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„SUN and Lamin B proteins contribute to heterochromatin maintenance at the nuclear periphery"

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I dedicate this thesis to:

My Lab, who gave me the opportunity to look into the scientific world.

My Colleagues and Friends, who were unbelievably helpful and always on my side.

My Family, who believes in me whatever I do.

Summary

The majority of the human genome contains transcriptionally inactive heterochromatin and only 20% actively transcribed euchromatin. The epigenetic landscape of heterochromatin and euchromatin is different and marked by defined modifications. In order to promote heritable transmission of gene expression states, epigenetic modifications have to be faithfully restored after cell division. Except for DNA methylation, the mechanisms involved in recapitulating the parental chromatin structure are ill-defined. It is known for over 50 years that heterochromatin is in close contact with the nuclear lamina. Yet, genome-wide mechanisms and the regulatory principles underlying peripheral heterochromatin are largely unknown.

The aim of this thesis is to elucidate these mechanisms of heterochromatin maintenance at the nuclear lamina. We investigated the contributions of lamin and SUN proteins in the maintenance of heterochromatin. For that, we are using a Chromatin *in vivo* Assay which allows us to artificial silence a reporter gene. We generated loss-of-function and truncated mutant cell lines, via CRISPR-Cas9. We systematically characterized LMNB and SUN2 cell lines in our reporter assay. This revealed that a loss-of-function LMNB2 mutant was unable to reactivate the reporter gene. Additionally, we obtained a significant increase of reactivating cells in a truncated mutant cell line of LMNB1. Surprisingly, we demonstrate for the first time that SUN2 specifically contributes to an epigenetic inheritance of heterochromatin. In summary, these data support evidence that the nuclear periphery influences heterochromatin maintenance. This opens future perspectives to understand heterochromatin maintenance in mechanistic and molecular details.

Zusammenfassung

Das menschliche Genom besteht zu 80% aus inaktivem Heterochromatin und nur 20% aktivem Euchromatin. Die epigenetischen Merkmale von Heterochromatin und Euchromatin sind durch unterschiedliche Modifikationen definiert. Diese Merkmale müssen nach der Zellteilung wiederhergestellt werden. Mit Ausnahme der DNA-Methylierung sind die Mechanismen die zur Rekapitulierung der parentalen Chromatinstruktur erforderlich sind weitgehend undefiniert. Es ist seit über 50 Jahren bekannt, dass Heterochromatin in engem Kontakt mit der Zellkernlamina ist. Mechanismen und regulatorische Prinzipien des peripheren Heterochromatins sind jedoch weitgehend unbekannt.

Das Ziel dieser Arbeit ist es Mechanismen von Heterochromatin an der Kernmembran aufzuklären. Wir untersuchten Lamin B- und SUN-Proteine auf ihren Einfluss bei der Rekapitulierung parentaler Chromatinstrukturen. Zu diesem Zweck verwendeten wir einen Chromatin-*In-vivo*-Test, mit dem wir ein Reportergen künstlich inaktivieren konnten. Wir haben mit der CRISPR-Cas9 Methode Knock-out und mutierte Zelllinien generiert. Danach charakterisierten wir diese Zelllinien in unserem Reporter-Assay. Unsere Ergebnisse zeigten, dass ein Knock-out von LMNB2 Proteinen das Reportergen nicht reaktivieren konnte. Andererseits haben wir eine signifikante Zunahme an reaktivierenden Zellen in einer mutierten Zelllinie von LMNB1 beobachten können. Des Weiteren zeigen wir, dass SUN2 die epigenetische Vererbung von Heterochromatin beeinflussen kann. Unsere Daten bestätigen, dass die Kernperipherie die Heterochromatin-etablierung nach der Zellteilung beeinflussen kann. Zukünftige Forschungsarbeiten werden unser Verständnis von Heterochromatin-etablierung in mechanistischen und molekularen Details erweitern.

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1 Introduction

1.1 The genome, chromatin and the nucleus

The 23 chromosomes of the human genome are comprised of over 3 billion base pairs which extended would span a distance of more than 2 meters (Collins *et al.*, 2004). By comparison, the nucleus of a human cell is on average only 6 µm in diameter. Hence, the genomic DNA is highly condensed to fit in a single nucleus (Griffiths *et al.*, 2002). In part, genomic compaction is achieved by wrapping the DNA around histone proteins forming the nucleosome, the basic unit of a chromatin fibre (Olins and Olins, 2003). Yet, this incredible achievement is not satisfactory alone. Cells have to organize their genome in a usable and functional way. Specific parts of the genome need to be easily accessible, at the times of need while other parts should be restricted to maintain genome stability. Gaining insights into genome organization in the context of three-dimensional nuclear space is essential to explain principles of gene regulation (Stanytė, 2017).

1.1.1 Chromatin organization: euchromatin and heterochromatin

Nearly 100 years ago scientists observed that the genome can be separated into two distinct types of chromatin: euchromatin and heterochromatin. In 1928, Emil Heitz was one of the first ones to observe these structures and suggested these terms (Heitz, 1928). He proposed that euchromatin is connected to gene activity and heterochromatin corresponds to genetically inert regions (Passarge, 1979). 30 years later, Frenster et al. successfully isolated euchromatin and heterochromatin from

human lymphocytes and confirmed Heitz's hypothesis. The majority of chromatin, around 80%, contains transcriptionally inactive heterochromatin and only 20% actively transcribed DNA (Frenster *et al.*, 1963). To sum up, these early studies revealed that chromatin is non-randomly packed and folded. Its structure and organization indicate its functional state.

In 1942, Conrad Waddington coined the term "epigenetics". He studied the aspects of development in the fruit-fly *Drosophila melanogaster* and defined epigenetics as changes of the phenotype without changes of the DNA sequence (Waddington, 1942). Nowadays we know that epigenetic mechanisms help to stabilize gene expression throughout development. Euchromatin and heterochromatin are consequences of these processes. Specifically, euchromatin is enriched in actively transcribed protein coding genes (Pueschel, Coraggio and Meister, 2016). In contrast, heterochromatin mostly consists of gene deserts enriched for repetitive elements, such as pericentromeric or transposable elements (Solovei, Thanisch and Feodorova, 2016).

1.1.2 Functional modifications of chromatin

Epigenetic mechanisms support heritable transmission of gene expression patterns by adapting chromatin. The epigenetic landscape of euchromatin and heterochromatin is very different and marked by defined modifications. Most of the known epigenetic modifications are reversible (Allis and Jenuwein, 2016). Back in the 1950's, chemical modifications of the DNA were already known (Hotchkiss, 1948). In particular, the methylation of cytosine at position 5 was proposed to have an influence on gene regulation (Holliday and Pugh, 1975). Seminal work using the drug 5-

azacytidine, which blocks DNA methylation, demonstrated changes in gene expression and phenotypes in fibroblast cell lines (Taylor and Jones, 1979). Soon thereafter, the functional connection between methylation of CpG dinucleotides and gene repression was established (Razin and Riggs, 1980).

Additionally, chromatin harbours different histone modifications (Turner, 1993). One of the first well studied modifications was histone acetylation (Allfrey and Mirsky, 1964). Euchromatin is marked with several histone acetyl groups to maintain its open conformation (Verdin and Ott, 2015). Furthermore, actively transcribed promoters carry histone 3 lysine 4 trimethylation (H3K4me3) marks (Schübeler *et al.*, 2004). While, heterochromatic DNA is heavily methylated and its histones are hypoacetylated (De Rubertis *et al.*, 1996). The best studied molecular hallmark of heterochromatin is histone 3 lysine 9 trimethylation (H3K9me3) (Tschiersch *et al.*, 1994; Nakayama *et al.*, 2001). This mark is recognized by the heterochromatin protein 1 (HP1) which helps to establish and maintain a repressive state (Lachner *et al.*, 2001). Enzymes that specifically bind histone modifications, “readers”, are frequently physically coupled to enzymes that catalyze the addition, “writers”, or removal, “erasers”, of modifications (Allis and Jenuwein, 2016). Nowadays there are more than 40 different histone modifications identified (Zhang and Pradhan, 2014). The combinatorial nature of histone modifications provides a logic for chromatin organization and gene regulation.

1.1.3 Heterochromatin maintenance

In order to promote heritable transmission of gene expression states, epigenetic chromatin modifications have to be faithfully restored after genome replication and cell division. Except for DNA methylation, the mechanisms involved in

recapitulating the parental chromatin structure are ill-defined. In the case of DNA methylation, both DNA strands are symmetrically methylated on CpG sequences. After genome replication, hemi-methylated DNA is specifically recognized by the DNA (cytosine-5)-methyltransferase 1 (DNMT1) enzyme, which catalyzes the methylation of new CpG's (Razin and Riggs, 1980). Its enzymatic activity is required to maintain a transcriptionally repressive state of genes in undifferentiated mouse embryonic stem cells (mESC) and to silence transposons (Li, Bestor and Jaenisch, 1992; Lyko, 2018). This autonomous mechanism guarantees that after every cell division the global DNA methylation pattern is maintained.

In addition, during DNA replication the interaction between histones and DNA is also disrupted. After replication, new histone-modifying complexes and their associated modifications have to be reestablished. This process is much more complex, due to the facts that more proteins and modifications are involved. The separation of parental histone complexes and the de novo chromatin formation have an enormous influence on the whole genome. Therefore, chromatin duplication includes histone disruption, transfer, along with reestablishment of specific modifications to maintain defined chromatin marks (Allis D., Caparros M. L., Jenuwein T., 2015). How the epigenetic landscape is passed on from one cell generation to the next remains poorly understood.

1.2 Structural components of the nucleus

1.2.1 The nuclear lamina

The nucleus is separated from the cytoplasm by the nuclear envelope (NE). It is a double membrane bilayer, described as inner and outer nuclear membrane (INM

and ONM, respectively) and separated by the perinuclear space (PNS) (Alberts *et al.*, 2002). The nuclear lamina is a filamentous layer located underneath the INM (Dechat *et al.*, 2010). This layer consists mainly of nuclear lamins which are classified as type V intermediate filament proteins (Gerace and Blobel, 1980; Goldman *et al.*, 1986; Mckeon, Kirschner and Caput, 1986). Lamins are important structural components of the nuclear envelope (Burke and Stewart, 2012). Based on their sequence homologies, expression patterns and biochemical features, lamins are subdivided into A- and B-types. The two isoforms of A-type lamins, lamin A and lamin C (LMNA/C), are derived from a single gene *LMNA* by alternative splicing (Lin and Worman, 1993; Gruenbaum and Foisner, 2015). LaminB1 and LaminB2 (LMNB) are the major B-type lamins and they are encoded by two separate genes (Peter *et al.*, 1989; Vorburger, Kitten and Nigg, 1989). At least one B-type lamin is expressed in all cells throughout development in most metazoans (Benavente, Krohne and Franke, 1985; Schatten *et al.*, 1985; Lehner *et al.*, 1987). In contrast, the expression of A-type lamins is developmentally regulated, being low in pluripotent cells (Röber, Weber and Osborn, 1989; Eckersley-Maslin *et al.*, 2013).

Genetic studies revealed that LMNA/C and LMNB proteins play critical roles in developmental regulation and organogenesis. *LMNB1/2* knockout mice die at birth with defects in their brains and lungs (Coffinier *et al.*, 2011; Kim *et al.*, 2011). In addition, *LMNA* knockout mice display cardiac and muscular abnormalities and die shortly after birth (Kubben *et al.*, 2011). Mutations in lamin genes lead to at least 15 distinct diseases, collectively known as laminopathies (Schreiber and Kennedy, 2013).

Overall, the achievements of the last year's show clearly that nuclear lamins are very important for nuclear architecture and its functions (Gruenbaum and Foisner, 2015). The entire nucleus is covered with a lamin network. This structure regulates

not only the shape of the nucleus but serves also as a platform for chromatin and numerous proteins involved in chromatin organization (Schirmer and Foisner, 2007).

1.2.2 The LINC complex

In addition to lamins, the linker of the nucleoskeleton and cytoskeleton (LINC) complex presents another well studied structural component of the NE (Lee and Burke, 2017). This highly versatile complex is present in all nucleated cells of plants, animals and fungal (Meier, 2016). The LINC complex consists of Klarsicht, ANC-1 and Syne homology (KASH) domain proteins in the ONM and Sad1 and UNC-84 (SUN) domain proteins in the INM. KASH proteins have cytoplasmic domains which interact with various elements of the cytoskeleton. In the PNS, they contain their characteristic KASH domain at the C-terminal (Starr and Han, 2002). In mammals, two SUN proteins, SUN1 and SUN2, are found in all somatic cells (Starr and Fridolfsson, 2010). Their N-terminal domain is located within the nucleus and can interact with LMNA and other proteins at the nuclear periphery (Haque *et al.*, 2010; Bone *et al.*, 2014). SUN proteins have their typical SUN domain in the PNS. Structural studies revealed that three KASH domains bind to homo-trimers of SUN proteins (Sosa *et al.*, 2012; Zhou *et al.*, 2012; Jahed *et al.*, 2018). Consequently, the LINC complex presents a physical link that spans both nuclear membranes. Therefore, it connects the NE, including nuclear lamins, to elements of the cytoskeleton (Padmakumar *et al.*, 2005; Crisp *et al.*, 2006; Haque *et al.*, 2006).

Despite these structural facts, the LINC complex harbours other different functions. For example, in meiotic cells SUN1 can bind telomeres through other proteins and plays a critical role in meiotic telomere attachment (Shibuya and

Watanabe, 2014). Furthermore, SUN2 also co-localizes with telomeres at the NE (Link *et al.*, 2014). In addition, the LINC complex associates with DNA repair mechanisms in mammalian cells (Lei *et al.*, 2012). Compared to lamin knockouts, SUN double knockout mice die also shortly after birth with defects in the nervous and muscle systems (Lei *et al.*, 2009; Zhang *et al.*, 2009). Furthermore, previous studies reported also an interesting role of SUN proteins in HIV infections (Luo, Yang and Gao, 2018). They indicate that the nucleoplasmic domain of SUN2 can inhibit HIV expression (Donahue *et al.*, 2016; Sun *et al.*, 2018)**.** Beyond being structural components of the inner nuclear membrane, these data suggest that SUN proteins can contribute to chromatin organization and gene regulation.

1.3 Heterochromatin and the nuclear periphery

Over five decades ago, early electron microscopy studies of vertebrate cell nuclei indicated that the nuclear lamina is closely associated with peripherally localized heterochromatin (Fawcett, 1966). These parts of the genome which interact with the periphery are defined as lamina-associated domains (LADs) (Guelen *et al.*, 2008; Kind *et al.*, 2013). They can reach up to 30% of the genome in mammalian cells and these domains range from 0.1 to 10 Mb (Guelen *et al.*, 2008). LADs are comparable to heterochromatin and similar characterized as gene poor and marked with histone 3 lysine 9 di/trimethylation (H3K9me2/me3) or histone 3 lysine 27 trimethylation (H3K27me3) transcriptionally silent histone modifications (Pickersgill *et al.*, 2006; Peric-Hupkes *et al.*, 2010). LADs can be classified into two categories: constitutive and facultative LADs (van Steensel and Belmont, 2017). Constitutive LADs tend to interact with the nuclear periphery in all cell types. Facultative LADs can

switch their nuclear position during development and usually consist of developmentally regulated genes (Harr, Gonzalez-Sandoval and Gasser, 2016). Depletion of enzymes, which label histones with heterochromatin marks, leads to a detachment of LADs from the periphery (Towbin *et al.*, 2012; Bian *et al.*, 2013; Harr *et al.*, 2015).

1.3.1 Anchoring heterochromatin to the nuclear lamina

In general, when genes are located at the nuclear periphery are either lowly expressed or transcriptionally silent (Kind *et al.*, 2015). Artificial gene anchoring to the nuclear lamina leads to a reduction in transcriptional activity (Finlan *et al.*, 2008; Reddy *et al.*, 2008). Nevertheless, genes can still be active while the locus is tethered to the nuclear periphery (Kumaran and Spector, 2008). A seminal study in *C. elegans* discovered the chromo domain-containing protein 4 (cec-4) which plays a specific role in heterochromatin regulation. Cec-4 is directly anchored to the nuclear lamina and has specificity to recognize H3K9me2 and H3K9me3 marks (Gonzalez-Sandoval *et al.*, 2015). As a result, it is possible that a heterochromatic state alone is sufficient to anchor chromatin to the nuclear periphery. Furthermore, recent studies indicate that even more nuclear lamina components are involved in chromatin anchoring to the nuclear envelope. For example, in mammals the lamin B receptor (LBR) is required for chromatin anchoring to the nuclear lamina (Solovei *et al.*, 2013; Harr *et al.*, 2015). LBR can interact with Xist (X-inactive specific transcript) and therefore promotes X chromosome anchoring to the nuclear lamina (Chen *et al.*, 2016) In the context of peripheral heterochromatin, nocturnal animals add a curious facet to that topic. Their retina rod cells express neither LMNA/C nor LBR proteins and thus have an inverted heterochromatin positioning within the nucleus. When both proteins are

overexpressed in these cells then heterochromatin localizes to the nuclear periphery (Solovei *et al.*, 2013).

To sum up, these results suggest that the location at the nuclear lamina does not silence gene expression, as transcription can still be activated there. It rather anchors silent genes and helps to maintain their heterochromatic state. Thus, the biological functions and the molecular mechanisms of heterochromatin anchoring to the nuclear lamina remain elusive.

1.4 Aim of this thesis

The structural and functional state of chromatin is essential for the vitality of cells. During cell division, the epigenetic marks of the whole genome need to be duplicated and segregated into each daughter nucleus. For over 50 years it is known that heterochromatin is in close contact with the nuclear lamina. The mechanisms for maintenance of heterochromatin at the nuclear periphery remain unknown.

The aim of this thesis is to elucidate the mode of heterochromatin maintenance at the nuclear lamina. Specifically, we investigated the contributions of Lamin and SUN proteins in the maintenance of heterochromatin following artificial initiation of reporter gene silencing using the Chromatin *in vivo* Assay.

2 Results

2.1 *In Vivo* heterochromatin maintenance reporter assay

What is the role of the nuclear periphery in heterochromatin maintenance? To address this question, we need a robust, as well as simple system to read out changes in heterochromatin maintenance in living cells. Therefore, we modified a well characterized mESC assay. In previous work, Hathaway *et al.* selectively recruited Heterochromatin Protein 1 alpha to induce gene silencing and described chromatin modifications at the *Oct4* gene. The heterochromatic state at the locus was heritable and stably transmitted through multiple cell divisions even when the initial stimulus was absent (Hathaway *et al.*, 2012).

In our assay, one *Oct4* allele is also genetically modified to study principles of heterochromatin maintenance. We introduced a Tet Operator site (TetO) upstream of the *Oct4* promoter and an in-frame nuclear GFP reporter replacing the first exon of *Oct4* (Figure 1A). In addition, we ectopically expressed a truncated form of Heterochromatin Protein 1 alpha containing only the chromo-shadow domain fused to FLAG-TetR-mCherry (HP1α). The TetR-TetO system allows us to tether HP1α to the *Oct4* reporter locus and to induce transcriptional silencing. Upon the addition of Doxycycline (Dox), TetR binding is released which enables analysis of heritable maintenance of heterochromatin and gene repression in the absence of the initial stimulus.

As a proof of principle, we tested the GFP expression of the *Oct4* reporter cell line in the absence of HP1α by fluorescence-activated cell sorting (FACS). We observed 88 percent (%) of GFP positive cells and only 12% of GFP negative ones

(Figure 1B). Most of the cells expressed GFP due to the fact that the *Oct4* promoter is active and the construct is correctly inserted into the genome.

Next, we determined if HP1α tethering is sufficient to induce *Oct4* reporter gene silencing. Consistent with Hathaway et al., TetR-dependent tethering of HP1α to the *Oct4* reporter allele yielded a homogeneous population of GFP negative cells (Figure 1B). Thus, HP1α recruitment was sufficient to induce a heterochromatic state at the reporter locus and silence GFP expression. Next, we sought to validate previous results that transmission of induced heterochromatin through cell divisions after the initial stimulus (Hathaway *et al.*, 2012). We cultured the cells for 7 days in medium with Dox and analyzed them again by FACS. In the presence of Dox, we observed that the reporter cell population remained GFP negative throughout many cell generations (Figure 1B). After validating that the modified reporter assay recapitulated heritable gene silencing, we will refer to the *Oct4* reporter cell line with HP1α tethering as wildtype (WT).

To determine whether the reporter cell line is sensitive to modifiers of heterochromatin maintenance, we used CRISPR-Cas9 to disrupt expression of DNMT1. DNMT1 methylates CpG dinucleotides and is a key player to maintain repressive DNA methylation. Western blot confirmed that our reporter KO cell line is DNMT1 deficient (Figure 1C). We compared the expression levels of GFP before and after treatment of Dox for 7 days. As expected, 95% of the cells are GFP-positive and showed a reactivation of the *Oct4* reporter allele. Hence, without DNMT1 the reporter cell line is not able to transmit induced heterochromatic silencing of the reporter locus.

We conclude from the results that our modified *Oct4* reporter cell line is first capable to induce silencing at the *Oct4* gene and that this heterochromatic state is heritably transmitted throughout multiple cell generations. Second, that we are able to

monitor changes in heterochromatin maintenance if we disrupt important mechanisms, like DNA methylation.

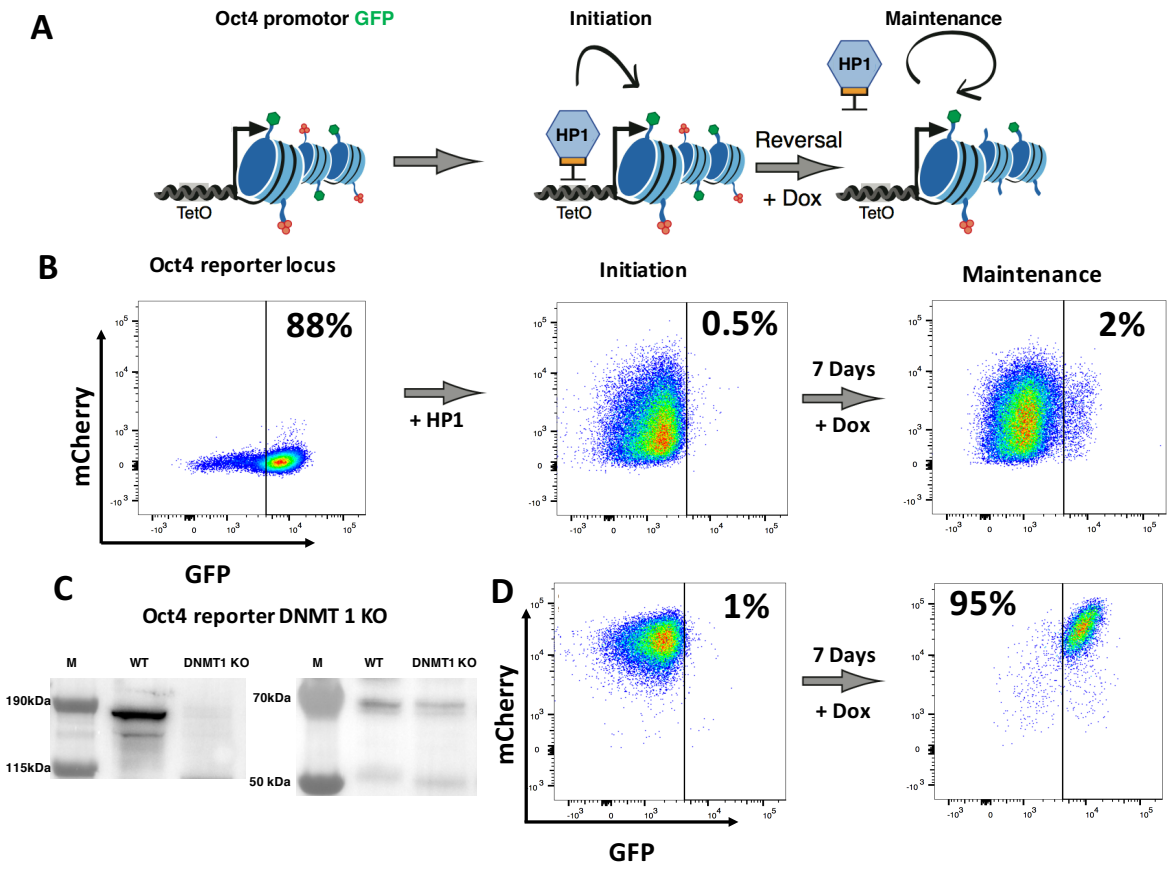

Figure 1. Design and characterization of heterochromatin maintenance *in vivo* assay.

A) Scheme of the modified *Oct4* reporter allele in mESCs. TetR fusion construct promotes reversible tethering of a truncated form of HP1α to a Tet Operator site upstream of the *Oct4* reporter allele. Doxycycline addition releases TetR binding to determine heritable maintenance of heterochromatin modifications and expression state in the absence of the initial stimulus. B) Flow cytometry plots show GFP expression before and during HP1α recruitment as well as after Dox-dependent reversal in the *Oct4* reporter cell line. Percentage (%) indicates a fraction of GFP-positive reporter cells. C) Western blot against DNMT1 in wild-type (WT) and DNMT1 CRISPR mutant *Oct4* reporter cell line. D) Flow cytometry plots before and after 7 days of Dox treatment of DNMT1 KO. % indicates a fraction of GFP-positive reporter cells. kDa, kilo Dalton; M, pre-stained marker; n: 50.000 event counts/plot

2.2 Generating hypomorphic and loss-of-function mutants of LINC-complex components and nuclear lamins

To determine if individual proteins of the INM contribute to the maintenance of heterochromatin in our reporter cell line, we used CRISPR/Cas9 technology to generate loss-of-function and truncated mutants of LMNB and SUN proteins (Pyzocha *et al.*, 2014). We decided to focus on these protein families which are both located at the nuclear periphery. The LMNB family of proteins, which is constituted by LMNB1 and LMNB2 in mESCs, together with LMNA/C proteins make up the NL (Figure 2A).. SUN proteins which are transmembrane proteins in the INM and are part of the LINC-complex (Figure 2A). In mESCs, this family consists of SUN1 and SUN2. All four proteins of interest are encoded by their own gene.

We started to design guide RNAs (gRNA) targeting each protein at either one spot to induce insertions, deletions or frame-shift mutations, or in two spots to excise a functionally important region. The gRNA design and the viral infection were performed as described in the Materials and methods section. We performed different strategies to ensure we can generate complete KO's as well as domain specific mutants.

First, we characterized the newly generated cell lines at the genomic level by identifying the genomic DNA sequence changes around the individual targeted sites via Sanger sequencing. After PCR genotyping, described in the Materials and methods section, PCR products were visualized on a gel and bands were excised for sequence analysis (data not shown).

The LMNB1 #B8 showed two PCR bands which we excised and submitted for Sanger Sequencing (Figure 2B). The sequencing data of the bigger PCR band, which had a similar size as the WT PCR band, indicated a 7 bp out-of-frame deletion. The

smaller band is roughly a 100 bp deletion. The WT band was around the expected size of 700bp (Figure 2B).

The LMNB2 #D9 showed one PCR band at the same size as the WT band (Figure 2B). The interpretation of raw sequencing data was not possible because the quality was poor. This was not a result of a technical defect or unclean sample preparation. This LMNB2 cell line was generated using one single gRNA thus, two alleles can carry different mutations and the raw sequencing data shows a mixture of both profiles. To overcome this problem, we used a published online software tool called: Tracking of Indels by DEcomposition (TIDE) (Brinkman *et al.*, 2014). With this program, we were able to separate the different alleles by calculations (data not shown). The LMNB2 #D9 had one allele with a 16 bp deletion and the other allele a 19 bp deletion. Both deletions were frame-shift mutations.

The SUN2 #A4 cell line was generated via a single gRNA and showed also one band (Figure 2B). We saw again a poor raw sequencing data and used TIDE. We calculated two frame-shift mutations, a 2 bp and a 20 bp deletion, respectively.

The SUN2 #D7 showed a smaller band in size compared to the WT band (Figure 2B). The sequencing data confirmed a 300 bp deletion. Furthermore, the SUN2 #H7 showed also a smaller band in size compared to the WT band and sequencing data confirmed a 100 bp deletion (Figure 2B). Both cell lines were targeted with two gRNAs. The PCR amplifications of a WT sample with the different genotyping primers had always the expected size and were also confirmed via sequencing (Figure 2B).

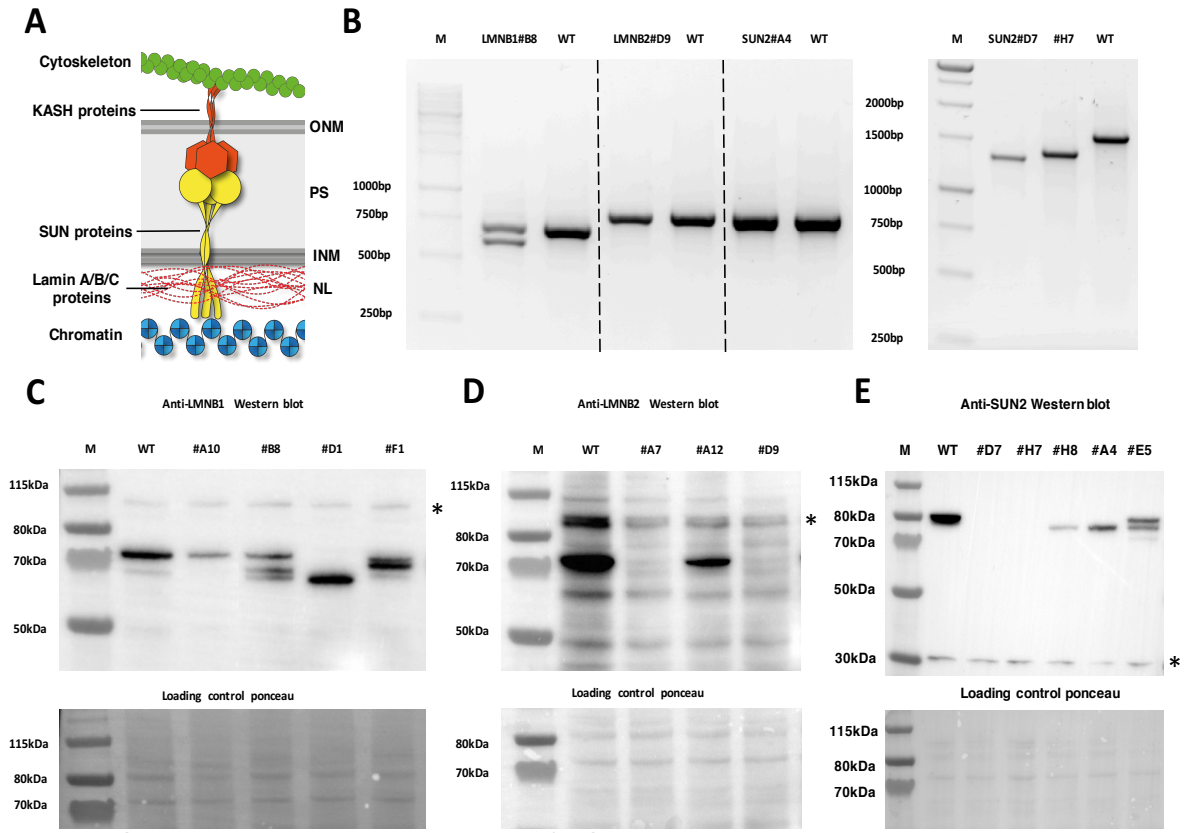

Figure 2. Generating and characterization of KO and mutant cell lines.

A) Schematic illustration of the LINC complex and the nuclear periphery. The LINC complex consists of KASH proteins in the ONM and SUN proteins in the INM. The cytoplasmic extensions of KASH proteins can bind to different cytoskeletal elements. In the PNS they interact and connect to each other through their KASH/SUN domains. The nucleoplasmic domains of SUN proteins interact with the NL. In mESC the NL consists mainly out of LMNB and LMNA/C proteins. B) Images of an agarose gel electrophoresis with genotyping products. On the first picture LMNB1, LMNB2 and SUN2 cell lines, with one gRNA targeted, next to the corresponding WT samples. On the second picture SUN2 cell lines, with two gRNA targeted, with the corresponding WT sample. C) Western blot with anti-LMNB1 antibody. WT, expected band around 70kDa, next to LMNB1 #A10, #B8, #D1 and #F1 samples. D) Western blot with anti-LMNB2. WT, expected band around 70kDa, next to LMNB2 #A7, #A12 and #D9 samples. E) Western blot with anti-SUN2 antibody. WT, expected band around 80kDa, next to SUN2 #D7, #H7, #H8, #A4 and #E5 samples. As a loading control for all Western blots a Ponceau S staining was performed on the membranes before incubation with an antibody. kDa, kilo Dalton; M, 1kb DNA marker on agarose gels and pre-stained marker for Western blots; WT wildtype *Oct4* reporter cell line. Asterisks (*) marks unspecific bands.

After we confirmed DNA editing at the genomic level, we proceeded to test whether these mutations actually affect protein expression. Therefore, we carried out Western blots with the respectively commercially available antibody. We started to test our LMNB1 cell lines. The WT sample had a clear and recommended band around 70 kDa (Figure 2C). We detected a weak unspecific band around 110 kDa in all samples (Figure 2C). LMNB1 #A10 had a weaker WT band in intensity and #D1 showed one strong smaller band. Interestingly, in the cell lines #B8 and #F1 we observed multiple bands present indicative of truncated LMNB1 proteins (Figure 2C). Thus, despite obtaining out-of-frame indels, CRISPR-Cas9 directed mutagenesis with a single gRNA yielded only truncated versions of LMNB1. No loss-of-function mutant of *LMNB1* could be recovered.

Next, we tested the LMNB2 cell lines. The WT lane gave a clear signal around 70 kDa and a strong unspecific background pattern (Figure 2D). By comparison, no signal was detected in the LMNB2 cell lines #A7 and #D9 (Figure 2D). We concluded, that in these cases a single gRNA targeting was suitable to generate KO's of *LMNB2*. LMNB2 #A12 contained a WT band but less intense (Figure 2D).

Finally, we sought to determine the protein expression in SUN2 cell lines. The WT lane gave a strong and expected band around 80 kDa (Figure 2E). We saw an unspecific band around 30 kDa in all samples (Figure 2C). The SUN2 cell lines #D7 and #H7, which were infected with two gRNAs, showed both no detectable signal (Figure 2E). Thus, CRISPR-Cas9 editing successfully modified *SUN2* expression in these cell lines. In contrast, SUN2 cell lines which were infected with a single gRNA had visible bands, albeit at a lower molecular weight. The cell lines #A4, #E5 and #H8 showed a smaller and less intense band (Figure 2E). These bands had a size of around 75 kDa. Further, the #E5 cell line had a WT band as well. So, the targeting

strategy with one gRNA was able to generate truncated versions of SUN2 but was not sufficient for loss-of-function mutants.

In summary, we were able to generate KO's for SUN2 and LMNB2 proteins via CRISPR-Cas9. For LMNB1 proteins we could obtain hypomorphic mutants with protein truncations. After having characterized expression changes, we went on to test the impact of LMNB1 and B2 as well as SUN2 mutations on heterochromatin maintenance with our reporter assay. Note that in the time period of this thesis we were not able to generate KO's or mutants for SUN1 proteins.

2.3 LMNB1 mutants and LMNB2 KO's show differential impact on heterochromatin maintenance

Nuclear lamins, as LMNB1 and LMNB2, are the most abundant proteins at the nuclear periphery of mESCs. Therefore, we wanted to investigate if a KO or mutant of them can also cause a reactivation phenotype in our *in vivo* assay.

In the absence of Dox, HP1 tethering induced *Oct4*-GFP silencing in LMNB1 and LMNB2 mutants similar to wild-type reporter cells (Figure 3A and 3B). Next, we measured GFP reactivation by FACS in LMNB1 and LMNB2 mutant reporter cells after treatment with Dox for 7 days. We obtained no significant increase of GFP positive cells in the LMNB2 KO #D9 over time (Figure 3A). The other LMNB2 KO cell line #A7 showed also not reactivation (data not shown). In sharp contrast to this observation, we observed that nearly 80% of cells reactivated in the LMNB1 mutant #B8 after 7 days of Dox treatment (Figure 3B). We did not expect such a fast response which was comparable to our positive control cell line DNMT1 KO. In other LMNB1 mutants no reactivation appeared (data not shown). In addition, we kept all the cell lines for several

weeks in culture and repeated this experiment. We measured a similar percentage of reactivation in these cell lines (data not shown).

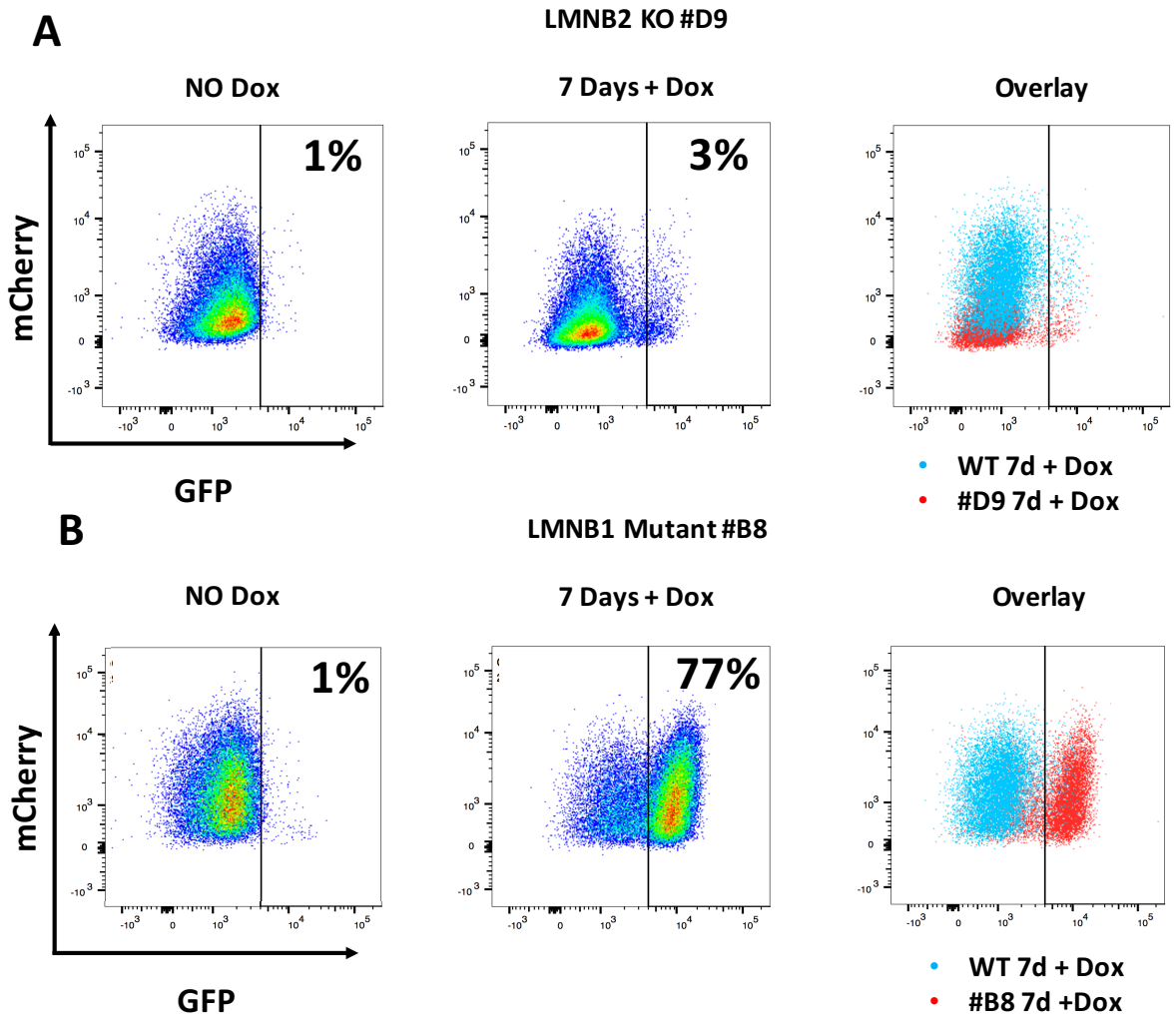

Figure 3. LMNB2 KO's have no effect on heterochromatin maintenance, although LMNB1 mutants have a negative effect.

A) and B) Flow cytometry plots show no and 7 days of Dox treatment. The overlay plots present in blue 7 days of Dox treatment of WT as control and in red the LMNB cell lines, respectively. All data were collected on the LSR Fortessa FACS machine. % indicates a fraction of GFP-positive reporter cells. n: 50.000 counts/plot

In summary, we find that a complete loss of one LMNB is unlikely to change heterochromatin maintenance at the nuclear periphery. Nevertheless, the LMNB1 mutant with his truncated structure revealed a dominant negative effect in our reporter assay. From previous studies it is known that *LMNA* is most frequently mutated within

lamin proteins, causing a variety of human laminopathies (Vlcek and Foisner, 2007). Most of these laminopathies are autosomal dominant. Furthermore, there are only a few B-type lamin mutations reported (Gruenbaum and Foisner, 2015). Thus, an altered version of LMNB proteins can influence the maintenance of heterochromatin at the modified *Oct4* locus.

2.4 Loss of SUN2 disrupts heterochromatin maintenance

Previous studies reported an interesting influence of SUN proteins in the context of HIV infection and latency. SUN2 maintained the repressive state of the integrated HIV genome and inhibited transcription of proviral DNA (Donahue *et al.*, 2016; Luo, Yang and Gao, 2018; Sun *et al.*, 2018). Therefore, we asked if SUN proteins have also an effect of heterochromatin maintenance in our *in vivo* assay.

To ensure we capture also early effects of our cell lines we already started with the treatment of Dox 14 days after viral infection. These first measurements were performed on the iQue Screener Plus FACS machine. This system allowed us to screen a big number of individual cell lines very fast. Similar to LMNB1 and B2, SUN2 mutant cell lines showed normal initiation of HP1-dependent repression in the absence of Dox (Figure 4B and 4C). Next, we tested how the *Oct4* reporter cell line would behave before and after treatment with Dox over time. Therefore, we measured our WT cell line on the iQue Screener Plus FACS machine after 14 days. We observed that the population of GFP negative cells is stably silenced (Figure 4A). Our results show that it is eligible to use the iQue Screener Plus FACS machine for our purpose.

To our surprise, both SUN2 KO's cell lines showed over the time course of two weeks an increase in reactivation. When we measured on day 7 we recognized a GFP

positive population in both cell lines. In the SUN2 #D7 we saw 17% and 36% in the SUN2 #H7, respectively (Figure 4B and 4C). Interestingly, the effect was even stronger after 14 days. The SUN2 #D7 cell line reached 32% of GFP positive cells (Figure 4B). In the SUN2 #H7 the reactivating population was even bigger with 45% and a separation between GFP negative cells and positive cells was clearly visible on the FACS plots (Figure 4C). These preliminary data showed us that SUN2 can influence heterochromatin maintenance at the *Oct4* locus.

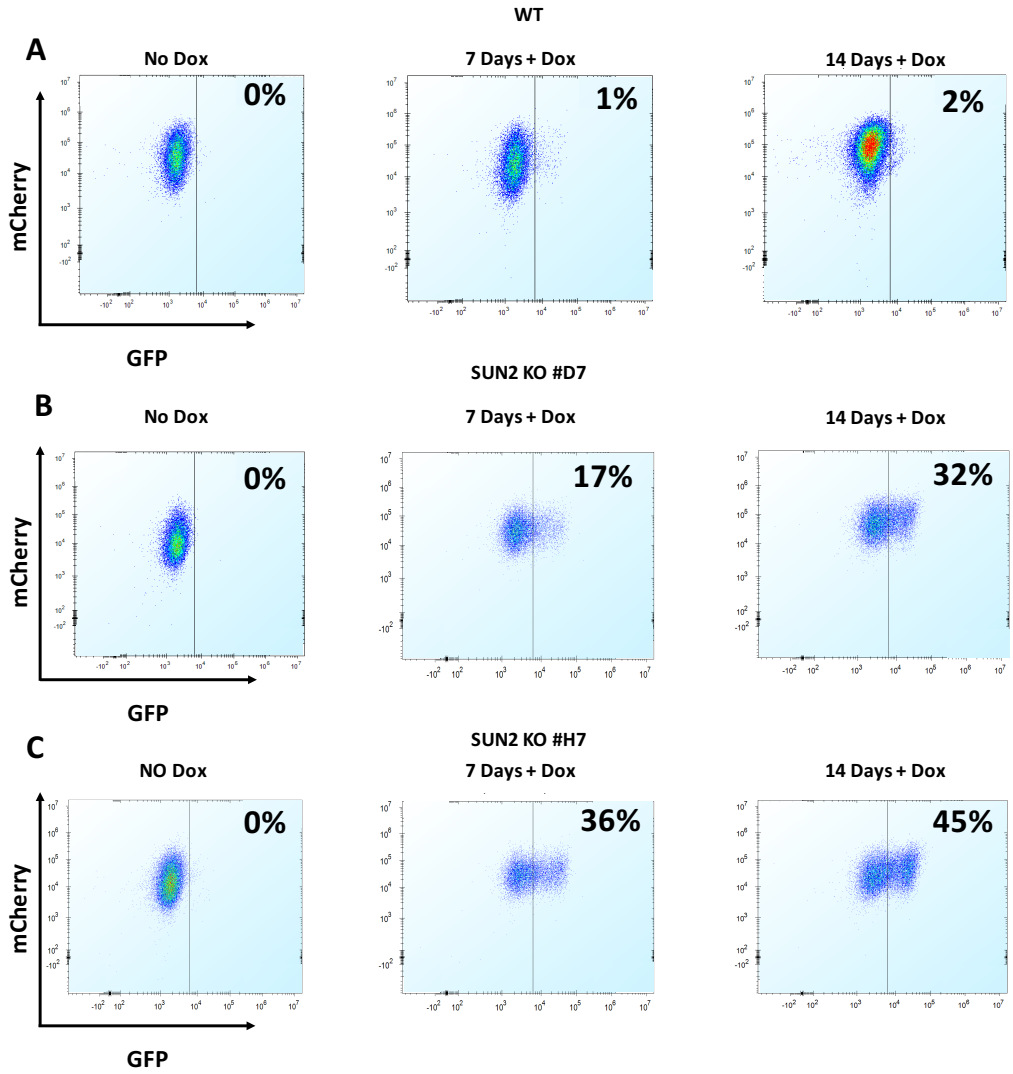

Figure 4. Loss of SUN2 disrupts heterochromatin maintenance.

A) Flow cytometry plots show GFP expression during HP1α recruitment without Dox as well as after 7 and 14 days Dox-dependent reversal in the *Oct4* reporter cell line (WT). B) Flow cytometry plots show no, 7 and 14 days of Dox treatment of the SUN2 KO cell line #D7. C) Flow cytometry plots show no, 7 and 14 days of Dox treatment of the SUN2 KO cell line #H7. % indicates a fraction of GFP-positive reporter cells. All data were collected on the iQue Screener Plus FACS machine n: 15.000 counts/plot

Surprisingly, a different outcome emerged when we repeated the Dox treatment one month later. Important to mention, we switched to the LSR Fortessa FACS machine. On day 7, we detected in both KO's less reactivation compared to the early time point measurement. This tendency continued also on day 14. The #D7 cell line reached 13% of GFP positive cells and the #H7 16% (Figure 5A and 5B). The control sample, WT showed expected GFP reactivation levels (Figure 5A and 5B).

Taken together, our results indicate that a complete loss of SUN2 at the INM promotes a reactivation of our *Oct4* reporter locus. Nonetheless, we further recognize the initial effect of disrupting heterochromatin maintenance is reduced after an extended period of culturing which may be linked to compensatory mechanisms.

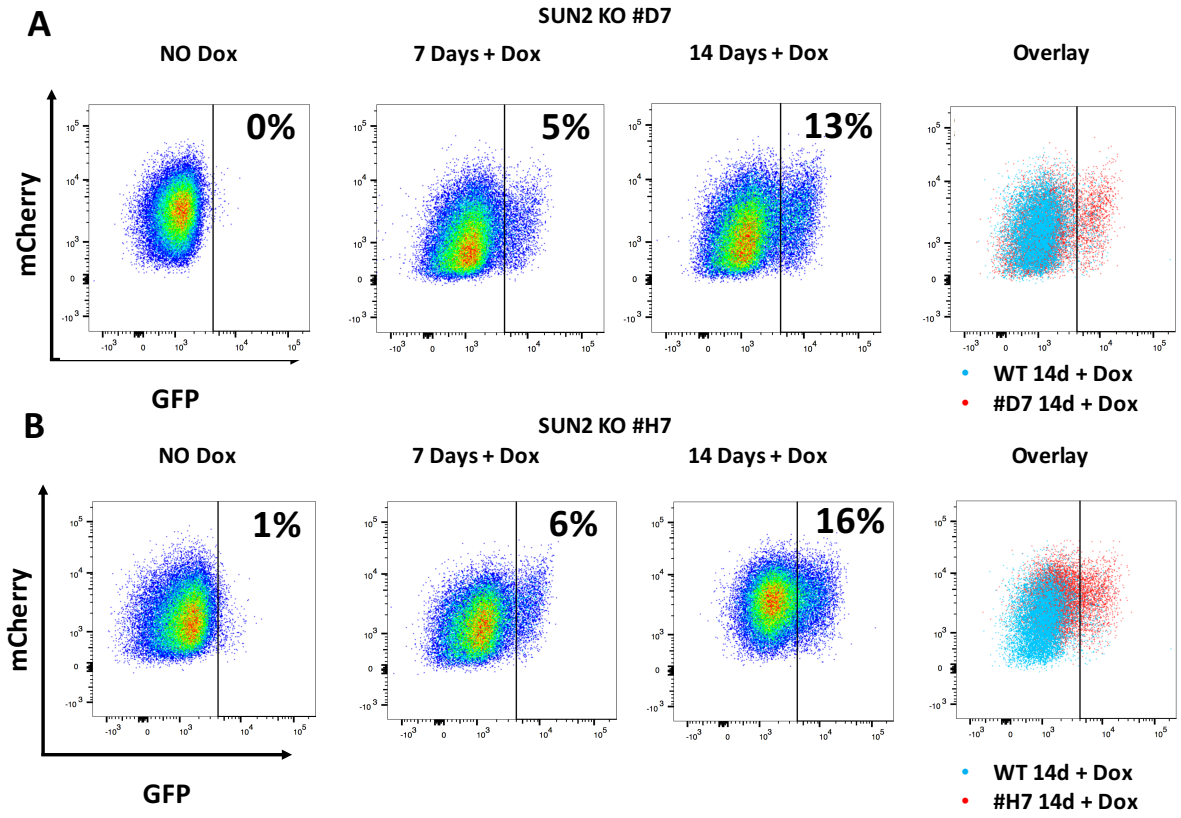

Figure 5. Reduced maintenance of Oct4 reporter gene repression after extended culture

A) and B) Flow cytometry plots show no, 7 and 14 days of Dox treatment. The overlay plots present in blue 14 days of Dox treatment of WT as control and in red the SUN2 cell lines, respectively. All data were collected on the LSR Fortessa FACS machine. % indicates a fraction of GFP-positive reporter cells. n: 50.000 counts/plot

2.5 SUN2 mutants have a dominant negative impact on heterochromatin maintenance

Although we observed that the negative effect of SUN2 KO's on maintenance decreases over time, we wanted to test the impact of SUN2 truncation on heterochromatin maintenance in our reporter system. The nucleoplasmic part of SUN2 is most likely a functional domain which interacts with chromatin or proteins at the nuclear periphery. The gRNA in these mutants was designed for this specific region. Therefore, we propose that our truncated versions of SUN2 have an altered amino acid sequence. These changes could result in abnormal chromatin interactions.

GFP expression was fully repressed in SUN2 mutants #E5 and #A4 in the context of HP1α tethering suggesting that initiation of heterochromatin is independent of SUN2 interactions. While WT reporter cells showed normal GFP reactivation levels at less than 5%, 7 days of Dox treatment led to more than 20% reactivation in both SUN2 mutant cell lines (Figure 6A and 6B). Reactivation was further increased after 14 days of Dox treatment reaching up to 58% in mutant #E5 and 55% in mutant #A4 (Figure 6A and 6B). Importantly, an extended culturing did not affect the observed phenotype (data not shown).

In summary, the reporter system revealed that SUN2 can play a role in heterochromatin maintenance. Based on our results, we conclude that SUN2 KO's have a stronger initial effect which over time reduces. In contrast, SUN2 mutants which harbour a deletion or a different amino acid sequence in their nucleoplasmic domain have a stronger dominant negative influence on maintenance. The reactivation remains over time on a maximum level around 50%.

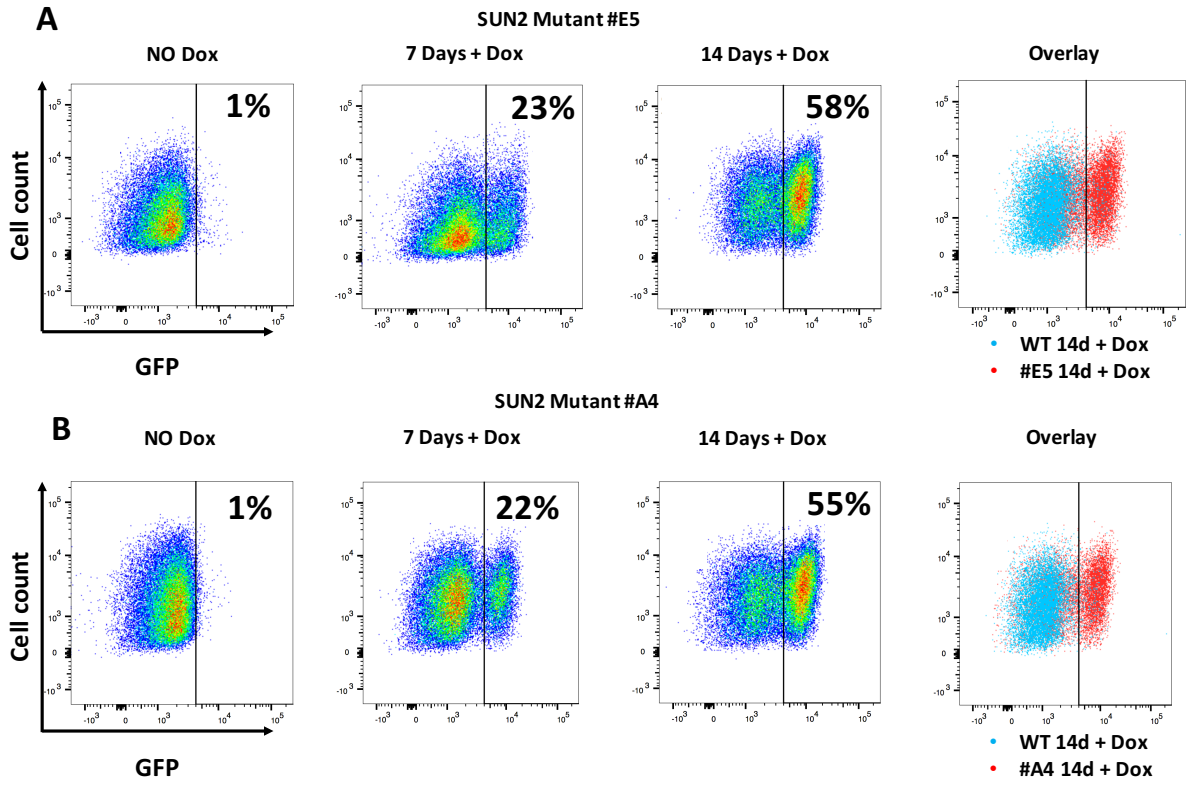

Figure 6. Truncated SUN2 mutants have a stronger negative effect on heterochromatin maintenance.

A) and B) Flow cytometry plots show no, 7 and 14 days of Dox treatment. The overlay plots present in blue 14 days of Dox treatment of WT as control and in red the SUN2 cell lines, respectively. All data were collected on the LSR Fortessa FACS machine. % indicates a fraction of GFP-positive reporter cells. n: 50.000 counts/plot

2.6 SUN2 mutations do not alter the heterochromatin marks

We sought to identify a global effect of SUN2 on heterochromatin, independent from our reporter system. Accordingly, we tested WT and SUN2 cell lines in an immunofluorescence assay for differences in markers of heterochromatin, H3K9me2 and H3K9me3. In addition, we co-stained with 4′,6-Diamidin-2-phenylindol (DAPI) and an antibody against LMNB proteins. DAPI is a fluorescent stain and binds specifically to DNA and resulted in a clear staining of the cell nuclei (Figure 7A). The shape and

size were not altered in loss-of-function and truncated SUN2 mutants (Figure 7E and 7I). Furthermore, DAPI staining is more intense in DNA dense areas, correlated with heterochromatin (Figure 7A, 7E and 7I). In addition, the anti-LMNB-antibody stained very specifically lamins at the nuclear envelope (Figure 7C, 7G and 7K). H3K9me3 was enriched in specific areas within the nucleus (Figure 7B). The overlay of DAPI and H3K9me3 images revealed a very high co-localization between DNA dense areas and H3K9me3 marked regions (Figure 7D). Interestingly, in the WT as well as in the SUN2 KO and mutant cell lines we detected a similar signal of H3K9me3 (Figure 7F and 7J). We neither saw a reduction in intensity nor a different shape (Figure 7H and 7L).

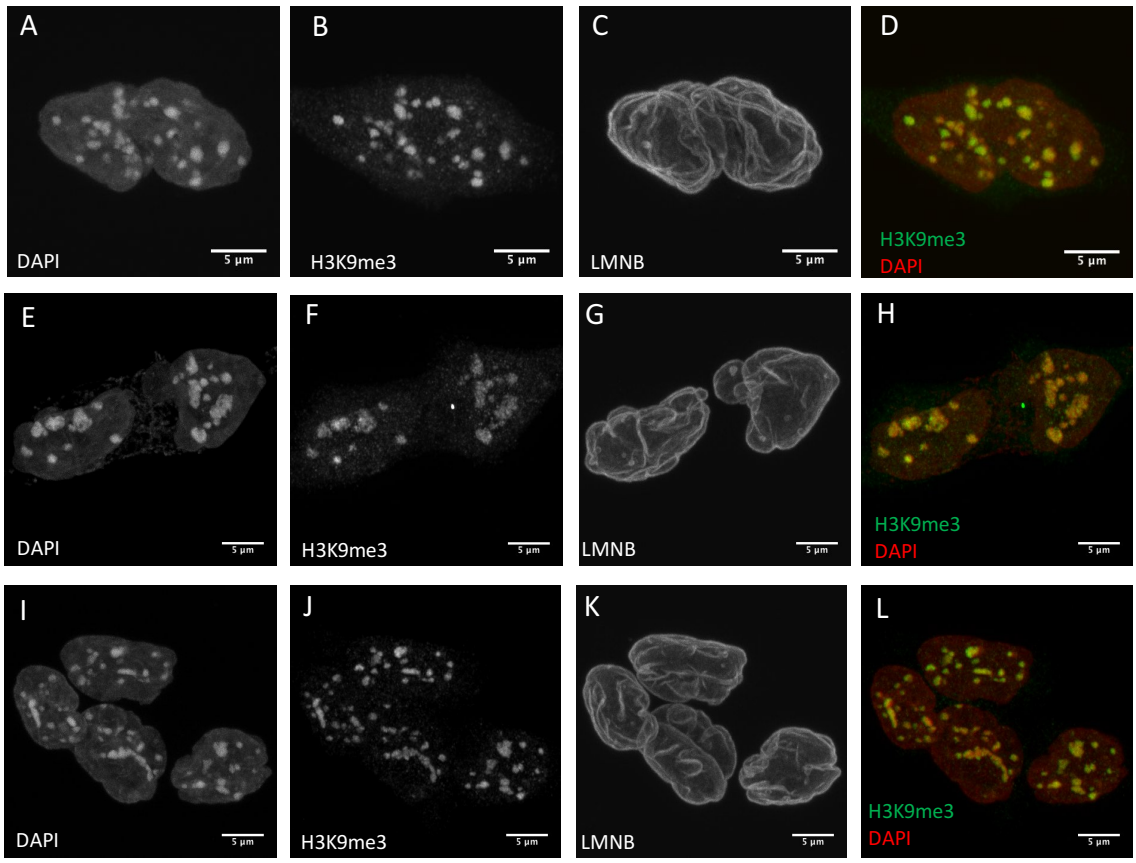

Figure 7. SUN2 KO's and mutants have no global effect on the heterochromatin mark H3K9me3. A) – D) Representative pictures of WT *Oct4* reporter cell line. E) – H) Representative pictures of SUN2 KO cell line. I) – L) Representative pictures of truncated SUN2 mutant cell line. A), E) and I) DAPI staining of mESCs nuclei. B), F) and J) Immunofluorescence staining of H3K9me3. C), G) and K) Immunofluorescence staining of LMNB proteins. D), H) and L) merge of DAPI and H3K9me3 immunofluorescence staining. All pictures are maximum intensity Z-projections.

Previous studies revealed that H3K9me2 specifically marks chromatin associated with the nuclear lamina in mESCs (Poleshko *et al.*, 2017). Therefore, we tested our cell lines for H3K9me2 marks (Figure 8). We co-stained with DAPI and antibodies against LMNB proteins. DAPI stained again specifically nuclei and DNA dense areas (Figure 8A, 8E and 8I). Further, the LMNB staining showed a clear signal for lamins (Figure 8B). The shape and intensity of LMNB proteins were not changed in loss-of-function and truncated SUN2 mutants (Figure 8F and 8J). The H3K9me2 signal was distributed in the whole nucleus (Figure 8C). To our surprise, the SUN2 KO and truncated cell lines showed a similar pattern of H3K9me2 (Figure 8G and 8K). An overlay of LMNB and H3K9me2 images revealed that H3K9me2 marked regions are enriched at the nuclear periphery (Figure 8D, 8H and 8L).

Together, we did not find differences in H3K9me3 or H3K9me2 suggesting that globally heterochromatin structure and distribution remains unaffected by SUN2 dysregulation.

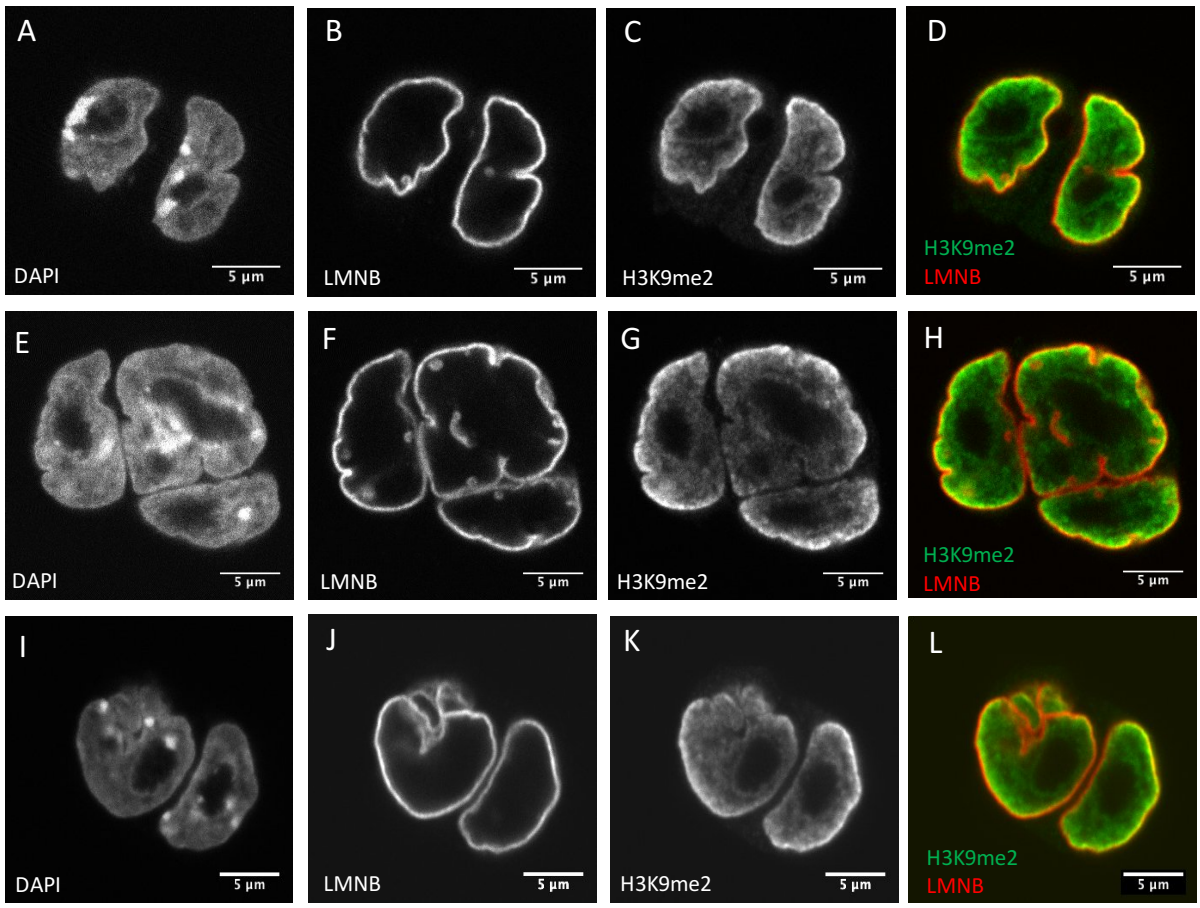

Figure 8. Loss-of-function and truncated SUN2 mutants have no effect on the peripheral heterochromatin mark H3K9me2.

A) – D) Representative pictures of WT *Oct4* reporter cell line. E) – H) Representative pictures of SUN2 KO cell line. I) – L) Representative pictures of truncated SUN2 mutant cell line. A), E) and I) DAPI staining of mESCs nuclei. B), F) and J) Immunofluorescence staining of LMNB proteins; the staining pattern is highly specific for the nuclear envelope. C), G) and K) Immunofluorescence staining of H3K9me2. D), H) and L) merge of H3K9me2 and LMNB immunofluorescence staining. All pictures are single plane images.

3 Discussion

It is known for over 50 years that heterochromatin is closely attached to the nuclear lamina. However, the relevance of this interaction for initiation and inheritance of gene silencing through a cell division is still controversial.

To investigate the contribution of the nuclear periphery to regulate heterochromatin we analysed mutants of INM components in a chromatin *in vivo* assay. CRISPR-Cas9 mutations of DNMT1 established that using this paradigm we are able to identify regulators of heterochromatin gene silencing in a candidate approach (Figure 1). Here we focussed on the roles of LMNB proteins and SUN proteins in heterochromatin regulation (Figure 2).

LMNB proteins are prominent components of the nuclear envelope. We decided to focus on LMNB proteins because in mESC these lamins are more abundant than LMNA/C proteins. Here we generated loss-of-function and hypomorphic mutants of LMNB1 and B2 (Figure 2). Initiation of silencing was not affected in either mutant cell lines (Figure 3). Similarly, loss of LMNB2 alone also did not change heterochromatin maintenance (Figure 3). This is consistent with previous studies reporting that a loss of all types of nuclear lamins which results in LAD detachment and altered chromatin interactions did not affect heterochromatin and gene expression (Kim, Zheng and Zheng, 2013; Zheng *et al.*, 2018). Hence, other proteins may compensate for the loss of LMNB2. In contrast, we found a LMNB1 mutant expressing a truncated version shows a dominant negative such that maintenance of silencing is significantly reduced in the reporter assay (Figure 3). The reactivation was similar to DNMT1 loss-of-function.

Currently, the effects of disrupting LMNB proteins are controversial. For

example, in human cell culture lines a downregulation of LMNB proteins resulted in apoptosis (Harborth et al., 2001). In contrast, a loss of B-type lamins in mESCs showed no obvious phenotype (Kim et al., 2011). There are two known human laminopathies associated with B-type lamins (Gruenbaum and Foisner, 2015). On the other hand, *LMNA* is frequently mutated with over 500 reported mutations (Vlcek and Foisner, 2007). Why the number of reported mutations differ so strong remains poorly understood. Thus, while a loss of lamins can be compensated, lamin mutations, like it is often the case in A-type laminopathies, severely disrupt heterochromatin maintenance.

SUN proteins are transmembrane proteins of the LINC complex. Their N-terminus is placed within the nucleus and can interact with chromatin and proteins at the nuclear periphery. SUN2 was not required for the initiation of silencing (Figure 4 and 5). However, SUN2 KO cell lines showed significant reactivation of heterochromatic gene silencing in the presence of Dox (Figure 4). This effect was attenuated over time suggesting compensatory mechanisms (Figure 5). Nonetheless, this is the first demonstration that SUN2 specifically contributes to an epigenetic inheritance of heterochromatin possibly by sequestering the reporter gene to the periphery (Figure 6).

Future experiments using Chromatin-IP against lamin proteins could test whether reporter gene interaction at LADs is affected by SUN2 mutations. As a next step, a genome-wide RNA sequence analysis in loss-of-function and truncated SUN2 mutants could give insights on the global changes of heterochromatin. If maintenance is globally impaired then repetitive elements, such as transposons, may be affected.

This study identifies for the first time the influence of the nuclear periphery on heterochromatin maintenance in an *in vivo* assay. These findings open many future questions.

First, can other factors at the nuclear lamina contribute to maintenance in our reporter system? In detail, can we find new proteins which lead to an increased reactivation? Which proteins can compensate for these effects?

Second, what is the biological significance of our assay? Do these proteins act on heterochromatin on a global level? Can we recapitulate the reactivation phenotype on the reporter genome-wide? Are we introducing an artificial effect based on our reporter design?

Finally, what are the molecular mechanisms for heterochromatin maintenance at the nuclear periphery? How do proteins interact with chromatin to stabilize gene repression?

To gain knowledge in all these questions will be an important future research perspective.

4 Materials and methods

4.1 Generation and culture conditions of mESCs

All mESCs used in this project were derived from haploid mESCs (Elling *et al.*, 2011). mESCs were cultivated without feeders in high-glucose-DMEM supplemented with 13.5% fetal bovine serum (Sigma), 10 mM HEPES pH 7.4, 2 mM L-Glutamin (Gibco), 1 mM sodium pyruvate (Sigma), 100 U penicillin/ml (Sigma), 0.1 mg streptomycin/ml (Sigma), 1x non-essential amino acids (Sigma), 50 mM beta-mercaptoethanol (Gibco) and recombinant LIF (37°C, 5% CO_2). For an attachment of the mESC, plates are coated with gelatin, ultrapure water with 0.1% gelatin (Millipore), for 30min at 37*C. Cells were passaged every 2 to 3 days and media were changed daily. Reversal of TetR fusion protein binding was achieved by the addition of 1 µg/ml doxycycline (final - Sigma, D9891) to ES cell culture medium.

4.1.1 Construct design and delivery

Knock out and mutants in the *Oct4* reporter cell line were obtained by CRISPR-Cas9 technology. CRISPR-Cas9 guide RNAs were designed using the online tool of the Zhang lab (http://crispr.mit.edu, Zhang, MIT 2013) and cloned in a modified lentiviral CRISPR-Cas9 expression vector expressing the guide RNAs by a U6 promoter and a wildtype hSPCas9 with either a Thy1.1 marker or a blasticidin selection marker.

All constructs were generated as lentiviral plasmids. Lentivirus was produced by polyethylenimine (PEI) co-transfection of the desired construct and two packaging vectors VSV-G (addgene #8454) and psPAX2 (addgene #12260) in HEK293T cells.

After 48-72 hours, the virus was collected. mESCs were then transduced with the virus for 48 hours in the presence of 8 µg/ml polybrene (Santa Cruz Biotechnology, SACSC-134220). After infection, mESCs were selected for an uptake of the desired construct. To achieve a homogeneous clone which carries the desired mutation, mESCs were plated sparsely on a 15cm plate. After 10 days of growing, single mESCs gave rise to homogeneous colonies. These colonies were picked and cultured for genotyping and further experiments.

CRISPR-Cas9 editing: The *SUN1* gene we targeted in Exon 3 with a single gRNA. For the *SUN2* gene we targeted Exon 2 with a single gRNA and Exon 2, Exon 3 with two gRNAs. The *LMNB1* gene we targeted in Exon 4 with a single gRNA and for the *LMNB2* gene we targeted Exon 2 with a single gRNA.

Table 1. List of plasmids

Plasmid #	**Description**	**target sequence**
1	Cas9 Thy1/APC/Cye7 LMNB1 sgRNA	GATTGAGTATGAGTACAAGC
2	Cas9 Thy1/APC/Cye7 LMNB2 sgRNA	AGCCAGCTCTGACTCGTACA
3	Cas9 Thy1/APC/Cye7 SUN1 sgRNA	AACAGCTTCGTACAGCAGTGGGG
4	Cas9 Thy1/APC/Cye7 SUN2 sgRNA	GCGCCTCACTCGCTACTCTCAGG
5	Cas9 BLAST SUN1 sgRNA	AACAGCTTCGTACAGCAGTGGGG
6	Cas9 BLAST SUN2 sgRNA	GCGCCTCACTCGCTACTCTCAGG
7	Cas9 BLAST LMNB1 sgRNA	GATTGAGTATGAGTACAAGC
8	Cas9 BLAST LMNB2 sgRNA	AGCCAGCTCTGACTCGTACA
9	Cas9 Thy1/APC/Cye7 SUN2 dgRNA 1	GCTCCAGGCCCGCCAGGGCG
10	Cas9 BLAST SUN2 dgRNA 2	GGGCAACGTATAAGGTGTGG

4.2 Genotyping of mESC

DNA from mESCs was obtained by lysis in Proteinase Kinase Buffer: (final: 10mM Tris-HCL, pH 7.5, 10mM EDTA, 10mM NaCl, 0.5% sarcosyl, fresh added 1mg/ml Proteinase K) at 55*C overnight. Afterwards, cells were treated with cold NaCl/Ethanol (final: 75mM NaCl ice cold in 100% ethanol, also fresh prepared) for min. 2h at RT, the DNA should precipitate. Then 3 wash steps with cold 70% Ethanol and air dried for min. 3h. Finally, the DNA was dissolved in TE buffer (final: 10 mM Tris-HCL, pH 8.0, 1 mM EDTA)

Genotyping PCR was performed with the corresponding forward and reverse primer, genomic DNA and an in-house Taq DNA Polymerase mix. All PCR products were loaded on agarose gels and band products were analyzed by Sanger sequencing.

For the interpretation of the raw sequencing data from two alleles with different mutations we used the published online software tool: Tracking of Indels by DEcomposition (TIDE) (Brinkman *et al.*, 2014). With this program, we were able to separate the traces from both alleles.

Table 2. Primers used for Genotyping

Primer	**5'-3' sequence**
MS LMNB1 geno sgRNA_sense	ACAAGACCCTGCCTGCATAC
MS LMNB1 geno sgRNA_antisense	ACACGTCCTCCCTCAGCTCT
MS LMNB2 geno sgRNA_sense	CCCCATTTCAACACCACAGT
MS LMNB2 geno sgRNA_antisense	TGAGGCTTTGGAGAAAAGGA
MS SUN1 geno sgRNA_sense	TCCATGACTCGGGTTTTCTC
MS SUN1 geno sgRNA_antisense	GCCATCTCACACACATCACC

MS SUN2 geno sgRNA_sense	ACCACACCTGTCCACAGTCA
MS SUN2 geno sgRNA_antisense	AAGGGAGCCATGCATATGAG
SUN2geno exon1and2_sense	TCTTCTGAGGCCTTGCTCTC
SUN2geno exon1and2_antisense	AGCAGTGCCTGTCAAATGTC

4.3 Flow cytometry analysis and sorting

Flow cytometry analyses were conducted either on an iQue Screener Plus (Intellicyt) or a LSR Fortessa (BD Biosciences) using ForeCyt or FlowJo software for data analysis. For fluorescent cell sorting a FACS ARIA III (BD Biosciences) was used. Selection of transgene expression by Thy 1.1 required surface staining with a Thy1.1 specific antibody. After incubation in PBS containing 1 % FBS with Fc-blocking antibody at 1:500 (Affymetrix eBioscience Anti-Mouse CD16/CD32 Purified) for 5 min at RT, mESCs were treated with Thy 1.1 antibody (Affymetrix eBioscience Anti-Mouse/Rat CD90.1 (thy-1.1) APC-eFluor 780) at 1:750 for 30 min.

4.4 Western blot

Nuclear extract from 10 x 10^6 mESCs was obtained by lysis in Buffer A (final: 25 mM Hepes pH 7.6, 5 mM MgCl2, 25 mM KCl, 0.05 mM EDTA, 10 % Glycerol, 1 mM DTT, 1 mM PMSF, 1x Complete Mini protease inhibitor) followed by collection in RIPA buffer (final: 150 mM NaCl, 1% triton, 0.5% sodium deoxy- cholate, 0.1% SDS, 50 mM Tris pH 8.0). Nuclear extracts were homogenized by sonication in a Diagenode Bioruptor and concentration was determined by Bradford assay (Biorad). 20-

40 µg/lane total protein was run on Novex Life Technology NuPAGE 4-12 % Bis-Tris gels in Invitrogen NuPAGE MES SDS Running Buffer and transferred on a Merck Chemicals Immobilon-P Membrane (PVDF 45 µm). The membrane was blocked (5 % non-fat dry milk in 1x PBS, 0.1 % Tween 20) and incubated with the following primary antibodies (5 % non-fat dry milk in 1x PBS, 0.1 % Tween 20):

Anti-Lamin B1 antibody - Nuclear Envelope Marker # ab16048 (Abcam)

Lamin B2 Mouse Monoclonal Antibody (clone E-3) # 332100 (Thermo Fisher Scientific (Life Tech))

SUN2 antibody [EPR6557] # ab124916 (Abcam)

DNMT1 Antibody (60B1220.1) # NB100-56519 (Novus Biologicals)

Finally, the membrane was incubated with corresponding secondary HRP coupled antibodies (5 % non-fat dry milk in 1x PBS, 0.1 % Tween 20), developed using Clarity Western ECL Substrate (Biorad) and imaged by a ChemiDoc XRS+ Imaging system (Biorad).

4.5 Immunofluorescence-microscopy

mESCs were grown on glass coverslips for immunofluorescence assays. For an attachment of mESCs, coverslips were coated with gelatin for 30min at 37*C. mESCs were plated on these coverslips, incubated o/n at 37*C, so that cells could settle and grow on coverslips. For fixation, we used our mESCs Medium + 4% Formaldehyde; 16% formaldehyde solution (ThermoFisher) dilute 1:4 in warm fresh medium. Blocking was performed for 1h at RT in the dark in blocking buffer (10% normal donkey serum + 1x PBS +1% BSA+ 0.3% TritonX). Then we applied diluted

primary antibody (1x PBS+1% normal donkey serum + 1% BSA + 0.3% TritonX) directly on coverslips and incubated o/n at 4°C in a wet chamber:

Lamin B1 Antikörper (B-10) # sc-374015 (Active Motif)

Histone H3K9me2 antibody (pAb) # 39239 (Active Motif)

Histone H3K9me3 antibody (pAb) #39162 (Active Motif)

Finally, the coverslips were incubated with corresponding secondary fluorophore-conjugated antibodies (1x PBS+1% normal donkey serum + 1% BSA + 0.3% TritonX +DAPI) for 1h at RT in the dark in a wet chamber.

Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate # A-21206 (Thermo Fisher Scientific), Donkey anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 647 conjugate # A-31571 (Thermo Fisher Scientific)

Last step, the coverslips were transferred on a microscope slide in mounting medium and seal with clear nail polish.

All images shown for comparison were acquired with a scanning confocal Zeiss LSM 880 AiryScan confocal microscopes equipped with a highly sensitive GaAsP detector (Zeiss), and a 63x/1.4 plan-apochromat Oil DIC objective (Zeiss). Lasers for confocal imaging were Laser Diode 405 25mW, an Argon 458, 488, 514 30mW, a DPSS 561 15mW and a HeNe 633 5mW.

Single plane and maximum intensity Z-projections images were processed using the open-source software Fiji.

5 Abbreviations

cec-4	Chromo domain-containing protein 4
CO_2	Carbon dioxide
Cas9	CRISPR associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
ChIP-seq	Chromatin immunoprecipitation followed by sequencing
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DNMT1	DNA (cytosine-5)-methyltransferase 1
Dox	Doxycycline
EDTA	*Ethylene-diamine-tetraacetic acid*
FACS	Fluorescence-activated cell sorting
GFP	Green fluorescent protein
gRNA	Guide RNA
H3K4me3	Histone 3 lysine 4 trimethylation
H3K9me2	Histone 3 lysine 9 dimethylation
H3K9me3	Histone 3 lysine 9 trimethylation
H3K27me3	Histone 3 lysine 27 trimethylation
HIV	Human immunodeficiency virus
HP1	Heterochromatin protein 1
HP1α	Heterochromatin Protein 1 alpha containing only the chromo-shadow domain fused to FLAG-TetR-mCherry
KASH	Klarsicht, ANC-1 and Syne homology domain
kb	Kilobase
LAD	Lamina associated domain
LBR	Lamin B receptor
LINC	Linker of the nucleoskeleton and cytoskeleton complex
LMNA/C	Lamin A and Lamin C
LMNB	Lamin B
Mb	Megabase
mESCs	Mouse embryonic stem cells
NE	Nuclear envelope
Oct4	Octamer-binding transcription factor 4
ONM	Outer nuclear membrane
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PNS	Perinuclear space
RNA	Ribonucleic acid
SUN	Sad1 and UNC-84 domain
TIDE	Tracking of Indels by DEcomposition
Tris	Tris(hydroxymethyl)aminomethane
Xist	X-inactive specific transcript

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