performed for both differentiation stages. Transcription start site (TSS) and transcription termination site (TTS) are highlighted. Y-axis shows log2ratios of ChIP/INPUT signals.*

A

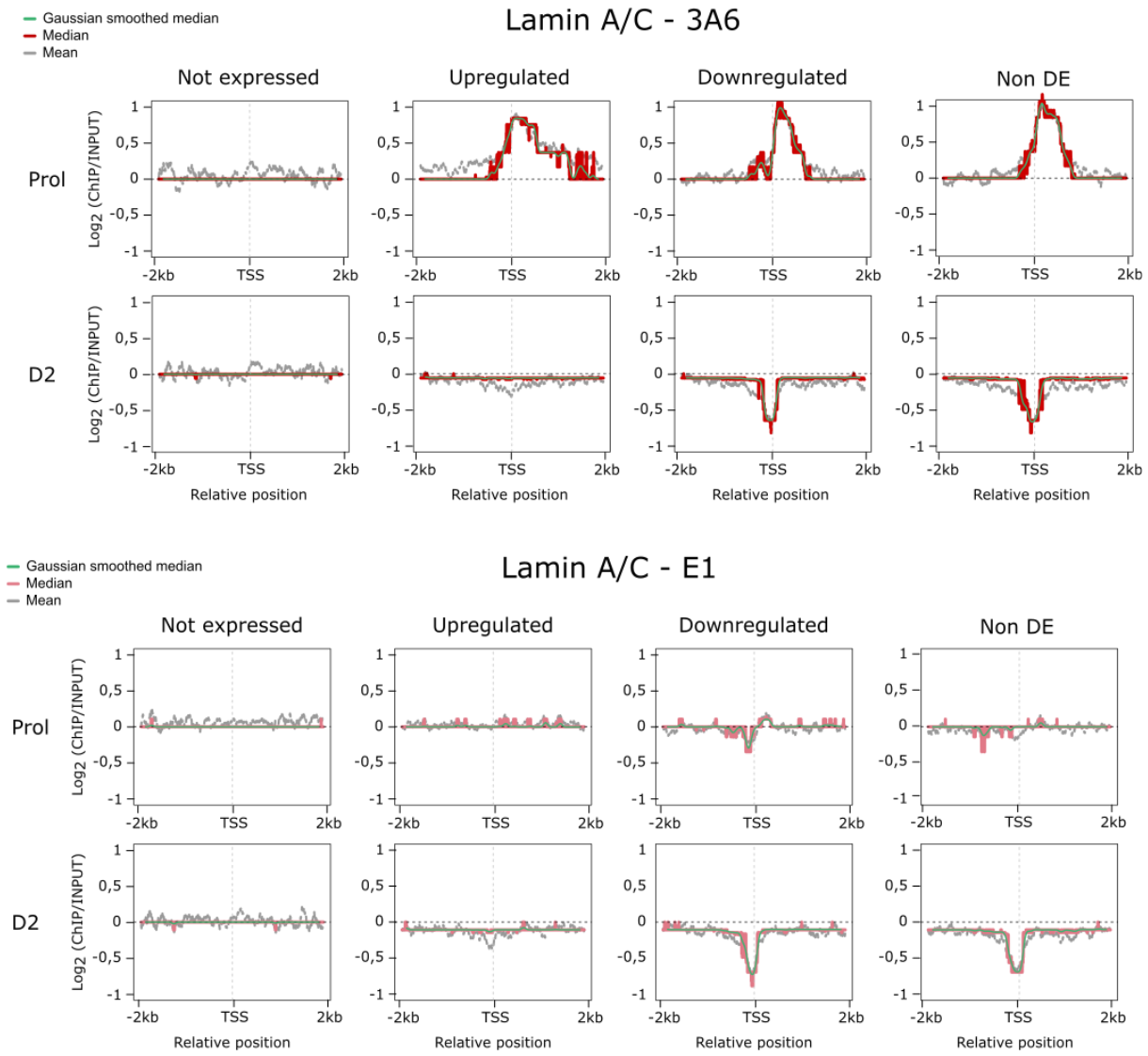


Figure 17. *(A) Unscaled profiles of Lamin A/C binding to the TSS of genes and their surrounding genomic sequences, obtained by ChIP-seq analysis with the Lamin A/C-3A6 and E1 antibodies, in LAP2alpha KO myoblasts. 500 genes were chosen for each of the analyzed categories: genes not expressed in all the analyzed stages of myoblast differentiation (not expressed) and genes upregulated, downregulated and whose expression was unchanged (non DE) during myoblast differentiation. The analysis was performed for both differentiation stages. Transcription start site (TSS) and transcription termination site (TTS) are highlighted. Y-axis shows log2ratios of ChIP/INPUT signals.*

2.15 LOSS OF LAP2alpha AFFECTS CHROMATIN ACCESSIBILITY IN MYOBLASTS

As potential mechanistic insight into the effects of Lamin A/C redistribution on chromatin, I hypothesized that increased binding of A-type lamins in regions containing genes involved in myoblasts functions may impair correct gene regulation. The deregulation of gene expression may be linked to steric hindrance or changes in histone marks that alter chromatin accessibility in those regions. To test this hypothesis, I performed a genome wide accessibility assay on LAP2alpha WT and KO myoblasts in Prol and D2 stages of differentiation, using the Assay for Transposase-Accessible Chromatin (ATAC)-seq. In this analysis, a transposase mediates the integration of adaptors in the chromatin on a genome-wide level, depending on chromatin accessibility. This means that the more a chromatin region is accessible for the transposase, the more the adaptors will integrate in that genomic region, resulting in more sequencing reads. Thus, a higher ATAC-seq signal correlates with higher accessibility.

The analyses were conducted in collaboration with Fatih Sarigöl (Max Perutz Labs). At first, we tested genome accessibility in LAP2alpha WT myoblasts during differentiation. When looking at the reads in the IGV browser, one sees a very high overlap in the accessible regions between Prol and D2 myoblasts (Figure 18A). To highlight possible differences between the differentiation stages, we performed a differential accessibility analysis with csaw, a Bioconductor package that uses a sliding-windows approach. Using a 300 bp sliding window analysis, we can see differential accessibility in the D2 versus Prol stage of myoblast differentiation. In particular, there is an increased accessibility in more than 18000 chromatin regions (windows) and decreased accessibility in around 22000 regions (Figure 18B, volcano plot on the left). The position and function of these regions is still undefined and will require further analyses.

When comparing genome-wide accessibility in LAP2alpha KO versus WT myoblasts, we again see a strong overlap of ATAC-seq reads between the samples in the IGV browser (Figure 18C). Thus, the main accessibility features of the genome are preserved in the absence of LAP2alpha. This is expected since the phenotype of LAP2alpha KO myoblasts and gene expression alterations are visible but subtle. Using the sliding window approach to test differential accessibility we see that, both in Prol and D2 stages, LAP2alpha-depleted myoblasts show regions of increased and decreased accessibility, compared to their WT counterparts. In Prol samples, we detect 385 regions whose accessibility is reduced in the absence of LAP2alpha and 451 regions with increased accessibility (Figure 18B, plot in the center). At differentiation stage D2, LAP2alpha KO myoblasts show mainly a reduction in accessibility compared to their WT counterpart (Figure 18B, plot on the right). This result is consistent with the hypothesis that loss of LAP2alpha induces changes in Lamin A/C binding to the chromatin that, in turn, lead to changes in chromatin accessibility. Nevertheless, the classification and functional characterization of the differentially accessible regions remains unknown, at this time.

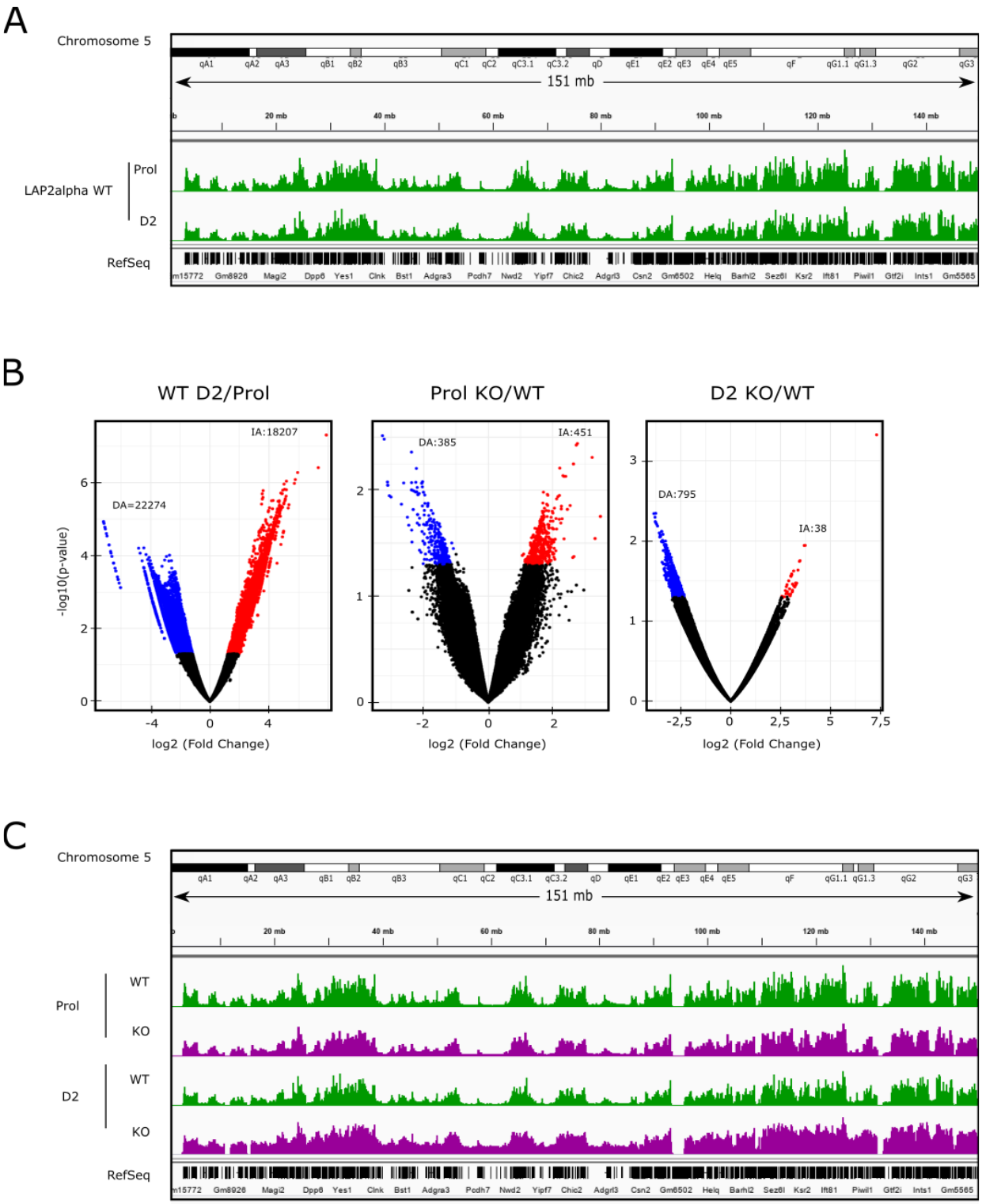


Figure 18. *(A) IGV browser visualization of mapped ATAC-seq reads on chromosome 5, in Prol and D2 LAP2alpha WT myoblasts. (B) Volcano plot summarizing the accessibility changes during differentiation, in LAP2alpha WT myoblasts (WT D2/Prol - left plot). Volcano plot representations of the differentially accessible regions in LAP2alpha KO myoblasts compared to LAP2alpha WT cells in the proliferating stage (Prol, middle plot) and after 2 days of differentiation (D2, right plot). IA= regions with increased accessibility; DA = regions with decreased accessibility. (C) Genome browser visualization of ATAC-seq reads aligned to the chromosome 5, for LAP2alpha WT and KO myoblasts, in both differentiation stages.*

CHAPTER 3: CONCLUSIONS AND DISCUSSION

In conclusion, in this thesis I discuss Lamin A/C and LAP2alpha binding to chromatin during myoblast differentiation, and its effects on gene expression.

I show that, in agreement with recent findings, Lamin A/C and LAP2alpha bind to both heterochromatin and euchromatin, in myoblasts. I see that the binding of these proteins to the chromatin is dynamic and changes during myoblasts differentiation, on a genome-wide level. I further describe LAP2alpha, but not Lamin A/C, enrichment on genomic regions containing a significant number of expressed genes, in both differentiation stages analyzed, pointing towards a role of LAP2alpha in the regulation of expressed genes. On the contrary, I report Lamin A/C binding to chromatin regions containing mainly not expressed genes, even in the EDD peaks detected outside cLADs. Accordingly, I find that Lamin A/C is consistently depleted at the TSS of expressed genes and, therefore, its binding to these regions negatively correlates with gene expression.

Given these results, I propose that A-type lamins and LAP2apha affect gene expression through local enrichment rather than through direct binding to the genes. In fact, the data show that both proteins are enriched in regions containing genes but are rarely bound to the gene bodies. This suggests that both proteins influence gene regulation through the formation of specific chromatin environments.

In myoblasts, loss of LAP2alpha leads to impaired differentiation. I report that, in LAP2alpha KO myoblasts, Lamin A/C distribution on chromatin changes on a genome-wide level. In the absence of LAP2alpha, A-type lamins bind to genomic regions containing a significant higher number of expressed genes, compared to LAP2alpha WT myoblasts, in the proliferating stage. These expressed genes include many genes relevant for myoblast differentiation and genes deregulated in LAP2alpha KO cells. I therefore hypothesize that the altered Lamin A/C binding to the chromatin affects its regulation and the expression of the genes within the bound regions. The changes in A-type lamin chromatin binding could lead, for example, to differences in chromatin accessibility. Here I report preliminary data showing that, in concordance with this hypothesis, loss of LAP2alpha is associated with changes in chromatin accessibility on a genome-wide level. A detailed characterization of the regions showing differential accessibility upon loss of LAP2lapha, though, is necessary and still missing.

In the next paragraphs I discuss the findings described in this thesis in the light of the current literature and other studies on Lamin A/C and LAP2alpha.

3.1 GENOME-WIDE INTERACTIONS OF Lamin A/C AND LAP2alpha WITH CHROMATIN

Lamins are the main components of the nuclear lamina, a proteinaceous structure located under the inner nuclear membrane and involved in vital cellular functions, including maintaining the mechanical properties of the nucleus (Dahl et al., 2008), regulating cell signaling, gene expression, and chromatin structure (Gruenbaum & Foisner, 2015). A- and B-type lamins display different expression patterns and subnuclear localization (Adam & Goldman, 2012). While B-type lamins are permanently farnesylated and therefore localize only at the nuclear periphery (Gerace & Blobel, 1980), A-type lamins have also been shown to form a soluble pool and therefore diffuse throughout the nucleoplasm (Hozak et al., 1995). The existence of a soluble pool of A-type lamins suggests the

possibility of their involvement in unique and specific lamina-independent functions. This is particularly interesting in relation to their binding to chromatin: since heterochromatin is enriched at the nuclear periphery, lamins in the NL can target those regions better, while a soluble pool of A-type lamins could also bind to different genomic loci. Nevertheless, distinguishing between the specific roles of peripheral and nucleoplasmic A-type lamins is very difficult, and separated functions of these two pools are not clearly identified, yet.

On a genome-wide level, lamins interaction with chromatin was initially characterized by Lamin B1 DamID experiments, and showed lamin binding to long, repressed, and gene-poor regions, called cLADs (Guelen et al., 2008). Since then, many studies reported different results, when analyzing A-type lamins. Lamin A/C, in fact, has been shown to bind also to euchromatic regions containing active genes (Gesson et al., 2016; Lund et al., 2013; Rønningen et al., 2015). According to these findings we report that, in myoblasts, Lamin A/C binds to both heterochromatic and euchromatic regions, in proliferating cells, and early during differentiation. The difference between the findings obtained with A- and B- type lamins could partially depend on the subnuclear localization of these proteins. Additionally, differences in A- and B-type lamins binding to chromatin could depend on the experimental methods used to identify the bound regions. Recently, in fact, a genome-wide ChIP-seq analysis of B-type lamins in epithelial cells reported Lamin B1 binding also to euchromatic regions (Pascual-Reguant et al., 2018). Differences in the results obtained with DamID and ChIP-seq have been ascribed to the ability of DamID to identify preferentially more stable interactions between lamins and chromatin, compared to ChIP-seq analyses (Naetar et al., 2017) and to a possible bias in the DamID signal normalization process towards stable chromatin interactions (Pascual-Reguant et al., 2018). Additionally, even within ChIP-seq analyses, the detection of lamin binding to euchromatin depends strongly on the sonication settings. In fact, the ability to pull down an adequate amount of euchromatin is fundamental to detect lamin binding to these genomic regions (Gesson et al., 2016; Pascual-Reguant et al., 2018).

Another difference between A- and B-type lamins, that accounts in part for their different functions, is their interaction with specific binding partners. Interestingly, A-type lamins specifically bind to one of the isoforms of the LAP2 family, LAP2alpha (T. Dechat et al., 2000). LAP2alpha is one of six isoforms encoded by the *Tmpo* gene and the only one that does not localize at the nuclear membrane (Berger et al., 1996). In fact, thanks to it specific C-terminal domain, LAP2alpha is soluble and diffuses throughout the nucleus (Thomas Dechat et al., 1998). LAP2alpha has also been shown to bind to the DNA, both directly and indirectly (Cai et al., 2001; Vlcek et al., 1999). This makes it the perfect partner for Lamin A/C, specifically in the nucleoplasm. LAP2alpha has, in fact, been shown to be involved in the regulation of A-type lamin distribution in the nucleus (Naetar et al., 2008) and of its binding to chromatin (Gesson et al., 2016). LAP2alpha is also able to bind to the DNA and its genome-wide distribution on chromatin has been only recently analyzed (Gesson et al., 2016; S. Zhang et al., 2013). In fibroblasts, LAP2alpha bound regions strongly overlap with those bound by Lamin A/C (Gesson et al., 2016). In concordance with these results, I report an extensive overlap between Lamin A/C and LAP2alpha binding to chromatin, in both the analyzed stages of myoblast differentiation. This is not surprising, as these proteins have been shown to interact with each other forming still structurally undefined complexes (T. Dechat et al., 2000; Furukawa et al., 1998). When analyzing their binding with the EDD peak caller (Lund et al., 2014), we see a significant overlap between Lamin A/C and LAP2alpha peaks, but we also identify some regions uniquely bound by each of these proteins. This suggests that Lamin A/C and LAP2alpha can also bind to chromatin independently or in association with other binding partners. Moreover, as previously discussed, Lamin A/C and LAP2alpha binding to chromatin is not sequence-specific (Meuleman et al., 2013; Stierlé et al., 2003) and therefore their exact binding sites may vary from cell to cell. Since in our

analyses we pool together millions of cells, the output obtained is the average binding signal of all of them. Because of this averaging, even in the regions where Lamin A/C and LAP2alpha EDD peaks overlap it is not possible to distinguish whether the two proteins are bound in a complex and whether they simply bind to the same region on the chromatin but in different cells. Single-cell analyses and ChIP-re-ChIP assays could give a better insight into Lamin A/C and LAP2alpha independent interactions or complex formation on chromatin.

Lamin A/C binding to euchromatin has been reported to change during cellular processes and to involve regions containing active genes (Lund et al., 2013; Rønningen et al., 2015). In adipocyte differentiation, A-type lamins have been shown to move away from genes that require to be activated. In these cells, Lamin A/C binds to the promoter and to the TSS of expressed genes of adipocyte precursors, but not their differentiated counterparts (Lund et al., 2013). During myoblast differentiation, we also see changes in both A-type lamins and LAP2alpha binding to chromatin. In these cells, Lamin A/C EDD peaks include thousands of genes. Nevertheless, only a small portion of them (around 7-8%) is expressed despite being mainly located in EDD peak regions outside cLADs. Contrary to the findings in adipocytes, we detect a consistent Lamin A/C depletion at the TSS of expressed genes, in all the analyzed differentiation stages. Depletion of Lamin A/C at the TSS of genes strongly correlates with their expression, while this pattern is absent in non-expressed genes. The reason for the discrepancy in the findings in adipocytes and myoblasts may depend on cell-specific mechanisms, but also on the different techniques used to produce the data. As seen before, in fact, the detection of Lamin A/C binding to specific chromatin regions seem to depend strongly on the method used to address these questions.

3.2 Lamin A/C AND LAP2alpha INFLUENCE GENE EXPRESSION BY LOCAL ENRICHMENT ON CHROMATIN RATHER THAN DIRECT BINDING TO SPECIFIC GENES

As discussed above, the data presented in this thesis show that Lamin A/C and LAP2alpha bind to chromatin regions containing many genes. Nevertheless, only a very small portion of these genes is directly bound by these proteins and among those, the majority is not expressed. Upon loss of LAP2alpha, Lamin A/C binding to chromatin changes. A-type lamins enrich in chromatin regions containing expressed genes and, among these, we find more than 40% of the genes deregulated in LAP2alpha KO myoblasts. This suggests that the deregulated Lamin A/C binding to chromatin could affect gene expression. This mechanism seems to rely on local Lamin A/C enrichment on the chromatin and not on direct binding to gene-bodies.

I, therefore, hypothesize that Lamin A/C and LAP2alpha affect gene regulation by creating specific chromatin environments rather than through direct binding to genes. There are several possible mechanisms through which the local binding of these proteins can affect gene expression. Preliminary data reported in this thesis show that loss of LAP2alpha leads to changes in chromatin accessibility in myoblasts. I hypothesize that local enrichment of Lamin A/C and LAP2alpha on chromatin is directly or indirectly involved in the regulation of its accessibility.

Lamin A/C have been shown to regulate chromatin dynamics by keeping chromatin motion slow and anomalous (Bronshtein et al., 2015). Their association with the chromatin could, therefore, directly affect the mobility of genomic loci in the nucleus and their regulation. Moreover, LAP2alpha has been involved in the maintenance of Lamin A/C dynamics and assembly state in the nucleoplasm,

and, in its absence, the soluble pool of A-type lamins forms more stable structures (Naetar et al., 2021). The changes in Lamin A/C assembly state in the nucleus of LAP2alpha KO myoblasts could translate into a more stable binding to chromatin, making the bound regions sterically more difficult to access for transcription factors and other proteins, and possibly explain the gene deregulation observed in these cells.

Alternatively, Lamin A/C and LA2alpha could regulate chromatin organization through the interaction with chromatin remodelers. It has been previously reported that Lamin A/C interacts with the Polycomb group (PcG) of proteins, which are important during differentiation for repressing genes that are not required in a specific cell state (Bracken et al., 2006). Lamin A/C was found to be involved in the assembly and correct localization of PcGs foci (Cesarini et al., 2015). One could speculate that Lamin A/C enrichment on long genomic regions containing unneeded genes could help recruit PcG proteins to these regions to inhibit their expression. Although I report that most of the genes contained inside Lamin A/C peaks in myoblasts are, indeed, not expressed, there is seemingly not a linear correlation between Lamin A/C binding and gene repression. In fact, upon loss of LAP2alpha, Lamin A/C binding to chromatin changes, and that the newly-bound genomic regions contain a high number of expressed genes, the majority of which is not affected in their expression after Lamin A/C binding. Nevertheless, in LAP2alpha KO myoblasts, Lamin A/C is found enriched in more than 40% of the genes deregulated upon LAP2alpha loss. This suggests involvement of Lamin A/C in this deregulation through a mechanism that is not “all or nothing”, but it is most likely one of the many layers responsible for the regulation of gene expression. The outcome of increased Lamin A/C binding could therefore depend on other lamin-independent mechanisms and on how much a specific gene depends on each of them for its functions.

LAP2alpha has also been shown to interact with proteins involved in chromatin remodeling, like the high mobility group N 5 protein (HMGN5). HMNG5 reduces chromatin compaction by destabilizing the histone H1 (Furusawa et al., 2015) and LAP2alpha influences its genome-wide distribution (S. Zhang et al., 2013). It is possible, therefore, that regions enriched in LAP2alpha recruit HMNG5 to these sites, influencing chromatin compaction and gene expression. In fact, LAP2alpha EDD peaks include many expressed genes and the binding of LAP2alpha to these regions could indirectly contribute to chromatin accessibility, therefore allowing their correct regulation by other factors. Upon loss of LAP2alpha, altered HMNG5 binding to chromatin could lead to the deregulation of some genes. A genome-wide ChIP-seq analysis of HMNG5 in LAP2alpha WT and KO myoblasts could elucidate the effect of LAP2alpha loss on HMNG5, in these cells.

Interestingly, unpublished data from our lab also suggest LAP2alpha interaction with other chromatin remodelers, in fibroblasts. Proximity-dependent biotin identification (BioID) screening revealed the interaction of LAP2alpha with CDH4 and Brg1, members of the Nucleosome Remodeling and Deacetylation (NuRD) complex and the SWItch/Sucrose Non-Fermentable (SWI/SNF) complex, respectively (Gesson, 2016). In addition to their specific chromatin remodeling functions, it has been shown that the interplay between these complexes and PcG proteins is necessary for the correct gene expression regulation during differentiation (Bracken et al., 2006). The NURD complex has been shown to facilitate the recruitment of the Polycomb Repressive Complex 2 (PRC2) during lineage commitment of embryonic stem cells (ESCs) and to facilitate gene repression (Reynolds et al., 2012). The SWI/SNF complex, instead, is known to both antagonize and facilitate the PcG complexes functions during differentiation (Ho et al., 2011). It is therefore even more tempting to speculate that both Lamin A/C and LAP2alpha can indirectly influence chromatin organization by creating local environments facilitating the interactions of these chromatin-regulating proteins. Binding of LAP2alpha and Lamin A/C on the same chromatin region could facilitate the recruitment of a

combination of these complexes to the DNA, whereas LAP2alpha (or Lamin A/C) specific enrichment to other regions may recruit only subgroups of these complexes, leading to a different transcription outcome. Such balance between active and repressive mechanisms could also account for the variable effect of Lamin A/C and LAP2alpha binding to chromatin on gene expression.

Another mechanism that could account for changes in gene expression in Lamin A/C enriched genomic regions involves changes in histone modifications. Mutations in Lamin A/C, in fact, have been shown to affect the epigenomic control of fibroblasts, in HGPS patients (Shumaker et al., 2006) and in other cell types (Perovanovic et al., 2016). Moreover, in LAP2alpha KO fibroblasts, newly bound Lamin A/C chromatin regions have been shown to have increased levels of both active and repressive histone marks (Gesson et al., 2016). Therefore, changes in Lamin A/C and, indirectly, LAP2alpha binding to chromatin may lead to changes in histone modifications. This can, in turn, depend on A-type lamins and LAP2alpha interaction with some of the chromatin remodelers described above, but can also be linked to their interaction with other, still unknown, binding partners.

Finally, the role of A-type lamins in gene expression could also be linked to their phosphorylation state. Recently, in fact, the binding of phosphorylated Lamin A/C to active enhancers was reported and associated with gene expression changes in progeria (Ikegami et al., 2020). Lamin phosphorylation is important during mitosis to disassemble the nuclear lamina (Peter et al., 1990). Nevertheless, A-type lamin phosphorylation has also been observed during interphase (Eggert et al., 1993) and the role of this subpopulation of lamins is still unknown. It is therefore plausible that different lamin subpopulations distribute differently on the chromatin and this results in different outcomes. It is not known, in fact, if phosphorylated interphase lamins bind to different partners compared to their unphosphorylated counterparts. This, too, could account for different gene expression outcomes. The behavior of phosphorylated lamins during myoblasts differentiation and the effect of LAP2alpha loss on their chromatin binding is currently unknown.

3.3 IMPLICATIONS OF CHROMATIN REGULATION BY Lamin A/C AND LAP2alpha IN THE SKELETAL MUSCLE

As discussed in the introduction of this thesis, both Lamin A/C and LAP2alpha are important factors in skeletal muscle differentiation. Mutations in Lamin A/C are responsible for class of diseases collectively called laminopathies, many of which include skeletal muscle defects (Nicolas et al., 2019). Additionally, in myoblasts, loss of LAP2alpha leads to impaired differentiation and gene expression deregulation (Gotic, Schmidt, et al., 2010). The mechanisms responsible for the defects observed in laminopathies have been extensively studied, but a comprehensive understanding is still missing. On the contrary, very little is known about LAP2alpha functions in myoblasts.

Here we propose that Lamin A/C and LAP2alpha are important factors involved in chromatin regulation, in myoblasts. We suggest that deregulation of their binding to chromatin is responsible, at least in part, for the phenotypes associated with Lamin A/C- and LAP2alpha-related defects. We propose that Lamin A/C and LAP2alpha bind to chromatin regions and create specific chromatin environments important for gene expression regulation. Local enrichment of these proteins could directly or indirectly affect chromatin accessibility, contributing to the correct regulation of these genomic regions during differentiation. Mutations in A-type lamins or LAP2alpha can affect this regulation by changing the distribution of these proteins on the chromatin. Alternatively, these

mutations can affect Lamin A/C and LAP2alpha ability to interact with other binding partners, which, in turn, can regulate chromatin accessibility.

In the future it will be important to properly distinguish the roles of the different pool of lamins, not only to identify specific functions carried out by the nuclear lamina and the nucleoplasmic pool of A-type lamins, but also to address the individual contribution of phosphorylated and unphosphorylated lamins to these processes. Moreover, it is fundamental to understand the mechanism through which A-type lamins and LAP2alpha influence chromatin regulation and gene expression. In this regard, the characterization of epigenetic features of Lamin A/C and LAP2alpha bound chromatin, in myoblasts, would help to gain a better insight in their effect on the genome. Additionally, the analysis of Lamin A/C and LAP2alpha interactions with chromatin remodelers in these cells could further shed light on the specific mechanism through which they influence gene expression.

MATERIALS AND METHODS

Immortalized Myoblasts (IM; p53KO)

Immortalized myoblasts derived from mice KO for the p53 tumor suppressor protein (kind gift of Gerahrd Wiche, University Vienna) were grown at 37°C, in 5% CO_2 atmosphere. Proliferating myoblasts were kept in Ham´s F10 nutrient mix (F-10, Gibco™) supplemented with 20% fetal bovine serum (FBS) and 100U/ml of Penicillin/Streptomycin (Pen/Strep). Proliferating myoblasts were kept at a cell confluence <= 70%.

Differentiation Experiments

Collagen coating

For myoblast differentiation experiments, all myoblasts, including proliferating cells, were cultivated on collagen coated plates. The coating was performed by diluting 500µl of collagen I (Corning® Collagen I, rat tail, 100 mg) in 50ml of sterile water with 57,5µl of glacial acetic acid. Plates were incubated with this solution for at least 30min at 37°C. The collagen solution was then removed, and the plate was rinsed in PBS before seeding the cells.

Differentiation

We define D0 myoblasts as proliferating myoblasts (cultivated on collagen coated plates) that have reached 80-90% confluence in the dish. To induce the differentiation process, proliferating myoblasts were cultivated until they reached 80-90% confluence in the plate (D0) and were afterwards kept in low serum conditions. The differentiation (low serum) medium consists of F-10 medium supplemented with 5% FBS and 100U/ml Pen/Strep. During the differentiation process, the differentiation medium was replaced every 24h.

Myoblasts Transfection for CRISPR-Cas9 genome editing

Myoblasts were grown on collagen-coated 10cm dishes and transfected at 40-50% confluence. The myoblasts were supplied with fresh growth medium 2hrs before transfection. The transfection was performed with polyethylenimine (PEI), using the plasmids pSpCas9(BB)-2A-GFP (PX458) from Addgene (here referred to as PX458-GFP) and its modified version containing the sgRNA specific for LAP2alpha, PX458-GFP-sgRNA3 (Naetar et al., 2021). 30µl of PEI were mixed to 300µl of Opti-MEM™ and 10-15ug of the desired plasmid. The mix was gently pipetted up-and-down a few times and incubated 15min at RT. The mix was added to the cells and uniformly distributed in the 10cm dish. Myoblasts were incubated overnight (O/N) with the transfection reagents and the medium was replaced after 8-10h with fresh growth medium.

Fluorescence-activated cell sorting (FACS)

Positive transfection was checked with a fluorescence microscope, as all plasmids used expressed GFP. A bulk sorting of the positively transfected cells was performed by the Bio-optics-FACS facility at the Max Perutz Labs, with the BD FACSAria-IIIu sorter. Myoblasts expressing GFP were seeded in a new plate, incubated for 36 to 48hrs and sorted again in the same way.

TIDE analysis

Analysis of the efficiency of CRISPR/Cas9 genome editing was performed by sequencing the edited genomic region and analyzing it with the online tool TIDE (https://tide.nki.nl/)

Immunofluorescence (IF)

Myoblasts were seeded at the desired confluence on collagen coated coverslips (see above "Collagen coating") in 12 well dishes. For the differentiation experiments, myoblasts were induced to differentiate on the coated coverslips. At the chosen time-point, cells were washed twice in PBS (w/ Ca^{2+} and Mg^{2+}), fixed with 4% paraformaldehyde (PFA) and washed twice in PBS (w/o Ca^{2+} and Mg^{2+}). Myoblasts were permeabilized in 50 mM NH_4Cl in 0,5% TritonX-100 in PBS. Cells were washed twice in PBS and then incubated in blocking solution (5% gelatin in PBS) for at least 30min at room temperature (RT). Primary antibodies were diluted in blocking solutions at the desired concentration and the cells were incubated in this antibody solution 1hr at RT. Myoblasts were washed twice in PBST (0,05% Tween 20 in PBS) and then incubated 1h at RT with secondary antibody dilution (fluorescent secondary Alexa Flour antibodies from Thermo Fisher scientific were diluted 1:400 in PBS). Myoblasts were washed twice in PBST and afterwards incubated 10min in a solution of 300nM 4',6-diamidino-2-phenylindole hydrochloride (DAPI) in PBS, at RT. Finally, after two additional washes in PBST, the coverslips were mounted on microscope slides with 77% glycerol with 20 mM Tris [pH 8.0] and 0.2mM of 1,4diazabizyclo [2.2.2] octane (DABCO).

Images were acquired at the Zeiss LSM 700 laser scanning confocal microscope, with a Plan-Apochromat 63x/1.4 Oil DIC objective.

SDS-PAGE electrophoresis and Western Blot (WB) analysis

Protein samples directly harvested in Laemmli buffer were run on 10 or 12% polyacrylamide gels at 25mA/gel. The proteins were then transferred to a 0,2μm pores-PVDF membrane at 80V for 2hrs. The membranes were blocked in a solution of 5% milk powder in PBS and incubated in this solution for at least 1h at RT. After a 5min wash in PBS, the membranes were incubated with the primary antibody solution (primary antibody diluted in PBS containing 2% bovine serum albumin (BSA) and 0,02% NaN_3) at 4°C, O/N. For antibodies used, see below. The membranes were then washed 3 times for 5min in PBST (0,05% Tween 20 in PBS) and incubated 2hrs at RT with the secondary antibody dilution. For signal detection, we used HRP-conjugated secondary antibodies, diluted in PBS. The membranes were briefly washed in PBS and the signal was detected by using Pierce™ ECL or ECL-plus western blotting substrates and visualized with a Bio-Rad ChemiDoc™.

Co-Immunoprecipitation (Co-IP)

Myoblasts were seeded in 15cm dishes and cultivated until reaching the desired confluency. Cells were washed twice in PBS (w/ Ca^{2+} and Mg^{2+}) and harvested in 3ml/dish of harvesting buffer (20mM Tris-HCl pH7,5, 100mM NaCl, 2mM EGTA, 2mM $MaCl_2$, 0,5%NP-40, 25U Benzonase, 1mM DTT and 1x EDTA-free proteinase inhibitors, in milli-Q H_2O). Cell lysates were incubated 10min on ice. At this stage, 50µl of the INPUT sample was mixed with 25µl of 3X Laemmli buffer and stored. The rest of the sample was further processed and centrifuged 10min at 4000RPM, at 4°C. The Supernatant was separated from the pellet, which was solubilized in 1x Laemmli buffer and is referred to as "Pellet". The supernatant was divided in 500µl aliquots and incubated O/N with the desired antibodies, at 4°C. Afterwards, pre-washed Pierce™ protein A/G magnetic beads (Thermo Scientific™) were added to each sample and incubated 4-5hrs at 4°C, rotating. Following this incubation, the supernatant was collected and stored, after adding the adequate volume of 3X Laemmli buffer. The beads, attached to the protein-antibody complexes, were washed three times in Harvesting buffer. After removing any leftover buffer, 50µl of 1x Laemmli buffer were added to each sample. The samples were vortexed,

incubated 10min at RT and then 5min at 95°C, shaking at 500 RPM. The samples were allowed to cool-down and the beads were removed with a magnetic rack. For western blot analyses, 5µl of each sample were loaded on polyacrylamide gels.

Primary antibodies

The antibodies used in this thesis were:

Mouse lamin A/C 3A6-4C11, Active Motif 39287, used as hybridoma supernatant, provided by the Max Perutz Labs Monoclonal Antibody Facility (1:500 dilution for WB, 1:10 dilution for co-IPs, 50µl/sample in ChIP assays); Mouse lamin A/C E1 , Santa Cruz Biotechnology (1:1000 dilution for WB, 1:100 dilution for IFs, 10µl/sample in ChIP assays, 30µl for co-IP); Goat lamin A/C N18, Santa Cruz Biotechnology (sc-6215, 60µl for Co-IP); Mouse LAP2alpha 1H11 provided by the Max Perutz Labs Monoclonal Antibody Facility (1:500 dilution for WB, 1:50 dilution for IF, 25µl/sample in ChIP assays, 24µl for co-IP); Mouse myogenin, F5D supernatant, Developmental Studies Hybridoma Bank (DSHB) (1:10 dilution for WB); Mouse myosin heavy chain (MyHC), MF-20 supernatant, DSHB (1:10 dilution for WB); mouse normal IgG (Millipore 12-371, 6µl for co-IP); rabbit normal IgG (Abcam ab46540, 6µl for Co-IP);

Messenger RNA sequencing (mRNA-seq)

Myoblasts were cultivated or differentiated at the desired stage and total RNA was extracted with the RNAeasy® mini kit from Quiagen, following the instructions of the manufacturer.

Total RNA was submitted to the Next Generation Sequencing facility at the Vienna Biocenter (https://www.viennabiocenter.org/vbcf/next-generation-sequencing/), which proceeded to the library preparation (polyA enrichment with the NEBNext® Poly(A) mRNA Magnetic Isolation Module followed by the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®) and sequencing on an Illumina platform (HiSeqV4) with a SR50 mode.

Reads were mapped to the genome using NextGenMap (Sedlazeck et al., 2013) and gene expression quantification was determined with RSEM (B. Li & Dewey, 2011).

Differential expression analysis

Differential gene expression analysis was performed using limma (Law et al., 2014). When compared to a control sample, we considered as differentially expressed those genes (DEGs) with a t least +/- 1,5fold change in expression levels, relative to the control sample, and p-values <= 0,05. Genes were considered as differentially expressed when these criteria were fulfilled in all three replicates.

Gene expression

We define expressed genes those with a minimum Fragments Per Kilobase Million (FPKM) value of 0,5 in all three datasets analyzed.

Chromatin Immunoprecipitation sequencing (ChIP-seq)

Myoblasts were cultivated at the desired confluency/differentiation on 15cm collagen coated dishes. Cells were washed with PBS (w/ Ca^{2+} and Mg^{2+}) and then incubated 10min at RT with 16ml of 1% MeOH-free Formaldehyde in PBS (w/o Ca^{2+} and Mg^{2+}) on a shaker. Formaldehyde was quenched by adding Glycine to a final concentration of 125mM and incubating 5min at RT, gently shaking. Myoblasts were washed twice in ice-cold PBS and harvested in 2ml of PBS containing protease inhibitors (cOmplete EDTA-free protease inhibitor cocktail tablets, Roche®) and PMSF. The collected cells were centrifuged 5min at 1200xg at 4°C and the pellet was resuspended in WASH buffer 1 (0,25% Triton™ X-100, 10mM EDTA, 0,5mM EGTA, 10mM HEPES, protease inhibitors and 0,1mM PMSF in milli-Q® water) and incubated 10min on ice. The samples were again centrifuges 5min at 1200xg at 4°C and the pellet was resuspended in WASH buffer 2 (200mM NaCl, 1mM EDTA, 0,5mM EGTA, 10mM HEPES, Protease inhibitors and 0,1mM PMSF in milli-Q® water). The samples were immediately centrifuged again with the same settings and resuspended in Lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl [pH 8,1], protease inhibitors and 0,1mM PMSF in milli-Q® water). The chromatin samples were incubated O/N at 4°C on a rotor. The day after, the chromatin concentration was checked at a Nanodrop™ spectrophotometer and samples were diluted to a final chromatin concentration of around 1µg/µl. The chromatin was then sonicated with the Diagenode´s BIORUPTOR® UCD-300 (power: HIGH, number of cycles: 6 for Prol myoblasts and 9 for D2 myoblasts). After sonication, chromatin was either snap-frozen and stored at -80°C or immediately processed.

For the IP, 50ug of chromatin were incubated with the desired antibody and diluted 1:10 with ChIP Dilution buffer (167,4mM NaCl, 16,72mM Tris-HCl [pH 8,1], 1,2mM EDTA, 1,1% Triton™ X-100, 0,001%SDS in milli-Q® water). The incubation was performed O/N at 4°C, rotating. At this stage, 12,5ug of chromatin were put aside as INPUT. The following day, 30ug of pre-washed Pierce™ protein A/G magnetic beads (Thermo Scientific™) were added to each sample and incubated 4-5hrs at 4°C, rotating. Afterwards, the supernatant was removed, and the beads washed with 1ml of the following buffers, in this order: RIPA buffer (150mM NaCl, 50mM Tris-HCl [pH 8,0], 0,1% SDS, 0,5% NaDOC and 1% NP-40, in milli-Q® water), High-Salt buffer (500mM NaCl, 50mM Tris-HCl [pH8,0], 0,1% SDS and 1% NP-40 in milli-Q® water), LiCl buffer(250mM LiCl, 50mM Tris-HCl [pH 8,0], 0,5% NaDOC and 1% NP-40 in milli-Q® water), and twice with TE buffer(10mM Tris-HCl [pH8,0] and 1mM EDTA in milli-Q® water). The supernatant was removed and 200µl of Elution buffer were added to each sample (and to the INPUT samples) and incubated 30 min at RT at 1200 RPM. From this step on the INPUT samples were processed together with the IP samples. The supernatant was then collected in a fresh Eppendorf and de-cross-linked by adding 10µl of 4M NaCl and incubating the solution O/N at 65°C at 300 RPM. The following morning 4µl of 0,5M EDTA, 8µl of 1M Tris-HCl pH 6,5 and 20ug Proteinase K (PK) were added to each sample and incubated 1hr at 55°C at 300 RPM. The DNA was then isolated using the DNA clean and concentrator kit by Zymo Research and eluted in 30µl of milli-Q® water.

The DNA was delivered to the Next Generation Sequencing facility at the Vienna Biocenter, which generated the library (NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®) and sequenced the samples on an Illumina platform (HiSeqV4), with SR100 mode.

ChIP-seq peak calling

The sequenced reads were mapped to the genome (Mus musculus GRCm38.86) using NextGenMap (Sedlazeck et al., 2013). The mapped reads were sorted and indexed using SAMtools (H. Li et al., 2009) and peaks were called with the Enriched domain detector (EDD) peak caller (Lund et al., 2014). Default parameters were applied.

Generation of gene profiles

The log2 ratios of the samples compared to the respective INPUTs were generated by using bamCompare (Ramírez et al., 2016). The average of the log2 profiles of 500 up-, down-, non-differentially expressed (non-DE) and not expressed genes were generated.

Gene binding

For each of the analyzed proteins, the binding to a gene was defined by dividing the gene in 10bp windows and calculating the log2ratio ChIP/INPUT in each window. Afterwards, the average binding value was obtained by summing the values of the individual windows and dividing them for the number of windows analyzed. For further analyses, we defined as bound the top 10% genes ranked according to their binding values.

Gene analysis

Classification of the genes found inside EDD peaks was performed using the gene ontology enrichment analysis on http://geneontology.org/ by searching for biological processes. Results were then plotted with the ggplot2 package, in Rstudio.

Random permutation tests

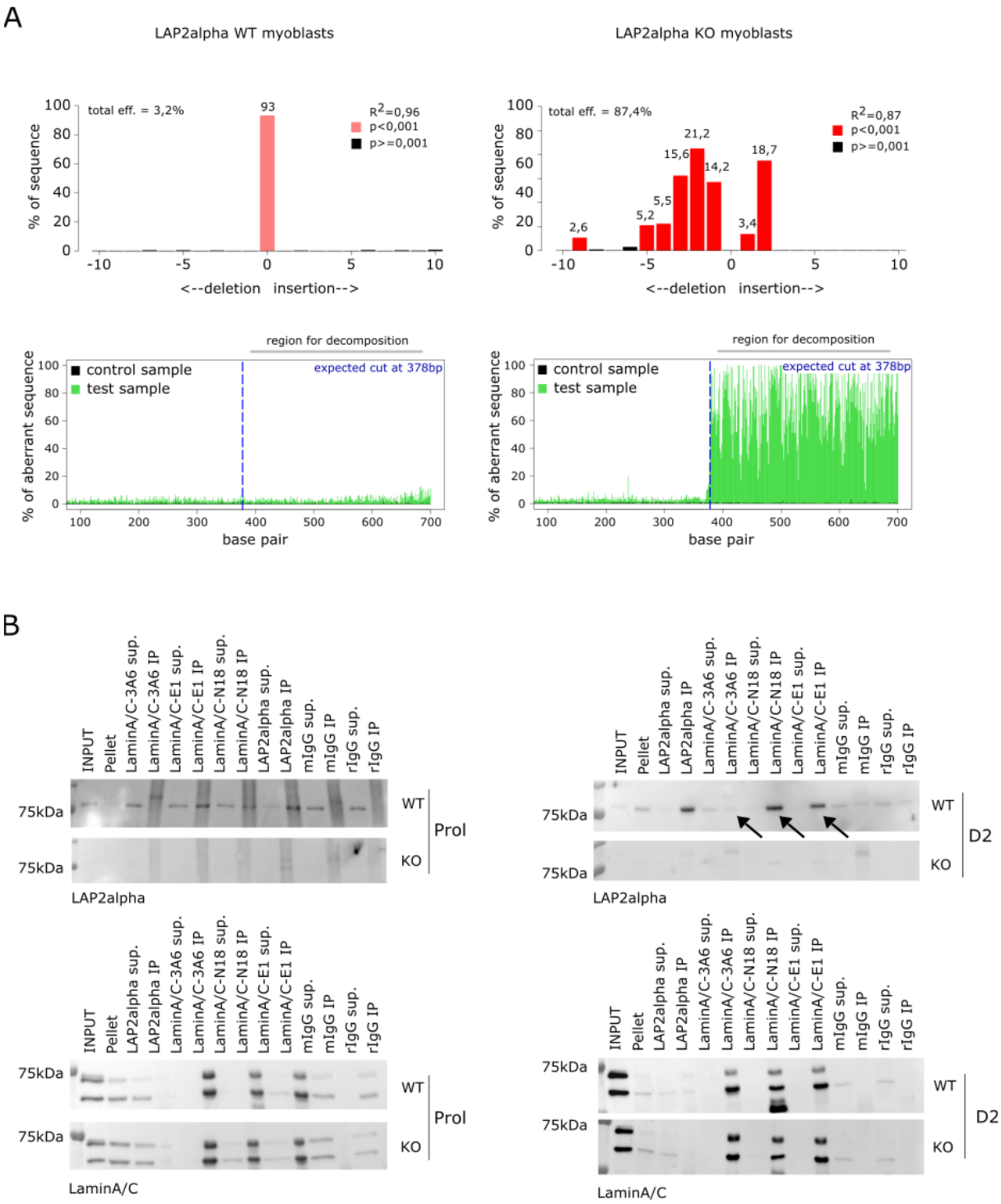
Random permutation tests were performed using the function overlapPermTest of the Bioconductor package regioneR, with the following parameters: ntimes= 1000, non.overlapping=TRUE, min.pctA=100, count.once=TRUE .

Assay for Transposase-Accessible Chromatin (ATAC)

Myogenic cells at the desired differentiation stage were washed in PBS, trypsinized and collected in 10%FCS (in PBS). Cells were counted at the CASY® cell counter. 2,5*10^6 cells/sample were centrifuged at 1200RPM for 5min, at RT. Afterwards, two washes in ice-cold PBS were performed and cells were spun-down each time at 4°C, at 1200RPM, for 5min. After the second wash, PBS was removed and cells were resuspended in 1ml of sucrose buffer 1A (0,16M sucrose, 3mM $CaCl_2$, 2mM Mg-acetate, 0,1mM EDTA, 10mM Tris-HCl [pH8,0], 0,5% NP-40 and 1mM DTT in milli-Q® water) and incubated 3min, on ice. Nuclei were spun-down at 700xg for 5 min, at 4°C and resuspended in 100µl of ice-cold nuclei resuspension buffer (50mM Tris-HCl [pH 8,0], 40% glycerol, 5mM $MgCl_2$, 1,1mM EDTA in milli-Q® water).

Nuclei were processed by the Next Generation Sequencing facility at the Vienna Biocenter, which proceeded to the tagmentation (using the Tn5 enzyme), library preparation (Nextera XT DNA library preparation kit), and sequencing on an Illumina platform, with PE50 (HiSeqV4) or PE75 (NextSeq550).

SUPPLEMENTARY FIGURES



Suppl. Figure 1. *(A) TIDE analysis of changes in the Tmpo locus after CRISPR/Cas9 genome editing. On the left the results show the gene editing in the control line (LAP2alpha WT myoblasts) transfected with the vector encoding the Cas9 protein but with no sgRNA. On the right the results show the editing in myoblasts transfected with a vector expressing the Cas9 protein and the sgRNA3, specific for the exon4 of the Tmpo gene (LAP2alpha KO*

myoblasts). The upper row depicts, for each of the cell lines, the insertions and deletions resulting from the gene editing. The lower row shows, after gene editing, how much the targeted region differs from the same genomic region in untreated myoblasts. (B) Co-Immunoprecipitation assays showing Lamin A/C and LAP2alpha interactions for the A-type lamin pools recognized by the various antibodies for lamin A/C. The antibody Lamin A/C-3A6 binds to the C-terminus of A-type lamins and the antibodies Lamin A/C-E1 and -N18 recognize the N-terminus. Proteins were immunoprecipitated in LAP2alpha WT and KO myoblasts with the different Lamin A/C antibodies, with an antibody specific for LAP2alpha and with control antibodies (IgGs). Membranes were blotted with LAP2alpha (1H11 antibody, upper row) and Lamin A/C (E1 antibody, lower row). The co-IPs were performed for Prol myoblasts (blots on the left) and D2 myoblasts (blots on the right). The arrows highlight the differential interaction of LAP2alpha with the pool of A-type lamins recognized by the Lamin A/C-3A6 antibody, compared to the other lamin antibodies used.

A

Genome coverage

	LAP2alpha	LaminA/C - 3A6	LaminA/C - E1
Prol	15,65%	15,27%	17,68%
D2	11,47%	19,94%	21,78%

B

Overlap with cLADs

	LAP2alpha	LaminA/C - 3A6	LaminA/C - E1
Prol	62,11%	45,61%	52,62%
D2	14,40%	47,95%	54,97%

C

Percentage of expressed genes inside EDD peaks

	LAP2alpha		LaminA/C - 3A6		LaminA/C - E1	
	Outside cLADs	Inside cLADs	Outside cLADs	Inside cLADs	Outside cLADs	Inside cLADs
Prol	23,16%	3,80%	13,76%	3,15%	11,53%	3,72%
D2	37,41%	2,35%	14,03%	3,78%	12,33%	3,70%

D

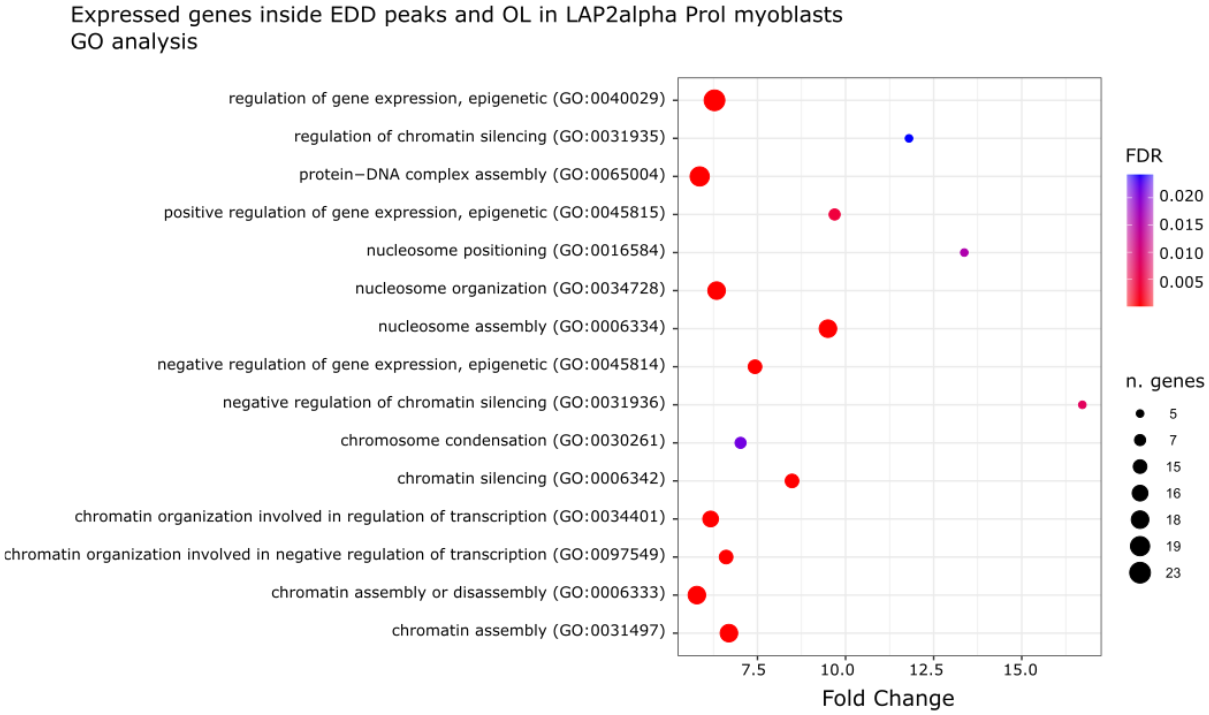
Directly bound genes inside EDD peaks

		Genes OL	Genes IL	Bound OL	Bound IL	% bound OL	%bound IL
LAP2alpha	Prol	3687	3448	482	509	13,07%	14,76%
	D2	7751	1277	830	192	10,71%	15,03%
LaminA/C - 3A6	Prol	4058	3082	558	613	13,75%	19,89%
	D2	4947	3838	657	826	13,28%	21,52%
LaminA/C - E1	Prol	3955	3897	503	824	12,72%	21,14%
	D2	4532	4588	619	972	13,66%	21,19%

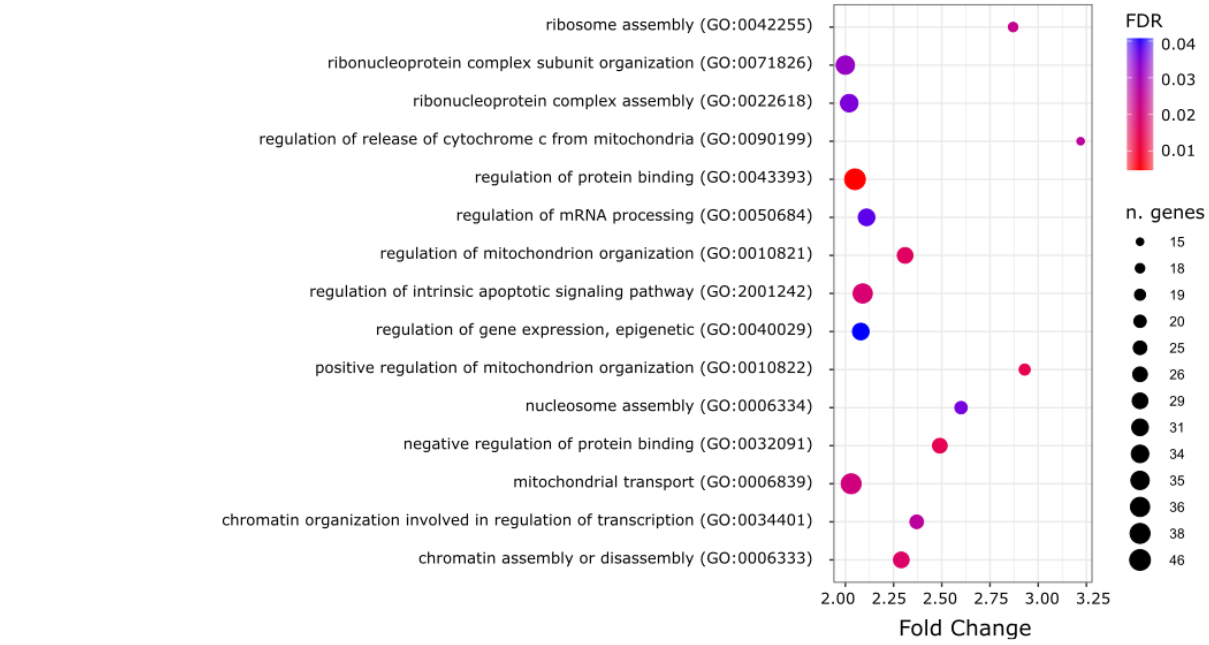
Suppl. Figure 2. *(A) Table showing the percentage of the genome covered by LAP2alpha, Lamin A/C-3A6 and Lamin A/C-E1 EDD peaks, for the differentiation stages analyzed (Prol and D2), in LAP2alpha WT myoblasts. (B) Table showing the percentage of LAP2alpha, Lamin A/C-3A6 and Lamin A/C-E1 EDD peaks overlapping with the reported cLADs, in LAP2alpha WT myoblasts. (C) Percentages of expressed genes found inside LAP2alpha, Lamin*

A/C-3A6 and Lamin A/C-E1 EDD peaks. The percentages are reported for both differentiation stages and are separately calculated for regions of the EDD peaks overlapping cLADs (inside cLADs) and not overlapping these regions (outside cLADs). (D) Table reporting the number of genes found inside LAP2alpha, Lamin A/C-3A6 and Lamin A/C-E1 EDD peaks. The 2 columns on the left show the total number of genes found inside EDD peaks in the different condition analyzed and are divided in genes outside cLADs (OL) and overlapping with cLADs (IL). The two middle columns report the total number of the genes found inside the EDD peaks analyzed and directly bound byLAP2alpha or A-type lamin. The genes are divided again for their location in region overlapping cLADs (inside cLADs, IL) or outside these regions (OL). The last two columns (on the right) also represent the bound genes inside EDD peaks, but as percentage of the total number of genes found inside these peaks.

A



B



Suppl. Figure 3. *(A) Bubble chart showing the results of the GO analysis of the expressed genes found inside LAP2alpha EDD peaks, outside cLADs (OL), in Prol myoblasts. (B) Bubble chart showing the results of the GO analysis of the expressed genes found inside LAP2alpha EDD peaks, outside cLADs (OL), in D2 myoblasts. For both (A) and (B) the charts display only the fifteen statistically most relevant classes of genes identified with GO analysis. The bubble sizes are proportional to the number of the genes found in the peaks for that specific GO class (in the legend as "n.genes"). The color of the bubbles represents their p-values adjusted after false discovery rate correction (in legend as "FDR").*

A

Genome coverage

	LaminA/C - 3A6	LaminA/C - E1
Prol	17,26%	15,14%
D2	15,27%	18,77%

B

Overlap with cLADs

	LaminA/C - 3A6	LaminA/C - E1
Prol	1,78%	38,91%
D2	40,91%	45,54%

C

		Overlap with LAP2alpha				
		Total length	bp overlap		% overlap	
			Prol	D2	Prol	D2
Lamin A/C-3A6 Prol	WT	415695000	230718000	172503000	55,50	41,50
	KO	470271000	70359000	227346000	14,96	48,34
Lamin A/C-E1 Prol	WT	481400000	268640000	158675000	55,80	32,96
	KO	411762000	190209000	182352000	46,19	44,29

D

Percentage of expressed genes inside EDD peaks

		LaminA/C - 3A6		LaminA/C - E1	
		Outside cLADs	Inside cLADs	Outside cLADs	Inside cLADs
LAP2alpha WT	Prol	13,76%	3,15%	11,53%	3,72%
	D2	14,03%	3,78%	12,33%	3,70%
LAP2alpha KO	Prol	40,34%	5,60%	17,55%	3,07%
	D2	17,84%	2,96%	15,66%	3,08%

E

Directly bound genes inside EDD peaks

		Genes OL	Genes IL	Bound OL	Bound IL	% bound OL	%bound IL
LaminA/C - 3A6	Prol	13864	143	1825	15	13,16%	10,49%
	D2	5038	2801	622	614	12,35%	21,92%
LaminA/C - E1	Prol	4672	2934	543	562	11,62%	19,15%
	D2	4943	3631	591	767	11,96%	21,12%

Suppl. Figure 4. *(A) Table showing the percentage of the genome covered by Lamin A/C-3A6 and Lamin A/C-E1 EDD peaks, for the differentiation stages analyzed (Prol and D2), in LAP2alpha KO myoblasts. (B) Table showing the percentage of Lamin A/C-3A6 and Lamin A/C-E1 EDD peaks overlapping with the reported cLADs, in LAP2alpha KO myoblasts. (C) Table displaying the overall length in bp of Lamin A/C-3A6 and -E1 identified peaks in proliferating LAP2alpha WT and KO myoblasts (left column). The table also show the overlap between these*

EDD peaks with the EDD peaks enriched in LAP2alpha, as bp overlap (column 2 for the Prol stage and 3 for D2) or % overlap (column 4) for the Prol stage and 5 for D2). (D) Percentages of expressed genes found inside Lamin A/C-3A6 and Lamin A/C-E1 EDD peaks. The percentages are reported for both differentiation stages, for LAP2alpha WT and KO myoblasts, and are separately calculated for regions of the EDD peaks overlapping cLADs (inside cLADs) and not overlapping these regions (outside cLADs). (E) Table reporting the number of genes found inside Lamin A/C-3A6 and Lamin A/C-E1 EDD peaks. The 2 columns on the left show the total number of genes found inside EDD peaks in the different condition analyzed and are divided in genes outside cLADs (OL) and overlapping with cLADs (IL). The two middle columns report the total number of the genes found inside the EDD peaks analyzed and directly bound by A-type lamins. The genes are divided again for their location in region overlapping cLADs (inside cLADs, IL) or outside these regions (OL). The last two columns (on the right) also represent the bound genes inside EDD peaks, but as percentage of the total number of genes found inside these peaks.

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