Regulation of Nuclear Gene Positioning during Muscle Differentiation

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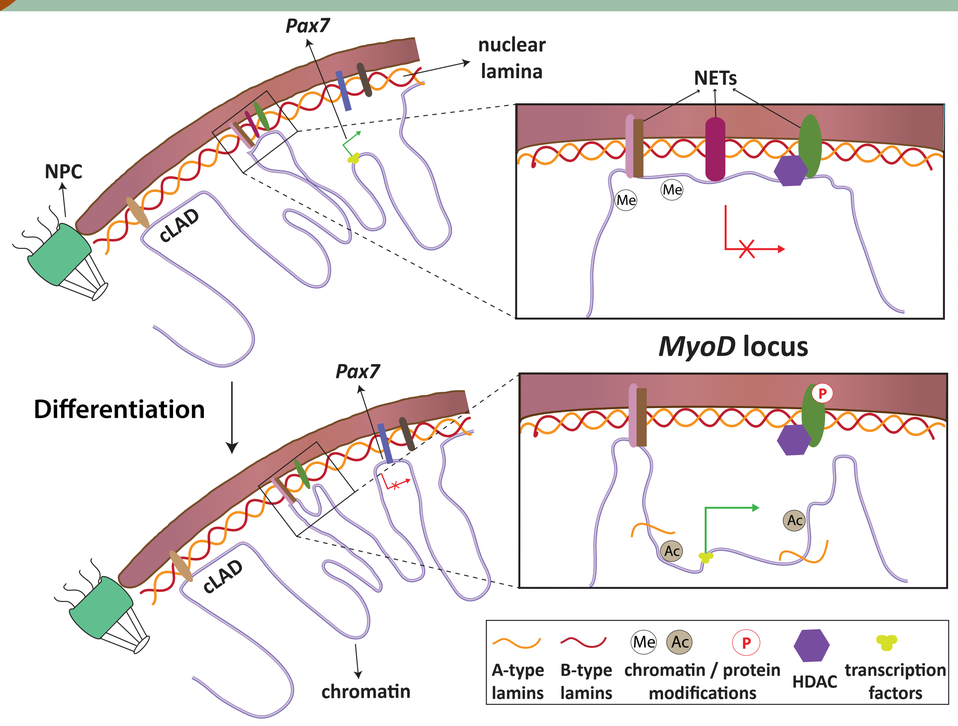
OVERWIEW

Nuclear lamins are major components of the lamina, a filamentous protein meshwork underlying the inner nuclear membrane of metazoan cells. The nuclear lamina is fundamental to maintaining chromatin architecture in the nucleus by providing anchorage sites for heterochromatic genomic regions, termed lamina-associated domains (LADs). Lamins, categorized into A- and B-type based on their biochemical properties, also interact with numerous nuclear envelope transmembrane proteins (NETs). These can, in turn, establish interactions with the genome and, together with lamins, contribute to nuclear chromatin organization. Apart from LADs, specific genes associate with the nuclear lamina in a more dynamic manner, a process that is essential for the coordinated temporal regulation of gene expression during development and differentiation. Whereas several studies have focused on understanding the establishment and anchorage of heterochromatic LADs to the lamina, the dynamic attachment of specific genes to the nuclear periphery remains poorly understood. Here, we aim to elucidate the detailed molecular mechanisms involved in nuclear positioning of specific genes during myogenic differentiation.

We developed a reporter system to easily track the localization of selected myogenic genes that have been shown to selectively undergo gain or loss of nuclear envelope association during muscle differentiation. Specifically, we inserted a short array of LacO repeats downstream of these genes using CRISPR/Cas9, to enable their microscopic visualization when bound by the Lac repressor fused to GFP. Systematic knock-out of several muscle specific nuclear envelope proteins, followed by evaluation of the peripheral positioning of the tagged loci, revealed certain NETs that contribute to peripheral tethering. Following the identification of the relevant tethers at the nuclear periphery, we plan to use the reporter cell lines to gain more mechanistic insights into the regulation of the release from or binding to the nuclear envelope during muscle differentiation.

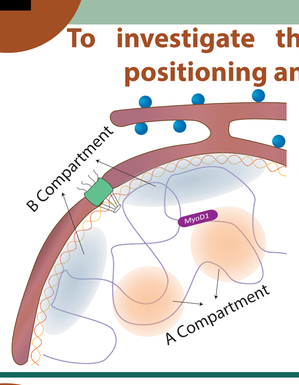
1 Working Hypothesis



Dynamic association of myogenic regulatory loci with the nuclear envelope (NE).
The dynamic attachment and release of specific genes from the NE is essential for the coordinated temporal regulation of gene expression during development and differentiation. In proliferating myoblasts, nuclear envelope transmembrane proteins (NETs) tether repressed genes (*MyoD*) to the nuclear periphery, while actively transcribed genes (*Pax7*) localize in the nuclear interior. Upon induction of differentiation, changes in anchor proteins and/or epigenetic modifications cause a rapid change of localization and expression patterns of these loci.

2 Aim of the Project

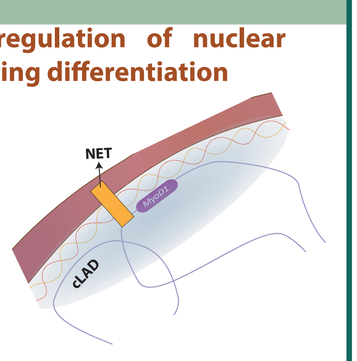
To investigate the mechanisms involved in the regulation of nuclear positioning and expression of myogenic genes during differentiation



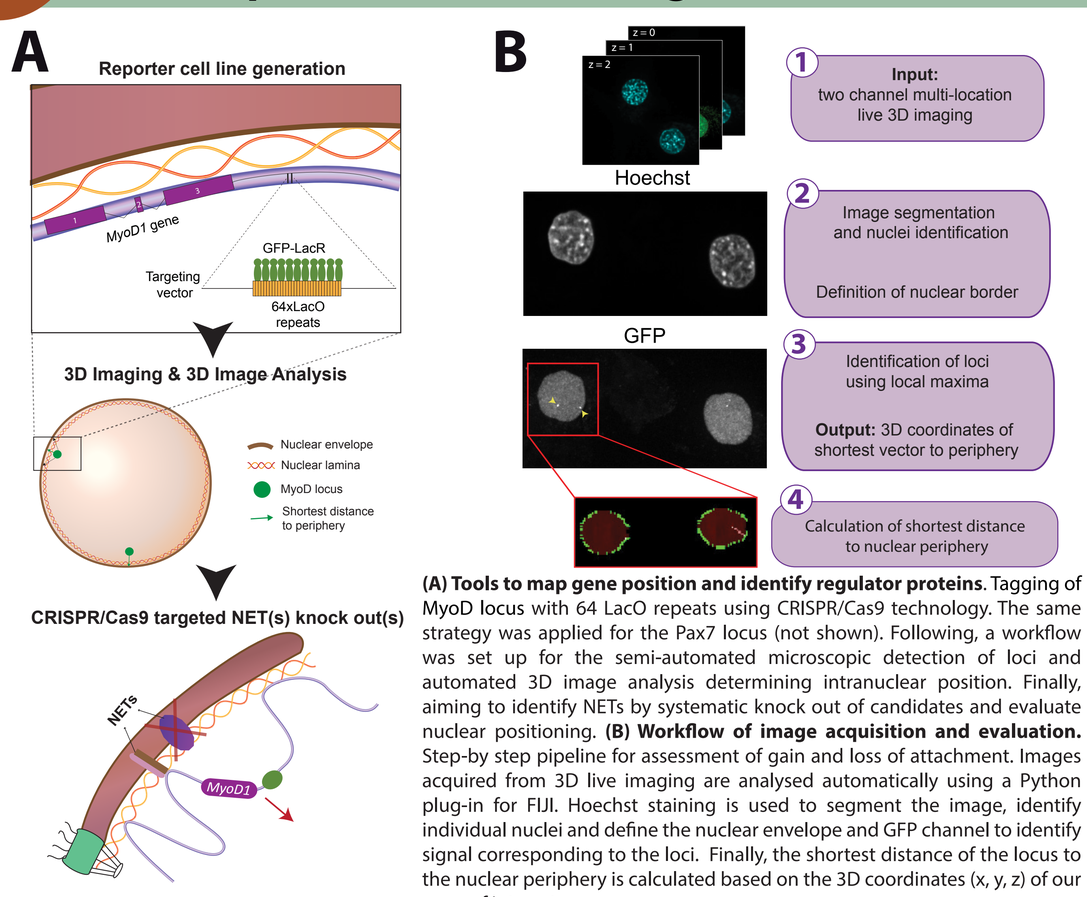
A. Is chromatin compartmentalization a regulator of gene positioning?

B. Which NE proteins are involved in association with the periphery? Are they different than the NETs mediating heterochromatin attachment?

C. Determine the pathways mediating the release from or binding to the nuclear envelope

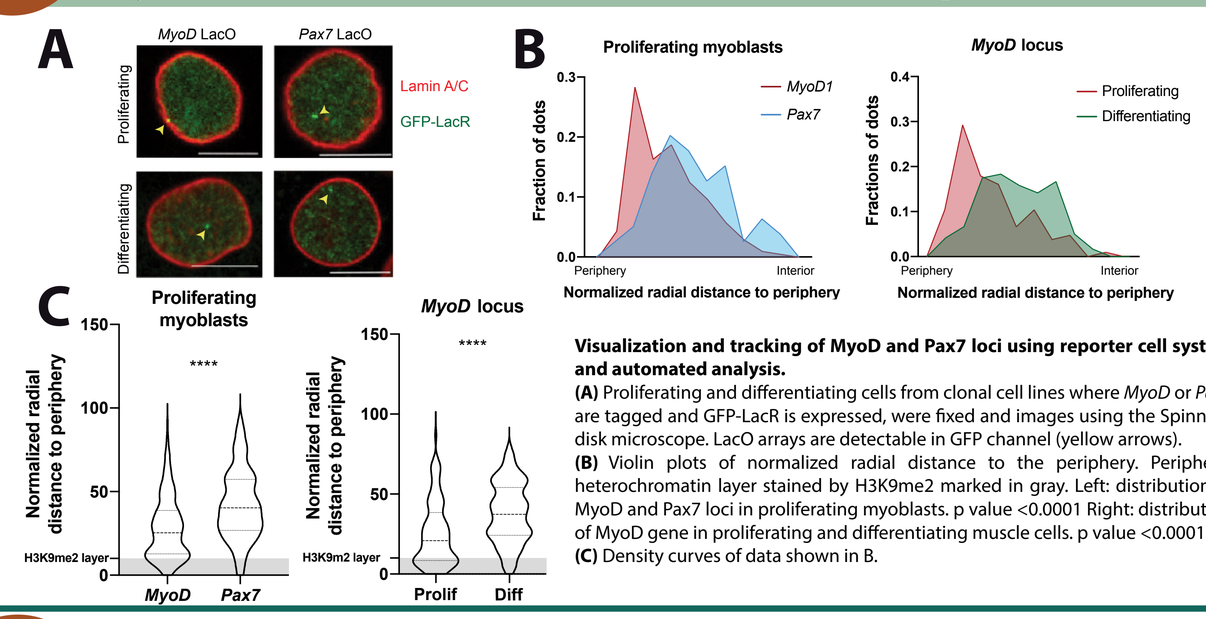


3 Reporter cell system to monitor and analyze nuclear position of loci during differentiation



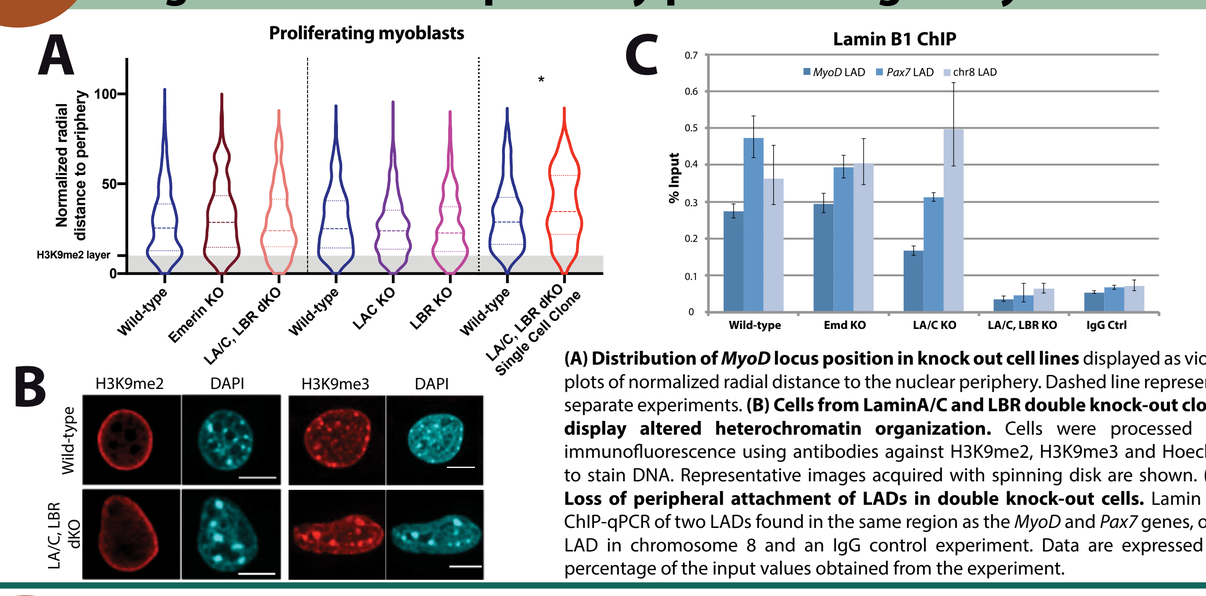
(A) Tools to map gene position and identify regulator proteins. Tagging of MyoD locus with 64 LacO repeats using CRISPR/Cas9 technology. The same strategy was applied for the Pax7 locus (not shown). Following, a workflow was set up for the semi-automated microscopic detection of loci and automated 3D image analysis determining intranuclear position. Finally, aiming to identify NETs by systematic knock out of candidates and evaluate nuclear positioning. **(B) Workflow of image acquisition and evaluation.** Step-by step pipeline for assessment of gain and loss of attachment. Images acquired from 3D live imaging are analysed automatically using a Python plug-in for FIJI. Hoechst staining is used to segment the image, identify individual nuclei and define the nuclear envelope and GFP channel to identify signal corresponding to the loci. Finally, the shortest distance of the locus to the nuclear periphery is calculated based on the 3D coordinates (x, y, z) of our gene of interest.

4 *Pax7* localizes more centrally in proliferating cells and *MyoD* moves to the nuclear interior during differetiation



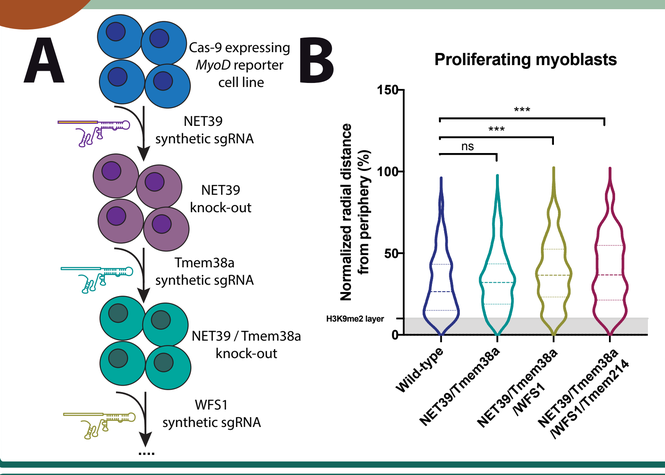
Visualization and tracking of MyoD and Pax7 loci using reporter cell system and automated analysis.
(A) Proliferating and differentiating cells from clonal cell lines where *MyoD* or *Pax7* are tagged and GFP-LacR is expressed, were fixed and images using the Spinning disk microscope. LacO arrays are detectable in GFP channel (yellow arrows).
(B) Violin plots of normalized radial distance to the periphery. Peripheral heterochromatin layer stained by H3K9me2 marked in gray. Left: distribution of MyoD and Pax7 loci in proliferating myoblasts. p value <0.0001 Right: distribution of MyoD gene in proliferating and differentiating muscle cells. p value <0.0001
(C) Density curves of data shown in B.

5 LA/C and LBR depletion affects heterochromatin organization and possibly positioning of *MyoD* locus



(A) Distribution of *MyoD* locus position in knock out cell lines displayed as violin plots of normalized radial distance to the nuclear periphery. Dashed line represents separate experiments. **(B) Cells from LaminA/C and LBR double knock-out clone display altered heterochromatin organization.** Cells were processed for immunofluorescence using antibodies against H3K9me2, H3K9me3 and Hoechst to stain DNA. Representative images acquired with spinning disk are shown. **(D) Loss of peripheral attachment of LADs in double knock-out cells.** Lamin B1 ChIP-qPCR of two LADs found in the same region as the *MyoD* and *Pax7* genes, one LAD in chromosome 8 and an IgG control experiment. Data are expressed as percentage of the input values obtained from the experiment.

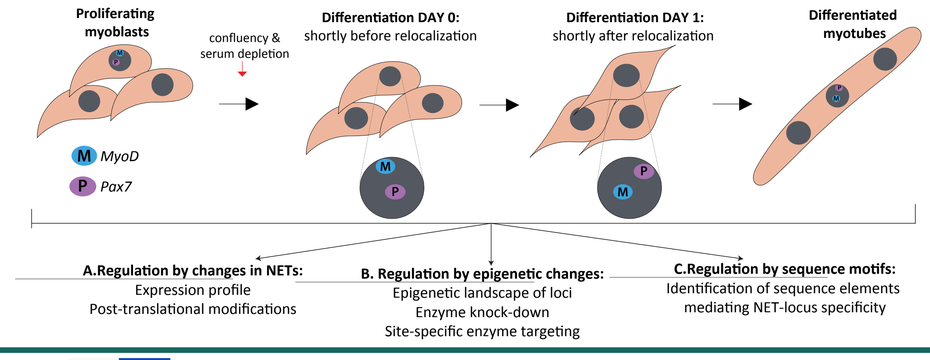
6 *MyoD* locus attachment to periphery is lost upon combined deletion of muscle specific NETs



(A) Experimental design utilized to perform multiple knock-outs of candidate proteins. Cell lines depleted of multiple candidate anchor proteins were generated from a MyoD reporter cell line constitutively expressing Cas9. Cells were transfected in a continuous manner with synthetic sgRNAs designed to target each gene of interest. Efficiency of knock-outs was estimated by assessment of gene editing (TIDE) and RNA levels (not shown). High levels (>80%) of gene editing were achieved for all targeted genes and downregulation of mRNA levels by >50% compared to wild-type.
(B) Distribution of *MyoD* locus position in wild-type and multiple knock out cell lines displayed as violin plots of normalized radial distance to the nuclear periphery. Peripheral heterochromatin layer stained by H3K9me2 marked in gray. Distributions were generated from at least 200 *MyoD* signals per sample in proliferating myoblasts. p values: ns=0.068, ***=0.0002, ***=0.0003 (Kolmogorov-Smirnov test)

FUTURE PROSPECTS

Determine factors and pathways contributing to the gain and loss of NE attachment



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