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Rapid Changes in Genome Organisation During Exit from Pluripotency and the Role of the Nuclear Envelope in Maintaining the Pluripotent State

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Declaration

I declare that this thesis is composed by myself and the work presented herein is my own, except where stated otherwise. This research has not been submitted for any other degree except the degree of Doctor of Philosophy at the University of Edinburgh.

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Lay Summary

Stem cells undergo a process called differentiation to give rise to the different types of cells that make up a fully developed organism. During differentiation, changes in the set of genes the cells express drive the changes in morphology that make different tissues. There are two stages of this: first the cells stop expressing the genes that maintain them as stem cells; second, they start expressing different sets of genes unique to each of the tissues they will become. The curious question is this – how do different cells come to express different genes, considering they all start from one single cell and also contain the same genetic information?

Cells have evolved many mechanisms to regulate gene expression thereby achieving the ability to tightly control which proteins are expressed in a certain cell type. One of these regulatory mechanisms, is the folding and positioning of DNA itself in 3D space within the nucleus, where the genome resides. The nucleus has a double membraned structure, called the nuclear envelope, defining its boundaries. This envelope acts as giant scaffold, interacting with certain parts of the genome to anchor them, thereby freeing up the space inside the nucleus to allow for DNA-DNA and DNA-protein interactions that are important for cellular functions. Typically, genes that are expressed are found in the nuclear interior and those that need to be silenced are stowed away at the nuclear periphery, anchored to the nuclear envelope.

The Schirmer lab has found that most of the proteins comprising the nuclear envelope are distinct for different tissues. Many of these tissue-specific nuclear envelope proteins direct the tissue-specific patterns of genome organisation and this in turn helps specify what genes are expressed in that particular tissue. This project is focused on the idea that even earlier, as a stem cell starts to differentiate to give rise to the different cell types, a similar change in nuclear envelope proteins directs genome organization and this is one of the mechanisms by which gene expression is controlled.

While we know that genome organization changes during differentiation, we do not yet know the earliest stages of this or how quickly genome organisation changes once differentiation begins. This thesis demonstrates that during the differentiation of mouse embryonic stem cell, one of the first changes in genome organization occurs with the repositioning of the *Rex1* locus from the nuclear interior, where the gene is expressed, to

the nuclear periphery. This repositioning occurs within the first hour of differentiation and is even reversible for the first few hours as cells cease to be stem cells. The cells continue to express REX1 protein for several hours after the gene is anchored at the nuclear periphery, suggesting that the repositioning of the gene is only one of the multiple steps required to shut the gene down. Interestingly, the composition of proteins in the nuclear envelope also changes in this same time frame with certain proteins starting to get expressed only after differentiation is induced in these cells. These new proteins and/or modifications such as phosphorylation on existing nuclear envelope proteins might be responsible for the anchoring of the *Rex1* locus to the nuclear periphery.

This thesis also demonstrates that when tissue specific nuclear envelope proteins like NET39, TMEM120A and TAPBPL are introduced in stem cells, these cells cease to be stem cells and start to differentiate. However, not all tissue specific proteins are able to tether the *Rex1* locus to the periphery, suggesting that different nuclear envelope proteins act as specific anchors for different parts of the genome. Together, these studies provide an insight into the dynamics of genome organization and the role of the nuclear envelope in establishing this organization during early stages of differentiation required before an organism can develop.

Abstract

The majority of Nuclear Envelope Transmembrane Proteins (NETs) are tissue specific and many of these facilitate tissue-specific genome organization. Genome organization changes dramatically during differentiation and these NETs impact this process: muscle-specific genome-organizing NETs NET39, WFS1 and TMEM38A are important for myogenesis (Robson et al, 2016) while fat-specific genome-organizing NETs TMEM120A and B are important for adipogenesis (Batrakou et al, 2015). Although during lineage specification of mouse embryonic stem cells (Peric-Hupkes et al, 2010), we do not yet understand the temporal dynamics of these changes nor the components of the nuclear envelope that orchestrate these changes during early stages of exit from pluripotency. In this thesis, I investigate the temporal dynamics of genome organization changes during pluripotency exit stimulated by LIF withdrawal. Using Fluorescence in-situ Hybridization (FISH) to label DNA, I demonstrate that some of the earliest changes in genome organization occur within the first hour of exit from pluripotency with the relocation of a locus containing three genes *Triml1*, *Triml2* and *Zfp42* (that encodes REX1, a well-known marker of pluripotency) from the nuclear interior to the nuclear periphery. The RNA and protein levels of these genes persist for several hours post exit, suggesting that reorganisation of the genome is among the very first of events occurring during lineage specification and is perhaps a higher order mechanism controlling differentiation as a change in genome organisation could affect the transcriptional profile of these cells. To try and identify the proteins involved in tethering the locus and the mechanism of release I also investigated the changes in the nuclear envelope composition as cells undergo an exit from pluripotency. I show that while certain proteins undergo post translational modifications such as phosphorylation, other new proteins are synthesised during the first two hours of exit. Using phospho-null mutants for LBR and LAP2α, I show that these play a role in the relocation of this genomic locus. Finally, I introduced tissue-specific genome-organizing NETs such as NET39 (muscle), TAPBPL (blood) and TMEM120A(fat) into embryonic stem cells and found that their introduction causes a forced exit from pluripotency. Interestingly, these NETs show specificity in their ability to affect the position of genomic loci encoding pluripotency factors like *Rex1* and *Nanog*, strengthening the idea that these tissue specific

NETs act as tethers to very specific genomic regions in order to maintain a tissue specific genome organization.

The results discussed here present for the first time, a temporal view of the changes in genome organisation during such early stages of *in vitro* differentiation. While *Rex1* repositioning has been studied in greater detail in this thesis, a more comprehensive study over the early stages of exit might reveal additional genomic loci that reposition during this phase. The rapid reorganisation of the genome following LIF withdrawal highlights the importance of tightly controlling and maintaining appropriate culture conditions for the study of pluripotency using embryonic stem cells as a model system. The study leads to conceptual advancement in stem cell biology by describing early events following exit from pluripotency and in the field of nuclear biology by identifying the NE composition in ES cells. Collectively the results demonstrate the role of the nuclear envelope in the maintenance of pluripotency and in orchestrating genome organisation changes during exit.

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Abbreviations

AC	Astrocytes
BAC	Bacterial artificial chromosome
bp	Basepairs
ChIP	Chromatin immunoprecipitation
cLAD	Constitutive lamina associated domain
CT	Chromosome territory
Da	Dalton
DamID	Dam methylase identification
DAPI	*4'.6'-diamidino-2-phenylindole*
DNA	Deoxyribonucleic acid
EDTA	*ethylenediaminetetraacetic acid*
EpiSC	Epiblast-derived stem cells
EPL	Primitive ectoderm like cells
ESC	Embryonic stem cells
FISH	Fluorescence in situ hybridisation
fLAD	Facultative lamina associated domain
GFP	Green fluorescent protein
h	Hour
HMGN	High mobility group N proteins
hPSC	Human pluripotent stem cells
ICM	Inner Cell Mass
IF	immunofluorescence
INM	Inner nuclear membrane
iPSC	Induced pluripotent cells
kb	Kilobase
lacO	Lac operator
LAD	Lamina associated domains
LBR	Lamin B receptor
LIF	Leukemia inhibitory factor

Mb	Megabase
min	Minutes
mRNA	Messenger RNA
NE	Nuclear envelope
NET	Nuclear envelope transmembrane proteins
NFW	Nuclease free water
NPC	Neural progenitor cells
NTS	Nick translation salts
ONM	Outer nuclear membrane
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
qRT-PCR	Quantitative real time PCR
RFP	Red fluorescent protein
RNA	Ribonucleic acid
SDS	Sodium dodecyl-sulfate
siRNA	Short interfering RNA
TAD	Topologically associated domains
U	Enzyme units
WB	Western blotting
ZGA	Zygotic genome activation

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Chapter 1

Introduction

The study of the nucleus dates back to 1831, when Scottish botanist Robert Brown first described the organelle in orchids while pioneering the use of light microscopes (R. Brown 1831). Following Brown's observation, a lively debate about the contents of the nucleus and its importance to life took prominence in the scientific community. It took about 100 years of work to establish that the nucleus is home to DNA and some proteins. Emil Heitz made one of the first significant observations on DNA compaction when he stained chromatin throughout various stages of the cell cycle. He reported that certain regions of the DNA stain more intensely than the rest and introduced the terms heterochromatin for the densely stained regions that remained compact during the cell cycle and euchromatin for regions that took up the stain lightly (Heitz 1928).

About 12 million bp of DNA is housed within a *Saccharomyces cerevisiae* nucleus (Jorgensen et al. 2007) and an even larger 3 billion bp genome resides in a human nucleus (Venter et al. 2001). It is by supercoiling and tight compaction that this vast amount of genetic material fits inside the nucleus. *This packaging must be achieved, however, without compromising the accessibility of DNA when it is required*. Furthermore, access to certain parts of the genome at a given time without compromising the status of the rest of it is key to regulating physiological processes. Therefore, it goes without saying that there are strict rules governing both the coiling and uncoiling of DNA. The DNA itself is arranged in the nucleus so that the heterochromatin is typically found in close proximity to the nuclear envelope and nucleoli while euchromatin dominates the nuclear interior. This was first observed in early electron microscopic images (Moses 1956). Since then genome organisation has been extensively studied with an increasing number of congenital and developmental disorders being linked directly or indirectly to disruption of this organisation.

In this chapter I will describe our understanding of non-random genome organisation followed by the discovery of tissue specific organisational patterns of genes and chromosomes. I will explain our present-day understanding of the hierarchical principles of chromatin folding and organisation into functional domains. I will then describe the role

of the nuclear envelope as a DNA-binding scaffold and elaborate on the specialised role of Nuclear Envelope Transmembrane Proteins (NETs) in facilitating tissue specific gene and chromosome positioning. I will describe the state of cells before they commit to lineages i.e. pluripotency and go on to provide a developmental time scale for changes in genome organisation, starting with how genome organisation in embryonic stem cells is different from that in adult cells and then delving into how dramatically this organisation changes during cellular differentiation. Specifically, I will talk about REX1, a well-known marker of naïve pluripotency, a state of pluripotency resembling the cells of the inner cell mass in a pre-implantation embryo, and summarise what we know about the protein and the organisation of the genetic locus that encodes this protein. I will elaborate on the state of embryonic stem cells as they undergo an exit from pluripotency and finally, I will integrate the various ideas discussed above to address the role of the nuclear envelope in orchestrating changes in genome organisation during differentiation and development.

1.1 The genome is non-randomly organised

Some of the earliest observations supporting the idea that DNA is not randomly floating about in the nucleus came from Carl Rabl at the end of the nineteenth century. During salamander cell division, he observed a clustering of centromeres on one side of the nuclear envelope, which presumably reflected the orientation of chromosomes from the preceding mitosis (Rabl 1885). However, it was Theodore Boveri who first proposed the idea that each chromosome visible in mitosis occupies a discrete and mostly non-overlapping territory in the nuclear space. He arrived at this conclusion from his observations of early stages of postzygotic development of *Ascaris megalocephala*, where he used protrusions of chromosome ends from prophase nuclei to mark out the order of chromosome territories (CTs) in interphase nuclei and continued to document the existence of these territories throughout mitosis, resulting in rather symmetrical arrangements of CTs in the daughter cells (Boveri 1909). In an unrelated study in 1979 where Zorn et al exposed small parts of the nucleus to UV microirradiation to study unscheduled DNA synthesis, they observed that the [^{3}H] thymidine incorporation into DNA could be often visualised on to individual chromosomes in the metaphase spread (Zorn et al. 1979). This further supported Boveri's theory about discrete CTs, though only providing indirect evidence. Irrefutable evidence proving the existence of these exclusive subdomains came to light in the 1980s when Fluorescence *in-situ* hybridization (FISH) was used to directly label CTs in human cells

with chromosome specific DNA probes (Manuelidis and Borden 1988) and eventually in plant cells with bacterial artificial chromosome FISH probes (Lysak et al. 2002). However, FISH only demonstrated the presence of CTs, yielding no insight into their arrangement.

The methods to analyse gene and chromosome positions have evolved over the years. To determine whether the distribution of CTs was random or non-random, it became important to define one or more reference points in the 3D nuclear space to score the position of each CT with respect to those reference points. These reference points were defined to consider two cases of non-random arrangement – radial arrangement and neighbourhood proximity arrangement. Radial arrangement was defined by dividing the nuclear space into a number of concentric shells (voxels) with equal volumes and scoring the likelihood of a CT being present in a particular voxel. Alternatively, measuring the distance of a CT to either the nuclear centre or the nearest point on the nuclear periphery or lamina could also be used to score the likelihood of a CT occupying a certain space in the 3D nuclear space. If a chromatin target was found with equal frequency in all voxels or at varying distances from the nuclear lamina, it would strongly argue for a random radial arrangement of the target. Neighbourhood proximity arrangement could be studied by scoring the distance between two chromatin targets of interest. If two chromatin targets always occupied neighbouring territories, then the distance between the two would remain fairly constant. However, defining chromatin targets themselves as reference points had a caveat in that it did not account for functional interactions of genomic loci and assumed that the territories were fixed in space, which of course is not true. Therefore, studying radial arrangement of CTs by measuring the probability of their occurrence in a certain voxel or by scoring their distance from the closest point on the nuclear periphery or lamina became the preferred method to study whether their arrangement was random.

Preferred nuclear locations for chromosomes were first suggested when it was observed that certain autosomes containing rDNA sequences (in humans these are chromosomes 13,14,15,21 and 22) participate in the formation of nucleoli and therefore remain associated with one another and are held in the same lateral orientation for several mitotic cycles (Bobrow and Heritage 1980). Eventually, 3D FISH experiments on human lymphocytes for chromosomes 18 and 19 confirmed non-random radial distribution of CTs. Chromosome 19 CT was consistently found in the nuclear interior while chromosome 18 preferentially occupied a territory close to the nuclear periphery. Interestingly this generally

correlates with the gene density of the chromosomes, with the gene rich chromosome 19 being in the nuclear interior and the gene poor chromosome 18 at the periphery (T. Cremer and Cremer 2001; M. Cremer et al. 2003; Croft et al. 1999). This gene density correlated radial distribution was documented for all chromosomes eventually (Boyle 2001). While such gene density correlated radial positions of chromosomes has been observed in other primates (Tanabe et al. 2002), radial organisation of territories is documented even in Hydra, suggesting that the radial positioning mechanism evolved at least 600 million years ago and has been evolutionarily conserved (Alexandrova et al. 2003).

Broadly speaking the CTs are maintained in a wide range of cell types. For instance, CTs for human chromosomes 18 and 19 are conserved in fibroblasts, keratinocytes, lymphocytes and epithelial cells from multiple tissues (M. Cremer et al. 2003; Murata et al. 2007; Boyle 2001). In mice, chromosome 14 is similarly positioned in lung and kidney cells (Parada, McQueen, and Misteli 2004). Similarly, porcine chromosomes 17, 13, 5 and X are similarly positioned in brain, lung and kidney tissues (Foster, Griffin, and Bridger 2012). However, certain CTs show a tissue specific arrangement. In freshly isolated mouse tissues, chromosome 5 CTs were preferentially found in the nuclear interior in hepatocytes, towards the periphery in small and large lung cells and in an intermediate position in lymphocytes, illustrating a tissue-specific spatial organisation of the genome (Parada, McQueen, and Misteli 2004).

Furthermore, a striking example of chromatin reorganisation during terminal differentiation was shown in mammalian retina adapted to nocturnal life. In the nuclei of rod cells of such mammals, all the heterochromatin is concentrated in the interior with euchromatin at the periphery (Solovei et al. 2009). It has been suggested that this might allow the nuclei to act as collecting lenses to channel minimal light for nocturnal mammals based on theoretical modelling simulations (Błaszczak, Kreysing, and Guck 2014). Such global nuclear reorganisation necessitates a profound reorganisation of radial chromatin arrangements rather than a change in just the CT proximity patterns.

The observation that genome organisation could be tissue specific led to increased curiosity as to why there might be a need for this and whether gene or chromosome positions could affect gene expression.

1.2 Genome organisation regulates gene expression

A gene density correlated CT position pattern hinted at the possibility that gene expression might be affected by gene or chromosome position itself. It is tempting to argue that parts of the genome that are tucked away at the nuclear periphery had sterically compromised accessibility and therefore would have reduced or even silenced gene expression. On the contrary, genes in the nuclear interior are accessible to the transcriptional machinery and therefore would be actively transcribed.

This argument was supported by the finding that peripheral heterochromatin is enriched in histone modifications that are associated with transcriptional repression, namely histone H4 lysine 20 di methylation (H4K20me2), histone H3 lysine 9 di methylation (H3K9me2) and histone H3 lysine 9 trimethylation (H3K9me3). This same peripheral heterochromatin is also depleted in modifications that are seen in actively transcribing genes or those poised for transcription, namely histone H3 lysine 4 di methylation and trimethylation (H3K4me2 and H3K4me3), histone H3 lysine 9 acetylation (H3K9Ac) and histone H3 lysine 36 trimethylation (H3K36me3) (Kind et al. 2013; J. Yao et al. 2011; Hirano et al. 2012). Considering the nuclear periphery is a largely repressive environment, gene positions play a crucial role in determining transcriptional output, along with epigenetic changes and the expression of tissue-specific transcription factors. In context of gene positions, movements of gene loci to and from the nuclear periphery correlate with their cell type specific transcriptional activity. Several groups have worked on forced tethers of specific genomic loci to the nuclear periphery to ask if this tethering would lead to a transcriptional repression of the locus. Surprisingly, upon tethering, some genes showed transcriptional repression while others did not, suggesting that the nuclear periphery is not entirely incompatible with active transcription (Reddy et al. 2008; Kumaran and Spector 2008; Finlan et al. 2008). In addition, peripheral heterochromatin is enriched in histone marks associated with facultative heterochromatin such as H3K9me2, rather than those associated with constitutive heterochromatin like H3K9me3 (Kind et al. 2013). There are also examples where peripheral genes are expressed, rather than repressed, as a mechanism to hasten the export of transcribed mRNAs to the cytoplasm. For example, in budding yeast, many genes are seen to be recruited to the nuclear pore complexes when they are induced (Short 2016).

Together these pieces of information suggest that although the nuclear periphery is not incompatible with transcriptional activity, it does act largely as a repressive environment, sometimes facilitating the formation of facultative heterochromatin so that genes that need to show cell type-specific or physiological state-dependent activation may move away from the periphery for increased expression. Building on the same idea, one might speculate that higher organisms have evolved to establish tissue specific genome organisation patterns as a higher order regulatory mechanism to control tissue-specific gene expression. While combined data from FISH, gene expression analysis and epigenetic profiling of chromatin clearly demonstrates a relationship between gene position and gene expression, these studies are limited to observing a small set of genes at a time. To understand if these implications hold true on a global level, it became important to develop methods that gave high throughput readouts and helped in the determination of global genome organisation patterns. These in combination with global gene expression analyses yielded a more comprehensive understanding of gene position-gene expression relationships in responses to changes in physiological and developmental transitions. The following section will summarise our present day understanding of the hierarchical principles of genome organisation, as understood from studies focusing on global genome organisation patterns, starting with the most basic level of chromatin compaction to form nucleosomes to the higher order structures formed by further compaction of these nucleosomes into functional domains that facilitate long range interactions.

1.3 Hierarchical Principles of Genome Organisation

At its smallest scale, DNA is compacted into 10- and 30 nm fibres seen in electron micrographs, by the wrapping of 146 base-pairs of DNA around an octamer of histone proteins to form nucleosomes. These nucleosomes function to structurally compact the DNA but also control gene expression by controlling accessibility to DNA binding proteins and transcription factors. At the intermediate scale of several tens of kilobases to a few megabases, chromosome capture techniques (3C) have identified several *cis* and *trans* interactions (Dekker 2002; Lieberman-Aiden et al. 2009) thus illuminating local 3D folding of DNA into spatial domains. In 2012, three seminal studies using high-throughput 3C methods revealed that human, mice and Drosophila chromosomes are composed of one

megabase large, self-interacting regions (Dixon et al. 2012; Nora et al. 2012; Sexton et al. 2012). On an average, genomic loci within the same self-interacting domain contact each other 2-3times more frequently than those located in two different domains. These self-interacting domains came to be defined as Topologically Associated Domains (TADs).

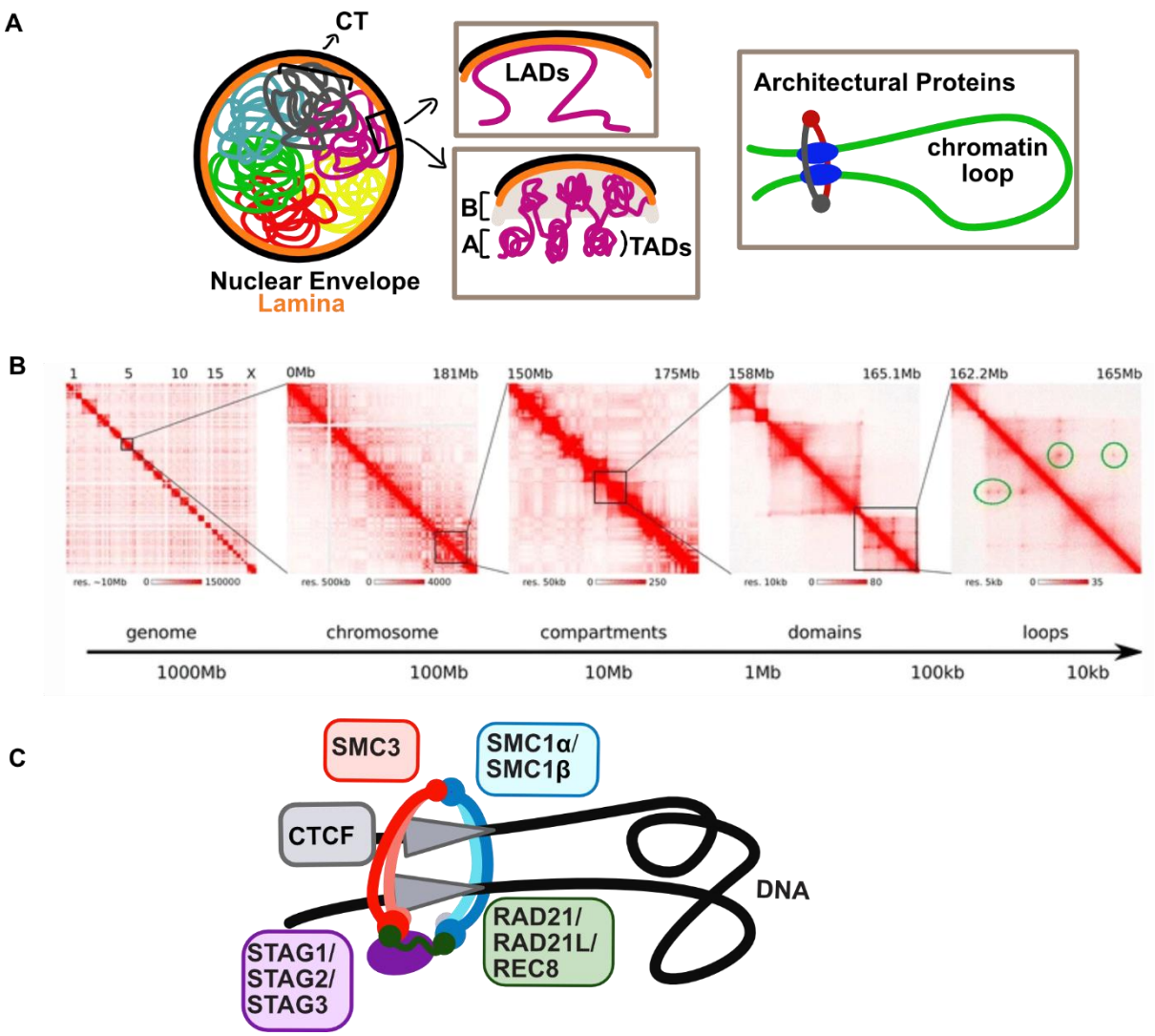

Figure 1: Hierarchical principles of genome organisation. A. The genome is non-randomly organised. Broadly, chromosomes occupy discrete territories (CTs) within the nucleus. At the periphery, the nuclear envelope anchors chromatin domains called Lamina Associated Domains (LADs). There are tissue-invariant LADs called constitutive LADs and other cell type specific LADs called facultative LADs. These LADs are typically repressed upon tethering. Chromosome domains are further organised into A and B compartments, which are enriched in transcriptionally active and inactive chromatin respectively. Local chromatin loops are stabilised by architectural proteins like CTCF and cohesin (Adapted from Sivakumar et al., 2019). B. HiC heatmaps for different scales of genome organisation from local loops that are a few kilobases to megabase domains which are further organised into compartments that make up whole chromosomes (reproduced from Szalaj and Plewczynski., 2018). C. Illustration showing the components of the vertebrate cohensin complex which acts in conjunction with CTCF, found at TAD boundaries (adapted from Sivakumar et al., 2019).

Unlike epigenomic compartments, TADs are not defined by chromatin states but by an elevated frequency of contacts within them. Functionally, TADs represent a particularly tissue-invariant scale of chromatin domains and are mostly conserved between different species (Dixon et al. 2012; Vietri Rudan et al. 2015). TADs themselves often have a nested and hierarchical configuration where smaller TADs can make up larger TADs and could be anywhere between ~40kb to ~3Mb with a median size of ~185kb. These smaller "loop domains" are special in that they can directly interact at their boundaries, thus forming a chromatin loop. In human cells, most loops were found to be stabilised by CTCF, an architectural protein binding to the CCCTC motif, which is also found at TAD boundaries (Rao et al. 2014). At the chromosomal scale, these TADs can be a part of multi-megabase interactions resulting in the formation of two spatial chromatin compartments, A and B, where greater interaction occurs within each compartment rather than across compartments. Interestingly, based on epigenetic modification, the spatial compartments also show a distinction in the type of chromatin occupying them such that the A compartment is predominantly comprised of actively transcribed chromatin while the B compartment is enriched in inactive chromatin (Figure 1A and B) (Lieberman-Aiden et al. 2009).

Classically described as an 'insulator', CTCF is a DNA binding protein with a central zinc finger domain flanked by largely unstructured C- and N-terminal domains whose function is poorly understood (Ohlsson, Renkawitz, and Lobanenkov 2001; Martinez and Miranda 2010). Currently the most supported biophysical model for loop extrusion suggests that at TAD and loop boundaries, CTCF functions with two subunits of a possible motor in a complex. In this model, the motor protein spontaneously engages chromatin and slides along the chromatin in opposite directions, either actively (Sanborn et al. 2015) or through diffusion (C. A. Brackley et al. 2018) to begin extruding a DNA loop. Loop extrusion then continues until the motor protein either spontaneously falls off or encounters an occupied CTCF binding site in the convergent orientation. Since the ring-shaped cohesin complex can remain topologically engaged and slide on both naked DNA and chromatin, this favours a linear tracking mechanism where cohesin is the motor protein. Vertebrate cohesin complex is a multi-subunit protein complex made up of a dimer of SMC proteins (SMC3-SMC1α/β) which is the core structural component. This forms a closed ring along with kleisin (RAD21/RAD21L/REC8) and STAG1/STAG2/STAG3 (Figure 1C). Disruption of

RAD21, a component of the cohesin complex resulted in a loss of loop domains without affecting the histone modification patterns, thus confirming that cohesin indeed is *at least one of the motor proteins* involved in loop formation. However, in the same study, a population of cohesin-independent loops was also observed to be frequently anchored around enhancer enriched genomic regions (Rao et al. 2017), suggesting that there might be other motor proteins or independent mechanisms at play. Similarly, targeted degradation of CTCF also results in a loss of TADs without affecting the segregation of the genome into A and B compartments, revealing that the compartmentalisation of mammalian chromosomes emerges independently of proper insulation of TADs (Nora et al. 2017). In conclusion, the roles of CTCF and cohesin in establishing genome organisation are starkly different. While cohesin seems to be the motor protein actively extruding loops, CTCF is a silent partner that is bound to its cognate sites marking the boundaries and waits for the loops to be extruded. In other words, while cohesin actively forms TADs, CTCF establishes TAD boundaries. Interestingly, by comparing evidences from single-molecule imaging, polymer simulation and Hi-C approaches, it has recently been suggested that TADs and loops are very likely dynamic structures that constantly form and break at a time scale of several minutes to tens of minutes in typical mammalian cells (Hansen et al. 2018). These dynamics might explain how the genome is reorganised during development and differentiation.

Although the loop extrusion model remains a hypothesis, it has proven to be remarkably resilient to both experimental testing and *in silico* simulations. Polymer simulations have shown the emergence of TADs and finer scale features of Hi-C data. In fact, the models have been able to explain a far wider array of observations such as why loops tend to not overlap and why the CTCF binding motifs at pairs of loop anchors lie in convergent orientations (Sanborn et al. 2015; Fudenberg et al. 2016). *In silico* modelling has also been used to test an alternate model for the establishment of spatial genome organisation that is CTCF and cohesin independent. Simulations studying DNA-DNA interactions, considering just two kinds of polymers – rigid ones representing heterochromatin and flexible ones representing euchromatin – showed a spontaneous segregation of heterochromatin to the nuclear periphery with euchromatin occupying the nuclear interior. This separation would also resemble the A and B compartments that the genome is split into (Cook and Marenduzzo 2009). With the addition of DNA binding proteins in the

simulation, a spontaneous aggregation of binding sites was observed that increased the local chromatin concentration. It is merely an extrapolation then that if there were proteins with bi- or multi-valency then they could effectively bridge out forming chromatin loops that will remain stable for as long as the factors are bound (Marenduzzo, Finan, and Cook 2006; Chris A. Brackley et al. 2016). In cells, transcription factors bind to DNA in a sequence specific manner and epigenetic marks may confer further specificity to such interactions. Such specific DNA-protein interactions might form specialised clusters effectively separated in 3D space. Thus, a new "transcription factor model" for the establishment of genome organisation has been proposed (Hnisz et al. 2017; Cook and Marenduzzo 2018). Since transcription is a common denominator in all kinds of organisms containing DNA as their genetic material, it seems that the transcription factor model might explain a first-degree organisation of the genome. However, architectural proteins like CTCF and cohesin confer stability to this order and might facilitate the dynamic reorganisation of the genome that is seen during development. Interestingly, SMC-condensin complexes, which are closely related to cohesin were recently found to extrude loops in both *B. subtilis* and *Caulobacter*, suggesting that this mechanism might be well conserved (X. Wang et al. 2017; Tran, Laub, and Le 2017). Furthermore, many components of the cohesin complex are evolutionarily conserved among eukaryotes with varying degrees of similarity, suggesting that the loop extrusion mechanism for establishing genome organisation might go back a long way (Sivakumar, de las Heras, and Schirmer 2019). It is thus likely that both proposed models work together in establishing genome organisation, with the transcription factor model explaining the establishment of specialised clusters and the loop extrusion model explaining the stability and dynamics of these clusters to facilitate differential interactions that are required to be established in response to various physiological stimuli. A large body of evidence suggests that TADs correspond to functional regulatory domains by primarily restricting promoter-enhancer contacts. The juxtaposition of promoters and enhancers by chromatin looping seems to be sufficient for gene activation (W. Deng et al. 2012). Specifically, enhancer-promoter pairing may determine the frequency at which transcriptional burst is initiated from a promoter (Bartman et al. 2016; Larsson et al. 2019). Thus, the main regulatory property of TADs is to delimit the genomic regions sampled by each locus to prevent the spurious activity of non-cognate enhancers in other TADs. Accordingly, deletion of a boundary between two TADs causes ectopic contacts and can

cause aberrant gene expression through improper enhancer-promoter contact (Flavahan et al. 2016). Loss of a TAD boundary between limb enhancers and the *wnt6* gene is sufficient to upregulate wnt6 expression and cause developmental defects in both mice and humans (Lupiáñez et al. 2015). This also suggests that since enhancers have limited intrinsic specificity for their target promoters, regulating contact probability with well-defined TAD boundaries appears to be crucial in regulation of gene expression. Interestingly, for the *Pitx1* gene that is important for hindlimb identity during mouse embryonic development, it was observed that the disruption of 3D organisation achieved by inversion of its enhancer element *Pen* leads to an ectopic *Pen-Pitx1* interaction in the forelimb and an ectopic patella formation in the arm (Kragesteen et al. 2018). Taken together, these studies illustrate how chromatin folding into TADs defines operational limits of regulatory landscapes and the importance of maintaining these boundaries and in turn the larger 3D genome organisation for normal physiological development (Robson, Ringel, and Mundlos 2019).

While TAD structures determine genome organisation within the nucleus, the nuclear envelope remains a major structural node that anchors a large amount of heterochromatin. In fact, it was in context of this structural scaffold that non-random gene positioning was first established. In addition to tethering away heterochromatin, the nuclear envelope also controls the release of specific genomic loci into the nuclear space to allow for its interactions with DNA and DNA-binding proteins. The next section discusses the nuclear envelope and its components in detail, highlighting the contribution of the Schirmer lab in understanding how tissue specific genome organisation may be brought about.

1.4 The Nuclear Envelope facilitates Tissue-Specific genome organisation

The Nuclear Envelope (NE) is a double membraned structure that encloses the eukaryotic nucleus which houses the genome. The outer and inner nuclear membranes (ONM and INM respectively) are interspersed by nuclear pore complexes which regulate transport between the nucleus and the cytoplasm. The ONM is contiguous with the endoplasmic reticulum and is connected to the cytoskeletal proteins. Communication between the cytoplasm and the nucleoplasm is established through protein complexes that span the luminal space between the two membranes, thus functionally connecting the ONM and the INM. Although the INM, ONM, nuclear pore complexes and the endoplasmic reticulum originate

from a continuous structure, they maintain their identities to a large extent through unique profiles of integral membrane associated proteins, together with specific lipid compositions and cholesterol (Ledeen and Wu 2004; Schirmer and Gerace 2005). In metazoans, the primary scaffold of the NE is provided by a fibrous protein meshwork called the lamina, composed of A type lamins (lamins A and C) and B type lamins (lamins B1 and B2). The B type lamins are expressed early in embryonic development and though their levels vary in different cell types, generally they persist ubiquitously throughout adult life. The A type lamins are thought to be expressed in a developmentally regulated manner. Thus, different cell types have different ratios of A and B type lamins and their isoforms (Broers et al. 1997).

The lamins are connected to the INM via many nuclear envelope transmembrane proteins (NETs). Although only a small number of NETs have been tested for their direct binding to the lamins, the Schirmer lab has identified that the NE of any given mammalian cell contains likely more than 100 different NETs. Most of these resist a detergent pre-fixation extraction that is characteristic of lamin-binding proteins (Korfali et al. 2010; Malik et al. 2010a; Wilkie et al. 2011). Comparing the nuclear envelope proteome between three different tissue types, namely liver, muscle and blood, revealed that under 20% of the total NETs are shared between the tissue types, highlighting that a vast majority of NETs are tissue restricted in expression (Korfali et al. 2012). Following on from this observation, several tissue specific NETs have been studied in greater detail in the Schirmer lab since. Muscle specific NETs NET39, TMEM214 and WFS1 (Robson et al. 2016), fat specific NETs TMEM120A and B (Batrakou et al. 2015; Czapiewski et al, unpublished data) and liver specific NET47 (Gatticchi et al. 2020) are just some examples of NETs that have been studied for their ability to regulate genome organisation in the respective tissue types. Such diversity in the NE proteome may potentially explain many previously unexplainable observations. For instance, complex disease pathologies like Emery-Driefuss muscular dystrophy have been traced to mutations in ubiquitous NETs like Emerin. However, the pathology manifests in select muscle types in the affected individuals and it hasn't been yet understood how a mutation in a ubiquitous NET might only affect the physiology of select tissues. Establishing that most NETs are tissue specific has opened up doors to investigating whether ubiquitous NETs like Emerin might act through tissue specific binding partners to carry out tissue specific functions.

Apart from being a barrier between the genome and the cytoplasm, the NE is also an important structural scaffold for tethering away a large amount of heterochromatin, thus laying the foundations for interphase chromosome topology. The development of LaminB1-DamID to construct maps illustrating global genome-NE interactions has been a milestone in establishing the importance of the NE in establishing genome organisation (Steensel and Henikoff 2000). DamID over many different cell types has revealed that certain lamina associated domains (LADs) are cell invariant (constitutive) while others are cell type specific (facultative), with their release from the NE often correlating with gene activation (Meuleman et al. 2013; van Steensel and Belmont 2017). The ability of LADs to allow or restrict certain TAD associations by constraining the extent to which genetic loci might diffuse into the nuclear space may be an important regulatory function of LADs beyond sequestration of repressed genes at the NE (Robson et al. 2017a). Although only a handful of NETs have been studied for their roles in maintaining genome organisation, the Schirmer lab has demonstrated several tissue specific NETs including muscle specific NETs like NET39 and TMEM38A, fat specific NET TMEM120A, blood specific NETs like TAPBPL and STT3A and the liver specific NET47, to have the ability to tether specific loci and even whole chromosomes to the nuclear periphery (Zuleger et al. 2013). Interestingly, tissue specific NETs were able to alter genome organisation even in a heterologous system (de las Heras et al. 2017). In fact, tethers established by NET47 are strong enough to reposition entire chromosomes to the periphery upon ectopic expression (Zuleger et al. 2013; Gatticchi et al. 2020). Thus, it seems that tissue restricted expression of NETs during development and differentiation plays a role in establishing tissue specific genome-NE tethers.

Muscle specific NETs like NET39, TMEM38A and WFS1 are expressed during the *in vitro* differentiation of myoblasts to myotubes. These NETs also direct specific myogenic genes like *Nid1* and *Cxcl1* to the nuclear periphery during myogenesis. Crucially, the NETs themselves seem to show specificity in which genetic loci they regulate, such that while *Nid1* repositioned in a NET39 dependent manner, *Cxcl1* was specifically regulated by WFS1. Finally, these muscle specific NETs were shown to be important for myogenesis itself, so that a knockdown of these NETs led to impaired myotube formation (Figure 2A; Robson et al. 2016). Similarly fat-specific NETs TMEM120 A and B were also shown to be important for adipogenesis (Figure 2B; Batrakou et al. 2015). Taken together these

studies suggest that the expression of tissue specific NETs during physiological development alters the genome organisation landscape significantly and thus tissue specific genome organisation patterns are maintained.

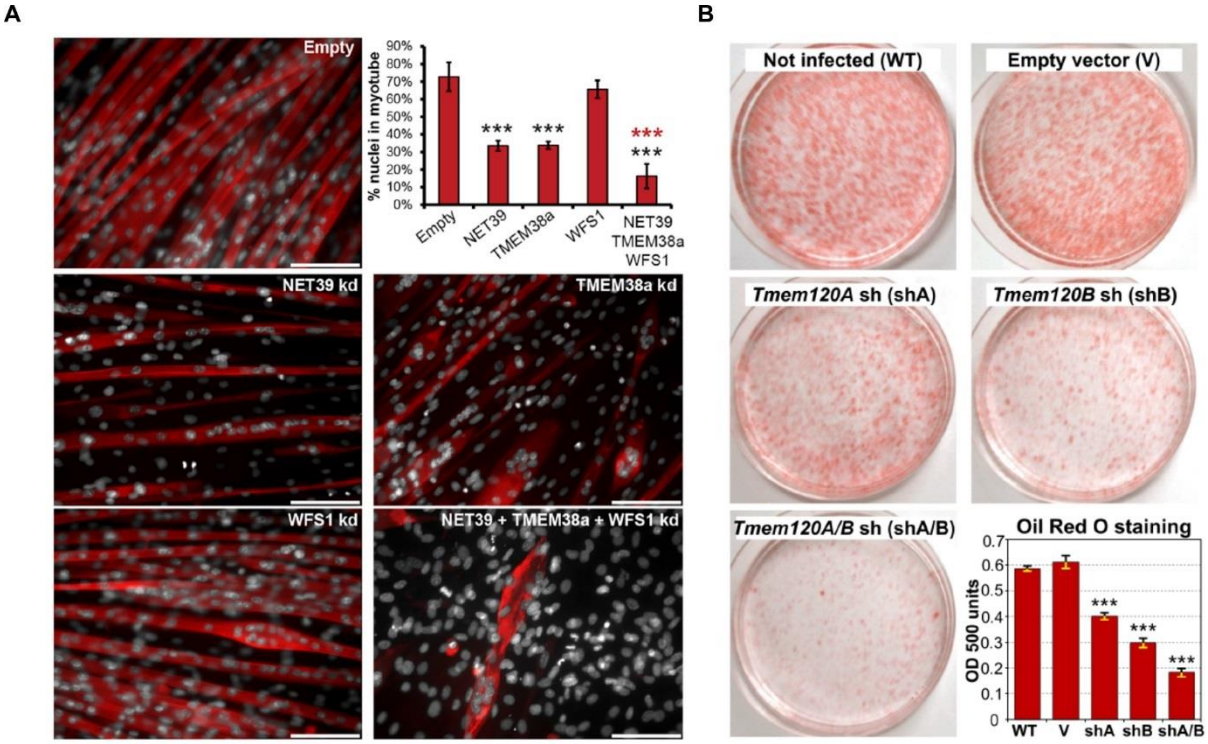

Figure 2: Tissue specific NETs are important for normal differentiation. A. Knockdown of muscle specific NETs TMEM38A, NET39 and WFS1 in C2C12 cells leads to significant impairment in myogenesis (Robson et al., 2016) B. Knockdown of fat-specific NETs TMEM120 A and B in 3T3L1 pre-adipocytes leads to reduced adipogenic potential (Batrakou et al., 2015). Figures are reproduced from the indicated publications.

Although we now seem to have some understanding of how tissue specific genome organisation might be maintained in lineage committed cells expressing tissue specific NETs, we do not yet know the answers to the following questions –

- ***How is genome organisation maintained before lineage commitment?***
- ***At what stage of development is tissue-specific genome organisation established?***
- ***What kind of physiological cues set in motion the changes in expression of the various NETs thereby affecting large scale genome organisation changes?***

Pluripotent embryonic stem cells from both mouse and humans have been used extensively in various studies to understand how genome organisation is different prior to lineage commitment. The following section discusses the idea of pluripotency, the culture

conditions for maintaining these embryonic stem cells and highlights the morphological and transcriptional changes as these cells exit pluripotency.

1.5 Maintenance of and Exit from Pluripotency

Soon after fertilization gives rise to a zygote, the first cleavage event occurs, giving rise to identical daughter cells and this marks the beginning of a series of events including cell differentiation, which eventually culminates in the formation of a fully developed organism. During mouse embryo development, the first cleavage events result in a loosely attached cluster of 8-16 cells called morula (from the Latin for mulberry, *Morus*). This cluster of cells is totipotent, with the cells being able to give rise to any cell type. Within hours, the morula undergoes compaction and cell polarisation accompanied by an intracellular rearrangement of microfilaments and basal localisation of mitochondria (Levy et al. 1986; Ducibella et al. 1977). The first differentiation event occurs in the blastula, when the outer layer of epithelial cells forms the trophoblast, that will eventually give rise to the placenta, and an inner cell mass (ICM) is yet unspecialised but constitutes the founder population of the pluripotent naïve epiblast cells which will give rise to the embryo proper (Figure 3). The second differentiation event separates the ICM into the hypoblast (primitive endoderm) and epiblast (ectoderm). Cells of the epiblast express three transcription factors – Oct4, Sox2 and Nanog – which form the core network of proteins that maintain their pluripotency (Yuan et al. 1995; Chambers et al. 2003). Soon after formation of epiblast and hypoblast the mouse (and human) embryo undergoes implantation into the uterus (Johnson and Ziomek 1983; J. Nichols and Gardner 1984; Rossant 1975). The cells of the epiblast proliferate at an extraordinarily rapid rate of 9 hours per cell cycle through adoption of a peculiar cycle with most cells cycling between the S phase and a short G1 phase (Solter, Škreb, and Damjanov 1971). During embryogenesis in mouse, at about 6.5 days post coitum, in a process called gastrulation characterised by the formation of the primitive streak, the cells progressively start differentiating to form one of the three embryonic lineages, ectoderm, mesoderm and definitive endoderm (M. Williams et al. 2012).

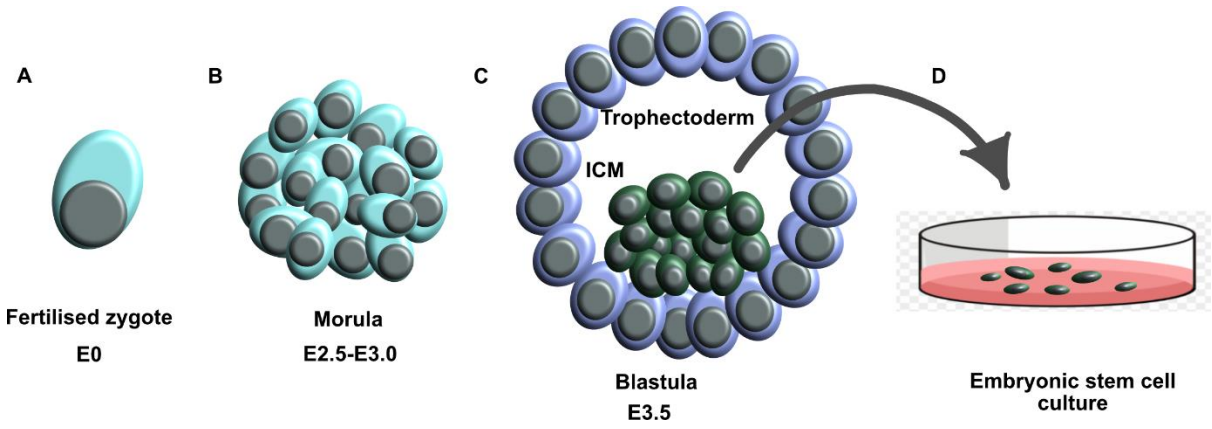

Figure 3: Illustration showing early stages of embryogenesis. The fertilised zygote goes through the initial cleavage to give rise to a cluster of cells called morula (A, B). Cells of the morula undergo further mitotic divisions to give rise to a Blastula (C). Cells of the blastula form two distinct populations. The outer layer of trophectoderm cells and the cells of the inner cell mass (ICM) which are the source of pluripotent embryonic stem cell cultures (D).

Pluripotency is the ability of a cell to act as the founder or stem cell for all embryonic and adult tissues along with the ability to self-renew. The epiblast cells of the pre-implantation embryo are the source of mouse embryonic stem (ES) cells (Martin 1981; Evans and Kaufman 1981). Like naïve epiblast cells, these ES cells, when put back into the blastocyst can undergo normal development and give rise to chimeric mice (A. Bradley et al. 1984). The post-implantation mouse embryos are a source of an alternative type of pluripotent cells, called epiblast-derived stem cells (EpiSC), which can be established under different growth conditions (Brons et al. 2007; Tesar et al. 2007). While these cells are unable to generate chimeric mice by blastocyst injection, they can show signs of early germ layer differentiation when grafted into post-implantation epiblasts (Huang et al. 2012). And likewise, Huang et al demonstrate that ES cells are incapable of contributing to chimeras when introduced into post-implantation embryos. Thus, the two pluripotent cell types can only integrate into the stage-appropriate embryonic environments. The different pluripotent characteristics exhibited by ES and EpiSC cells are called naïve and primed pluripotency.

When mouse ES cells were first established, the culture conditions were empirical and they were maintained by co-culture with growth arrested mouse embryonic fibroblasts (MEFs) (Robertson 1997). The discovery of LIF as a cytokine that was able to sustain the pluripotency of these cells in the absence of the feeder layer was the first step towards establishing a defined culture condition to maintain these cells *in vitro* (Smith and Hooper 1987; Smith et al. 1988; Williams et al. 1988). During embryonic development, Leukemia

Inhibitory Factor (LIF) and the LIF receptor are expressed by the trophectoderm and ICM of the blastocyst in a complementary manner (Jennifer Nichols et al. 1996). The ability of LIF to sustain pluripotency depends on the addition of serum to the medium. LIF is an interleukin 6 (IL-6) class cytokine that activates Signal Transducer and Activator of Transcription 3 (Stat3), a transcription factor that inhibits the differentiation of ES cells (H. Niwa et al. 1998; Matsuda 1999). Serum and LIF withdrawal leads to spontaneous differentiation of pluripotent cells into neurons (Ying, Stavridis, et al. 2003).

Phenotypically, ES cells in culture share many qualities with the epiblast cells, including a rapid cell cycle (Stead et al. 2002) and the expression of core pluripotency factors Oct4, Sox2 and Nanog. Gain of function experiments emphasise the importance of these core transcription factors in pluripotency. Nanog overexpression supports self-renewal even in the absence of LIF (Chambers et al. 2003), while ectopic expression of Oct3/4 and Sox2 with additional transcription factors Klf4 and cMyc is sufficient to reprogram embryonic and adult fibroblasts into pluripotent cells (Takahashi and Yamanaka 2006). Knock-down or knock-out of Oct4 and Sox2 in ES cells leads to a loss of pluripotency and differentiation. The loss of Nanog leads to reduced self-renewal ability but compromises cell viability (Ivanova et al. 2006; Mitsui et al. 2003; Chambers et al. 2003; 2007). Pluripotent ES cells do not grow as a homogenous population in serum/LIF culture conditions. Heterogeneity is observed in terms of expression of *Rex1, Stella, Nanog, Esrrb* and *Klf4* (Chambers et al. 2007; Hayashi et al. 2008; Toyooka et al. 2008; van den Berg et al. 2008; Hitoshi Niwa et al. 2009). Such heterogeneity could be attributed to batch variations in serum, and therefore efforts were made towards developing serum-free culture conditions.

Since simultaneous withdrawal of serum and LIF led to spontaneous neuronal differentiation, it was thought that inhibition of this process while withdrawing serum from the medium might help in establishing serum free conditions. To that effect, BMP4, a growth factor previously found to inhibit neuralisation of Xenopus embryo (Dale et al. 1992), was added to the medium in combination with LIF and was found to facilitate serum-free propagation of ES cells (Ying, Nichols, et al. 2003). Further studies revealed that the direct inhibition of FGF/ERK pathway, which triggers differentiation of ES cells, combined with a partial blockade of glycogen synthase kinase 3 (Gsk3) enabled ES cell propagation without BMP or LIF (Burdon et al. 1999; Ying, Stavridis, et al. 2003; Ying et al. 2008). Today, a widely adopted culture system for ES cells is the 2i/LIF condition, comprising the

addition of LIF along with two small molecule inhibitors that target MEK (PD0325921) and Gsk3 (CHIR99021), that ensures a largely homogeneous population of cells in terms of gene expression and morphology (Wray et al. 2011). This uniform condition of self-renewal is termed the 'ground state' (Ying et al. 2008). Transcriptome analysis comparing ES cells cultured in serum/LIF and 2i/LIF reveals that about 25% of the active genes show 2-fold or greater differences between the two conditions. Several ectodermal and mesodermal specification genes that are expressed in serum are repressed in 2i. Interestingly, ES cells transferred between the two culture conditions switch their transcriptional profile. It was also found that although serum induces a metastable state of cells within a heterogeneous population with regards to Rex1 expression, the Rex1 positive population of the cells retain naïve pluripotency and developmental potential (A. Smith 2010; Marks et al. 2012).

The analogy between ES cells and the epiblast has led to these cells being using widely as a surrogate for early epiblast to study and characterise the properties of stem cells and the various differentiation pathways. However, ES cells (extracted from e 3.5-4.5 mouse embryos) are different from the EpiSC (extracted from e6.0-e6.5 mouse embryos) in growth factor requirements, gene expression profiles, morphology, metabolism, DNA methylation patterns and X chromosome activation status (Jennifer Nichols and Smith 2009). Between these naïve and primed states of pluripotency, is a characteristically distinct phase where cells lose the expression of naïve state factors like Esrrb, Rex1, Klf4 and Klf2 and gain expression of transcription factors such as Otx2, Oct6 and Sox3, as well as maintaining core pluripotency factors like Oct3/4 and Sox2 (Hoffman, Wu, and Merrill 2013). During this period, global gene expression reveals that the transcriptome is significantly divergent from both naïve and primed pluripotency (Mohammed et al. 2017). Events in the window that captures the transition between naïve and primed pluripotency are poorly understood.

During *in vitro* differentiation, upon withdrawal of LIF or 2i, naïve ES cells embark on a path to lineage commitment. However, this is not a one-step process. Naïve ES cells do not respond to differentiation signals directly following 2i withdrawal (Mulas, Kalkan, and Smith 2017). Typically, for directed differentiation, pluripotent cells need to go through the development of embryoid bodies (EBs) by aggregation, which is a stage that expresses genes indicative of lineages from all three germ layers (Itskovitz-Eldor et al. 2000). Once

EBs are formed, they can then, in theory, be differentiated to any lineage of choice. The exit of naïve cells from their pluripotent state to form EBs and the associated change in gene regulatory networks is analogous to the formative phase observed during embryo development.

Spontaneous differentiation by LIF withdrawal leads to an exit from pluripotency in monolayer cultures where cells show a characteristic morphological flattening. Furthermore, gene expression profiles start to show divergence within the first week of LIF withdrawal with mesoderm and endoderm genes being upregulated at 7- or 14-days post LIF withdrawal and a concurrent downregulation in genes like Stat3 and Tbx3 which are known to be important in the maintenance of pluripotency (Heo et al. 2005). This suggests that most biological processes directing cell differentiation might be determined within the first two weeks during spontaneous differentiation. When ES cells were induced to form primitive ectoderm like (EPL) cells in culture by LIF withdrawal, it was seen that as cells exit from pluripotency over the first 6 days, they exit heterogeneously and show differential gene expression. While *Oct4* mRNA is sustained up to 6 days post LIF withdrawal, *Rex1* is downregulated 4 days post withdrawal and concurrently, *Fgf5* expression gradually upregulated, starting at 2 days post LIF withdrawal. *In situ* hybridisations in whole mouse embryos also shows a differential expression of genes like *Crtr1, Rex1, Psc1* and *Oct4*, which are all expressed in pluripotent ICM cells, as cells start committing to various lineages. For instance, *Crtr1* and *Rex1* were downregulated at ~4.75 days post coitum marking the stage of transition of pluripotent cells in vivo but this transition is accompanied by the maintenance of *Oct4* and *Psc1* expression and the upregulation of *Fgf5* (Pelton et al. 2002). During *in vitro* differentiation, at the protein level, REX1 is downregulated within 4 days post LIF withdrawal (S. Zhang et al. 2019). This suggested that *Rex1* is a naïve pluripotency marker.

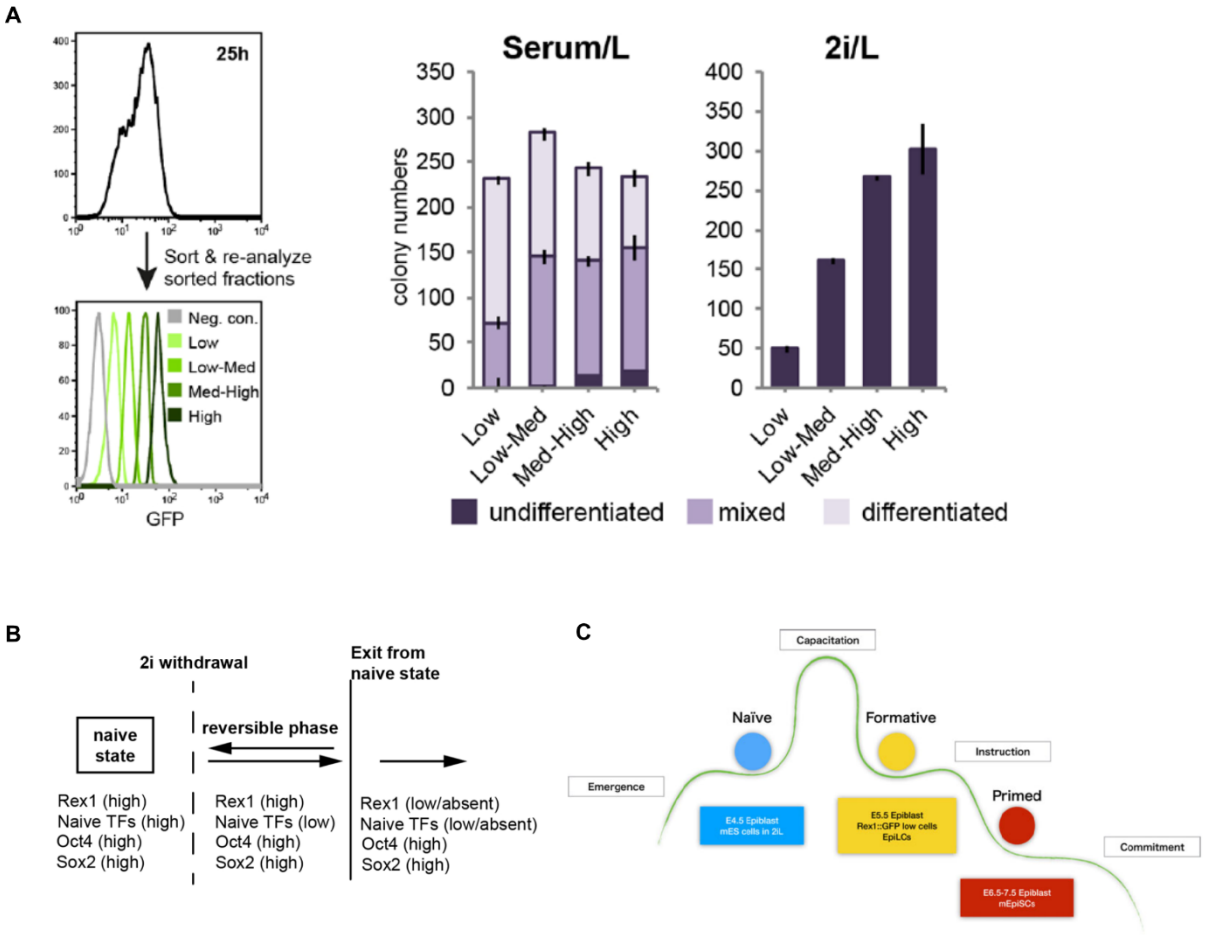

Figure 4: Exit from pluripotency is a multistep process. A. Cells exit pluripotency asynchronously. Rex1::GFPd2 cells subjected to 2i withdrawal for 25h show a heterogeneous population with cells expressing varying levels of GFP from low to high. When replated into serum/LIF or 2i/LIF culture conditions, cells expressing medium to high levels of GFP are able to form undifferentiated colonies (Kalkan et al., 2017). B. 2i withdrawal leads to an exit from naïve pluripotency characterised by high expression of Rex1, Oct4 and Sox2. There is a window of ~25h following 2i withdrawal where Rex1 expression is still high and the exit is reversible. Rex1 downregulation marks an irreversible exit from naïve pluripotency (Kalkan et al., 2017). C. Stages of exit resemble in vivo lineage commitment. ES cells cultured in 2i/LIF resemble e4.5 epiblast cells. These cells are unresponsive to differentiation cues. Upon 2i withdrawal, these cells exit pluripotency and go through a formative stage characterised by low Rex1 expression. This stage resembles the e5.5 epiblast cells. The introduction of differentiation cues at this stage leads to a primed state that resemble e6.5-7.5 epiblast cells that can then undergo lineage commitment (Kinoshita and Smith., 2018).

By knocking in a destabilised GFP to one of the Rex1 alleles, the Smith lab generated a new resource (Rex1::GFPd2 cells) for tracking the identities of naïve cells exiting pluripotency. Early stages of exit from pluripotency were monitored after withdrawal of 2i in serum free medium. Oct4 protein expression persisted up to 48 hours post 2i withdrawal,

while rare cells expressing Sox1 were first detected at 48h and eventually form large clusters by 72h. RT-qPCR for pluripotency genes at various time points during the first 48h post 2i withdrawal confirmed the persistence of Oct2. Other factors like Nanog and Esrrb show a downregulation within 9h post release and Rex1 and Klf2 by 16h post 2i release, suggesting that pluripotency factors display individual downregulation kinetics upon 2i withdrawal or that some are upstream of others and affect their transcriptional output. In the same study, flow cytometry also revealed that by 25h post release, Rex1 promoter activity is heterogeneously downregulated yielding a population ranging in GFP expression from low to high, indicating strongly at a heterogeneous exit of a stem cell population from pluripotency. Finally, by 48h, all cells lose GFP expression. Strikingly, when ES cells subjected to 2i withdrawal were sorted on the basis of GFP expression and re-plated in either serum/LIF or 2i/LIF culture conditions, they were able to reverse their state of exit and form undifferentiated colonies of cells. However, the degree of differentiation observed was proportional to the time the cells had spent in 2i release, suggesting that ES cells become increasingly committed as the 2i withdrawal is prolonged but for the first 16h of release, the exit is largely reversible. By 25h post 2i withdrawal, in the Rex1 heterogeneous population of cells, only those that retain GFP expression (i.e. Rex1 promoter activity) show self-renewal ability (Figure 4A; Kalkan et al. 2017). In a nutshell, exit from pluripotency due to 2i withdrawal is not a one-step process. Downregulation of naïve transcription factors alone is not sufficient to push these cells over the edge into lineage commitment. There is a window of ~25h hours when Rex1 promoter activity is retained (measured by GFP expression in these cells) and as long as this is so, they could always revert back to their undifferentiated state (Figure 4B). The cells that lose Rex1 expression are competent for multi-lineage specification and respond more rapidly to induction than either ground state ES cells or those that have high Rex1 expression post 2i withdrawal (Mulas, Kalkan, and Smith 2017). This seems to suggest that cells that have lost Rex1 expression are perhaps in the formative state of pluripotency and have undergone capacitation (Figure 4C). One could also argue that Rex1 downregulation led to the capacitation. Similar to mouse ES cells, human naïve pluripotent cells (hPSCs) also do not respond to differentiation cues. When they are capacitated by withdrawal of growth factors, they attain epithelioid morphology and undergo spontaneous differentiation in an asynchronous manner by day12 after withdrawal. The cells express the neuroectodermal

marker SOX1 and endodermal markers CXCR4 and SOX17 by 7 days post growth factor withdrawal suggesting that they attain capacitation and somatic lineage competence in this duration. However, the role of Rex1 in enabling this capacitation has not been studied yet (Rostovskaya, Stirparo, and Smith 2019).

Another intermediate state of cells reported within a heterogeneous ES cell culture is that of a 2-cell like embryonic stage. These are cells constituting less than 1% of an ES cell culture that resemble a 2-cell stage embryo in their transcriptional profile. Two-cell like cells seem to have greater developmental plasticity and greater nuclear reprogrammability than ES cells, they maintain *Oct4* mRNA levels and have very high levels of *Zscan4* transcripts. However, this state is not similar to the cells exiting pluripotency by *Rex1* downregulation that enter the formative state of pluripotency (Rodriguez-Terrones et al. 2018). On the other hand, culturing mouse ES cells in specific conditioning medium leads to them attaining a reversible conversion to an alternative pluripotent phenotype of EPL cells. These cells require LIF but marker expression indicates that they are distinct from both naïve ES cells and primed EpiSC. Interestingly, EPL cells may be induced by L-proline, which activates the mammalian target of rapamycin (mTOR) pathway (Washington et al. 2010), which has been previously implicated to play a role in naïve stem cell exit (Betschinger et al. 2013) with a knockdown leading to embryonic lethality (Murakami et al. 2004). Together, this suggests that the mTOR pathway might be important in the transition from naïve to formative phase of pluripotency. Interestingly, ES cell progression to lineage competence depends on two transcriptional repressors, TCF3 and RBPJ, and one activator, ETV5. Co-deletion of *Etv5* and *Tcf3* or deletion of *Rbpj* delays naïve state exit after 2i withdrawal as measured by *Rex1* expression. Elimination of all three factors traps ES cells in their undifferentiated state from which they can rarely exit, even in presence of strong differentiation stimuli. The triple knockout cells retain expression of naïve pluripotency factors such as Nanog, Klf4 and Oct4 and lack neuronal differentiation marks like Sox1 and Tuj1, in spite of being cultured in neuronal differentiation medium for 8 days (Kalkan et al. 2019). Finally, two of the three core pluripotency factors, namely Oct4 and Sox2, seem to undergo subnuclear retargeting during the transition from naïve to formative pluripotency. Within the first 48h following 2i/LIF withdrawal, these two factors form nuclear foci that colocalise with heterochromatic regions formed by large scale chromatin reorganisation to mediate differentiation associated gene silencing. This nuclear

compartmentalisation may change the localised concentration of the transcription factors in the nucleoplasm and might modulate the transcriptional output (Verneri et al. 2020).
In summary, pluripotency is classically considered to be the capacity of cells to differentiate into any lineage with no predetermination. However, we now know that this is not a one step process as naïve pluripotent cells do not respond to differentiation stimuli. It seems that the capacitation of pluripotent cells is a pre-requisite for multi-lineage differentiation. As cells are induced to differentiate *in vitro*, they first need to undergo an exit from pluripotency facilitated by LIF or 2i withdrawal. As the exit stimulated by withdrawal of LIF and 2i/LIF are not identical, it is important to study both systems. Exit from pluripotency comprises of a global reorganisation of transcription factor localisation and the gene regulatory network to attain a state that is neither similar to naïve pluripotency nor primed pluripotency. This formative phase is reversible for the first ~16 hours of exit and is characterised by the downregulation of *Rex1* (A. Smith 2017). In light of these findings, the next section reviews REX1 as a marker for naïve pluripotency, its contribution to maintaining pluripotency in both human and mouse ES cells and the genomic environment of *Rex1/Zfp42*.

1.6 REX1 – a marker of naïve pluripotency

One of the proteins highlighted above as being important for pluripotency is REX1 or reduced expression 1, which is a 37kDa zinc finger protein encoded by the *Zfp42/Rex1* gene (Gene IDs: mouse, 22702; human, 132625). The gene was first isolated and identified in F9 murine teratocarcinoma stem cells, where upon retinoic acid induced differentiation, the steady-state mRNA level of REX1 was rapidly reduced (Hosler et al. 1989). Using *in situ* hybridization to cellular RNA, REX1 was detected clearly in cells of the inner cell mass in a day 4.5 mouse blasctocyst (Figure 5A). In the same study, REX1 was also detected in undifferentiated ESCs and meiotic germ cells of the adult mouse testis (Rogers, Hosler, and Gudas 1991). During mouse embryo development, Rex1 is expressed in the pluripotent cells of the ICM until 4.75 when the cells start transitioning towards lineage commitment and thereafter Rex1 is detected at very low levels in the extra embryonic ectoderm in a day 5.25 embryo following which it is entirely lost (Figure 5B; Pelton et al. 2002). Subsequently REX1 was detected in several mouse and human ESCs (Richards 2004; Carpenter et al.

2004; Sato et al. 2003), CD34 positive hematopoietic stem cells (Goolsby et al. 2003), human multipotent stem cells isolated from the bone marrow (D'Ippolito 2004) and CD133 positive umbilical cord blood cells (Baal et al. 2004), certain cultured human cancer cells and even human teratocarcinoma cells (Mongan, Martin, and Gudas 2006). In fact, in mice, REX1 expression is several folds higher than median value in the ES cells while it is negligible in the ~60 other tissues including adult stem cell progenitors (Figure 6). Together this suggested that REX1 was a marker for multipotency and pluripotency and REX1 has been mostly studied as such.

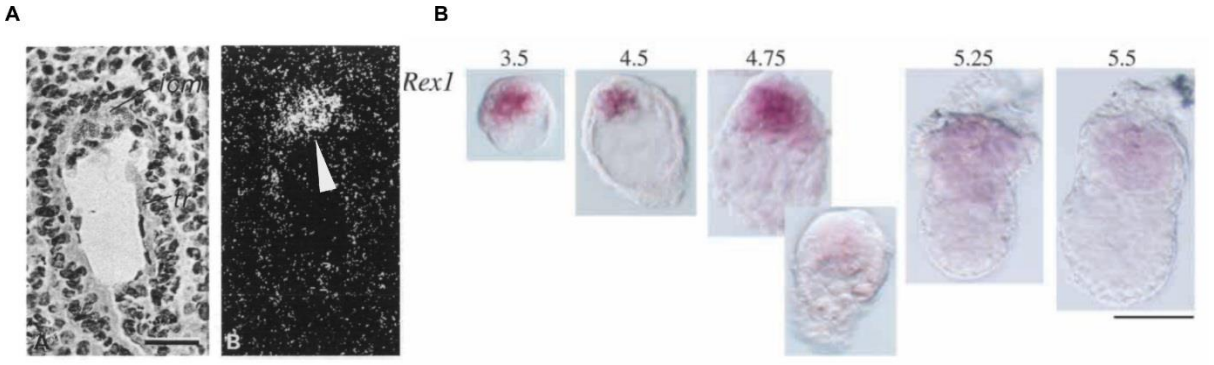

Figure 5: ICM cells show high Rex1 expression. A. Light field micrograph of an e4.5 mouse embryo stained with hematoxylin-eosin (left) and a dark-field illumination of the same section hybridised to the Rex1 probe (right). The silver grains (white dots) show Rex1 expression in cells of the inner cell mass (ICM) that are the source of pluripotent ES cells in culture (Rogers, Hosler and Gudas., 1991). B. ICM cells show high Rex1 expression in e3.5, 4.5 and 4.75 embryos. As these cells under lineage commitment Rex1 expression is downregulated (Pelton et al., 2002).

Although REX1 was identified independently due to its stark expression profile showing a downregulation upon differentiation of F9 cells, sequence comparison showed that it is related to YY1 (Yin Yang 1), a well characterised and evolutionarily conserved transcription factor (J. L. Brown et al. 1998; Y. Shi, Lee, and Galvin 1997; Sui et al. 2004). The amino acid sequence similarity between REX1 and YY1 transcription factors is limited mostly to the zinc finger domains, where the identity is about 75%. Considering that all four zinc finger domains of YY1 bind the DNA major groove (Houbaviy et al. 1996), it was proposed that REX1 is also likely to bind to the major groove (Mongan, Martin, and Gudas 2006). Another mammalian gene that is related to YY1 is YY2, which seems to be a retroposed copy duplicated from YY1, based on its intron-less structure and location within the intron of Mbtps2, an X chromosomal gene (Nguyen et al. 2004; Luo et al. 2006). A comprehensive analysis of all YY1 related sequences identified from the genome

sequences of both vertebrates and invertebrates shows that REX1 and YY2 evolved rather recently and are found only in placental mammals. The zinc finger domains of YY2 and REX1 have been under different selection pressures than YY1, leading to a divergence in the sequences thereby weakening their DNA binding affinity and even changing the DNA binding motifs for REX1 (J. D. Kim, Faulk, and Kim 2007). The structural differences along with the germ cell specific expression of REX1 suggests a potential placental-mammal specific functional role for the protein.

The promoter element of *Rex1* contains an octamer motif – ATTTGCAT – which acts as a binding site for core pluripotency-related octamer transcription factor members of the POU domain family of DNA-binding proteins. This element isolated from the endogenous gene was able to promote the expression of bacterial lacZ gene in mouse embryos in morula, showing the first sign of *in vivo* evidence for its activity (Hosler et al. 1993). ES cells express three members of the POU family of transcription factors: Oct3/4, its alternative spliced form Oct5, and Oct6, in addition to the ubiquitously expressed Oct1 (Schöler et al. 1989; Okamoto et al. 1990; Schöler, Ruppert, et al. 1990; Rosner et al. 1990; Schöler, Dressler, et al. 1990). Not just the core pluripotency factor Oct3/4 and Oct6, but also Nanog, Dax1, Nac1 and Klf4 were seen to occupy the *Rex1* promoter (Ben-Shushan et al. 1998; J. Kim et al. 2008). Interestingly, a knockdown of Nanog in ES cells results in a downregulation of Rex1 mRNA. Furthermore, in P19 cells, where neither Nanog nor Rex1 are normally expressed at any detectable levels, transfection with Nanog-GFP leads to an induction of Rex1 as detected by RT-PCR analysis. Together, these pieces of data suggest that Nanog positively regulates the expression of Rex1. Similarly, it was also demonstrated that Sox2 and Oct3/4 are able to positively regulate Rex1 expression (W. Shi et al. 2006). A comparison of promoter occupancy of 9 known pluripotency factors revealed that while numerous targets are shared by an extended set of pluripotency factors namely Oct4, Sox2, Nanog, Klf4, Dax1, Zfp281 and Nac1, the targets of c-Myc and Rex1 largely fell into a separate cluster and, interestingly, most genes in this cluster are implicated in protein metabolism rather than developmental processes (J. Kim et al. 2008). A proteome-based approach to determine changes in expression of multiple proteins due to REX1 depletion in human ES cells showed that it could affect several processes including translation, RNA metabolism, cardiac differentiation and even mitochondrial organisation (Son et al. 2015).

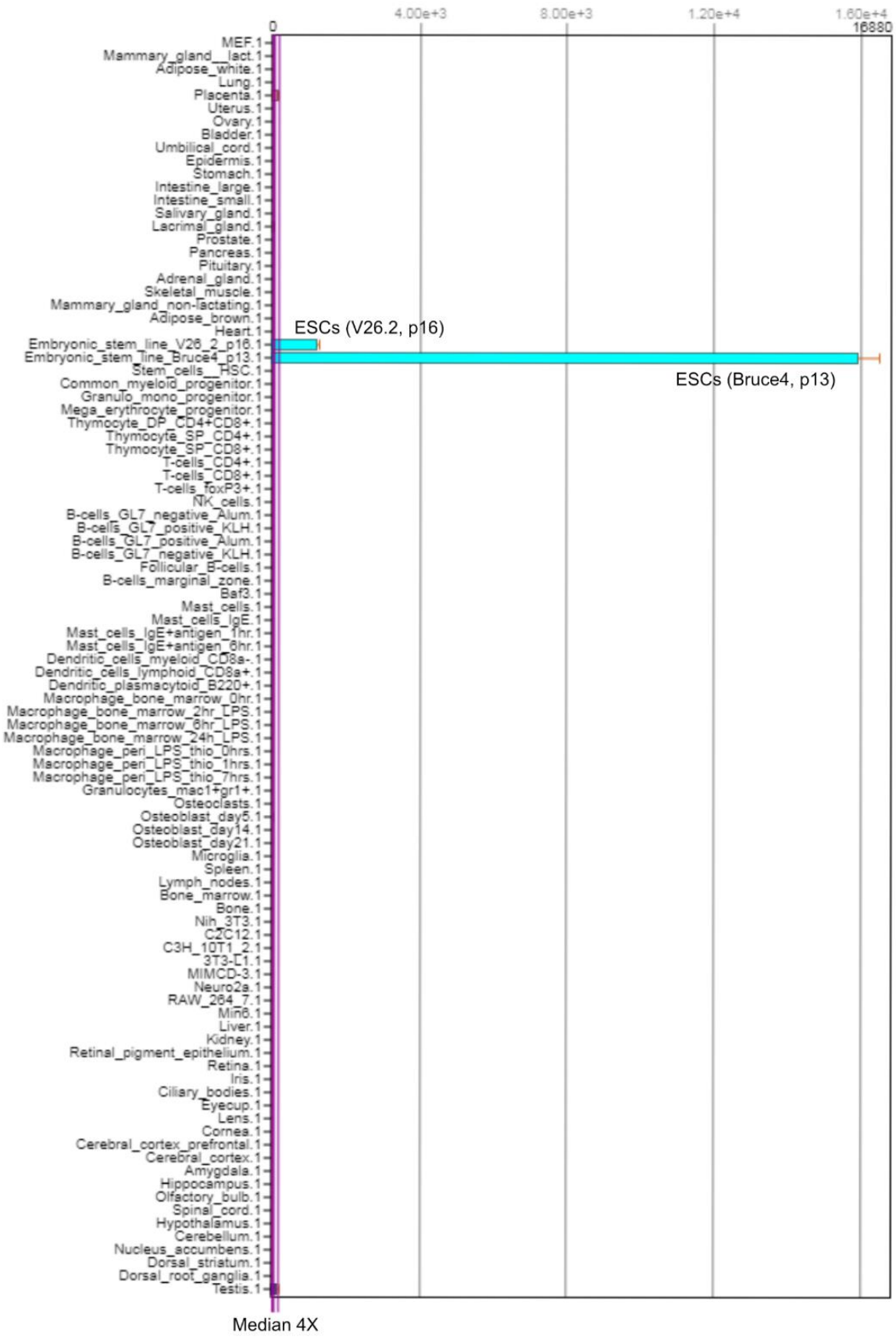

Figure 6: Rex1 expression is a marker of pluripotency. Microarray data for Rex1 expression in over 60 mouse tissues was downloaded from BioGPS (Wu et al., 2009). The purple line marks 4X median value. Rex1 expression is several folds higher than the median expression value in two different pluripotent ES cell lines, V26.2 and Bruce4. All other lineage committed cell types including hematopoietic stem cells, erythroid and myeloid progenitors show low to no Rex1 expression.

There seems to be a difference in the role of REX1 in human and mouse pluripotent cells. In human ES cells, depletion of REX1 using multiple REX1-specific shRNAs disrupts self-renewal properties and promotes spontaneous differentiation, in addition to inducing apoptosis and reducing proliferation. REX1 depleted human ES cells also form immature teratomas *in vivo*, with no mature mesoderm tissues. In the same cells, co-transfection of an exogenous REX1 expression vector also significantly increased cyclin B1 and B2 promoter activity, suggesting that Rex1 may have a role in maintaining normal cell cycle progression in the highly proliferating ES cells. Furthermore, depletion of endogenous REX1 with shRNAs prevents the reprogramming of fibroblasts with canonical reprogramming factors, while addition of ectopic REX1 enhances reprogramming efficiency (Son et al. 2013). Together these pieces of data suggest that in human ES cells, REX1 is important for maintaining their undifferentiated state and that it is indispensable for reprogramming.

Curiously, Rex1 expression in certain human cancer cell types has fuelled the idea that it might be a marker of cancer stem cells. The knockdown of Rex1 using siRNAs was shown to improve the chemotherapeutic effects in gliomas (B.-S. Kim et al. 2011). REX1 was also found to be expressed in several cervical cancer cell lines and it was shown to facilitate the migration and invasion of cervical cancer cells *in vitro* and distant tumour metastasis *in vivo* during the progression of a cervical carcinoma (Zeng et al. 2019). REX1 deficiency in hepatocarcinoma cells has also been reported to induce p38 MAPK signalling and an activation of oxidative stress responses, collectively contributing to enhanced stemness and metastatic capabilities of these cells (Luk et al. 2019).

In mouse ES cells, however, the contribution of functional REX1 during pluripotency is controversial. A targeted deletion of *Rex1* in F9 embryonic carcinoma cells results in impaired differentiation into the visceral endoderm when induced by retinoic acid (Thompson and Gudas 2002). In MESPU13 (M13) line of ES cells, persistent knockdown of *Rex1* over 7 days using RNA interference led to spontaneous differentiation of the cells into endoderm and mesoderm lineages. Conversely, an overexpression of *Rex1* also caused these cells to lose their self-renewal characteristics, suggesting that a normal expression level of *Rex1* is important for ES cell self-renewal and that REX1 might act as a dose-dependent regulator (J.-Z. Zhang et al. 2006). In great contrast, in E14tg2A cells, over expression of *Rex1* neither induced differentiation in the presence of LIF nor maintained

self-renewal in the absence of LIF. From the same parent line, *Rex1*$^{-/-}$ pluripotent ES cells could be established and showed no difference in the kinetics of differentiation induced by LIF withdrawal for 4 days or during the formation of embryoid bodies. These cells contributed to the formation of whole embryos after blastocyst injection, indicating they possess all functional aspects of pluripotency. Finally, both male and female *Rex1*$^{-/-}$ homozygotic mice were normal and fertile (Masui et al. 2008). Collectively, the data from this study strongly suggests that Rex1 is dispensable for maintenance of pluripotency in mouse embryonic stem cells. The differences in observation could be potentially due to the use of different mouse stem cell lines in the two studies. It could also be that Yy1, which is expressed widely in various tissue types and has pleiotropic functions including direct and indirect transcriptional activation and repression (Gordon et al. 2006), functionally compensates for *Rex1* and masks any phenotype in *Rex1*$^{-/-}$ ES cells. Interestingly, as discussed in the earlier sections, undifferentiated mouse ES cells can shift their identities to generate different cell populations such as inner cell mass like *Rex1*$^{+}$*/Oct3/4*$^{+}$ cells and primitive ectoderm like *Rex1*$^{-}$*/Oct3/4*$^{+}$ cells under serum/LIF culture conditions (Toyooka et al. 2008). This kind of heterogeneity could also contribute to the differences observed in the Rex1 overexpression phenotypes in the different studies.

In mice, *Rex1* is thought to be an epigenetic regulator for genomic imprinting during early mouse development, an epigenetic mechanism that silences one parental allele of a small subset of genes. During mouse embryo development, the paternal X chromosome is inactivated in the pre-implantation stage embryo and extra-embryonic tissues by imprinting. This is later activated upon the formation of an epiblast where the cells then randomly inactivate either the maternal or paternal X chromosome. Rnf12, a gene located in close proximity to Xist is an E3 ubiquitin ligase, and plays a crucial role in the first stage of paternal X chromosome inactivation by imprinting. Curiously, it was found that REX1 degradation by RNF12 was crucial to initiate this inactivation (Gontan et al. 2012; 2018; F. Wang and Bach 2019). ChIP experiments in mutant mice with disrupted *Rex1* transcription demonstrated that *Peg3* and *Nespas*, two imprinted genes, are downstream targets of Rex1 and that Rex1 is required for maintaining the allele-specific DNA methylation of the *Peg3* (Jeong Do Kim et al. 2011). *Peg3* is highly expressed in the placenta and is well known for its involvement in controlling foetal growth rates (Curley et al. 2004). In the placenta, Rex1 is also detected on the surface of the parietal yolk sac

adjacent to where the embryo lies (Jeong Do Kim et al. 2011). The parietal yolk sac, along with the visceral yolk sac is known to be very critical for early foetal-maternal interaction (Miri and Varmuza 2009). Therefore, it might be possible that Rex1 may control a set of genes involved in early foetal-maternal interactions.

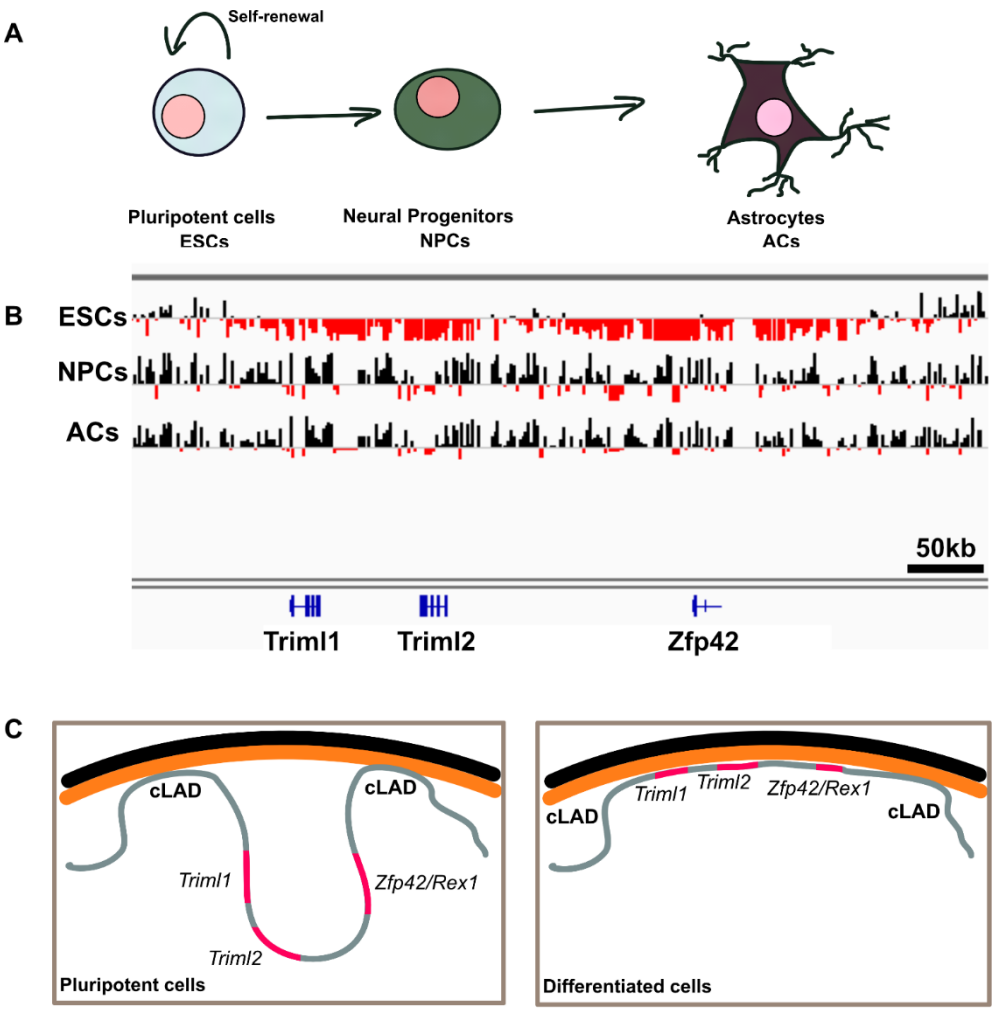

Figure 7: *Rex1* genomic locus repositions during *in vitro* differentiation. A. A functional basis for relocation of genes was studied in three different stages of *in vitro* differentiation – Pluripotent ES cells (ESCs), neural progenitor cells (NPCs) and terminally differentiated Astrocytes (ACs). B. DamID traces showing the Rex1 genomic locus containing genes Triml1, Triml2 and Zfp42, encoding Rex1, in the three stages of differentiation. Signal above the line represents regions in contact with the nuclear periphery (black) and signal below the line represents a region found in the nuclear interior (red) (Peric-Hupkes et al., 2010) C. Illustration depicting the DamID data. In ESCs, the locus containing the genes and their intergenic regions is found in the nuclear interior and it repositions to the periphery as the cells commit to neural lineages. On either ends the locus is flanked by constitutive LADs (cLADs).

In a study that looked at global genome organisation at various points during the differentiation of ES cells along the neural lineages, namely pluripotent ES cells,

multipotent neural progenitor cells and terminally differentiated astrocytes, it was found that the *Rex1* genomic locus repositions from the nuclear interior to the nuclear periphery during differentiation (Figure 7A and B; Peric-Hupkes et al. 2010). *Zfp42*, encoding Rex1, has two additional genes *Triml1* and *Triml2* in close proximity and this whole region is flanked by constitutive LADs on either end. However, in pluripotent ES cells where the genes are transcribed, the locus containing the three genes and their intergenic regions loops into the nuclear interior and upon differentiation, this loop is neatly tethered to the periphery (Figure 7C), suggesting that the nuclear envelope tether regulates and/or facilitates its transcriptional activity. It was recently shown that the high mobility group N (HMGN) proteins HMGN1 and HMGN2, members of the HMGN family of proteins that are ubiquitously expressed and bind to nucleosomes to weaken the binding of linker histone H1 and promote chromatin decompaction (Catez et al. 2002; Murphy et al. 2017), regulate *Rex1* expression in mouse ES cells. ChIP-seq experiments with antibodies specifically recognising OCT4, NANOG and SOX2, which are known to regulate *Rex1* expression, revealed a high specific occupancy of all three transcription factors to a region located at the 5' end of the *Rex1* gene (Figure 8), suggesting that this region is potentially a superenhancer.

ES cell lines were derived from *Hmgn1*$^{+/+}$ *Hmgn2*$^{+/+}$ (WT) and *Hmgn1*$^{-/-}$ *Hmgn2*$^{-/-}$ (double knockout) mouse embryos. No specific differentiation phenotype was reported for the double knockout line in the study. The loss of HMGN1 and HMGN2 reduces the binding of the transcription factors to the superenhancer and a correlated reduction of histone marks characterising active chromatin was also observed at the superenhancer. Using chromatin conformation capture (3C), this presumed superenhancer was shown to interact with the promoter of *Rex1* itself, thereby suggesting that the HMGN proteins regulated *Rex1* expression by regulating the epigenetic landscape of the superenhancer (Zhang et al. 2019). The neat genomic loop formed in the nuclear interior by the *Rex1* locus in ES cells where it is transcribed and the presence of the presumed superenhancer on its 5' end makes this an interesting locus to study.

Development involves very tight spatio-temporal regulation of gene expression. Dynamic physiological state dependent changes in chromatin folding allow for the stringent regulation of gene expression. The following section discusses the plasticity of genome organisation and touches upon the epigenome during differentiation and development.

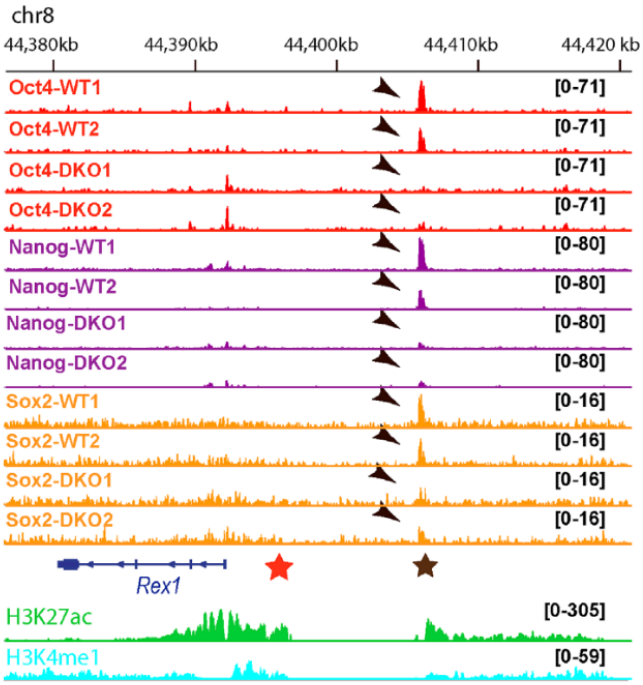

Figure 8: The 5'region of Rex1 gene has a putative superenhancer that is occupied by naïve transcription factors Oct4, Nanog and Sox2. Cells with a double knockout (DKO) for chromatin modifiers HMGN1 and HMGN2 leads to a loss of transcription factor occupancy at the superenhancer (Zhang et al., 2019).

1.7 Genome Organisation in pluripotency and differentiation

Zygotic genome activation (ZGA), an event that marks the transition from maternal dependence to activation of zygotic transcription so that it is self-reliant, is also the mark of establishment of 3D genome organisation in the zygote. Recently, single nucleus Hi-C was used to study chromatin states during oocyte to zygotic transition in isolated maternal and paternal nuclei of G1 phase mouse zygotes. It was found that the chromatin is organised into weak TADs and loops and strikingly, while paternal chromatin segregates into A and B compartments, maternal chromatin showed no such segregation (Flyamer et al. 2017). Furthermore, maternal and paternal genomes are spatially segregated even after the fusion of the pronuclei (Du et al. 2017). Contrary to only weak TAD domains, characteristic LADs are established right after fertilisation at the two-cell stage and the formation of these LADs facilitates major reorganisation in genome organisation (Borsos et al. 2019). In bovine pre-implantation embryos, microscopic evidence has shown the emergence of radial genome organisation concurrent with ZGA (Koehler et al. 2009). In mouse embryos, by the 2- to 4-cell stages, TADs and compartments can be detected, which supports that idea that genome

organisation is established concurrently with ZGA (Gassler et al. 2017; Ke et al. 2017). However, how ZGA and 3D chromatin organisation are coordinated remains to be studied. Except human embryos, transcription is not required for loop and TAD formations in embryos (X. Chen et al. 2019; Hug et al. 2017; Ke et al. 2017). Also, while CTCF is detected in both mouse oocytes and early embryos, CTCF is undetectable in human embryos, suggesting that in humans, ZGA and embryonic CTCF expression is perhaps necessary for TAD border insulation (reviewed by Vallot and Tachibana 2020). ZGA is triggered by transcription factors such as Zelda in *Drosophila* (Liang et al. 2008), Nanog, Pou5f1 and SoxB1 in zebrafish (M. T. Lee et al. 2013) and Oct4 (homolog of Pou5f1) in humans (L. Gao et al. 2018). These factors compete with histones to bind to open chromatin and establish the chromatin state of pluripotent cells (Veil et al. 2019).

On the basis of predominantly histological evidence many stem cell progenitors from neoblasts in planaria to hematopoietic progenitors in mouse were classically described to have 'open chromatin', mostly due to the lack of densely stained heterochromatin (reviewed by Mattout and Meshorer 2010). Using electron microscopy, it was shown rather strikingly that peripheral heterochromatin was mostly absent in pluripotent human embryonic stem cells compared to those that had undergone retinoic acid induced differentiation (Park et al. 2004). A similar pattern of largely homogeneous chromatin was also found in the cells of the ICM in mouse blastocyst, confirming that the 'open chromatin' state is physiologically relevant (Ahmed et al. 2010). Curiously, stochastic optical reconstruction microscopy (STORM) revealed the presence of discrete nanodomain "clutches" of nucleosomes in the interphase nuclei of both serum-grown and 2i-grown ES cells with 2i-grown cells having lower number of nucleosomes per clutch. The presence of these clutches suggested an established global chromatin structure in pluripotent cells. Interestingly, in neuronal progenitor cells derived from ES cells, the number of nucleosomes per clutch was the highest, showing further evidence in support of the open chromatin state of ES cells (Ricci et al. 2015). These studies combined with the higher accessibility of chromatin in pluripotent cells to MNase digestion (Morozumi et al. 2016) and a reduction of DNase-I hypersensitive sites during the differentiation of ES cells to neural progenitor cells (T. Deng et al. 2013) confirmed without doubt that the chromatin in the pluripotent state of cells is far more accessible and "open" and that as differentiation progresses, the chromatin is subjected to further condensation. As for the establishment of

genome organisation itself, 3D-FISH in mouse ES cells using whole chromosome paints revealed, similar to differentiated cells, the presence of chromosome territories (Mayer et al. 2005). There is also widespread transcription of repetitive elements and coding regions, confirming the hyperdynamic state of chromatin (Efroni et al. 2008). An established higher order chromatin architecture in mouse ES cells also demonstrated by mapping topological-associated domains (TADs) using Hi-C approaches (Dixon et al. 2012). Higher order chromatin architecture is also achieved by the spatial clustering of genomic sites that act as binding sites for transcription factors such as Nanog and Oct4. Such clustering allows for an increase in the local density of binding sites and leads to higher transcription rates (de Wit et al. 2013). As is seen in other cell types, this higher order chromatin architecture is maintained by architectural proteins like cohesin subunit Smc1 and Mediator (Phillips-Cremins et al. 2013).

As discussed before, since serum/LIF and 2i/LIF culture conditions were shown to both maintain pluripotent cultures with the population being more heterogeneous in serum/LIF culture conditions, it is of interest to see whether genome organisation is different between these states. To address this, the 3D genome organisation was studied in these two closely related states of pluripotency by subjecting the cells to serum to 2i interconversion, where the culture conditions were switched from one to the other. It was observed that while most promoter-promoter, promoter-enhancer and enhancer-enhancer interactions were conserved between the two states, certain extremely long-range intra- and inter-chromosomal interactions are only detected in serum/LIF ES cells and some of these interactions involve the *Hox* genes. The genomic loci involved in these long-range interactions were prominently characterised by H3K27me3 and H3K4me3 bivalence and several of these overlapped with promoters of transcription factors involved in cell-fate determination. Interestingly, these interactions are lost in a time dependent fashion during serum/LIF to 2i transition (Joshi et al. 2015). It was proposed that these interactions are observed due to the primed state of cells in the serum/LIF conditions. Furthermore, these long-range promoter-promoter interactions at the Hox gene clusters in primed pluripotent cells depend on PRC1 and PRC2, components of the polycomb repressor complex, which are thought to introduce H3K27me3 modifications and further recruit PRC1 to sustain the 3D chromatin structure at the Hox gene promoters. In the same study it was also found that several enhancers transition to an active chromatin state in the PRC knockout cells, which

also correlates with an upregulation of genes they contact (Schoenfelder et al. 2015). Together, these studies might suggest that although these long-range interactions are dispensable for pluripotency itself, in the naïve pluripotent cells, the polycomb repression machinery physically constrains developmental transcription factor genes and their enhancers in a silenced but poised spatial network. By carefully modulating the 3D architecture of these specific genes, the cells may be able to fine-tune their transcriptional state during the transition from naïve to primed pluripotency and commit to further lineage specification. The epigenome of 2i-grown ES cells also seems unique when compared to that of serum-grown ES cells and EpiSCs. Specifically, 2i-grown ES cells exhibit global DNA hypomethylation and significantly lower H3K27me3 deposition at promoters (Marks et al. 2012; Habibi et al. 2013). The pericentromeric heterochromatin (PCH) in naïve ES cells is enriched for H3K27me3. Removal of DNA methylation leads to enhanced deposition of H3K27me3 at the PCH in these cells with no major effects on satellite transcript levels. In EpiSCs however, this leads to abolishment of transcriptional repression. Thus it suggests that during the transition from naïve to primed states of pluripotency, the epigenetic state of PCH is modified towards a more repressive state (Tosolini et al. 2018). The nuclear envelope (NE), through its diversity, contributes to tissue specific genome organisation but whether it has a role in maintaining genome organisation in pluripotent cells is an interesting concept to explore. As discussed in the earlier sections, nuclear envelope transmembrane proteins along with lamins act as a major scaffold upon which most of the transcriptionally silent heterochromatin is anchored and thus the NE plays a key role in determining and orchestrating genome-NE interactions. Lamin A/C in particular has been shown to assist in establishing some genome organisation (Solovei et al. 2013) and is expressed later in differentiation so reports of its absence in ES cells (Röber, Weber, and Osborn 1989) seemed to support the idea of its subsequent expression assisting in the changing genome organisation. Accordingly, Lamin A/C was initially used as a marker for *in vitro* differentiation and its absence in ES cells has been used to explain the genomic plasticity in these cells (Constantinescu et al. 2006; Melcer et al. 2012). However, Lamin A/C was later shown to be present using western blots in over 9 independent ES cell lines and in ICM cells, suggesting that the earlier reports were likely mistaken due to epitope masking (Eckersley-Maslin et al. 2013). Moreover, Lamin A/C localisation in ES cells depends on Lamin B1 (Guo et al. 2014). These reports confirm that Lamin A/C is present

in ES cells and its absence is not an appropriate marker for differentiation. Although it is likely that Lamin A/C is massively upregulated during differentiation and therefore much more detectable.

Lamin B1-DamID was used to map the lamina associated domains (LADs) in mouse ES cells, demonstrating that LAD organisation was already established in the pluripotent state of cells (Peric-Hupkes et al. 2010). When ES cells were depleted of A and B type lamins and subjected to Emerin-DamID, the LAD profiles looked strikingly similar to the LaminB1-DamID profiles, suggesting that the larger docking of heterochromatin to the NE does not depend on lamins (Amendola and Steensel 2015). One caveat of this study is that while Lamin B1 and B2 were knocked out, Lamin A/C depletion was achieved through RNA interference which does not guarantee a complete abolishment of the protein and it was at least partially present during establishment of genome organisation. In another study, where a triple knockout cell line was generated so that none of the lamins were expressed, a global decondensation of constitutive heterochromatin and detachment of facultative heterochromatin was observed. This detachment of peripheral heterochromatin was enough to affect inter-TAD interactions without compromising overall TAD structures (Zheng et al. 2018). This suggests that global genome organisation in ES cells is established to a large degree by the peripheral organisation of heterochromatin, which in turn is lamin-dependent.

Differentiation of pluripotent cells and lineage commitment is accompanied by large scale genome organisation changes and these dramatic changes are accomplished within a few cell divisions. Some of the initial studies to study genome organisation in ES cells suggested that at the chromosome territory (CT) level, ES cells have no special organisation compared to differentiated cells. However, a recent study quantified the frequency of chromosome intermingling in undifferentiated ES cells, cells exiting pluripotency and terminally differentiated NIH3T3 fibroblast cells. It was observed that the fraction of the individual CTs which interacted with other heterologous chromosomes, characteristic of long-range interactions between genes and their regulatory elements, was higher at the onset of differentiation and in differentiated cells. Furthermore, the intermingling regions showed an accumulation of RNAPII suggesting that these were active transcriptional sites (Maharana et al. 2016). From these results it seems that ES cells have well organised CTs but interactions between CTs are defined better as lineage commitment takes place. Upon

closer look at genomic loci by 3D-FISH, the NANOG gene locus was shown to have a more interior position in human ES cells compared to differentiated lymphoblastoid cells. Similarly, OCT4 shifted from an interior position in the CT in the human ES cells to the surface of the territory in lymphoblasts (Wiblin 2005). In another study, during the retinoic acid induced differentiation of mouse ES cells, the HoxB locus, containing genes that control the body plan of a developing embryo, is seen to decondense. Concurrently, HOXB1 and HOXB9 genes move from within the chromosome territory to its periphery and are expressed (Chambeyron 2004). Similarly, in a study comparing three chromosomes of differing sizes and gene densities but also containing loci that encode pluripotency and lineage regulators like NANOG, OCT4 and CDX2, it was found that the radial position of whole chromosomes remains largely unchanged during bovine development from a zygote into a blastocyst. However, when investigating individual loci, it was observed that the loci for both NANOG and CDX2 were shown to relocate to the surface outside the CT in correlation with their stage specific expression. This change in gene positions resulted in a corresponding change in the shape of the CT from a regular to an irregular shape (Orsztynowicz et al. 2017). Taken together these studies suggest that while at the CT level, genome organisation is no different in pluripotent cells, organisation is dynamic at the single gene level with developmental genes repositioning to facilitate their expression or repression during differentiation.

Global NE-genome interactions during the *in vitro* differentiation of mouse ES cells into neural progenitor cells and terminally differentiated astrocytes were mapped using Lamin B1-DamID to identify which regions reposition to and from the nuclear periphery during lineage commitment. The study showed that several developmentally important genes were under regulation by the NE, where they were released in a stage-specific manner from the NE for expression and other loci containing pluripotency genes were tethered to the periphery for repression as the cells exit pluripotency. For instance, neuron specific *Pdch9* is released from the NE concomitant with its transcriptional activation in the neural lineages, while the locus containing *Zfp42/Rex1*, a prominent marker of naïve pluripotency, is tethered to the NE to facilitate its repression as lineage specification progresses (Peric-Hupkes et al. 2010). This study illustrated the role of NE in regulating the expression of key developmental genes during differentiation. Various studies have documented a functional basis for the repositioning of developmental genes. During muscle

differentiation, the locus encoding *Titin*, a protein that plays an important role in muscle contraction, is released from the NE in myoblasts to the nuclear interior in myotubes where it is expressed (Robson et al. 2016). As discussed in the earlier sections, the main structural components of the NE seem to be fairly constant across cell types but its composition is dynamically remodelled during various physiological processes. The inner nuclear membrane (INM) in particular, shows enormous tissue diversity in its composition with hundreds of nuclear envelope transmembrane proteins (NETs). This tissue diversity of the INM facilitates a tissue specific genome architecture during differentiation and development. For instance, the repositioning of several muscle specific genes like Nid1, Ptn and Cxcl1 is directly affected by muscle NETs TMEM38A, WFS1 and NET39 (Robson et al. 2016). Analogously, during adipocyte differentiation, several genes reposition to and from the periphery concomitant with their transcriptional status in a manner dependent on TMEM120A, a fat specific NET (Czapiewski et al, unpublished date). Interestingly, recent efforts to look at whether NETs merely regulate genes or also other regulatory elements revealed that several enhancers are under TMEM120A regulation during adipocyte differentiation and several myogenic enhancers are under NET39 regulation during muscle differentiation. For instance, the predicted enhancer for *Klf9*, a regulator of PPARG that is well studied for its roles in adipogenesis, is released from the nuclear periphery during 3T3L1 differentiation into adipocytes *in vitro*. Similarly, Col1a2, a gene upregulated in obesity, is seen to interact with its predicted enhancer in differentiated adipocytes (Schirmer lab unpublished data). Together these studies highlight the importance of the nuclear envelope in orchestrating genome organisation during differentiation and suggest that through controlled release of genomic loci into the nuclear interior or recruitment of loci to the NE, the NE plays a role in fine tuning various developmental programs.

A comparison of the Hi-C data from human ES cells with four other human-ES-derived lineages shows that ~36% of the genome switches between A and B compartments upon differentiation. Consistent with experimental evidence seen thus far, the B compartment that typically contains transcriptionally inactive heterochromatin sees an expansion upon differentiation and regions that switched from A to B compartments were repressed. Interestingly, TADs were seen to switch compartments as whole units, rather than fragments of TADs repositioning separately, suggesting that the higher order domain organisation was not broken but instead these domains simply function as a single unit

through the course of differentiation (Dixon et al. 2012). Similar switching of marker genes between A and B compartments to maintain their stage-specific transcriptional activity was also observed in adipogenesis and myogenesis models recently (He et al. 2018). Furthermore, during neuronal differentiation, long-range interactions between active A compartment TADs become less pronounced while inactive B compartment TADs show stronger interactions reflecting the formation of heterochromatin. In ES cells, the long-range interactions involve Polycomb-bound genes and some others bound by pluripotency transcription factors. Upon neuronal differentiations, these networks are reorganised to be replaced by cell-type specific transcription factors bound and enhancer-promoter interactions (Bonev et al. 2017). A look at sub-TAD structures at higher resolution revealed that when as ES cells exit pluripotency and commit to neuronal lineages, as many as 83 ES-cell-specific interactions are lost and an additional 165 neural progenitor cell specific interactions are gained, suggesting that within the nucleus, higher order chromatin organisation in the form of TADs and chromatin loops are dynamic too. In the same study, it was observed that >80% of the significant interactions in ES cells were anchored by some combination of architectural proteins like CTCF, Med12 or Smc1, with different combinations working at different length scales to fulfil distinct roles in genome organisation. CTCF and Cohesin were seen to anchor various constitutive interactions that were detected during the different stages of lineage commitment (Phillips-Cremins et al. 2013). Taken together, these studies highlight how constitutive interactions are maintained by key architectural proteins like CTCF and Cohesin but facultative interactions that change during differentiation might be controlled and anchored by other such proteins, many of which have yet to be described.

While we now understand that genome organisation changes dramatically during differentiation, there is surprisingly little work done to understand the temporal dynamics of these reorganisations. In the next section, I will elaborate on some seminal studies that provided insight into the temporal dynamics of genome reorganisation and tried to answer the following question:

How quickly can the genome reorganise itself in response to a stimulus?

1.8 Temporal dynamics of genome organisation

Although dramatic changes in genome organisation have been recorded at various stages of differentiation and development, these changes have often been studied over several days. During embryonic development, changes in organisation are studied over the course of a few cell divisions during which various groups of cells commit to the different lineages. However, considering that the 3D genome structure is quite static at a global level while many physiological responses are rapid, whether the genome can be reorganised rapidly and without cell division to facilitate such responses has been of interest for a while.

In *Arabidopsis*, a rapid repositioning of the light-inducible loci like GUN5, RBCS and chrolophyll a/b binding proteins (CAB) locus is seen from the nuclear interior to the nuclear periphery upon light activation within 3 hours and this relocation of the locus correlates with its transcriptional activation (Feng et al. 2014). In *Drosophila* Kc167 cells, a rapid reorganisation of TAD structures was observed upon a 20-minute heat-shock treatment, where new inter-TAD interactions were established and architectural proteins were seen to be redistributed from TAD boundaries to intra-TAD regions (L. Li et al. 2015). In mammalian systems, early studies addressed these questions directly by subjecting cells to serum starvation to induce quiescence and a reversible growth arrest. Surprisingly it was found that a number of chromosomes including chromosome 10, 13, and 18 show altered nuclear positions upon serum starvation of human dermal fibroblasts within 15 minutes post starvation, suggesting that changes in genome organisation can be rapid. Considering that the movements are rapid, it was expected that these movements could not be due to random diffusion and must be an active process requiring ATP and are perhaps dependent on the actin-myosin motors and indeed this was found to be true (Mehta et al. 2010). This further supports the idea that rapid physiological responses could, in theory, be regulated by rapid gene repositioning to and from the nuclear periphery to control its expression. Rapid and reversible chromatin condensation is also seen in chondrocytes subjected to osmotic challenge for 15 minutes and changes in osmolality have been shown to alter gene expression and metabolic activity in a wide range of tissues including those of renal, respiratory and cardiovascular systems (Irianto et al. 2013). Recently hyperosmotic stress was shown to cause a reversible change in local chromatin organisation with weakened TAD boundaries and a perturbation in A/B compartment organisation so that there was an

increase in B-like compartments and increased interactions between them (Amat et al. 2019). In response to heat shocking CHO cells for 30 minutes, the Hsp70 gene locus moves towards the nuclear interior and shows increased association with nuclear speckles in a promoter dependent fashion (Hu, Plutz, and Belmont 2010). Recently, it was elaborated that this movement is Lamin A/C dependent in DLD1 cells and that it is potentially mediated by the motor protein Nuclear Myosin 1 (NM1) via its interaction with Lamin A – Emerin at the nuclear envelope (Pradhan, Nallappa, and Sengupta 2020). Together these observations suggest that chromatin is rapidly reorganised in response to various stimuli and considering the time scales at which these studies have been carried out, it seems that such rapid reorganisations can happen without the cells having to go through cell division. One mechanism by which such rapid changes could be orchestrated by the NE was proposed by the Schirmer lab and the idea of "*constrained diffusion*" of genomic loci, where the regions that need to be tightly regulated are bound by the NE so that the loops formed by these loci do not extend too far into the nucleus to facilitate transcription of the genes or other long-range interactions. Upon the need for a transcriptional shut down or abolishment of the long-range interactions to facilitate rapid responses to physiological stimuli, these loci can then be quickly tethered back to the periphery (Robson et al. 2017a). The development of live cell imaging methods to track genomic loci using fluorescently tagged dCas9 or single particle tracking of chromatin has now enabled us to better understand rapid changes in genome organisation, thus going beyond just capturing snapshots of the cells at various early stages during response to various physiological stimuli (Shaban and Seeber 2020).

Motor proteins like actin and myosin have been associated with the cytoskeleton for many decades but recently evidence has been mounting to suggest that there is a nuclear pool of actin and myosin and that these proteins serve important functions in the nucleus (Percipalle and Vartiainen 2019). In mouse embryonic fibroblasts, propulsive forces for cell migration are provided by actin isoforms with β-actin retaining a unique nuclear function that prevents myogenic differentiation by regulating global transcription (Tondeleir et al. 2012). It is quite likely then, that in these cells the nuclear β-actin pools play a major role in the organisation of the genome. The Grosse lab demonstrated that nuclear actin polymerisation was required for nuclear expansion and consequently for chromatin decondensation after mitosis (Baarlink et al. 2017) while the Fisher lab suggested a role for nuclear actin

dynamics in DNA replication (Parisis et al. 2017). In Drosophila cells, double stranded breaks in heterochromatin have been shown to relocalise to the nuclear periphery for repair by a directed motion along nuclear actin filaments assembled by the Arp2/3 complex at the repair sites (Caridi et al. 2018). These studies, consistent with well-established actin/myosin functions in the cytoplasm, strongly argue their supporting the movement of genomic loci and even whole chromosomes for rapid changes in genome organisation to stimuli.

1.9 Outstanding questions

The use of pluripotent embryonic stem cells to establish global genome organisation patterns prior to lineage commitment has been instrumental in the understanding that genome organisation is, in fact, dramatically different between pluripotency and lineage commitment. Studies looking at the reorganisation of LADs and TADs during stem cell exit from pluripotency have highlighted the dynamic state of the genome as cells start committing to a particular lineage. Previous data from the Schirmer lab have illustrated that the nuclear envelope composition itself is tissue-specific and this has helped explain how tissue-specific genome organisation is achieved during and maintained upon terminal differentiation. However, there are several distinct stages between pluripotency and terminal differentiation and we do not have a comprehensive understanding of the global changes in genome organisation that take place as cells progress through these early stages. It is now known that there are large scale transcriptional changes as cells exit their pluripotent state and this exit, though it lasts several hours as a reversible state during *in vitro* differentiation, is a unique state that allows us to probe some of the earliest changes in genome organisation, transcriptome and metabolome of the cells. And these early changes lay the foundations upon which gene networks are reoriented towards successful lineage commitment. It is therefore of interest to the field of stem cell biology to understand the temporal dynamics of changes in genome organisation as cells exit pluripotency and commit to lineages. But ***how quickly does the genome reorganise during exit from pluripotency?*** Changes in genome organisation do not always imply dramatic movements of genomic loci to and from the NE. The tethering of certain genomic loci at the periphery could also affect distal regions present on the same chromosome by propagation effects along the polymer. ***How far away from a tether point do genes cease to be affected by the***

positioning? Can minor changes in gene positions caused by such distal tethers affect TAD formation enough to prevent or facilitate long range interactions that might affect gene expression?

Although a functional basis for relocation of genes during lineage specification has been reported, the components of the nuclear envelope that might have a role in orchestrating these changes during early stages of exit from pluripotency remain unknown. ***What does the NE proteome of pluripotent cells look like and how different is it from lineage committed cells? How does the NE proteome change during exit from pluripotency?*** Drawing from the diversity of NETs observed in different tissue types, it would appear that as cells progress from their pluripotent state through differentiation into defined lineages, they acquire different NET signatures which might bring about the coordinated changes in genome organisation that support lineage commitment. Such changes in peripheral genome organisation aided by NETs most definitely affect TAD formations within the nuclear space and thereby also affect long range gene-enhancer interactions. Thus, NET mediated establishment of peripheral genome organisation could affect complete interphase chromatin topology.

Finally, changes in genome organisation are often studied in a gene centric manner but unpublished data from the Schirmer lab has shown that several regulatory elements including enhancers and miRNAs are under NET regulation in both adipogenesis and myogenesis. Thus, it would seem that the role of NETs in maintaining genome organisation goes far beyond establishing chromosome topology and the diversity of the NET proteome between various tissue types might be the key to understanding tissue specific gene expression profiles and that mutations in these specific NETs might also explain tissue specific disease pathologies.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Bacterial strains and genotypes

DH5alpha F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF) U169 hsdR17(rK- mK+) λ–

2.1.2 Buffers and solutions

Table 1: Composition of buffers used

Name	Composition
Denaturation buffer	70% formamide, 2× SSC
Hybridisation buffer	For DNA FISH: 50% formamide, 2× SSC, 1% Tween20, 10% Dextran Sulphate For RNA FISH: 10% formamide, 2X SSC, 1% Tween20, 10% Dextran sulphate
SSC, 20x	3M NaCl, 300mM Sodium Citrate pH 7.2
Immuno-FISH blocking buffer	4% BSA, 4X SSC, 0.1% Tween20
FISH wash buffers	Buffer1: 2X SSC Buffer 2: 0.1X SSC Buffer 3: 4X SSC, 0.1% Tween20
LB	1% tryptone, 0.5% yeast extract, 10mM NaCl, pH 7.4
PBS (pH 7.4)	65mM $Na2PO_4$, 8.8mM $KH2PO_4$, 137mM NaCl, 2.7mM KCl
TE	10mM Tris-Cl, pH 8.0, 1mM EDTA
TAE	40mM Tris-acetate, 1mM EDTA
GMEM	Glasgow's Modified Essential Medium (ThermoFisher #11710035)
Opti-MEM	Opti-MEM® I Reduced Serum

	Medium (Gibco, 31985062)
ESGRO-2i Medium	2i medium for ES cell culture (Sigma-Aldrich #SF016-100)
SDS-PAGE buffer	25 mM Tris pH 8.3, 192 mM glycine, 0.1% SDS
2X Iso buffer	10% PEG 8000 (NEB #B1004A), 200mM Tris, pH 7.5, 20mM $MgCl_2$, 20mM DTT (Invitrogen #Y00147), 0.4mM dNTP mix, 2mM NAD (NEB #B9007S)
10X Nick Translation Salts (NTS)	0.5 M Tris pH7.8, 0.05M MgCl2, 0.01M BME, 500ug/ml BSA
2X Laemmli buffer	4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125M Tris HCl, pH 6.8
NE1 hypotonic lysis buffer	20mM HEPES-KOH pH 7.9, 10mM KCl, 1mM MgCl2, 0.1% Triton X100, 20% glycerol

2.1.3 Primary Antibodies

Table 2: List of primary antibodies and dilutions used

Antigen	Host	IF dilution	WB dilution	Band size (kDa)	Source
Nanog	Rabbit	1:400	1:1000	~40	Abcam
Oct4	Rabbit	-	1:1000	~45	Abcam
H3	Mouse	-	1:2000	17	Abcam (10799)
LaminA/C (3262)	Rabbit	-	1:1000	70	(Schirmer et al, 2001)
GAPDH	Rabbit	-	1:5000	~37	
Nup153	Mouse	-	1:1000	153, 214	Abcam QE5
LBR (11745)	Rabbit		1:1000	~70	

2.1.4 Secondary Antibodies

Table 3: List of secondary antibodies used at the indicated dilutions

Antibody	Host	Dye	Dilution	Source
Anti-mouse	Donkey	Alexa 488	1:2000	Invitrogen #A21202
Anti-mouse	Donkey	Alexa 568	1:2000	Invitrogen #A10037
Anti-rabbit	Donkey	Alexa 488	1:2000	Invitrogen #A21206
Anti-rabbit	Donkey	Alexa 568	1:2000	Invitrogen #A10042
Streptavidin	Recomb	Alexa 488	1:100	Invitrogen #S-11223
Streptavidin	Recomb	Alexa 548	1:100	Invitrogen #S-11226
Anti-digoxigenin	Mouse	Alexa 488	1:100	Jackson labs #200-542-156
Anti-digoxigenin	Mouse	Alexa 594	1:100	Jackson labs #200-582-156
Anti-rabbit	Donkey	IRDye® 800CW	1:1000	Licor #926-32213
Anti-rabbit	Donkey	IRDye® 680RD	1:1000	Licor #926-68073
Anti-mouse	Donkey	IRDye® 800CW	1:1000	Licor #926-32212
Anti-mouse	Donkey	IRDye® 680RD	1:1000	Licor #926-68070

2.1.5 Probes used for FISH

DNA-FISH

All the BACs and Fosmids of the RP23, RP24 and WI1 libraries used for DNA-FISH were sourced from BACPAC Genomics (Oakland, California) and the BMQ library BAC from Source Biosciences. Abbreviated names for libraries merely refer to the published names they were given by their creators.

Table 4: List of probes used for DNA-FISH

Probe name	Chr	Species	BAC/FOSMID	Gene(s)/region
RP23-449D9	8	Mm	BAC	Triml1, Triml2, Zfp42
RP24-141P13	6	Mm	BAC	Nanog
BMQ-358G08	6	Mm	BAC	Ptn
WI1-2049I09	6	Mm	FOSMID	Nanog L1 (first fLAD upstream of the Nanog promoter)
WI1-1027O11	6	Mm	FOSMID	Nanog R (cLAD downstream of Nanog)
RP24-165N12	6	Mm	BAC	Nanog L2 (second bigger LAD upstream of the Nanog promoter)

Further smaller probes used for testing the interaction between *Rex1*, *Triml1* and *Triml2* genes were generated using PCR. The primers used for each of the genes are listed below (Tables 5, 6 and 7).

Table 5: List of primers used to design Triml1 probes

Primer name	Sequence
Triml1-fos-F1	AATGGACAGTGACAACGC
Triml1-fos-R1	AGCCTTCTCTCTAGCACTTG
Triml1-fos-F2	GTCAGAGGTATCAAACTC
Triml1-fos-R2	GCTGTGACTGAAGAGGACTT
Triml1-fos-F3	ATCAGGTGCTGTGTTG
Triml1-fos-R3	TGTGTTACAGCTCCTTAG

Table 6: List of primers used to design Triml2 probes

Primer Name	Sequence
Triml2-fos-F1	AAGAAGTCCACATCG
Triml2-fos-R1	TACCTGTTCCTACTACTG
Triml2-fos-F2	GCCAAGCATAGAAAG
Triml2-fos-R2	AGAGAGCCACATTAAGTC
Triml2-fos-F3	ATGTGTTCAGTAGAAGG
Triml2-fos-R3	CAAAATTGTAGGTGGAC
Triml2-fos-F4	TTGGAGGTCAGAATC
Triml2-fos-R4	AGAAGGTAGACAAACAGTG

Table 7: List of primers used to design Zfp42/Rex1 probes

Primer name	Sequence
Zfp42-fos-F4	TCAGATGTGTTGCCAAG
Zfp42-fos-R4	TCTTTGCCATTCCTCC
Zfp42-fos-F3	TGAAGGCTACCTAATACC
Zfp42-fos-R3	AGTTCAATTTCTGGGGA
Zfp42-fos-F1	GTTGACTACTGCCAAAG
Zfp42-fos-R1	TCACACATCAAGGCTG
Zfp42-fos-F2	AGCCTGCTCTTTTCTGAAG
Zfp42-fos-R2	GAGTGGCAGTTTCTTCTT

RNA-FISH

RNA-FISH probes for the mouse *Zfp42* gene encoding REX1 were designed using the Stellaris Designer for custom probes. The Zfp42 transcript was used as input sequence to generate 25 oligos which were then tested using BLAST for specific binding to the target sequence. These oligos, listed in Table 8 below, were then ordered from Integrated DNA Technologies IDT).

Table 8: Oligos designed to probe Zfp42/Rex1 transcripts in RNA-FISH

Oligo Name	Sequence
Seq 1	TCCCTTTTTAGATGGACGAA
Seq 2	AGCCACAGTGGAAATCTAGG
Seq 3	ATCCCAAGAAGAAACTGCCA
Seq 4	TAGTCCATTTCTCTAATGCC
Seq 5	AAACACCTGCTTTTTGGTCA
Seq 6	CCTTCTTGAACAATGCCTAT
Seq 7	ATGATGCACTCTAGGTATCC
Seq 8	GGGTTCGGAAAACTCACCTC
Seq 9	AAAGGAAATCCTCTTCCAGA
Seq 10	GAACCTGGCGAGAAAGGTTT
Seq 11	TCGAGAAGGGAACTCGCTTC
Seq 12	CCTTTGTCATGTACTCCAAA
Seq 13	CTGCAAGTAATGAGCTCGCC
Seq 14	CTTGAGGACACTCCAGCATC
Seq 15	TTTTATCCCTCAGCTTCTTC
Seq 16	CGTGGACAAGCATGTGCTTC
Seq 17	TGAGGACAATCTGTCTCCAC
Seq 18	TCTGTCATTAAGACTACCCA
Seq 19	GTACATAATGCGTGTATCCC
Seq 20	GGAATACCAAAAGAGGCCTT
Seq 21	TTTCCTGTTGGAACTATACC
Seq 22	CTTTTGAAACTGCTTTCACC
Seq 23	CCCCTTCAATAGCACATATA

Seq 24	ACTGATTTTCCGACGTATGC
Seq 25	GGCCATTCTTTAGGATTATC

2.1.6 Mammalian Cells

Mouse embryonic stem cells of the E14TG2a line, used for most experiments in this study were a gift from the Voigt lab (Wellcome Centre for Cell Biology, University of Edinburgh). The CGR8 line of mouse ES cells were a gift from Guillaume Blin at the Scottish Centre for Regenerative Medicine in Edinburgh. The Rex1::GFP line of cells with a destabilised GFP under the regulation of the *Rex1* promoter were obtained from Prof. Austin Smith (Living Systems Institute, University of Exeter).

2.2 Mammalian Cell Culture

2.2.1 Cell maintenance

Serum/LIF culture conditions: E14 and CGR8 cells were cultured in GMEM supplemented with 20% FBS (ThermoFisher #A3160402), 1X non-essential amino acids (ThermoFisher #11140050), 1X sodium pyruvate (ThermoFisher #11360070), 50µM 2-Mercaptoethanol (ThermoFisher #31350010) and 2ml recombinant LIF made by expressing a plasmid expressing human LIF as published in Smith et al. 1988. LIF activity was assayed using Western blots to look for Nanog and Oct4 expression and to omit batch variation, all experiments in this study were performed using the same batch of recombinant LIF. Cells were passaged every second day in a 1:12 dilution by trypsinisation and grown on plates coated with 0.1% gelatin (SIGMA) in PBS.

2i/LIF culture conditions: E14 and Rex1::GFP cells were cultured in the LIF containing ESGRO-2i medium which was supplemented with the provided GSK3β and Mek inhibitors to keep the cells pluripotent. Cells were passaged every second day using Accutase (ThermoFisher #00-45555-56) and grown on gelatin coated plates.

Stable cell lines: The tet-ON stable cell lines generated using Gibson assembly and Gateway cloning (described in detail below) for E14 cells expressing NET50, TAPBPL and NET39 were maintained in the standard serum/LIF conditions. For selection the clones were subjected to Puromycin at 1µg/ml and the induction of NETs was brought about by

1µg/ml doxycycline treatment for 48h. Cells were passaged every second day by trypsinisation as standard serum/LIF cultures.

LIF/2i withdrawal to stimulate *in vitro* differentiation: For LIF withdrawal, cells were cultured in serum/LIF culture conditions as mentioned above. To stimulate *in vitro* differentiation, cells were trypsinised and replated in medium containing all the components of a complete medium except recombinant LIF. Similarly, for 2i withdrawal experiments, cells grown in LIF containing ESGRO medium mentioned above were replated in medium lacking only the GSK3β and Mek inhibitors.

All cells were cultured at 37ºC in humid atmosphere with 5% CO_2. In all cases, *in vitro* differentiation was stimulated by LIF withdrawal, except where clearly mentioned as 2i withdrawal.

2.2.2 Transfection

Lipofectamine 2000: For ectopic expression of NETs in ES cells, E14 cells were transfected using Lipofectamine 2000. 2µg DNA and 9ul Lipofectamine 2000 were separately added to two 50ul aliquots of Opti-MEM and incubated for 5 minutes at room temperature. Following a quick vortexing, the aliquots were combined and incubated for a further 20 minutes at room temperature. Meanwhile cells were trypsinised, neutralised and seeded onto gelatin coated plates at a density of 0.5million cells per well of a 6 well plate. Following the 20-minute incubation the DNA-lipovesicle mixture was added to the cells while they were still in suspension to maximise the chances of the vesicle uptake.

Nucleofection: For the expression of LAP2α and LBR WT and mutants in ES cells, E14 cells were nucleofected with the Lonza Mouse Embryonic Stem Cell Nucleofector Kit (#VPH-1001) on the Amaxa Nucleofector 2b Device (second generation) using the programme A-023. Nucleofection was seen to be much more efficient than standard vesicle mediated transfection with over 95% cells expressing the plasmid after 24h.

2.3 Nucleic Acid Methods

2.3.1 Sequencing plasmid DNA

The entire sequence of cDNAs cloned into expression vectors or mutated using site-directed mutagenesis was verified by sequencing. Standard BigDye Terminator Cycle Sequencing (ThermoFisher #4337455) reactions were sent out for sequencing either to the Edinburgh Genomics (Kings Buildings, University of Edinburgh) or the sequencing facility at MRC Institute of Genetics and Molecular Medicine, University of Edinburgh.

2.3.2 RNA extraction

ES cells were collected by trypsinisation and then centrifugation at 400g for 3 minutes. The pellets were resuspended directly in Trizol and incubated for a minimum of 5 minutes at room temperature. 200µl chloroform was then added to the mixture and it was vortexed for 20s. Following another 5-minute incubation at room temperature, the mixture was spun down at 12,000g for 20 minutes at 4ºC. The aqueous phase was carefully extracted and mixed with 500ul isopropanol, mixed and incubated at room temperature for 5 minutes. A subsequent spin for 10 minutes at 13,600g at 4ºC left a pellet containing the RNA, which was washed with 70% ethanol. Finally, the pellet was resuspended in an appropriate amount of RNase-free water and left to solubilise at room temperature for 5 minutes. To eliminate the trace Trizol contaminants, the RNA was re-precipitated by adding 2.5 volumes of ethanol and 0.1 volume of 3M sodium acetate, pH 6.5. Following an incubation of 5 minutes at room temperature, it was centrifuged again at 13,000g, 15 minutes. The pellet obtained after this was washed again with 70% ethanol as described above and the RNA obtained was re-solubilised in an appropriate amount of RNAse free water for 5 minutes. The quality and concentration were assessed using a NanoDrop 2000c spectrophotometer and by running the product on the gel.

2.3.3 Quantitative real time PCR (qPCR)

cDNAs were generated using the Thermoscript II RNase H- reverse transcriptase as per manufacturer's instructions. Briefly, following annealing to a polyA primer at 65°C, 5 µg of total RNA was incubated with 1X Thermoscript RNAse H- reaction buffer, 10 U RNAsin, 10mM DTT, 1µM dNTPs mix and 200 U Thermoscript II RNAse H– Reverse Transcriptase. Tubes were vortexed, centrifuged briefly, and incubated at 42°C in a thermocycler with the heated lid at 80°C for 2 h. After heat inactivation of the reverse transcriptase by a 15 min incubation at 70°C, RNA was removed by incubating samples with 1 µL of 7 mg/ml RNAse A for 45 min at 37°C. cDNA was diluted with 90 µl of water and the reactions stored at -20°C.

For qPCR, 20 µl reactions containing 8.4 µl of appropriately diluted cDNA (normally serial dilutions of 5-fold), 800nM forward and reverse primers and 1x LightCyclerR 480 SYBR Green I Master were carried out in a 96-well LightCycler 480 Multiwell Plate. The plates were briefly vortexed and centrifuged. Polymerase chain reactions were carried out in a LightCycler 480 using the program detailed in Table 9. Primers used are listed in Table 10.

Expression data was analysed using LightCycler 480 Software v1.5.0.39. Primers for real-time PCR were designed using RealTime PCR web tool (http://eu.idtdna.com/Scitools/Applications/RealTimePCR) with default parameters except for amplicon size, which was set to 100 bp minimum, 150 bp optimum and 200 bp maximum. The specificity of the primer pair to the target sequence was checked using Primer BLAST.

Table 9: Thermocycling conditions for qRT-PCR

Segment	No. of cycles	Temperature (°C)	Duration
1 (Initial Denaturation)	1	95	5 minutes
2 (Amplification)	45 cycles	95	10 seconds
		55	15 seconds
		72	20 seconds

Table 10: qPCR primer sequences

Gene	Species	Forward	Reverse
Triml1	Mm	TGCTTACCTGCTTTTGTCCG	CTCCGTCTTCTTTCCCACTT
Triml2	Mm	AAAGAGATGATCGAGGCTGAG	TTTCTTGGGACTTCTGCATG
Zfp42	Mm	CACGGAGAGCTCGAAACTAA	CAGCCATCAAAAGGACACAC

2.3.4 Site Directed Mutagenesis

Human wild-type LBR-GFP and LAP2α-GFP (sourced from Roland Foisner at the Vienna BioCentre) were found to lose phosphorylation during exit from pluripotency. To study the importance of these phospho-sites in the tethering of the *Rex1* locus to the periphery, phospho-null mutants were generated using site directed mutagenesis. LBR had two phospho-sites, ser-71 and ser-86, that were mutated to alanine sequentially with S71A

being generated first. Similarly, LAP2α has three phospho-sites, ser-66, ser-67 and ser-422. Serines 66 and 67 were mutated to alanine using the same primers and this was followed by the introduction of an alanine instead of ser-422. The primers used for mutagenesis are listed in Table 11 and the PCR reaction mixture in Table 12. The reaction was set up on ice with the components added in the same order as listed in Table 12. The thermo cycling conditions are listed in Table 13.

Table 11: Primer sequences for site directed mutagenesis

Protein	**Mouse phosphorylation site**	**Mutation**	**Primer Sequence**
LAP2α	S66, S67	S66,67A	F:CGCGCTCTTCGTCAGCGGCGAAGTCCGGGGGCC R:GGCCCCCGGACTTCGCCGCTGACGAAGAGCGCG
	S422	S422A	F:GGACTTTTCTTGGAGGAGCCAGGAATTCAGTTTCTTG R:CAAGAAACTGAATTCCTGGCTCCTCCAAGAAAAGTCC
LBR	S71	S71A	F:CTGGAAGGGGCACTGGAAGTTGAGCCACCT R:AGGTGGCTCAACTTCCAGTGCCCCTTCCAG
	S86	S86A	F:CGACCAGGGGCTCGGGAGCGTGACC R:GGTCACGCTCCCGAGCCCCTGGTCG

Table 12: PCR reaction mixture for site directed mutagenesis

Component	**Amount per reaction (µl)**
Distilled water (dH2O)	40.5
10× PfuUltra II reaction buffer	5.0
dNTP mix (25 mM each dNTP)	0.5
DNA template (25 ng/µl)	1.0

Primer #1 (10 µM)	1.0
Primer #2 (10 µM)	1.0
PfuUltra II fusion HS DNA polymerase	1.0
Total reaction volume	50.0

Table 13: Thermocycling conditions for mutagenesis

Segment	No. of cycles	Temperature (°C)	Duration
1 (Initial Denaturation)	1	95	2 minutes
2 (Extension)	30 cycles	95	20 seconds
		60	20 seconds
		72	2 minutes
3 (Final elongation)	1	72	3minutes

2.3.5 Gibson assembly and Gateway cloning

Gibson assembly, a robust exonuclease-based method to assemble DNA seamlessly in the intended orientation, was used to clone the NETs into a tet-ON expression construct. The reaction contains a 5'exonuclease to generate overhangs, a polymerase to fill in the gaps of the annealed single strands and a DNA ligase to seal the nicks (Gibson et al. 2009). The NETs – NET50, TAPBPL and NET39 – tagged with either GFP or RFP were amplified from Image Clones and assembled into the LR Entry vector pENTR2B2 using primers listed in Table 14. Primers were designed using the in-built Gibson assembly tool in SnapGene.

Table 14: Primers for Gibson Assembly to insert indicated NETs into LR entry vector

Insert	Primer sequence
NET39 EGFP	F: CGGAACCAATTCAGTCGACTGATGCCAGCTTCCCAGAGC
	R:ACAAGAAAGCTGGGTCTAGATATCTTACTTGTACAGCTCGTCCATGCC
NET50 mRFP	F: CCGGAACCAATTCAGTCGACTGATGAACTGGGAGCTGCTGC

	R: ACAAGAAAGCTGGGTCTAGATATCAGGCGCCGGTGGAG
TAPBPL mRFP	F: GCCGGAACCAATTCAGTCGACTGATGGGCACACAGGAGGG
	R:ACAAGAAAGCTGGGTCTAGATATCAGGCGCCGGTGGAG

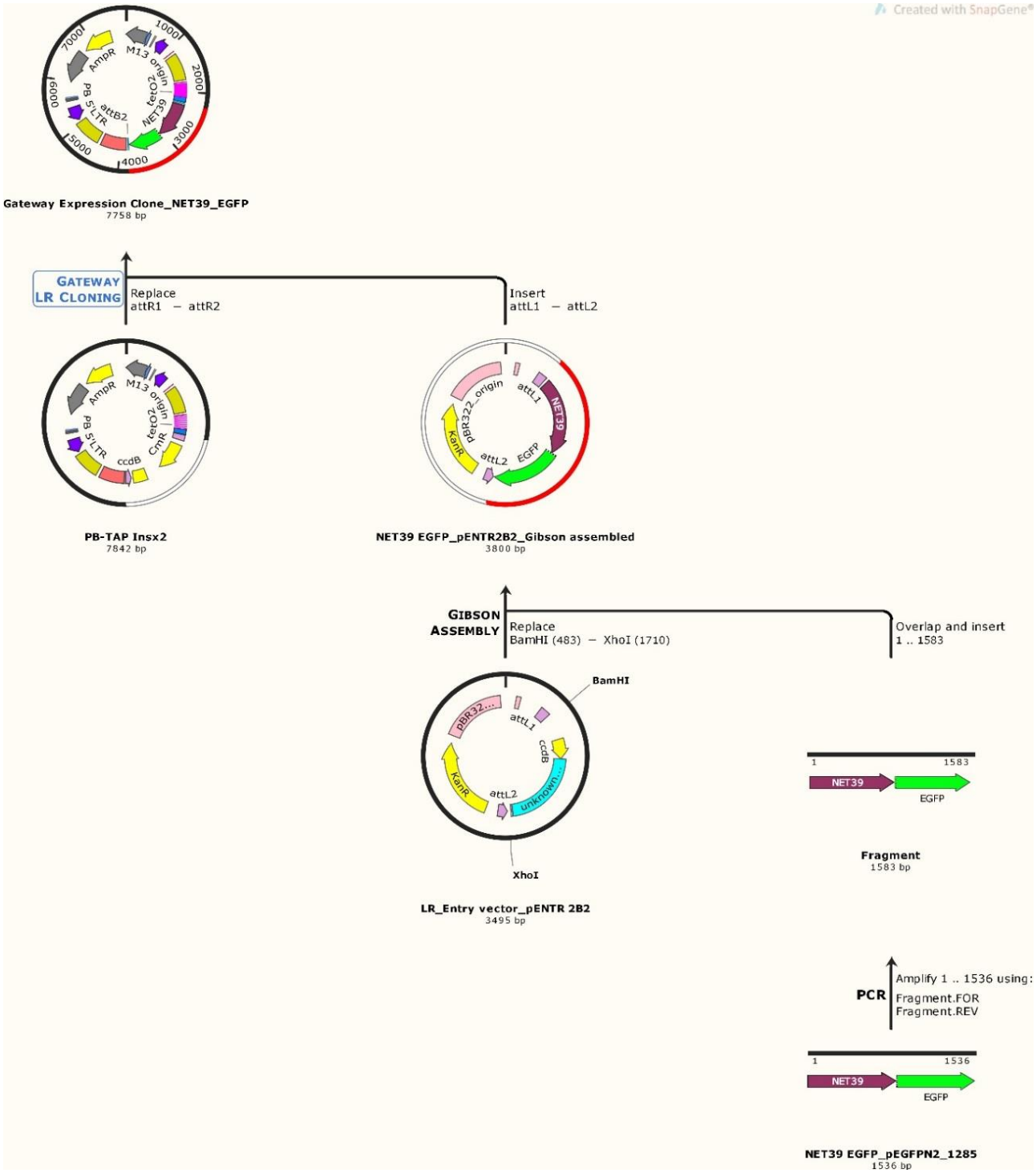

Figure 9: Cloning strategy to generate inducible stable cell lines expressing tissue-specific NETs. GFP/RFP tagged NET was amplified from the Image Clone and Gibson Assembly was used to ligate the fragment into an LR entry vector pENTR 2B2. Gateway LR cloning was then used to move the insert into the expression vector PB Tap-Insx2 yielding the final tet-ON expression vector for the tagged NET. Image generated using Snapgene.

For Gibson assembly, the vector backbone (pENTR 2B2) was opened up with restriction enzymes BamH1 and Xho1 and the digested fragment was extracted from the gel. Assembly reaction was set up with 75ng vector and a 3-fold excess of the insert using the reaction mixture stated in Table 15. Post assembly, the ligated product was transformed into DH5α for selection. Colony PCR and sequencing of the insert was used to screen the clones obtained. DNA amplified from the selected clone was then extracted and used for Gateway reaction.

Table 15: Reaction mixture for Gibson Assembly

Component	**Amount per reaction (µl)**
NFW	7.95
Iso buffer	4
Taq. Ligase (NEB #M02208S)	2
Phusion (NEB #M0530L)	0.25
T5 exonuclease (NEB #M0363S)	0.8
Total volume	15

Using the manufacturer's protocol (ThermoFisher LR cloning kit), the LR reaction was carried out to swap the insert in to the destination/expression vector PB-Tap Insx2. A flow chart depicting the cloning strategy for generating the tet-ON NET39 expression construct is shown in Figure 9. Plasmids for gateway LR cloning to insert gene of interest were a gift from Keisuke Kaji at the Scottish Centre for Regenerative Medicine, University of Edinburgh.

2.3.6 Probe labelling for FISH

Nick Translation: BACs and Fosmids are large molecules of DNA containing ~200kb and ~70kb inserts respectively. Such large molecules of DNA were labelled with Biotin or Digoxigenin using Nick Translation reaction, the components of which are described in Table 16. The reaction was incubated at 16^{o}C for 90 minutes followed by a heat inactivation at 75^{o}C for 10 minutes. 3µl of the reaction was run on an agarose gel to check that the smear was centred around 200-500bp. The rest was made up to 100µl with nuclease free water and purified through the Quick Spin column for radiolabelled DNA Sephadex G-50 (Roche

#11273973001) columns using the manufacturer instructions. Typically, 5µl of this labelled probe was used per slide for FISH.

Table 16: Reaction mixture for Nick Translation

Component	Amount per reaction
10X NTS	4µl
0.5mM dATP (Promega)	5µl
0.5mM dCTP (Promega)	5µl
0.5mM dGTP (Promega)	5µl
Bio-16-dUTP (if using digoxigenin then dig-11-dUTP 1.5ul + 0.5mM dTTP 2ul) Roche #11093070910 (Biotin) Roche #11093088910 (Dig)	5µl
Template DNA (BAC/Fosmid)	10µl (3-4µg DNA)
DNase 1 (dilute 1:30 in ice cold NFW and use this) (Roche #04716728001)	1µl of the diluted enzyme
DNA polymerase 1 (Invitrogen #18010-017)	1µl
NFW	4µl

End labelling: Probes generated for RNA-FISH were 20-mers and are too small to be labelled by nick translation. Therefore, they were end labelled using terminal deoxynucleotidyl transferase (TdT) which catalyses the addition of deoxynucleotides to the 3'hydroxul terminus of DNA molecules. The reaction was set up using the mixture stated in Table 17 and incubated at 37ºC for 60 minutes. Addition of 2.5µl of 0.2M EDTA was used to stop the reaction. All the 25 oligos for RNA-FISH were individually labelled and the reactions were pooled in the end. The labelled probes were purified by addition of equal volume of phenol: chloroform: isoamyl alcohol, vigorous mixing and centrifugation at 13,600 rpm for 5 minutes. The aqueous layer was extracted and used directly for hybridisation. 100µl of the labelled probe mix was used per slide.

Table 17: Reaction mixture for End Labelling.

Component	Volume (µl)
NFW	25
5X TdT reaction buffer	10
Oligo (1uM)	2
Biotin 11-UTP (Thermo #R0081, diluted to 5uM)	5
Diluted TdT (Thermo #EP0161, diluted to 1.5U/ul)	5
Total volume	50

2.4 Microscopy Methods

2.4.1 Immunofluorescence

Adherent cells were grown on fibronectin-coated coverslips and washed in PBS to remove cellular debris and remaining serum prior to fixation with 4% para-formaldehyde (PFA), 1X PBS for 10 minutes at room temperature. Following fixation cells were washed twice in PBS. Cells were then permeabilised for 10 min with 0.2% Triton-X-100 in PBS and then washed 3 times in PBS. Coverslips were blocked in 4% bovine serum albumin (BSA), 1X PBS for 20 min at RT and subsequently incubated with the appropriate primary antibody (dilutions listed in Table 2). Following 3 washes in PBS, coverslips were incubated with goat secondary antibodies conjugated with either Alexa Fluor® dyes (summarised in Table 3) and 4,6- iamidino-2 phenylindole, dihydrochloride (DAPI) at a final concentration of 2 µg/ml (1:2,000). Coverslips were then extensively washed in PBS multiple times over the course of 15 minutes and then mounted on slides with Fluoromount G (EM Sciences).

2.4.2 Fluorescence *in situ* Hybridisation (FISH)

Sample Prep: For 3D FISH, cells were grown on fibronectin coated slides, fixed with 4% PFA for 10 minutes, permeabilised with 0.5% Triton-X-100 for 20 minutes. For 2D FISH and RNA-FISH, cells were trypsinised, neutralised and collected by centrifugation at 400g, 3 minutes. They were then treated with 70mM KCl for 7 minutes at 37ºC and fixed with 3:1 methanol: acetic acid. The cells were then dropped on slides from approximately 30-40cm above.

Probe preparation: For DNA-FISH, appropriate amount of labelled BAC/Fosmid was dispensed along with 5µl Cot1 DNA per slide and 1µl salmon sperm DNA per slide. 3 volumes of Ethanol and 0.1 volume of sodium acetate pH 5.2 were added to the tube to precipitate the DNA overnight at 4ºC. The next morning, the probe was centrifuges at 13,600rpm for 30 minutes to precipitate the probe. The pellet was resuspended in 50µl hybridisation buffer per slide at 37ºC.

For RNA-FISH, end labelled oligos were precipitated by the addition of 0.1 volume of sodium acetate pH 5.3, 3 volumes of ethanol and 1µg glycogen (GlycoBlue Invitrogen #AM9515) at 4ºC overnight. The next morning, the probes were precipitated by centrifugation at 13,600 rpm for 30 minutes and the pellet was resuspended in the hybridisation buffer for RNA-FISH as mentioned in Table 1.

DNA-FISH: Cells were pre-equilibrated in 2X SCC and treated with RNase A (100µg/ml) in 2X SSC at 37oC for 1 hour. Following washing in 2X SCC, cells were dehydrated with a 70%, 90% and 100% ethanol series. Slides were then air dried. For denaturation the slides were submerged into a Coplin jar containing preheated Denaturation buffer (70% formamide, 2X SSC, pH 7.0) for 20 minutes in a water bath. Simultaneously, the probes were denatured in the same conditions. After the incubation, the probes were transferred to 37ºC for pre-annealing. The slides were removed from the denaturation buffer and a second ethanol dehydration series was i*mmediately* performed using ice cold 70% ethanol for the first step followed by room temperature 90% and 100% ethanol. The slides were air dried again. 50µl probe in hybridisation buffer was dispensed on each slide and pre-cut clean strips of autoclave bags were used to cover the slides ensuring no air bubbles remained. Hybridisation reactions were then left to anneal at 37°C overnight in a humidified chamber. After incubation, the slides were washed thrice for 5 minutes each in Buffer1 at 45ºC, followed by 3 similar washes in Buffer 2 and one final wash in Buffer 3. Samples were then blocked with 4% BSA, 2X SSC for 30 minutes at room temperature. Fluorophore conjugated Streptavidin or anti-Dig antibodies in the same blocking solution were then added (dilutions mentioned in Table 3) to the samples and incubated for 45 minutes at room temperature. Following 3 washes of five minutes each in Buffer 3, the slides were mounted with coverslips in Vectashield containing DAPI.

RNA-FISH: The slides were rinsed in 2X SSC at room temperature and washed twice in 50% formamide, 2X SSC at 37ºC. They were washed twice more in 10% formamide, 2X SSC at room temperature. The probes were denatured at 80ºC for 20 minutes and placed immediately on ice. 50µl probe was dispensed per slide and these were covered with coverslips previously wiped and cleaned with RNase ZAP. After ensuring that the air bubbles had been expelled, hybridisation reactions were incubated overnight in a humidified chamber. The following day, slides were washed in twice for 3 minutes each in 2X SSC at room temperature and rinsed once in 4X SSC, 0.1% Tween20. Fluorophore conjugated streptavidin was used for detection and here onwards the protocol follows exactly from the DNA-FISH protocol described above.

2.4.3 Incucyte imaging

Transfected cells expressing NETs were sorted using FACS (explained below) and 20,000 cells were seeded in each well of a 12 well plate to be imaged using the Incucyte S3 Live-cell analysis system at the Callagher lab in the MRC Institute for Genetics and Molecular Medicine. Phase contrast images of the cells were taken at a magnification of 20X every 3 hours for 72 hours. The images were analysed using the Incucyte software using masks that detect cells and the output was generated as percentage confluence for each well for every time point.

2.4.4 Alkaline Phosphatase Assay

The tet-ON ES cell lines expressing NET50, TAPBPL and NET39 were seeded at 20000 cells per well of a 12 well plate. Doxycycline was used at 1µg/ml to induce NET expression in these cells. As a control for the assay, standard E14 cells were seeded in duplicates and one of the two wells was subjected to LIF withdrawal for 6 days, the same duration for which NET expression was induced. At 6 days post induction, the alkaline phosphatase assay was performed using Millipore's Alkaline Phosphatase Detection Kit (Sigma Aldrich #SCR004) following the instructions provided by the manufacturer.

2.4.5 Microscopy and analysis

Images were acquired on a Nikon TE-200 microscope using a 1.45 NA 100x objective, Sedat quad filter set, PIFOC Z-axis focus drive (Physik Instruments) and a Prime 95B camera (Photometrics) run by Metamorph image acquisition software. For 2D FISH, image analysis was done on the plane of focus for the alleles, which often is one plane due to how the sample is prepared. For deconvolution analysis for 3D FISH, Z-stacks were acquired at

intervals of 0.2 µm from the 1 µm above to 1 µm below the imaged nucleus. Image stacks were deconvolved using AutoQuant X3 (*Media Cybernetics*, RRID:SCR_002465) software. Distance from the nuclear periphery for the FISH images was measured using ImageJ. A minimum of 50 loci were analysed per sample. Since the data is non-parametric, statistical analysis was performed using Mann-Whitney test (for 2 groups) or Kruskal-Wallis ANOVA (for comparing >2 groups). Data are presented as scatters overlaid with the median and interquartile range and taken as statistically significant at $p<0.05$.

2.5 Fluorescence Activated Cell Sorting Methods

2.5.1 Cell cycle analysis

Rex1::GFP cells were subjected to 2i withdrawal for various durations over the course of 72 hours. At the appropriate time points, cells were collected by trypsinisation and centrifugation at 800g, 3 minutes. They were washed with PBS once and then fixed with 2% PFA, 5 minutes at room temperature. The cells were then stained with the cell permeable DNA binding dye Hoechst (diluted 1:10000) for 20 minutes at room temperature. The cells were then spun down at 800g, 3 minutes followed by two washes in PBS. Finally, the cells were resuspended in PBS and run on the FACS Aria IIu flow cytometer to analyse the cell cycle.

2.5.2 Sorting and Analysis

Sorting was used to enrich the population of transfected cells expressing tissue-specific Nets and for enriching the GFP+ cells in the Rex1::GFP population of cells subjected to 2i withdrawal. In both cases, the cells were collected by trypsinisation and following a wash with PBS to remove cellular debris were resuspended in complete medium with reduced serum (2% FBS instead of the regular 20%) since FBS interferes with sorting. The cells were then sorted using a four-laser FACS Aria IIu flow cytometer (Becton Dickson). The gating strategy is explained in Figure 29. The sorted population was collected in 100% FBS and either plated in gelatin coated plates containing complete medium or used for further experiments.

2.6 Protein Extraction and Western Blotting

Protein samples for western blotting were prepared from lysing equal number of cells for each sample directly in the standard Laemmli buffer. This was followed by sonication (Ultrasonic Atomizer VCX 130 ATFT for 30s at 20% amplitude with a 1s on, 1s off pulses

to shear the DNA. Lysates were then boiled for 10 minutes at 95°C to further denature the samples. Cell lysates were separated on 8-12% Tris-glycine-SDS or Bis-Tris gels. Subsequently the gels were transferred onto nitrocellulose membranes (Odyssey 926-31092) by means of semidry transfer (Pierce G2 Fast Blotter ThermoFisher Scientific). After transfer the membrane was blocked in western blot blocking buffer (5% milk powder in PBS with 0.05% Tween-20) for 30 min. Subsequently, the membrane was incubated with the primary antibody diluted in western blot blocking buffer at the dilutions indicated in Table 1 for 60 min at room temperature or overnight at 4°C. Three washes in PBS, 0.05% Tween-20 were then followed by incubation with the secondary antibody conjugated to an IRDye® for 60 min at room temperature. Following 3 washes additional washes in PBS, 0.05% Tween-20, the membranes were detected on a Li-COR Odyssey Quantitative Fluorescence Imager.

2.7 Mass Spectrometry Methods

2.7.1 Sample Preparation

Since this study uses cell lines instead of primary cells or tissues, variability arising from biological replicates are less likely. Therefore, cells were collected over three different passages but pooled together before the samples were sent for mass spectrometry. A crude isolation of intact nuclei was performed using a hypotonic lysis buffer NE1 (Skene and Henikoff 2017), the composition for which is mentioned in Table 1. Cells from three different passages were collected by trypsinisation and washed with ice cold PBS. They were then collected by centrifugation at 600g, 3 minutes. Ten million cells were resuspended per ml of cold hypotonic lysis buffer supplemented with 10mM sodium fluoride and 10mM sodium orthovanadate (phosphatase inhibitors) and incubated on ice for *strictly* 5 minutes only. Samples were checked under the microscope to confirm that the isolation leaves intact nuclei and centrifuged at 600g, 3 minutes to collect the nuclear pellet. The pellet was very gently washed with ice cold PBS. 5% of this pellet was used for a quick test for checking the retention of phosphoproteins and the rest (50 million intact nuclei per sample) was sent for mass spectrometry to the proteomics facility at the Stowers Institute for Medical Research, Texas.

2.7.2 Detection of Phosphoproteins using ProQ Diamond

One million whole cells and the intact nuclei obtained from the same number of cells were lysed in equal volumes of Laemmli buffer. The denatured lysates were separated on two

10% Bis-tris gels. One gel was stained with Coomassie Blue to detect the total proteins and the other was stained with Pro-Q Diamond Phosphoprotein Gel Stain (ThermoFisher Scientific) using the manufacturer protocol. ProQ Diamond stain selectively stains phosphoproteins in acrylamide gels, without the need for blotting and is compatible with mass spectrometry, allowing analysis of phosphorylation state of entire proteomes.

2.7.3 Detection of phosphoproteins by mass spectrometry

Samples were digested using 0.1µg/µl Trypsin (Promega #V5111) and processed for phosphopeptide enrichment using the Sequential enrichment from Metal Oxide Affinity Chromatography (SMOAC) approach (ThermoScientific). Both Fe-NTA with iron-chelate resin spin columns (Thermo Scitentific#A32992) and TiO spin tips (ThermoScientific #A32993) were used to enrich complementary fractions of phosphopeptides from the trypsin digested samples. The samples were split into 5 technical replicates and run on MudPIT DDA| Fe-NTA – Velos Orbitrap. The MS/MS datasets were searched against *M. musculus* protein sequences downloaded from NCBI (released on 2018.06.27). Peptide level information was assembled into protein level information using DTASelect v1.9 (Tabb, McDonald, and Yates 2002). Peptides and proteins detected were compared using CONTRAST. Combining all runs, proteins had to be detected by at least 2 peptides. Proteins that were subsets of others were removed using the parsimony option in DTASelect on the proteins detected after merging all runs. Proteins that were identified by the same set of peptides (including at least one peptide unique to such protein group to distinguish between isoforms) were grouped together, and one accession number was arbitrarily considered as representative of each protein group. The Stowers Institute in-house quantitative software, **NSAF7**(v 0.0.1), was used to create the quantitative contrast report on all detected peptides and non-redundant proteins identified across the different runs. This contrast report was compared against a reference list of NETs discovered in the Schirmer lab to identify the NETs that are expressed in the E14 line ES cells with and without LIF withdrawal. Of the ones that were expressed in both samples, those gaining or losing phosphorylation were of specific interest for this study.

Chapter 3

Genome Organisation changes during exit from pluripotency

Changes in genome organisation during differentiation and lineage commitment has been of interest to the scientific community for many years. That the organisation changes during differentiation has been known for over a decade and the global changes in LAD (Peric-Hupkes et al. 2010) and TAD (Dixon et al. 2015) organisation have been mapped during differentiation of embryonic stem cells into various lineages. It is now known that there is a functional basis for the reorganisation of the genome during linage commitment such that genes like *Pcdh9* which are only expressed in the brain, are released from the nuclear periphery as neuronal differentiation progresses, while other genomic loci like those encoding pluripotency factor *Rex1* are tethered to the periphery (Figure 7). However, the temporal dynamics of these changes remain unknown.

In this chapter I will address the dynamics of this reorganisation as mouse embryonic stem cells (ES) exit pluripotency. I will first show results to demonstrate that LIF withdrawal leads to exit from pluripotency and spontaneous differentiation of ES cells. I will then discuss how various pluripotency loci behave during differentiation and how relocation to and from the nuclear periphery is not the only mechanism that might control gene expression. The major focus of this chapter will be the study of temporal dynamics of genome organisation changes during exit from pluripotency. I will present evidence to show that the tethering of the *Rex1* locus to the periphery is a common mechanism during ES cell exit from pluripotency but the dynamics may be cell line dependent. Finally, I will discuss the effects of gene position on gene expression and present a hypothesis to explain why the rapid relocation of the locus to the periphery might be important.

3.1 Rapid and reversible tethering of *Rex1* to the nuclear periphery marks exit from pluripotency

3.1.1 LIF withdrawal leads to exit from pluripotency

Mouse ES cells are a good model to study early changes post exit from pluripotency. As explained in chapter 1, the continued propagation of ES cells in culture requires the addition of either 2i (a GSK3β and MEK inhibitor cocktail) and/or LIF (leukemia inhibitory factor) in presence of serum to activate STAT3. CHIP-seq data shows that STAT3 occupies several target sites across the genome, including the sites at *Oct3/4*, *Sox2* and *Nanog* loci (Ho et al. 2011), suggesting a key role for this transcription factor in maintaining the pluripotency network. Constitutive activation of STAT3 is also able to confer a LIF-independent self-renewal programme (Matsuda 1999). Curiously, knockout of LIF is not detrimental to development, but results in sterility of female mice, due to the requirement of LIF for blastocyst implantation (Stewart et al. 1992). However, knockouts of the downstream effectors of LIF all result in either embryonic or perinatal death (reviewed by Onishi and Zandstra 2015). Together this seems to suggest that the in vivo roles of LIF are diverse and potentially can be carried out by other factors as well. Withdrawal of LIF results in spontaneous differentiation of ES cells into all three lineages (K. Yao et al. 2014).

Thus, to study the changes in genome organisation during early stages of exit from pluripotency, standard E14Tg2A mouse ES cells were cultured in presence of recombinant LIF in a feeder-free manner. These cells, when subjected to LIF withdrawal, exit pluripotency and eventually form Embryoid Bodies (EBs), which can then in theory be differentiated into any lineage of choice with the appropriate stimuli (Figure 10A). For the first 24h post LIF withdrawal, the expression of core pluripotency factors like OCT4 and NANOG persists in the population as shown by western blotting. Subsequently, complete exit from pluripotency leading to EB formation results in a massive downregulation of these transcription factors so that the cells may eventually commit to defined lineages (Figure 10B).

Nanog is a naïve marker of pluripotency and is among the first such markers to be steadily downregulated during 2i withdrawal, along with Esrrb, Klf2 and Tfcp211 (Leeb et al.

2014). Upon 24h of LIF withdrawal, Nanog expression is asynchronously downregulated such that some cells retain the protein while others do not and by 48h, most cells show no Nanog expression (Figure 10C). This is considered to indicate that this is a driving factor because it parallels the well-known observation that the initial transition of cells from pluripotency is asynchronous (Kalkan et al. 2017). It must be noted however that the loss of key transcription factors such as Nanog and Klf2 is not sufficient for exit of ES cells from the naïve state. Although the loss of Nanog and Klf2 preceded Rex1 downregulation, self-renewal capacity is fully retained as long as Rex1 promoter activity is maintained (Kalkan and Smith 2014).

Morphologically, the cells continue to grow in well-defined colonies, with the number of spontaneously differentiated flatter cells becoming more prominent in culture as cells spend longer in exit conditions (Figure 10D).

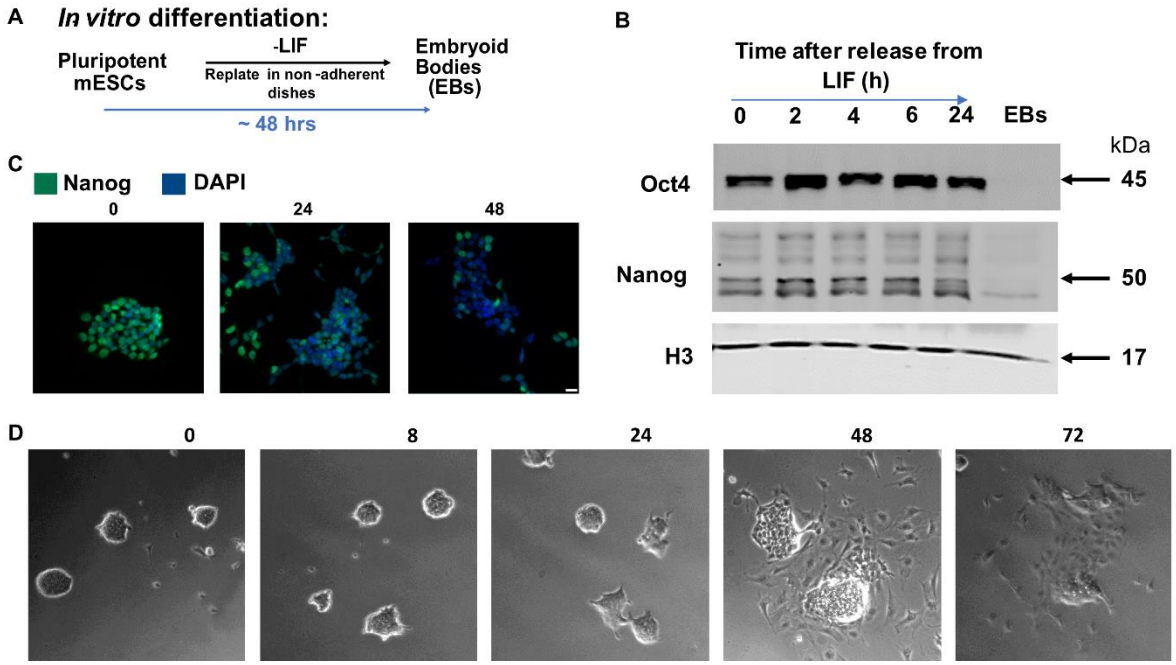

Figure 10: LIF withdrawal causes exit from pluripotency. A. Schematic describing the design for stimulating exit from pluripotency by LIF withdrawal. Cells were grown in serum/LIF conditions for at least 5 passages before all experiments. For stimulating in vitro differentiation, cells were replated in medium lacking LIF. Replating mESCs on non-adherent dishes in medium lacking LIF leads to the formation of embryoid bodies over roughly 48 hours. B. Naïve transcription factors Oct4 and Nanog are expressed over the first 24h post LIF withdrawal but their expression is lost in embryoid bodies. C. As cells exit pluripotency they do so asynchronously. At 0h post LIF withdrawal, Nanog expression is homogeneous across the population. 24h post LIF withdrawal, most cells express Nanog but some cells start to lose Nanog expression and the heterogeneity in the population is clearer at 48h. Scale bar represents 10µm. D. Phase contrast images of cells subjected to LIF withdrawal for 0, 8,24, 48 and 72h. In pluripotent culture conditions, cells grow in well-defined colonies but exit from pluripotency is accompanied by characteristic morphological flattening of cells.

3.1.2 Changing gene groups identified by DamID suggest a functional consequence for relocation of genes

One of the first genome-wide studies highlighting the changes in genome organisation during differentiation of ES cells came from the van Steensel lab where they performed a Lamin B1-DamID on ES cells (ESCs), neuronal progenitor cells (NPCs) and terminally differentiated astrocytes (ACs) to study which genomic regions are associated with the nuclear periphery in the form of LADs and how they change as lineage commitment progresses. They found that about 40% of the genome was composed of LADs ranging from about 40kb to 15Mb in size. While the genome-lamina interactions were mostly similar across the cell types, they found that certain genomic regions reposition to and from the lamina as lineage commitment progresses and this is a highly orchestrated stepwise reorganisation (Peric-Hupkes et al. 2010). For instance, the genomic locus encoding the pluripotency factor Rex1 (gene name *Zfp42*), containing two other neighbouring genes *Triml1* and *Triml2*, repositions from the nuclear interior in ES cells to the periphery in differentiated cells. Analogously, the *Ptn* gene encoding neurite growth-promoting factor 1 (NEGF-1) is found at the nuclear periphery in ES cells, and is released upon differentiation into neuronal lineages where the gene is expressed (Figure 11A and B). Thus, a functional basis for the relocation of genes in response to physiological stimuli and development was proposed to be one mechanism by which orchestration of genome organisation controls gene expression.

On the other hand, not all pluripotency loci move dramatically to and from the nuclear periphery to regulate their transcriptional state. One example of this is the *Oct4* gene, which is never found at the periphery even in differentiated cells (Figure 11C). While this agrees with previous studies reporting the absence of a role for the nuclear envelope in transcriptional regulation of *Nanog* and *Oct4* by tethering mechanisms in human ES cells (Wiblin 2005), it is entirely possible that the regulation in human and mouse ES cells is not directly comparable. However, the neat repositioning of the *Rex1* locus (for simplicity the entire locus containing genes *Triml1*, *Triml2* and *Zfp42* will be called the *Rex1 locus* in this study) provides an exemplary genomic locus to use for tracing the temporal dynamics of nuclear envelope mediated changes in genome organisation during lineage commitment.

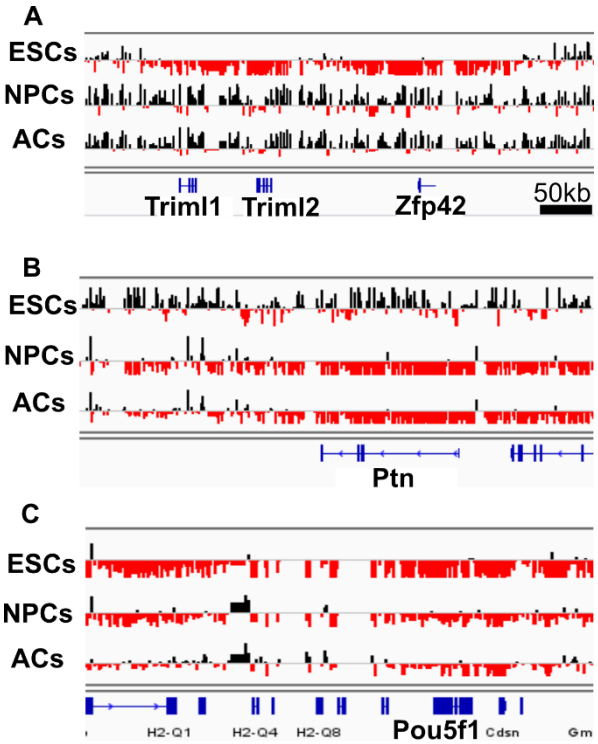

Figure 11: DamID traces for the genomic loci studied here. LaminB1 DamID data shown over three stages of lineage commitment – pluripotent mouse ES cells (ESCs), neural progenitor cells (NPCs) and terminally differentiated astrocytes (ACs). A. The *Rex1* genomic locus containing genes *Triml1, Triml2* and *Zfp42* repositions from the nuclear interior to the periphery in differentiated cells. B. The *Ptn* gene is found at the nuclear periphery in ESCs and moves to the nuclear interior as cells differentiate. C. The *Pou5f1* gene that encodes Oct4 remains in the nuclear interior in all three cell types. DamID data generated by Peric-Hupkes et al., 2010 is displayed using IGV.

3.1.3 Rex1 locus repositions to the nuclear periphery within an hour of LIF withdrawal

To understand the temporal dynamics of *Rex1* repositioning during exit, ES cells cultured in serum/LIF conditions were subjected to LIF withdrawal and the genomic loci encoding *Rex1* and *Ptn* were labelled with BACs in a 2D DNA-FISH experiment done at various time points following exit. At *T0*, the *Rex1* locus is found in the nuclear interior at a median distance of 3µm from the edge. Surprisingly, during the first hour of differentiation, the *Rex1* locus repositions to the nuclear periphery. Within 30 min the number of loci seen at the periphery (≤150nm from the DAPI edge) increases from 1% at *T0* to 23% at *T30* (Figure 12A top, and 12B left). The *Ptn* locus is found at the periphery with the first quartile at 0.3µm from the DAPI edge in cells at *T0*. Over the first hour of exit due to LIF withdrawal, the locus continues to remain at the periphery with insignificant shifts in the median values (Figure 12A bottom, and 12B right).

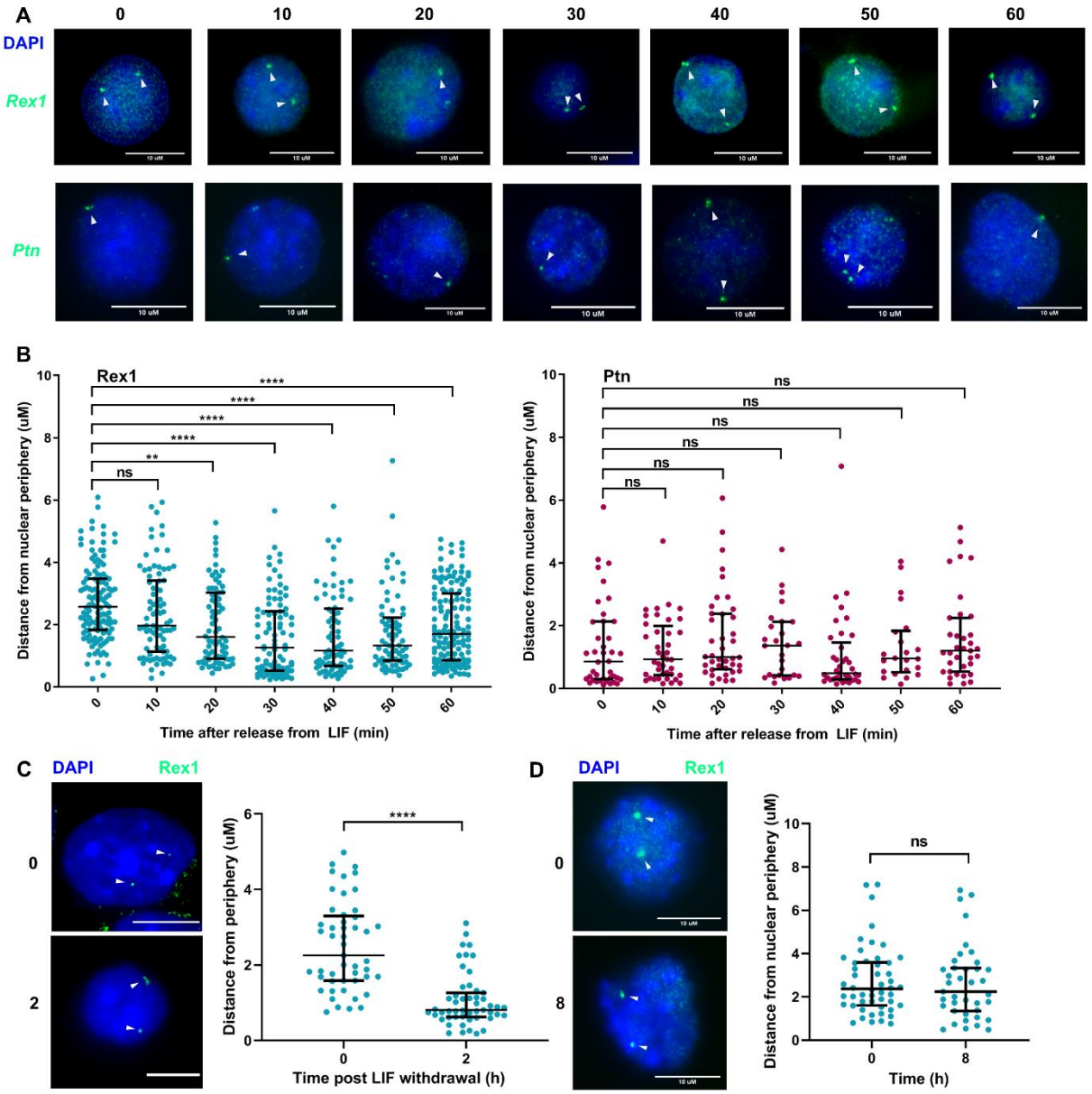

Figure 12: The *Rex1* locus rapid repositions during exit. A. Representative 2D FISH images showing the *Rex1* and *Ptn* locus positions in nuclei subjected to LIF withdrawal for 0, 10, 20, 30, 40, 50 and 60 minutes. The white arrows indicate the loci. Scale bars represent 10µm. B. Quantitation of distance from nuclear periphery for FISH images shown in A. The *Rex1* locus repositions from the nuclear interior to the periphery within an hour after LIF withdrawal while the *Ptn* locus is retained at the periphery in this time frame. **p<0.01, ****p<0.0001, ns p>0.05. C. Representative image planes with the alleles in a 3D FISH for *Rex1* in cells subjected to LIF withdrawal for 2h (left) and quantitation for distance from periphery showing scatters overlaid with median and interquartile range (right). Scale bar represents 10µm and **** p<0.0001. D. Representative 2D *Rex1* FISH images for cells subjected to trypsinisation and re-plated in serum/LIF culture conditions for 8h (left) and quantitation for distance from periphery (right). Ns p>0.05, scale bar represents 10µm

As it has been argued that the results obtained from 2D FISH are as reliable as those from 3D FISH, we elected to see if our results could be replicated by 3D FISH. As background,

for 2D distance measurements, analysis is done either on single images selected by a representative slice or generated by maximal intensity projections. While the vertical distance information is lost in this process, this greatly simplifies image processing steps and reduces the computational resources needed for the analysis. 3D measurements provide the vertical distance information but this is also subject to lower resolution in the z-axis and optical aberrations. In a recent study from the Misteli lab, they compared the suitability of 2D versus 3D distance measurements in the analysis of various features of spatial genome organisation. They found a high degree of agreement between 2D and 3D analysis, with higher convergence of measurements as the interrogated distance increases (Finn et al. 2017). In other words, for studies such as this one, where the locus travels a median distance of almost ~2µm, 2D and 3D measurements would expectedly reveal similar results. However, for studies looking at loci separated by very small distances say gene-enhancer interactions, 3D measurements would more accurately reflect the changes.

While 3D distance measurements are more accurate than 2D measurements these are subject to noise in the z-axis due to the aberrations mentioned above, which can be ameliorated only by sampling at much higher frequency in z, which in turn requires greater computational power for both imaging and analysis. Thus, they concluded that for comparing distances between cells to obtain trends, especially when the repositioning is as dramatic as a movement to and from the periphery, 2D distance measurements are as suitable as 3D measurements. As a confirmation of this, *Rex1* repositioning was also confirmed in a 3D FISH experiment. As anticipated, the locus repositions to the nuclear periphery 2h post LIF withdrawal. At *T0,* the locus is found at a median distance of 2.3µm and the first quartile is at 1.6µm from the nuclear periphery i.e. 25% of the loci closest to the periphery are ≤1.6µm from the DAPI edge. At *T2,* most data points cluster neatly around the median value of 0.7µm from the periphery. The first quartile is at 0.5µm i.e. 25% of the datapoints are found ≤500nm from the DAPI edge and 10% of the loci are within 200nm from the nuclear periphery (Figure 12C). Since the results of 2D and 3D FISH agree well with each other, for simplicity, most of the FISH done in this study was analysed in 2D.

Since LIF withdrawal for the formation of EBs during *in vitro* differentiation requires the cells cultured in serum/LIF culture conditions, to be trypsinised and replated into medium without LIF and left for aggregation, it needed to be confirmed that the repositioning of the

locus was due to LIF withdrawal and not due to the replating process. Trypsinisation rounds up cells and nuclei which could potentially alter genome organisation, though this is never been experimentally tested or demonstrated. Therefore, the cells were trypsinised and replated in complete medium containing LIF and DNA-FISH was used to trace the *Rex1* locus. It was found that the locus remains at the nuclear interior even after 8h of replating (Figure 12D) suggesting that the repositioning of the *Rex1* locus is a characteristic event of exit from pluripotency.

3.1.4 Rex1 repositioning is reversible during the early stages of exit

The asynchronous loss of REX1 expression marks a progression in pluripotent status that precedes a decline in classical markers like OCT4. However, for as long as cells continue to express REX1, the cells retain the ability to regain ground state pluripotency (Kalkan and Smith 2014; Kalkan et al. 2017). Thus, during the ES cell entry into differentiation, there is a substantial interval of about 24h or longer between the loss of naïve pluripotency and the manifestation of differentiation. During this interval many changes are seemingly reversible (A. Smith 2017). To test if *Rex1* repositioning was also similarly reversible, when cells were subject to LIF withdrawal for 2h and the medium was re-supplemented with LIF for an additional 2h. The initial recruitment of the locus to the periphery upon LIF withdrawal was abolished when LIF was re-supplemented, confirming both, that the repositioning observed is indeed LIF dependent and also that this tethering is reversible during the first 2h of exit (Figure 13). Such rapid reversible changes in genome organisation have not been described previously during exit. It suggests that the tethering of this locus to the periphery is of importance and perhaps one of the earliest events that sets the stage for departure from ground state pluripotency.

When cells are maintained in serum/LIF conditions, the *Rex1* locus is found anywhere between 0.5 to 6µm from the nuclear edge with a median value of about 3µm in a population of cells (Figure 12B, *T0*). As cells exit pluripotency due to LIF withdrawal for an hour, the scatter of the data is between 0.5µm to 4µm, with most datapoints clustering towards the X-axis indicating a recruitment to the periphery with a median value of ~1.8µm distance from the nuclear edge (Figure 12A and B). This represents an asynchronous recruitment of the *Rex1* locus in different cells and could also be attributed to the

heterogeneity in the population. To eliminate the possibility that the heterogeneity of the population contributes to the scatter in the datapoints, the recruitment of the locus was tested during exit set up by 2i withdrawal, where the heterogeneity is considered minimal.

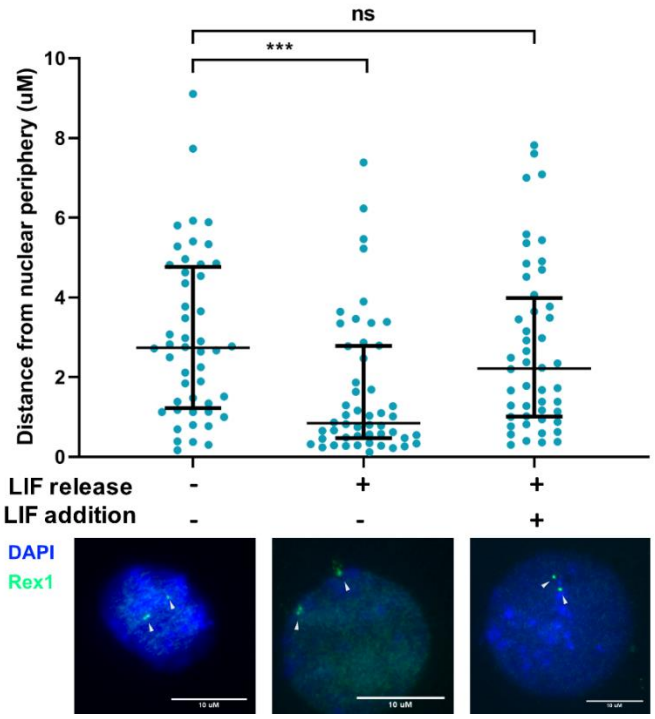

Figure 13: *Rex1* tethering is LIF dependent and reversible. Quantitation of distance of the *Rex1* locus from the periphery in cells subjected to exit by LIF withdrawal (LIF release) for 2h and then re-supplemented with LIF (LIF addition) for 2h (top). ***p<0.001, ns p>0.05. Re-introducing LIF in the medium leads to the locus being released from the periphery. Representative FISH images (bottom), scale bar is 10µm.

3.1.5 2i withdrawal stimulates Rex1 repositioning

ES cells grown under serum/LIF conditions are morphologically different from when they are grown in 2i/LIF conditions. In serum/LIF, ES cells grow in colonies with well-defined boundaries with rare cases of flatter cells seen at the edges. In 2i/LIF conditions, the cells grow as uniform near-spherical adherent clusters and are less spread out (Figure 14A). During exit, the *Rex1* locus moved from a median value of about 2µm to 0.5µm with 20% of the loci found ≤300nm from the nuclear edge and three quartiles of the data points under 1.4µm from the edge. However even at *T0*, the scatter of the datapoints reveals that the locus resides in a range of 0.25µm to 6µm from the periphery in the various different nuclei

scored, which is similar to when cells are cultured in serum/LIF (Figure 12A, *T0*). Upon exit (Figure 14B, *T2*), the broader range is similar with the most interior locus scored at ~6µm from the edge but the one closest to the periphery is 0.1µm away. However, the case of 2i withdrawal, most data points cluster towards the X-axis showing a strong repositioning phenotype (Figure 14B, *T2*).

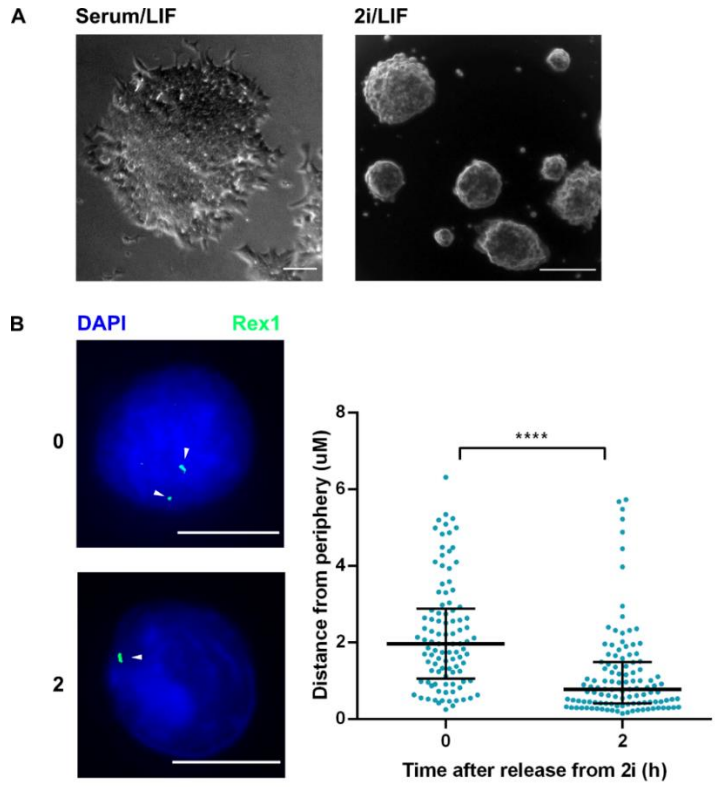

Figure 14: *Rex1* repositioning is seen in 2i withdrawal induced exit. A. Phase contrast images of cells grown in serum/LIF and 2i/LIF culture conditions. In both conditions pluripotent cells grow in well-defined colonies but the population is more homogeneous in 2i/LIF conditions with fewer spontaneously differentiated cells seen at the colony edges. Scale bar is 100µm. B. Representative FISH images for *Rex1* in cells subjected to 2i withdrawal for 2h (left) and quantitation of distance from periphery (right). The locus repositions from nuclear interior to the periphery in this time frame. ****p<0.0001.

3.1.6 Rapid repositioning of the Rex1 locus may depend on actin-myosin motor proteins

Nuclear actin was long described to be the result of thermodynamic wandering. However, it is now known that nuclear actin does not simply result from diffusion but has key roles in regulating many nuclear functions. And the same holds true for nuclear myosin,

specifically myosin I (NM1) (Pederson and Aebi 2002). The recruitment of target loci towards relatively stably positioned Cajal bodies is inhibited in presence of a dominant-negative mutant of beta-actin, showing a key role for nuclear actin in facilitating long range chromatin movements (Dundr et al. 2007). Using drugs that inhibit actin polymerization or myosin function, it was shown that both actin and NM1 act in concert to promote RNA polymerase I transcription (J. Ye et al. 2008). A similar approach using drugs to prevent actin polymerisation and inhibit NM1 function has been used previously to show the dependence of rapid single gene and whole chromosome movements on actin polymerisation and NM1 activity (Mehta et al. 2010; Pradhan, Nallappa, and Sengupta 2020).

To test if the rapid recruitment of *Rex1* to the nuclear periphery depends on actin polymerisation, ES cells were treated with Latrunculin, which sequesters free actin monomers or Jasplakinolide, which stabilises F-actin and prevents its depolymerisation, before subjecting them to LIF withdrawal for 2h. In both cases, *Rex1* repositioning fails to occur (Figure 15A). Similarly, when cells were treated with butanedione monoxime (BDM), which is a non-selective myosin ATPase inhibitor, *Rex1* failed to relocate to the nuclear periphery upon LIF withdrawal (Figure 15B). Together this suggests that actin polymerisation and myosin functions are important for the relocation of the locus to the nuclear periphery during exit.

It must be noted, however, that treating whole cells with inhibitors for actin polymerisation or myosin function can have a wide spectrum of effects. Thus, while these experiments are consistent with the dependence of the rapid relocation of this locus on motor proteins, it remains possible that this is an effect of the drugs treatment causing a complete freeze-down of transport machinery. However, while the effects of one drug alone may be due to off target or secondary effects, that both Jasplakinolide and Latrunculin treatment should show the same effects strengthens the possibility that actin polymerisation indeed has a role in *Rex1* repositioning. Classical ways to address this question would either involve depletion of the protein using a knockdown or knockout approach, microscopy or *in vitro* methods, which come with their own caveats. While the knockout of β-actin in mice results in embryonic lethality (Tondeleir et al. 2012), conventional *in vitro* depletion experiments for proteins with such widespread functions could also be ineffective as that would most likely lead to cell death and/or additional phenotypes such as inhibition of differentiation.

Therefore, using chemical ways to inhibit motor protein function, especially when confirmed using more than one drugs, yielded strong data to support our hypothesis that rapid relocations of genomic loci may be more targeted and depend on the nucleoskeleton.

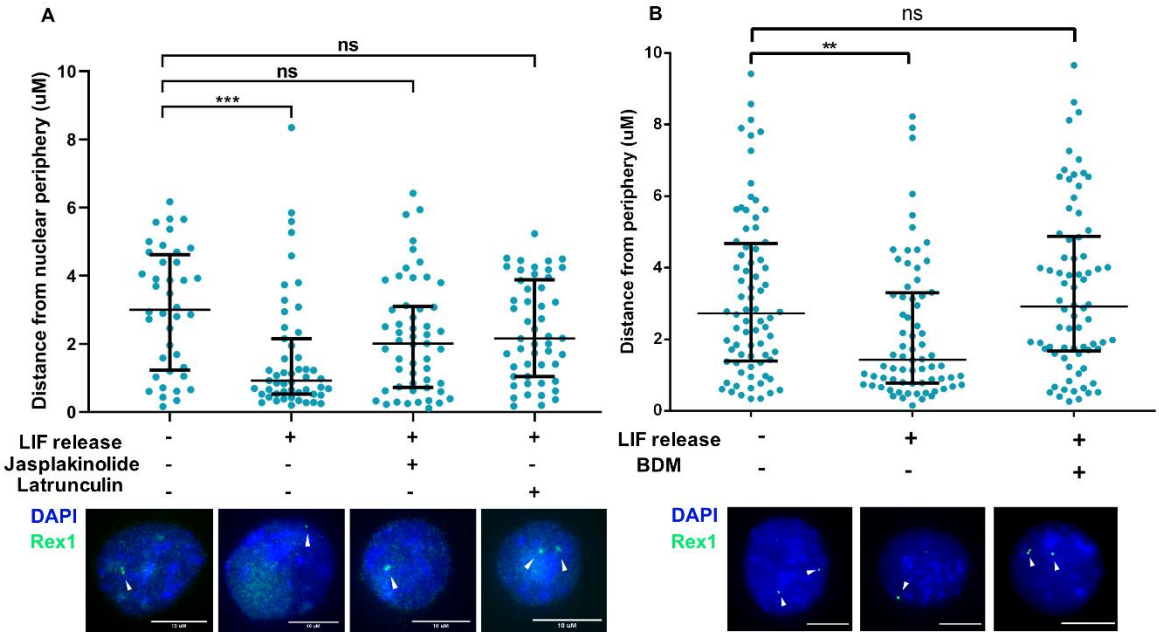

Figure 15: *Rex1* repositioning depends on motor proteins. A. Quantitation for distance of *Rex1* from the periphery (top) in cells subjected to LIF withdrawal after 1µm Latrunculin A or 1µm Jasplakinolide treatment, inhibitors of actin polymerisation. Inhibiting actin polymerisation prevents locus repositioning to the periphery. ***p<0.001, ns p>0.05. Representative FISH images (bottom), scale bar is 10µm. B. Quantitation for distance of *Rex1* from periphery (top) in cells treated with 1mM BDM, a non-selective myosin ATPase inhibitor, before they are subjected to LIF withdrawal for 2h. BDM treatment affects locus repositioning. **p<0.01, ns p>0.05. Representative FISH images (bottom), scale bar is 10µm.

3.1.7 Temporal dynamics for *Rex1* repositioning are cell line dependent

E14Tg2a cells have been widely used for a number of studies on pluripotency, exit and *in vitro* differentiation. This male mouse ES cell line was derived from mouse strain 129/Ola inner cell mass, isolated by blastocyst trypsinisation. The rapid repositioning of *Rex1* is an interesting observation and to establish whether this is unique to exit of E14Tg2a cells or whether it is a general mechanism that takes place during the exit of other stem cell lines as well, the experiments were also carried out in CGR8 ES cells, which is a germ-line competent cell line, also established from the inner cell mass of a 3.5 day male pre-

implantation mouse embryo of the 129 strain. These cells were maintained in the same culture conditions as E14Tg2a cells in medium containing serum/LIF and exit was induced by LIF withdrawal.

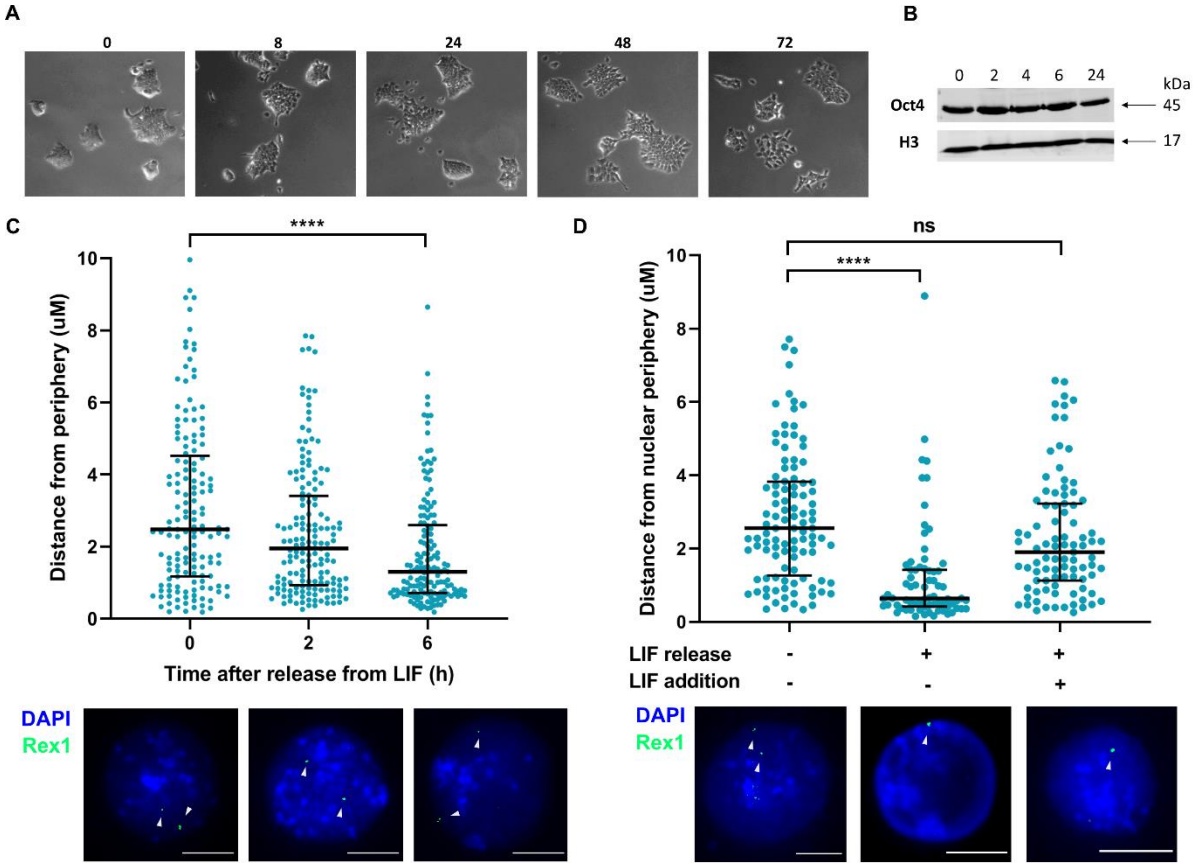

Figure 16: Temporal dynamics of *Rex1* repositioning are cell line dependent. A. Phase contrast images of CGR8 line of mouse ES cells subjected to LIF withdrawal for 0, 8, 24, 48 and 72h. Characteristic morphological flattening of cells observed during exit is observed upon LIF withdrawal. B. Naïve transcription factor Oct4 is expressed over the first 24h post LIF withdrawal. C. Quantitation of *Rex1* distance from periphery in cells subjected to LIF withdrawal (top). The locus repositions 6h after LIF withdrawal. ****p<0.0001. Representative FISH images (bottom), scale bar is 10µm. D. *Rex1* repositioning is LIF dependent and reversible in CGR8 cells. Distance from periphery measurements for *Rex1* (top) in cells subjected to LIF withdrawal for 6h (LIF release) and re-supplemented with LIF for 2h (LIF addition). ****p<0.0001, ns p>0.05. Representative FISH images (bottom), scale bar is 10µm.

As seen for E14Tg2a cells, CGR8 cells also undergo exit from pluripotency due to LIF withdrawal and the onset of differentiation is reflected by changes in colony morphology over 72h (Figure 16A). Just as in E14Tg2a cells, steady Oct4 expression is retained for the first 24h post LIF withdrawal (Figure 16B). However, when subjected to LIF withdrawal, while E14tg2a cells show Rex1 recruitment to the periphery within the first hour, in CGR8 cells the relocation is observed at 6h post exit. In CGR8 cells, at *T0*, three quartiles of the

datapoints lie over 1.4µm from the periphery. At *T2,* the distribution of datapoints is similar to the *T0* sample with only a slight shift in the median. It is only at *T6* that 25% of the datapoints cluster below 500nm from the periphery (Figure 16C). In contrast, in E14Tg2a cells, within the first hour of exit, over 20% of the loci were recruited to the periphery (Figure 12A and B). Although the temporal dynamics of repositioning are different in the two cell types, the reversibility in *Rex1* recruitment is consistent during the first few hours post exit (Figure 16D).

3.1.8 Investigating effects of gene position on gene expression

As discussed in Chapter 1, gene position tends to affect gene expression. Although the nuclear periphery is not incompatible with transcription, it largely functions as a repressive environment so that genes that move towards the periphery in response to a physiological stimulus often become repressed. That *Rex1* is tethered to the nuclear periphery during in vitro neurogenesis has been previously shown using both DamID and FISH approaches. In the same study it was also shown that when pluripotent ES cells form neural progenitor cells, there is a downregulation in *Rex1* expression (Peric-Hupkes et al. 2010). However in this study, neural progenitor cells were derived using a well-established protocol for ES cell differentiation in N2B27 medium which promotes neural fate commitment, typically over a 4-5 day period (Ying and Smith 2003). Similarly, REX1 downregulation during exit from pluripotency and subsequent lineage commitment, both *in vivo* and *in vitro*, has been reported in various other studies, making REX1 a marker for naïve pluripotency (Pelton et al. 2002; Mulas, Kalkan, and Smith 2017; Kalkan et al. 2017). However, in all these cases differentiation has been studied over a period of several hours to a few days.

Since the *Rex1* locus is tethered to the periphery within 2h post LIF withdrawal, I was interested in whether this relocation leads to repression of any of the three genes within this locus namely *Triml1, Triml2* and *Rex1*. Therefore, in an attempt to answer this a qPCR experiment was designed to quantify Rex1 mRNA over the first few hours of exit. It is important to note, however, that RNA degradation itself is a mechanism to regulate gene expression. In bacteria, mRNA turnover is rapid with most mRNA half-lives typically being only 2-3 minutes, allowing the cell to respond quickly to alterations in its environment. In eukaryotes, however, different mRNAs are degraded at different rates such

that the half-lives of mRNAs in mammalian cells vary from less than 30 minutes to approximately 20 hours (Cooper 2000).

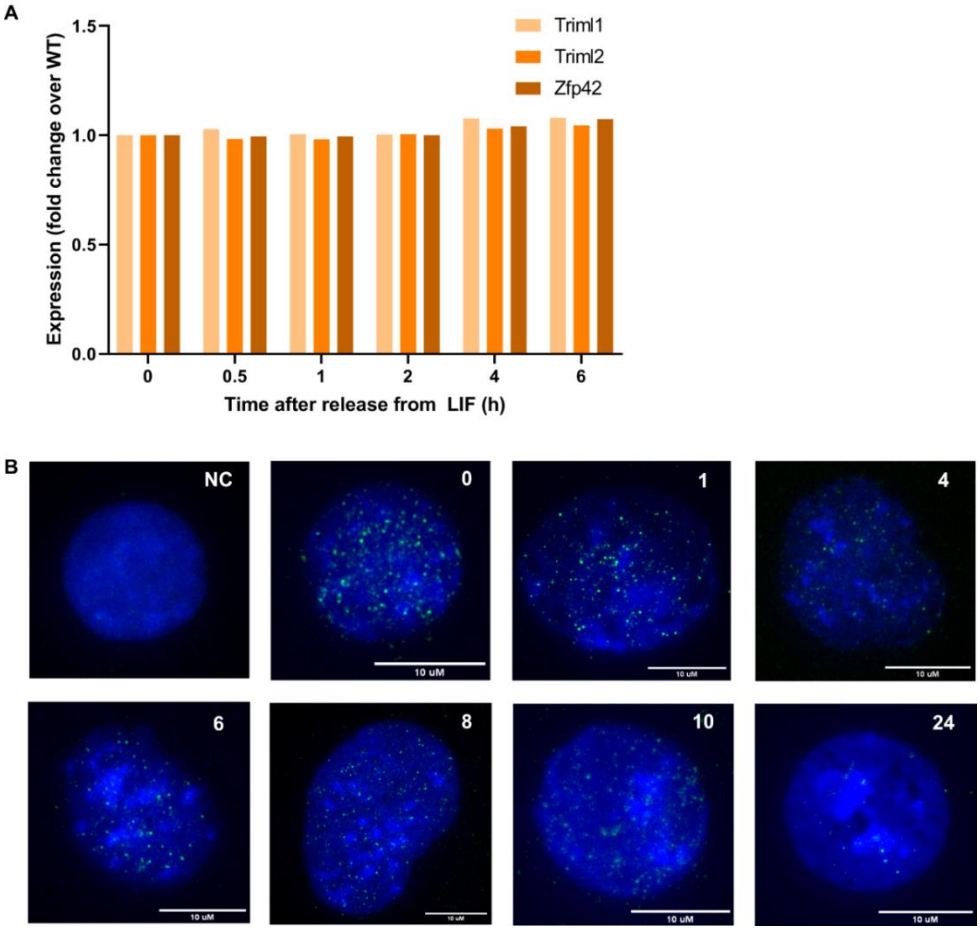

Figure 17: *Rex1* promoter activity persists for several hours after the locus repositions to the nuclear periphery. A. qPCR for the Triml1, Triml2 and Zfp42 (encodes Rex1) in cells subjected to LIF withdrawal for 0, 0.5, 1, 2, 4 and 6h. Expression was normalised over GAPDH and normalised values for fold change over WT is shown here. B. RNA-FISH images for Rex1 in isolated nuclei of negative control (NC) and cells subjected to LIF withdrawal for 0, 1, 4, 6, 8, 10 and 24h. Green dots represent Rex1 mRNA, blue = DAPI. At 0h post LIF withdrawal, there are several Rex1 transcripts and the signal goes down by 24h into exit. Scale bar = 10µm.

A conventional qPCR experiment performed using RNA extracted from whole cell lysis showed no difference in gene expression for any of the three genes over the first 6h of exit (Figure 17A). This agrees with previous studies where Rex1 downregulation was reported to occur about 16h post exit induced by 2i withdrawal. In the same study, at a later stage of 25h post 2i withdrawal, Rex1 was seen to be asynchronously downregulated (Kalkan et al. 2017). Using whole cell lysis to extract RNA takes into account both freshly transcribed nuclear and longer lasting ribosomal-associated translating cytoplasmic RNA. In an

attempt to quantify only the nuclear *Rex1* transcripts, RNA-FISH was performed on isolated nuclei. The negative control (NC) showing an example of a nucleus stained without hybridisation (with the RNA-FISH probes) confirms that the signal observed in the other samples is not due to background fluorescence. The pan-nuclear transcripts detected in ES cell nuclei at *T0* reflect the high transcriptional activity of the locus. By 8-10h post LIF withdrawal, there is a marginal decrease in transcripts but by 24h, the decrease in signal is massive (Figure 17B), confirming a transcriptional repression of the locus albeit much longer after tethering.

3.1.9 Monitoring Rex1 promoter activity during exit

It is not entirely surprising that a genomic locus that is being tethered to the periphery would not immediately change its transcriptional activity as other studies have shown that gene repositioning acts in concert with other factors (Robson et al. 2016). The results from qPCR and RNA-FISH use transcripts as a read-out. As mentioned above, mechanisms like RNA turnover affect such read-outs. In other words, the results from these experiments confirm that the transcripts are retained up to ~24h post LIF withdrawal, but without knowing the turnover rates in these particular cells under these particular conditions, it is not possible to determine from these results whether the promoter is indeed active during this entire period. To study the loss of naïve identity and track individual cells in their transition to formative pluripotency, a knock-in of destabilised GFP to the *Rex1* locus was generated by the Smith lab (Kalkan et al. 2017). This Rex1::GFPd2 cell line is an ideal system to monitor the promoter activity as the cells exit pluripotency, as the half-life of this destabilised GFP is ~2h. These cells have been used in detailed studies tracking the transition from ground state pluripotency by the Smith lab. It is now known that up to 16h after 2i withdrawal, the cells remain uniformly GFP-positive and can revert to self-renewal efficiently. It is also known that by 25h GFP expression profile is heterogeneous and that the newly formed GFP-low cells can respond to stimuli inducing the formation of the three embryonic germ layers (Kalkan et al. 2017; Mulas, Kalkan, and Smith 2017). These results suggest that the Rex1 promoter does indeed remain active for several hours post 2i withdrawal.

Rex1::GFPd2 cells were cultured in 2i/LIF conditions and subjected to 2i withdrawal for 72h. In agreement with the results from the Smith lab, the population is homogenously GFP-positive up to 22h post 2i withdrawal. At 48h, newly formed GFP-negative cells are

detected and finally by 72h post 2i withdrawal, the population mostly consists of GFP-negative cells (Figure 18A). The exit from pluripotency is also reflected in cell cycle dynamics. Rex1::GFPd2 cells cultured in 2i/LIF show a large number of cells in S and G2/M stages of the cell cycle. This shifts slightly by 22h post 2i withdrawal where the histogram shows a marginal decrease in the G2/M population. By 72h, most cells are found in the G1 (Figure 18B).

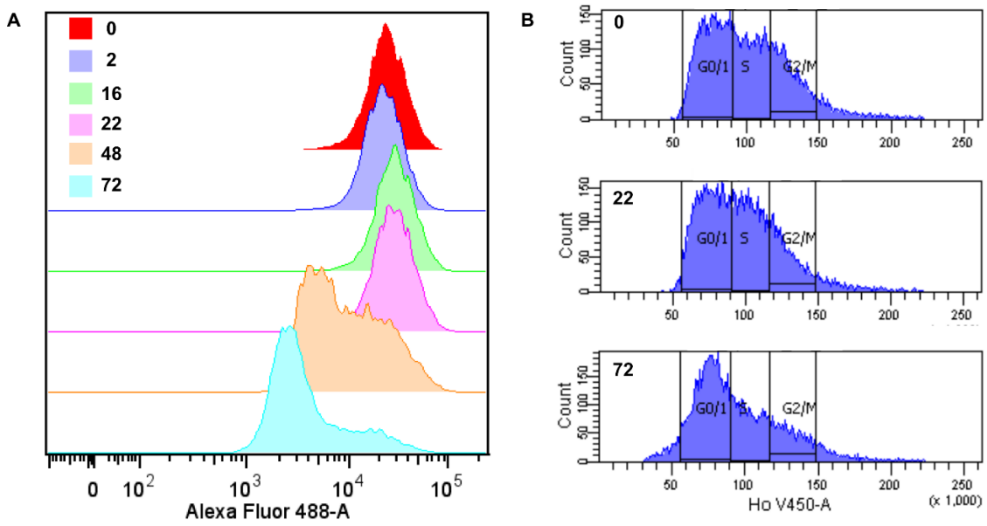

Figure 18: *Rex1* promoter activity measured in Rex1::GFPd2 cells. A. Rex1::GFPd2 cells generated by the Smith lab (Kalkan et al., 2017) were subjected to 2i withdrawal for 0, 2, 16, 22, 48 and 72h. GFP expression is indicative of an active promoter. Histogram overlays for GFP signal in the samples is shown here. The cells homogeneously express GFP up to 22h post LIF withdrawal. At 48h the population is heterogeneous for GFP expression, which is almost entirely lost by 72h post 2i withdrawal. This experiment reproduces the results of Kalkan et al., 2017. B. Rex1::GFPd2 cells subjected to 2i withdrawal for 0, 22 and 72h were stained with Hoechst and analysed for cell cycle based on DNA content. At 0h and 22h post 2i withdrawal, most cells are actively cycling and a high percentage of these are found in S and G2/M. 72h into exit, there is a very high percentage of cells in G0/G1 stage of the cell cycle indicating spontaneous differentiation.

Cell cycle and the mechanisms required for executing cell fate decisions are intricately linked. Pluripotent mouse ESCs have very short G1 phase, which nearly doubles during neural stem cell differentiation (Roccio et al. 2013). In both human and mouse ES cells, longer G1 phases typically correlate with pluripotency exit and initiation of differentiation (Calder et al. 2013; Coronado et al. 2013; Soufi and Dalton 2016). In fact, in human ES cells, G1 phase cells have an increased susceptibility to differentiate compared to the equivalent S or G2 phase cells (Sela et al. 2012). The precise mechanism behind the importance of G1 phase in controlling cell fate decisions has yet to be determined but

several hypotheses have been put forth. The complement of transcription factors present in the G1 phase may yield in higher responsiveness to differentiation cues. The G1 phase may also have a more permissive chromatin state, both in terms of higher order genome organisation and epigenetic modifications to allow a cell to respond specifically to certain stimuli (reviewed by Hardwick and Philpott 2014).

Since the Rex1::GFPd2 cells come from a specific subclone of E14tg2a and, to my knowledge, have not been used for genome organisation studies, it was of interest to me to establish whether these cells also show a rapid relocation of the *Rex1* locus as seen in E14 and CGR8 cells. FISH for the *Rex1* locus reveals a repositioning from the interior to the periphery by 2h, with 50% of the loci found ≤500nm from the DAPI edge. Thus, these cells resemble the E14 parent line in the repositioning phenotype for *Rex1* (Figure 19). Since the half-life of this destabilised GFP is ~2h, the continued maintenance of promoter activity reflected by GFP expression in these cells (Figure 18A) is a better measure of transcriptional output compared to the qRT-PCR and RNA FISH experiments described in the previous section.

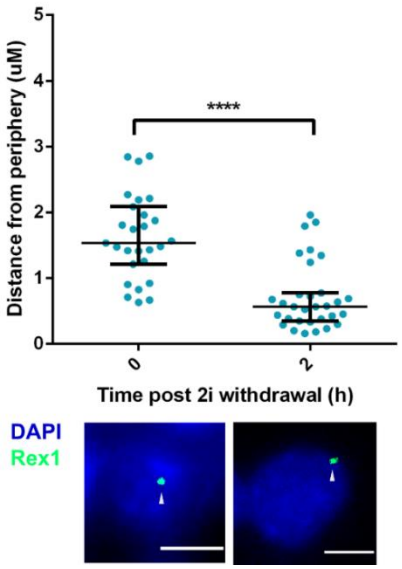

Figure 19: *Rex1* locus rapidly repositions to the periphery in Rex1::GFPd2 cells during exit. Distance from periphery measurements for *Rex1* in the Rex1::GFPd2 cells subjected to 2i withdrawal for 2h (top). ****p<0.0001. FISH was done in 3D and representative image planes with the Rex1 allele (green) are shown in the bottom panel. Scale bar is 10µm.

Taken together, these results suggest that the rapid movement of the *Rex1* locus to the nuclear periphery does not lead to an immediate transcriptional shut down. If there are minor differences in transcriptional activity due to steric effects, the changes are minimal and below the detection threshold of the experiments presented here.

Considering how quickly the gene relocates to the periphery, it is also evident that it is one of the first events determining the transition from naïve to a formative state of pluripotency. It is also known that while *Rex1* expression is asynchronously downregulated in a population of ES cells exiting pluripotency, cells that retain *Rex1* expression are the ones with the ability to self-renew when 2i/LIF is re-supplemented into the medium and the cells that lose *Rex1* expression are perhaps those that have undergone capacitation (Kalkan et al. 2017). It is therefore an interesting question to ask why the locus repositions so long before its transcriptional repression and the next section discusses a plausible hypothesis that might answer this question.

3.1.9 The Rex1 locus may act as an enhancer

While REX1 has been shown to be important in maintaining the undifferentiated state of human ES cells, it seems to be dispensable for mouse ES cell pluripotency based on the fact that *Rex1*$^{-/-}$ mice are viable and fertile. Furthermore, *Rex1* over expression fails to maintain pluripotency in the absence of LIF in mouse ES cells (Masui et al. 2008). Similarly in rats, REX1-deficient rESCs and rats were viable and apparently normal (Meek et al. 2020).

The results presented in the previous sections show that that the locus is tethered to the nuclear periphery within an hour of subjecting cells to LIF withdrawal to stimulate *in vitro* differentiation, but the promoter activity is maintained uniformly in cell populations for much longer leading to its asynchronous downregulation. We also know that during the first 24h, exit is reversible and that loss of Rex1 that marks an irreversible transition from naïve to formative state of pluripotency (Kalkan et al. 2017). In agreement with this, when LIF is re-supplemented back into the medium, the tethering phenotype of the *Rex1* locus is quickly reversed. Though it seems that the tethering of the locus itself marks the beginning of the events leading to exit, the rapidity and reversibility of this event is intriguing. Considering these pieces of information together, it leads to the following question:

Why is the Rex1 locus subjected to positional regulation at all?

One possible explanation for is that the locus potentially contains regulatory elements and it is the precise regulation of these that requires rapid changes in locus position. I chose to look at whether this locus might contain enhancers, which are canonically defined as short (100-1000bp) noncoding DNA sequences that drive transcription independent of their relative distance, location or orientation to their cognate promoters (Schaffner 2015). Enhancers tend to loop to and associate with adjacent genes in order to activate their transcription. While most of these interactions occur within a distance of ~50kb of the enhancer, many interactions can potentially occur at greater distances up to several megabases (Ong and Corces 2011; Sanyal et al. 2012). Even with the contraction in space provided by topologically associated domains, the distances between genes and their enhancers can be quite large. Several models have been proposed to facilitate such long range interactions including the idea that Pol II bound to upstream regulatory elements might move along the DNA pulling the enhancer with it to come into contact with the promoter or that protein chain linkers might oligomerize and facilitate loop formation (Furlong and Levine 2018). Interestingly, enhancers can also co-activate two different target genes (Fukaya, Lim, and Levine 2016).

The 5' end of the *Rex1* gene is occupied by naïve transcription factors OCT4, NANOG and SOX2, thus making it a potential superenhancer, defined as a genomic locus typically containing a cluster of enhancers capable of acting on several different gene targets. Using 3C, the authors also show that the superenhancer interacts with the promoter of *Rex1* itself, thereby regulating its expression (S. Zhang et al. 2019). However, the authors do not discuss the other genomic loci that are potentially targeted by the superenhancer.

The accurate and precise regulation of gene expression is key to developmental programming. Enhancers play a central role in orchestrating spatiotemporal precision in gene regulation during development. For instance, failure of enhancer activation by chromatin remodelling factor Brg1 leads to a failure in mesoderm induction (Alexander et al. 2015). During development, superenhancers which consist of clusters of enhancers that are densely occupied by transcription factors are often found next to genes that define cell identity (Whyte et al. 2013). The presence of a superenhancer in the *Rex1* locus occupied by master transcription factors like OCT4, NANOG and SOX2, suggests the potential importance of this enhancer in maintaining ES cell state. Thus, it is possible that the tight

regulation of the superenhancer function is necessary for defining the transcriptional landscape for pluripotency and rapid tethering to the nuclear periphery is the regulatory mechanism for controlling its function.

EnhancerAtlas is a database for identifying predicted enhancers annotated using information collected from eight types of experimental approaches including P300 binding sites, POL2 binding sites, histone modifications, transcription factor binding sites, CHIA-PET, etc (T. Gao et al. 2016). Using this database, I identified an additional pluripotent state-specific predicted enhancer within the intronic element of the *Rex1* gene in E14 mouse ES cells and in mouse induced pluripotent cells (Figure 20A). Further, Bonev et al identified in their HiC datasets, an ES specific interaction with an adjacent region (containing genes Triml1 and Triml2) which was also tested using FISH (Bonev et al. 2017). A visualisation of the region generated using 3D genome browser (Y. Wang et al. 2018) is shown here (Figure 20B).

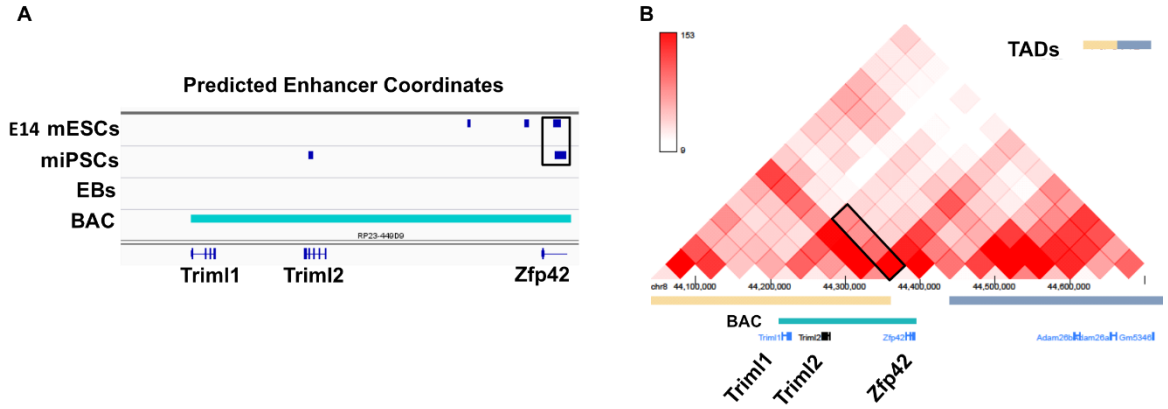

Figure 20: *Rex1* locus might contain an enhancer. A. IGV screenshot showing the presence of a putative enhancer within the intronic region of the Zfp42 gene that encodes Rex1. The sequence is a predicted enhancer in E14 mouse ES cells (E14 mESCs) and mouse induced pluripotent stem cells (miPSCs) but not in embryoid bodies (EBs). The bacterial artificial chromosome (BAC) used for hybridisation to the *Rex1* genomic locus spans the three genes and their intergenic region and contains the putative enhancer. B. HiC heatmap of the TAD containing the three genes Triml1, Triml2 and Zfp42, visualised using 3D genome browser. The HiC maps were generated by Bonev et al, 2017. The BAC marks the region that was probed in the FISH experiments.

This led to the following hypothesis illustrated in Figure 21:

The loop formed by the locus into the nuclear interior allows for the interaction of the enhancer with its target genes, among which are Triml1 and Triml2. This interaction is abolished upon the tethering of the locus to the periphery.

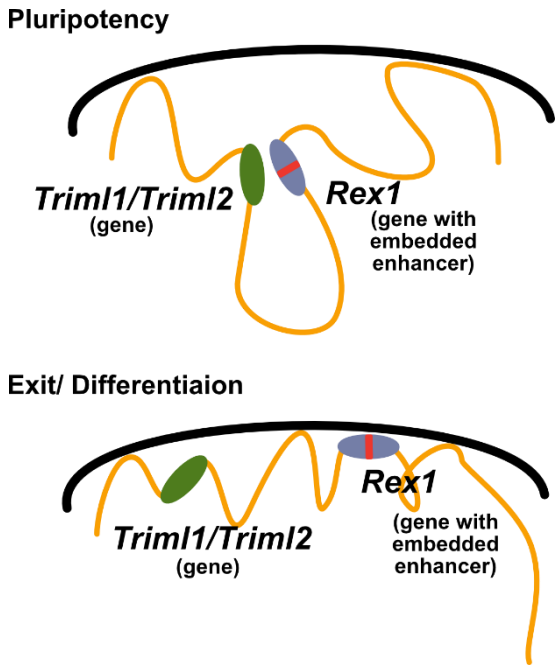

Figure 21: Illustration showing the hypothesis that the putative enhancer embedded (red) within the Rex1 gene might interact with the two other genes in the locus in ES cells when the loops extends into the nuclear interior (top). As cells exit pluripotency the tethering to the periphery might sterically inhibit this interaction (bottom).

Determining the position of an enhancer is much more difficult than for a gene because enhancers tend to be only ~1/1000 the size of genes. This means that labelled probes to cover this small area will not allow for many individual probed hybridising and thus a very weak signal. Most commercially available probes in the form of fosmids are ~50-70kb in length while BACs could range from ~150-250 kb in length, which are too big to probe genomic distances of 15-20kb. The entire probe spanning the three genes and the intergenic region is a BAC of about ~200kb in length. Thus, using this BAC as a template, I generated PCR amplicons spanning the length of about 20kb covering each of these three genes individually. Ideally, I would have liked to use a probe for the predicted enhancer element alone but since that is under 4kb in length, the probe would not have been detectable in a FISH experiment. Using these probes in DNA-FISH, I sought to determine if *Rex1*, and thus potentially the embedded enhancer within it, interacted with either *Triml1* or *Triml2*

and crucially, if this interaction is abolished within the first two hours of exit. By measuring the distance between the probes in ES cells subjected to LIF withdrawal for 2h, I found that while the median distance between the probes does not change for *Rex1-Triml1* interaction (Figure 22B) the median distance for *Rex1-Triml2* interaction changes from about 0.2µm to about 0.4µm (Figure 222C) when ES cells are subjected to LIF withdrawal, suggesting that the *Rex1-Triml2* interaction is abolished when the locus is tethered to the periphery, which was measured in the same experiment using the BAC for the entire locus in the same experiment (Figure 22A). However, this failed interaction does not result in a downregulation of Triml2 transcripts in the first 6h during exit (Figure 17). It would be interesting to follow Triml1 and Triml2 expression over a longer time course to establish whether the loss of gene-putative enhancer interaction leads to transcriptional downregulation.

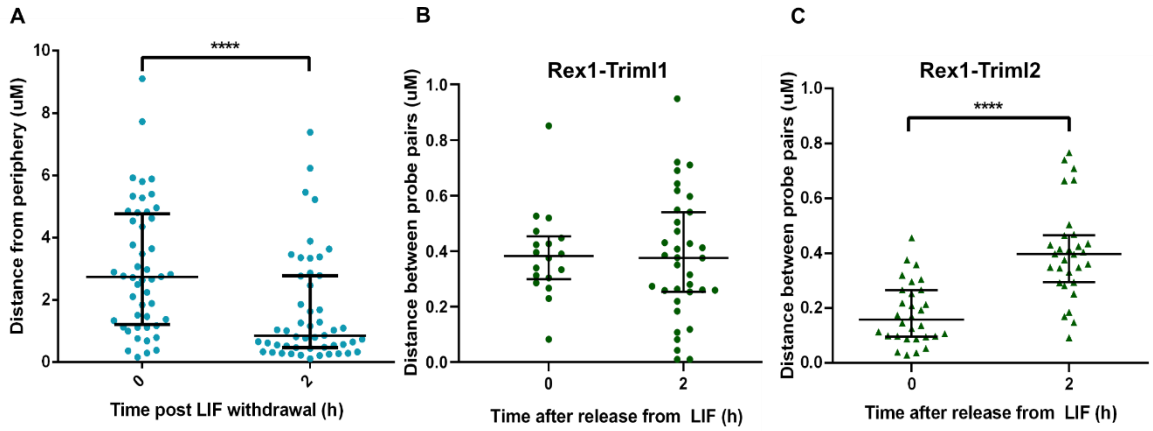

Figure 22: *Rex1* interaction with Triml2 is abolished when the locus is tethered to the periphery. A. Distance from periphery measurements for the entire *Rex1* locus probed with the BAC indicated in Figure 20A in cells subjected to LIF withdrawal for 2h. **** p<0.0001. B. Distance between probe pairs for *Rex1* and *Triml1* genes in cells subjected to LIF withdrawal for 2h. Higher distance indicates a lack of interaction between the genes. C. Distance between probe pairs for *Rex1* and *Triml2* genes in cells at 0 and 2h post LIF withdrawal. Higher distance between the probes at 2h post LIF withdrawal represents a loss of interaction between the two genes when the locus is tethered to the nuclear periphery. ****p<0.0001.

Similar nuclear envelope mediated regulation of enhancers has also been observed in other systems in the Schirmer lab such as adipogenesis and T-cell activation, where enhancer position within the nucleus is determined by the presence of absence of tissue specific nuclear envelope proteins (data not shown). Together, these results insinuate a crucial role for the temporal regulation of *Rex1* locus position during exit from pluripotency.

3.2 Distal tethers affect the position of *Nanog* locus

One of the core pluripotency factors, Nanog was first described as a pluripotency sustaining factor for its ability to bypass the requirements of LIF. The ectopic overexpression of Nanog was sufficient to maintain self-renewal of ES cells in the absence of LIF (Chambers et al. 2003). Eventually it was also described as the upstream regulator of Rex1 expression (W. Shi et al. 2006). As a core transcription factor for pluripotency, Nanog functions to integrate various signalling cues to affect the transcriptional landscape of ES cells for appropriate response to the stimuli. Towards this end it was shown that Nanog achieves LIF independent self-renewal by activating the LIF responsive genes, specifically *Esrrb*, thus suggesting that the intersection of Nanog-Esrrb signalling with LIF signalling represents a major node for the maintenance of pluripotency (Festuccia et al. 2012). Recently, the molecular mechanism by which Nanog might control the transcriptional network was elaborated on. It was shown that Nanog drives the recruitment of other core transcription factors like Oct4, Sox2 and Esrrb to thousands of regulatory sites where it promotes chromatin accessibility by Brg1 recruitment, thus controlling the transcriptional network (Heurtier et al. 2019).

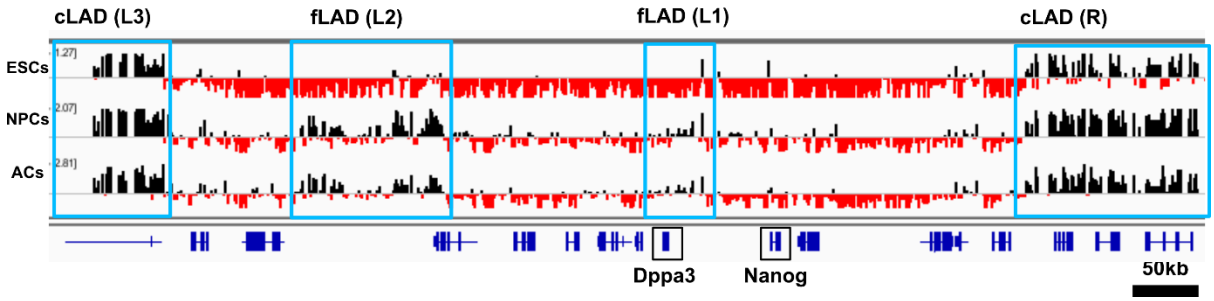

Figure 23: LaminB1 DamID traces for the *Nanog* locus in pluripotent ES cells (ESCs), neural progenitor cells (NPCs) and terminally differentiated astrocytes (ACs). The Nanog gene is found in the nuclear interior in pluripotent and differentiated cells. On one end it is flanked by a constitutive LAD (R) and on the other end, it is flanked by two facultative LADs (L1, that contains the *Dppa3* gene, and L2, which is in an intergenic region) and a constitutive LAD (L3). Data generated by Peric-Hupkes et al., 2010 and displayed using IGV.

During exit from pluripotency induced by 2i withdrawal, Nanog is downregulated by 9h into exit, earlier than Rex1 downregulation is observed (Kalkan et al. 2017). While Rex1 expression seems to be controlled by dramatic gene position changes to and from the nuclear periphery, it seems to not be the case with the Nanog locus. From DamID traces obtained during the differentiation of ES cells along neuronal lineages, it is clear that the

Nanog gene itself is not recruited to the nuclear periphery upon lineage commitment (Figure 23). In a different study comparing the position of Nanog in human ES cells and differentiated lymphoblasts, *Nanog* was never seen to be *at* the nuclear periphery. However, both its chromosome territory and the locus itself were found to be significantly closer to the nuclear periphery in differentiated cells (Wiblin 2005). The results presented here from experiments on E14tg2a mouse ES cells show a similar situation where the locus does not relocate entirely to the periphery but the median distance of the locus to the periphery decreases within the first hour of *in vitro* differentiation, a time window where the positional regulation of *Nanog* has not been studied before. When ES cells were subjected to exit from pluripotency by LIF withdrawal, within the first hour, the *Nanog* locus moved from a median distance of 3.4µm to 2.5µm from the periphery. Less than 2% loci are found ≤500nm from the periphery (Figure 24A and B). The movement towards the nuclear periphery is small but significant.

Upon closer inspection of the genomic locus, I found that while *Nanog* itself is never found at the periphery, it is flanked on one end by a constitutive LAD (R) and on the other by two facultative LADs which are seen in both, neural progenitor cells (NPCs) and astrocytes (ACs). The facultative LAD closest to *Nanog* (L1) contains the *Dppa3* gene, a primordial germ cell specific gene important for the maintenance of pluripotency and embryogenesis (S. Zhao et al. 2019). The second facultative LAD (L2) is found in an intergenic region about 250kb from the Nanog locus (Figure 23). I hypothesised that the movement of the Nanog locus towards the periphery could be due to distal tethers formed by the recruitment of either of these facultative LADs to the periphery during exit from pluripotency.

To test this hypothesis, I subjected ES cells to LIF withdrawal for 2h and used DNA-FISH to look at the early recruitment of facultative LADs to the periphery. At 2h post LIF withdrawal, the constitutive LAD (R, for simplicity) remains at a median distance of 1µm from the periphery (Figure 25C) and the facultative LAD containing the *Dppa3* locus (L1, for simplicity) remains at a median distance of 2µm from the periphery (Figure 25B). However, the other facultative LAD, L2, moves from a median distance of 2.5µm to 1µm from the periphery during this period, with 25% of the loci ≤400nm from the DAPI edge (Figure 25A). Thus, it is possible that this recruitment results in the *Nanog* gene edging towards the nuclear periphery.

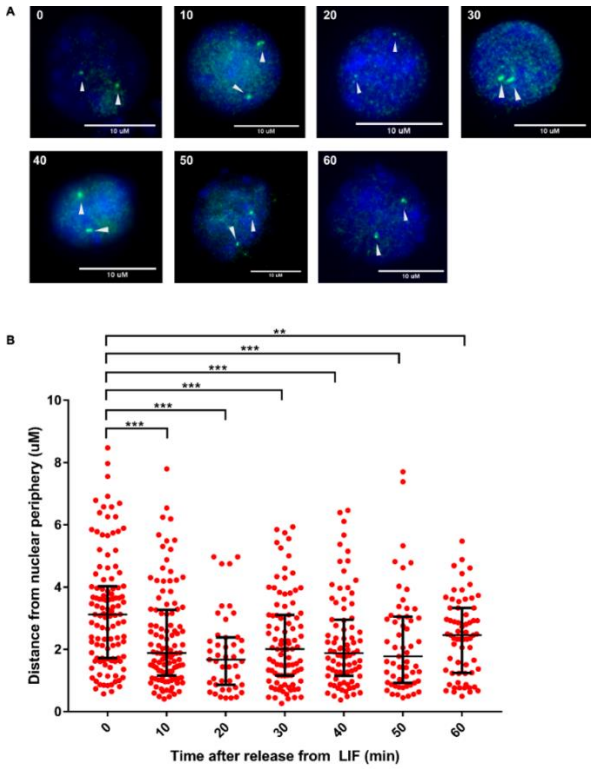

Figure 24: *Nanog* gene moves closer to the nuclear periphery during exit. A. Representative FISH images for cells subjected to LIF withdrawal for 0, 10, 20, 30, 40, 50 and 60min. White arrows indicate the *Nanog* locus in Green. Blue = DAPI. Scale bar represents 10µm. B. Distance from periphery measurements for images shown in A. The locus shows significant movement towards the nuclear periphery. **p<0.01, ***p<0.001.

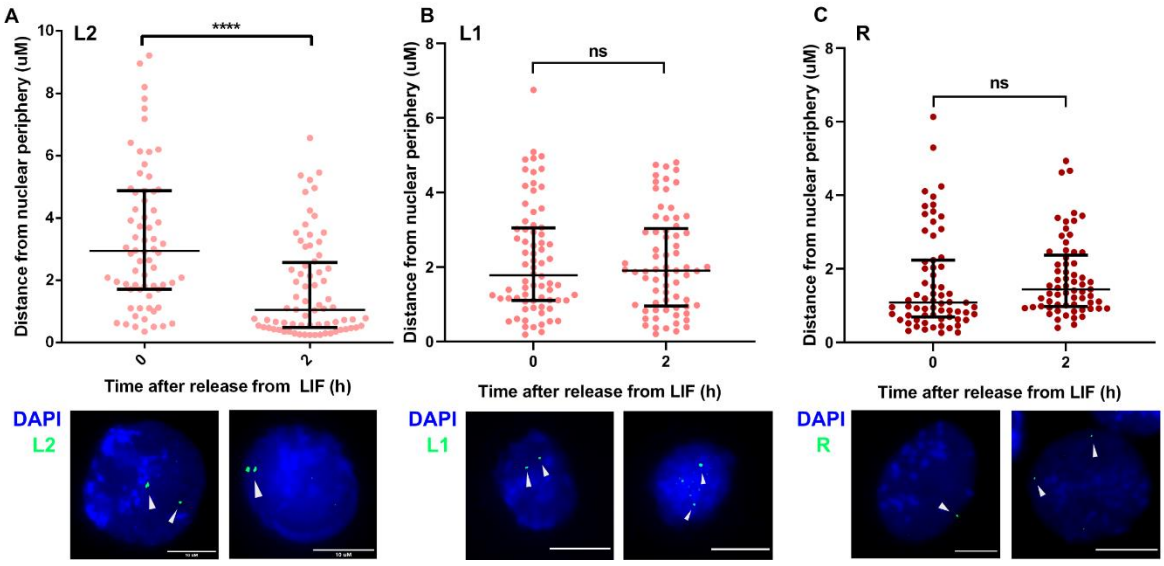

Figure 25: Identification of the potential distal tether that might cause *Nanog* to move towards the periphery. The LADs on either ends of the Nanog gene were tested for their position in the nuclear interior in cells subjected to LIF withdrawal for 0 and 2h. The facultative LAD L2 repositions to the nuclear periphery within 2h of exit while (A: top panel shows distance measurements and bottom panel shows representative FISH images) while the LAD that contains Dppa3 (L1) and the constitutive LAD (R) do not reposition to the periphery in this time frame (B and C: distance measurements shown in the top panel and representative images in the bottom panel).

Whether this slight shift in the position of Nanog affects its microenvironment such that its transcriptional status is in turn affected remains to be studied. It is possible that the slight shift towards the periphery is enough to inhibit gene-enhancer interactions such that transcriptional output might be regulated. The rapid recruitment of L2 to the periphery within 2h post LIF withdrawal, considering it is an intergenic region, is also intriguing. It will be of interest to see if the region might be home to regulatory elements that might have a role to play in maintaining the pluripotent status of ES cells. It could also be that the tethering of L2 functions to change the microenvironment or the TAD structure of the Nanog locus itself. Towards this end, a CRISPR mediated deletion of L2 in ES cells would allow the study of whether the region itself has a role in the maintenance of pluripotency and whether its deletion leads to a change in higher order chromatin organisation around the Nanog locus. Genome organisation is conventionally studied in a gene-centric manner. However, less than one percent of the genome is made up of protein coding regions. Intergenic regions house several important regulatory elements and their importance in maintaining the physiological state of cells is now gaining prominence (Lupiáñez et al. 2015). The nuclear envelope is a major tethering node and with its diversity and tissue specificity, has the unique ability to preferentially tether different parts of the genome in a cell state specific fashion. A previous study from the Schirmer lab revealed how the constrained release of enhancers and genes from the nuclear lamina facilitated their interaction and thus allowed gene expression during T cell activation, thus illustrating a case where the nuclear envelope played a regulatory role in controlling the enhancer activity (Robson et al. 2017b). Thus, investigating the differences in nuclear envelope composition will potentially reveal specific tethering mechanisms for not just target genes, but also other regulatory elements like microRNAs, enhancers and insulators.

3.3 Discussion

In this chapter, I have demonstrated the temporal dynamics of changes in genome organisation during exit from pluripotency. I have described a simple model to study exit from pluripotency using LIF withdrawal. Using this model, I have demonstrated the relocation of the *Rex1* locus, containing genes *Zfp42/Rex1, Trim1 and Triml2*, to the nuclear periphery within an hour of LIF withdrawal. This method of studying in vitro differentiation is different from how exit is studied in the Smith lab in that I use LIF withdrawal to stimulate in vitro differentiation whereas they use 2i withdrawal. The two

culture conditions are different and one downside of using serum/LIF cultures is that the starting population is heterogeneous. However, I replicated the results in cells cultured in 2i/LIF medium and have shown that the rapid repositioning of the locus is conserved in both types of exit i.e. one stimulated by withdrawing LIF in serum/LIF cultures and one stimulated by withdrawing 2i in the 2i/LIF cultures. Further, using Rex1::GFPd2 cells, the Smith lab demonstrated the reversible state in exit during the first 16-25h following 2i withdrawal. Similarly, in my experiments, I find that *Rex1* repositioning is LIF dependent and is reversible at least in the first 2h following LIF withdrawal. It would be interesting to trace the time beyond which this reversibility is lost.

Intriguingly, while the repositioning itself is observed in CGR8 ES cells as in E14tg2a ES cells, the temporal dynamics of repositioning are different with the locus showing a delayed recruitment to the nuclear periphery in CGR8 cells. There are no morphological differences observed or reported between E14 and CGR8 cells. Individual studies on pluripotency and differentiation done on either E14 or CGR8 cells, show that both cell types are pluripotent and express similar loss or gain of markers during differentiation. However, the various cellular processes occurring during exit from pluripotency have not been directly compared between the two cell lines. Some studies show minor differences between the two cell lines. For instance, in a study comparing the regulation of serine/threonine kinases Pim1 and Pim3 by LIF, it was observed that the relative expression of *Pim1* and *Pim3* measured by RT-PCR was much higher when E14 cells were subjected to LIF release for 24h followed by LIF addition for 1h, as compared to CGR8 cells subjected to the same conditions (Aksoy et al. 2007). In another instance, both E14 and CGR8 cells were reported to express toll like receptor (TLR) 2 and not TLR4 but the FACS profiles show much higher signal intensity in CGR8 cells. In the same study, the effects of LPS treatment are much more detrimental in CGR8 cells compared to E14 cells, and the rescue following antibody treatment is also more significant in CGR8 cells (Taylor et al. 2010). These two examples provide some evidence suggesting that while the mechanisms and cellular responses to various treatments are largely similar between the different ES cell lines, there could be minor differences in expression levels of various individual components, including transcripts and proteins between the cell lines in spite of them being generated from the same parent mouse strain.

Unravelling the tethering mechanism would ideally require capturing actin polymerisation around the locus during *Rex1* relocation. But capturing this polymerisation with a classical antibody approach or a tagged protein approach for live cell imaging may prove difficult considering signal intensity from cytoplasmic actin would be way stronger than that from nuclear actin, thereby making it difficult to study the nuclear polymerisation process closely. Pull-down approaches might be one way to study the direct association of the locus with nuclear actin or myosin but given the rapidity of relocation, it might be tricky to find an intermediate time during this relocation when the locus might be physically associated with the motor proteins. Also, considering the asynchronous recruitment of the locus in the population, pull-down experiments done over whole populations of cells might not provide indisputable evidence. Finally, *in vitro* approaches to study actin polymerisation or NM1 activity will prevent the simultaneous study of *Rex1* recruitment to the nuclear periphery. Thus, whether actin polymerisation or NM1 function drive the recruitment is a difficult question to study using any of the classical approaches used in biology. However, using inhibitors to prevent motor protein functions points to yet another scenario where nuclear actin and myosin have crucial functions.

The inner 'nucleoskeleton' is now generating interest for its proposed roles in genome organisation and gene regulation in cell differentiation and development (Xie and Percipalle 2018). Accumulating evidence demonstrates the importance of nuclear actin in differentiation. For instance, cytochalasin D treatment leads to rapid accumulation of nuclear actin and promotes the differentiation of mesenchymal stem cells into osteogenic lineages (Sen et al. 2015). During the differentiation of HL-60 cells into macrophages, actin translocation to the nucleus leads to the transcription of macrophage related genes (Xu et al. 2010). The nuclear β-actin pool significantly contributes to neuronal development by regulating genome organisation and activation of neuronal programs during neurogenesis (Xie et al. 2018). Interestingly, nuclear actin levels also affect gene expression. Manipulating the nuclear pool of actin by introducing a construct containing actin-NLS leads to gene activation in HeLa cells (Yamazaki, Yamamoto, and Harata 2015) while such overexpression in HaCaT keratinocytes leads to the repression of adhesive and cytoskeletal genes (Sharili et al. 2016). Nuclear actin has been shown to be closely associated with several chromatin remodelling complexes (Xie et al. 2020) and therefore, it is not surprising that it plays such key roles in orchestrating genome organisation. While several studies

indicate the role of motor proteins in orchestrating local genome organisation changes, it is yet to be elucidated if these proteins might have a role to play in establishing higher order chromatin organisation during development and differentiation.

Considering the recruitment of *Rex1* happens within the first two hours of LIF withdrawal and both its mRNA and protein continue to be detected until much later, the alternative hypothesis for a role of the locus as an enhancer was investigated. I found that the intronic sequence of *Rex1* has a predicted enhancer and it and interacts with *Triml2* in ES cells. Upon tethering to the periphery this interaction is abolished. Thus, I postulate that the enhancer could have several other target genes in ES cells and it is this activity that must be tightly regulated to facilitate exit from pluripotency, however, this has yet to be tested as does the functionality of the predicted enhancer.

Finally, I have investigated the temporal dynamics of the repositioning of Nanog locus towards the nuclear periphery and shown that this repositioning, while subtle, is also rapid and occurs within the first two hours post exit. While Nanog is never found at the nuclear periphery, I have demonstrated that its relocation towards the periphery depends on a distal tether in a non-coding region. Following on from that, I have discussed the importance of the nuclear envelope mediated tethers in modulating the activity of regulatory elements. The changes in the composition of nuclear envelope upon exit induced by LIF withdrawal are described in the following chapter.

Chapter 4

Study of the Nuclear Envelope Proteome in ES cells

Over a decade of work from the Schirmer lab has been dedicated to identifying the components of the nuclear envelope in different tissue types and understanding the role of these diverse proteins in genome organisation. Surprisingly little is known about NETs expressed in pluripotent cells. Some ubiquitous NE components have been looked at in association with differentiation. For instance, the composition of NE itself is shown to change with differentiation, with an increase in LaminA/C expression in association with cellular differentiation (Lebel et al. 1987). However, using *Xenopus* embryos and mammalian cell cultures as models, it was shown that total lamin concentration, rather than lamin type governs nuclear size, highlighting the importance of lamins in determining nuclear shape and size for normal development (Jevtić et al. 2015). In mice, while embryonic development was shown to be complete even in the absence of lamins and emerin, the gene expression fidelity during primitive endoderm commitment depended on these proteins (E. R. Smith et al. 2017). *In vitro*, a triple-knockout of Lamin B1, B2 and A in mouse ES cell cultures had no effect on their differentiation into all the germ layers (Y. Kim, Zheng, and Zheng 2013). Curiously, in mouse ES cells, peripheral genome organisation i.e. lamina-associated domain (LAD) organisation is independent of all types of lamins (Amendola and Steensel 2015). The one lamin in *C. elegans* is important during germline development for maintaining nuclear morphology and chromatin compaction, in addition to being responsible for the appropriate localisation of inner nuclear membrane proteins emerin and MAN1, which in turn also have roles in orchestrating genome organisation and gene expression (Margalit et al. 2005). Furthermore, zygotic knockouts of several nucleoporins have been generated in mice leading to embryonic lethality. Deletion of NPC subunits like Nup62/98/133/155 results in lethality at later developmental stages, Nup50 mutants die at gestation, and Nup214 mutants die before implantation (Borsos and Torres-Padilla 2016). Thus, various studies have looked at NE components to understand their roles and functions in pluripotency and early development. However, there has yet to be a comprehensive effort towards exploring the diversity of NETs in pluripotent cells.

That genome organisation changes during lineage progression is now well known. My own results from Chapter 3 demonstrate the rapid relocation of the *Rex1* locus from the nuclear interior to the periphery during the early stages of exit from pluripotency, way before lineage commitment is observed *in vitro*. Identifying the components of NE that tether the locus to the periphery in ES cells is therefore of interest to the fields of both, stem cells and nuclear organisation as it will add to and further explain roles of the specific proteins that are able to orchestrate these early changes that mark exit from pluripotency, a cell biological event key to setting the stage for eventual lineage commitment. This chapter explains the rationale, sample preparation and results for mass spectroscopy to identify the NETs in ES cells. Furthermore, the results presented here include NETs that gain or lose phosphorylation, a post-translational modification previously shown to be able to make and break connections between the NE and chromatin, during early stages of exit from pluripotency. Finally, using mutagenesis, the NETs responsible for mediating the *Rex1* locus tether at the nuclear periphery will be revealed.

4.1 Sample Preparation and Experimental Design for Mass Spectrometry

Understanding the nuclear envelope composition of ES cells is key to explaining mechanisms governing the genome organisation changes that take place during exit from pluripotency, lineage commitment and eventual tissue specification. As shown by previous studies in the Schirmer lab, most NETs are tissue restricted. The results presented here and other studies from the lab performed on adult tissues indicate that the diversity in NE composition is crucial in establishing tissue specific genome organisation. However, no study has yet looked comprehensively at the nuclear envelope of cells in their pluripotent state or in early stages of lineage commitment.

Tethering of the *Rex1* locus to the periphery within 2h of LIF withdrawal seems to suggest that this tethering activity is important for setting the stage to eventual exit from pluripotency, which *in vitro* can take a little more than 24h. As a first step to identifying the mechanism by which this tether is made, it was important to look at the proteins at the nuclear envelope of ES cells and how this composition changes during early stages of exit from pluripotency. Since we know that the locus is tethered within the first 2h following LIF withdrawal, this time point was picked to assess the changes in NE composition compared to cells that have been maintained in pluripotent culture conditions. When

considering the mechanism by which the locus is tethered and assuming it is a direct tether, one of the following scenarios is likely.

a. The physical repositioning of the locus to the periphery is mediated by actin/myosin motor proteins and the tether to an existing NET should depend on the proximity of the locus to the periphery, or
b. Once the locus physically repositions to the periphery, specific affinity between the NET for either a sequence motif or for a protein sitting on the *Rex1* locus likely drives the repositioning. If the tether is an existing NET, then this NET must undergo post translational modifications during exit from pluripotency which then enables its binding to the locus, or
c. If the tether is a previously absent NET, then LIF withdrawal leads to a change in NE composition so that one or more of the new NETs play a role in tethering the locus to the periphery, or
d. Changes in epigenetic marks at various loci depending on LIF withdrawal leads to large-scale repositioning of chromatin to the NE based on an affinity mechanism between NETs and heterochromatin.

Distinguishing between likely scenarios, particularly *b* and *c*, requires mass spectrometry of the nuclear envelope of ES cells before and after LIF withdrawal. Also, since supplementing the media with LIF seems to reverse the tether (Figure 13), it suggests that the tethering mechanism is controlled and reversible and this would most likely involve post-translational modifications on existing NETs. Thus, scenario *c* seems less likely than *b*. Several nuclear lamina-chromatin interactions have been previously described to occur through phosphorylation of NETs such as LBR and Lap2β, and lamins themselves (Mattout-Drubezki and Gruenbaum 2003).

The procedure for isolation of nuclear envelopes takes ~10 hours and we were concerned about the possible loss of relevant phosphorylation. The short half-lives of phosphatase inhibitors in aqueous solution offers little insurance against loss of phosphoproteins. To circumvent this, crude isolation of whole nuclei was performed using a hypotonic solution supplemented with phosphatase inhibitors in under 10 minutes. The samples were checked under a microscope to ensure that the lysis was complete with a retention of whole nuclei. Whole cell (WC) and nuclear fraction (N) lysates were separated on an SDS-PAGE gel and stained with Coomassie Blue (Figure 26A). Identical amounts of lysates separated by gel

electrophoresis were stained with ProQ diamond phosphoprotein gel stain (Figure 26B), which selectively stains phosphoproteins on acrylamide gels and allows for in-gel detection of phosphate groups attached to tyrosine, serine or threonine residues. Phosphoprotein staining shows a minimal loss of phosphorylation in the nuclear fraction. The lysates from were also subjected to western blotting which revealed intact pools of nuclear proteins like histone H3, Oct4, Lamin A/C and nucleoporin Nup153 with a loss of the cytoplasmic protein GAPDH (Figure 26C), suggesting that the isolation of nuclei, however crude, did enrich for nuclear targets. Similar isolation of whole nuclei was performed on ES cells at zero and two hours post LIF withdrawal and sent to the mass spectrometry facility at the Stowers Institute for Medical Research to be analysed both for proteins unique to each sample and for phosphoproteins.

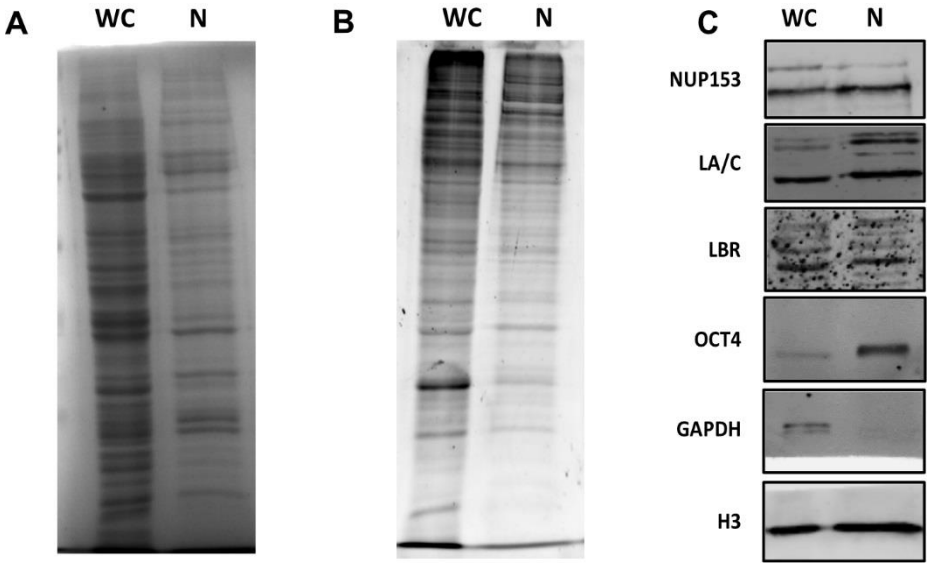

Figure 26: Sample preparation for mass spectroscopy. Whole cell (WC) and isolated nuclear (N) fractions of ES cells prepared in presence of phosphatase inhibitors were separated by gel electrophoresis and stained with Coomassie Blue for total proteins (A). B. Identical amounts of lysates as in A stained with ProQ Diamond phosphoprotein stain that identifies phospho groups attached to serine, threonine and tyrosine residues. C. Western Blots showing the presence of nucleoporin Nup153, Lamin A/C, LBR, Oct4, GAPDH and histone H3 in both the whole cell and nuclear fractions.

4.2 Mass Spectrometry Analysis: a summary

Organellar proteomes have been of interest for nearly two decades now. The tricky aspect of studying organelle proteomes is the fact that most protein constituents are in constant exchange with the rest of the cell. Proteins in the nuclear subdomains contain both resident and transient proteins. Perturbations of the cell might change the organelle proteome considerably. In several proteomic analyses of nuclear structures, unexpected proteins are

commonly observed (Andersen et al. 2005; Jurica and Moore 2003; Saitoh et al. 2004; Andersen and Mann 2006). To be more comprehensive in identifying NETs, the Schirmer lab used a method originally developed in the Blobel and Gerace labs to isolate the nuclear envelope by first isolating whole nuclei by Dounce homogenisation and subjecting them to two rounds of DNase and RNase digestion with several rounds of density gradient centrifugation to float first contaminating membranes and second released chromatin. To ensure a clean NE prep, Digestions were followed by rapid staining of nuclei with DAPI and extended if most of the DAPI signal had not been washed out. NETs were identified using subtractive proteomics using microsomal fractions to subtract out the potential contaminants followed by bioinformatic analysis by membrane topology prediction (Korfali et al. 2009). Thus, ~2000 proteins were found per run in leukocyte NEs (Korfali et al. 2010) and ~700 proteins in skeletal muscle NE fractions (Wilkie et al. 2011). Several of these were then confirmed to be NE resident by immunofluorescence (Korfali et al. 2010; Malik et al. 2010b; Wilkie et al. 2011) and a subset of 954 NETs were compared for their tissue specificity (Korfali et al. 2012).

Correspondingly, the proteomic dataset obtained from crudely isolated whole nuclei of pluripotent ES cells in theory might be expected to have more proteins than isolated NEs and indeed, a total of 3780 proteins were identified. The relative abundance of these proteins was estimated by distributed normalised spectral abundance factor values (Y. Zhang et al. 2010). This approach measures relative abundance of a protein as a function of the spectral counts and length of each protein. For peptides that are shared among different protein isoforms, this approach involves distributing the spectral counts among protein isoforms to provide an accurate and reproducible quantitation. Consistent with expectations based on precious work on nuclear composition, when the proteins in the ES whole nuclear sample were ordered according to their distributed normalised spectral abundance values, the most abundant proteins were the nucleolar protein nucleophosmin followed by the different histone variants and undifferentiated embryonic cell transcription factor 1, Utf1. However, GAPDH and β-actin were also among the top 5% of the proteins detected in this sample due to their sheer abundance in the cells, suggesting the presence of cytoplasmic contaminants. In mass spectrometry there is the additional problem in that as the number of proteins in a sample increases, the more abundant proteins begin to swamp the spectra for lower abundant proteins. Smaller proteins also yield fewer peptides so that

even if they are much less abundant than a much larger protein the larger protein could have a larger total number of spectral counts. Since NETs are far less abundant than any such proteins mentioned above, their identification can prove more challenging and it should be noted as a caution of this data that less abundant NETs could have been missed.

We employed two methods for analysis. Since NETs all contain at least one transmembrane domain, their identification among the pool of nuclear proteins can potentially be performed using membrane topology prediction. Therefore, we first, we applied a Transmembrane Hidden Markov Model protein topology prediction algorithm to identify all proteins with at least one predicted transmembrane domain in the sample. This yielded a total of 189 transmembrane proteins from both samples - cells maintained in presence (T0) and absence of LIF (T2). Of these, 107 of these proteins were previously known NETs. While the results contained many of the classical NETs like Sun2 and LAP2β, it also included several ER and mitochondrial proteins that are likely contaminants like lectin and mannose binding protein 1 (Lman1), cytochrome b5a and b (Cyb5a, Cyb5b) and the NADPH-cytochrome P450 reductase (Por). We checked cellular compartment distribution using GO and found 55 proteins to be potential contaminants lacking nuclear localisation i.e. they came from plasma membrane, mitochondrial membrane and other cytosolic organelles. Of these 55 proteins, 15 were ER proteins that could potentially transiently reside in the NE making them potential NETs. Thus, using this method, over 20% of the total proteins recovered were *definitely* not NETs.

Our second analysis method expected to more reliably determine NETs within this pool, was to take the list of putative NETs that were identified from the clean NE preps in the three tissue samples previously studied in the Schirmer lab as a reference list. Since the NETs are far less abundant in the ES nuclear fractions, we relaxed all the criteria based on spectral counts and peptides identified. Instead, we decided to consider all the NETs identified in the samples as long as they were detected in at least 3 of the 5 technical replicates run, irrespective of the low spectral counts. Since this analysis involves a reference list of bona fide NETs, we decided to use results from this method alone, which are described below.

Of the 954 putative NETs identified in previous studies from the Schirmer lab (Figure 27A; Korfali et al. 2012), only 122 NETs are identified in both samples together. Of these, merely 55 were found in ES cells *before* LIF withdrawal (T0). This observation might be consistent

with the idea that majority of the NETs known so far are indeed tissue restricted and are expressed at later stages of differentiation. However, it is entirely possible that other NETs that are less abundant might be missed in the whole nuclear analysis. A look at the distribution of these 55 NETs with regards to which parent tissue each of these was originally identified in reveals that 24 of these were originally identified as ubiquitous NETs, 4 exclusively in liver, 2 in muscle and 10 in blood. The rest were expressed in at least two of the three parent tissues (Figure 27B top panel). Again, a high percentage of the NETs found in ES cells being previously described as ubiquitous NETs is, both expected and reassuring of the hypothesis that the NE proteome complexity increases dramatically with the addition of tissue specific NETs in differentiated cell types and this might enable lineage transitions by orchestrating genome organisation, mechanotransduction and other related functions.

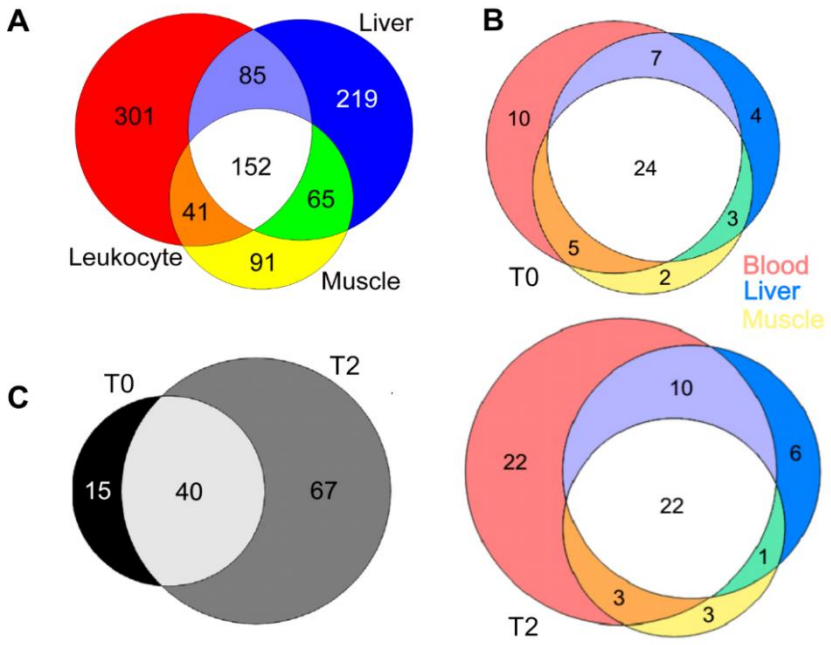

Figure 27: Summary of mass spectrometry data. A. Venn diagram showing the overlap in NETs discovered in nuclear envelope preps from Liver, Muscle and Leukocyte fractions. Less than 20% NETs are expressed ubiquitously in these three tissue types. Data reproduced from Korfali et al., 2012. B. Summary of NETs discovered in ES cells subjected to 0 (T0; top) and 2h (T2; bottom) of LIF withdrawal. The colours in the Venn diagram depict the distribution of NETs between the three parent tissues they were originally identified from. Over 40% of the NETs identified in each sample were previously identified as ubiquitous NETs. C. Venn diagram showing the numbers of NETs overlapping between cells subjected to 0 (T0) and 2h (T2) of LIF withdrawal.

Interestingly, a total of 107 NETs are discovered in ES cells that have been subjected to LIF withdrawal for 2h (T2). Between the two samples, 40 NETs are shared in expression,

leaving 15 NETs being specific to the pluripotent state of cells and 67 others that are induced in cells at the early stages of exit form pluripotency (Figure 27C). The list of NETs specific to T0, T2 and those that are shared between the two is provided in the Appendix.

4.3 Phospho- changes during early differentiation

In the context of the study presented in this thesis, identifying the NETs present in ES cells subjected to 0 and 2h of LIF withdrawal is primarily to identify candidate NETs that might facilitate the rapid relocation of the *Rex1* locus to the periphery. In theory, any of the 67 NETs uniquely identified in *T2* (Figure 27C; complete list provided as an appendix) or those that change phosphorylation will make for good candidates to test in further experiments for their role in tethering the *Rex1* locus to the nuclear periphery. However, as mentioned before, since the tethering at the periphery is both rapid and reversible, it is likely that the mechanism involves post translational modifications. Therefore, efforts were focused on testing some of the candidate proteins that show changes in phosphorylation.

Table 18: NETs that show phosphorylation changes in the first two hours of exit

UniProt ID	Gene Name	MW	Phosphorylation	Residues detected
A2A8U2	Tmem201	72.5	LOSS	609S,610S,612S,613S,614T
Q3U9G9	Lbr	71.4	LOSS	71S, 86S
Q80WJ7	Mtdh	63.8	LOSS	423S
Q61033	Tmpo (Lap2a isoform)	75.2	LOSS	66S,67S,422S
Q8K3Z9	Pom121	121	GAIN	319S,322S,325S,355S,409S,412S,413S,416S,441S,446T,450S
Q99P72	Rtn4	126.6	LOSS	165S,167S,171T,489S,648T,649S,651S

Of the 40 NETs that are detected in both samples, only 9 showed changes in their phosphorylation status. Of these, 3 proteins were previously annotated as NETs on the basis of predicted transmembrane domains in them but with advances in membrane topology predictions no longer qualify as NETs. Thus, we ended up with only 6 NETs that change phosphorylation status (Table 18) narrowing down the possible candidates to be tested by a huge margin. Since the analysis was done in a gene centric manner, thymopoietin (gene name TMPO) shows up in the list of NETs but a look at the phosphopeptides detected reveals that it is the nucleoplasmic isoform LAP2α and not the classical NET LAP2β that loses phosphorylation during exit. Interestingly, LAP2α also lost phosphorylation. Both the

LAP2α and LAP2β bind lamins and chromatin. NETs like TMEM201, MTDH, and LBR seem to lose phosphorylation while others like POM121 are phosphorylated upon exit from pluripotency. Since LBR and LAP2α are both described extensively in literature for their chromatin binding abilities, these candidates were prioritised in this study.

4.4 LAP2α phosphorylation status affects *Rex1* tethering to the periphery

LAP2α is an alternatively spliced isoform of the TMPO gene that has at least six identified isoforms in mammals (Foisner and Gerace 1993; Harris et al. 1994; 1995; Berger et al. 1996). All the LAP2 isoforms, except LAP2α and LAP2ζ, contain a C-terminal transmembrane domain which allows for their integration into the inner nuclear membrane with preferential binding interactions with the B type lamins. LAP2α has a unique 500 amino acid long C-terminal region with no transmembrane domain and is located predominantly in the nucleoplasm and its interactions with lamins A and C have been demonstrated *in vitro* (Markiewicz et al. 2002). Notably, studies have focused on investigating particular interactions between lamin and LAP2 subtypes. Thus, it remains unclear whether LAP2α can bind B-type lamins or that LAP2β can bind A-type lamins. In the nucleoplasm, LAP2α and Lamin A/C exist in a stable complex such that their distribution depends on each other (K. Furukawa et al. 1995; Dechat 1998; Dechat et al. 2000). In the absence of LAP2α, lamin A/C is absent from euchromatic regions and this is associated with changes in epigenetic histone marks in euchromatin. While the epigenetic changes in euchromatin did not significantly affect gene expression, the loss of lamin A/C in heterochromatic regions correlated with increased gene expression (Gesson et al. 2016). This complex, through its interactions with DNA, has been proposed to control higher order chromatin organisation and epigenetics and therefore gene expression.

The localisation of LAP2α in the nucleoplasm depends on domains located in both the N- and C-terminal α-specific regions. A nuclear targeting domain in LAP2α (amino acids 270-615) is responsible for linking this protein to nuclear structures and thereby stabilising its localisation in the nucleoplasm. The domain structure of LAP2α is shown in Figure 28A. Studies from the Foisner and Gerace groups have together shown that LAP2α contains at least two different chromatin binding sites – one located within the LAP2α-specific nuclear targeting domain (amino acids 270-615) and another in the N-terminal LAP2 common region (amino acids 1-85) (Kazuhiro Furukawa, Fritze, and Gerace 1998; Vlcek et al. 1999). Furthermore, this 1-85 common region also contains the 40 residue long structural

motif common to all LAP2 isoforms called the LEM domain, which interacts with a small 10kDa DNA cross-linking protein called barrier-to-autointegration factor (BAF) that can mediate its interaction with chromatin (Segura-Totten and Wilson 2004). In addition to its DNA bridging activity, BAF can also compact chromatin, which might contribute to the formation of heterochromatin and thus affect gene expression (C. M. Bradley et al. 2005).

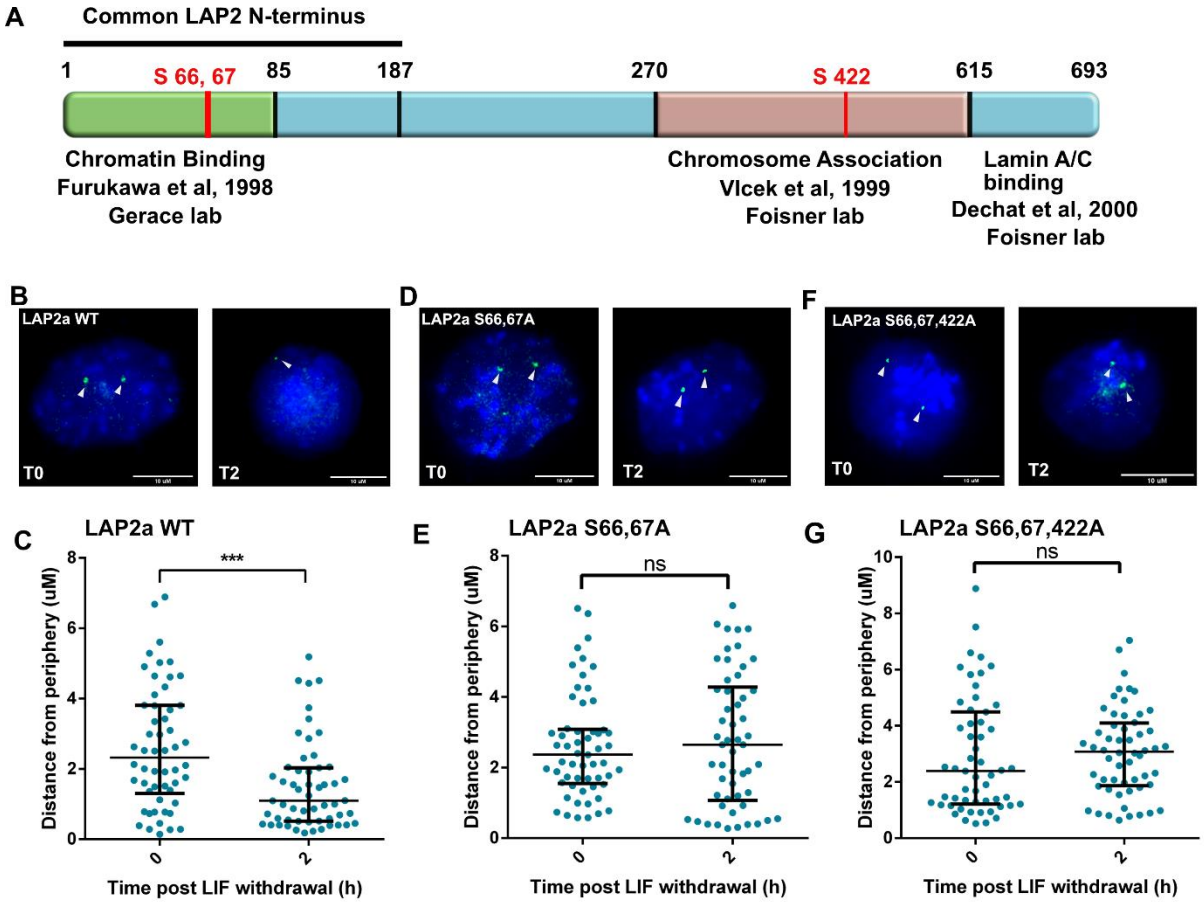

Figure 28: LAP2α phosphorylation status affects *Rex1* repositioning. A. Illustration showing the domain structure of LAP2α. The three serines mutated to alanine – 66, 67 and 422 – are within domains previously shown to be important for chromatin binding. B. Representative FISH images for *Rex1* (green) in cells transfected with LAP2α WT construct, subjected to 0 and 2h of LIF withdrawal. C. Distance from periphery quantitation for images in B. ***p<0.001. D. Representative FISH images for *Rex1* (green) in cells transfected with LAP2α S66, 67 A mutant construct, subjected to 0 and 2h of LIF withdrawal. E. Distance from periphery quantitation for images in D. ns p>0.05. F. Representative FISH images for *Rex1* (green) in cells transfected with LAP2α S66, 67, 422A mutant construct, subjected to 0 and 2h of LIF withdrawal. G. Distance from periphery quantitation for images in F. ns p>0.05. Scale bars represent 10μm.

Mass spectrometry revealed a loss of phosphorylation at serines 66, 67 and 422 on LAP2α in the first 2h of differentiation induction (Table 18). While S66 and S67 are part of the N-

terminal chromatin binding region of the protein, S422 is within the nuclear targeting domain. To test the importance of these residues on the relocation phenotype observed for the *Rex1* genomic locus during exit, they were mutated to alanines and the resulting phospho-null mutants were overexpressed in ES cells. All the LAP2α WT and mutant constructs were GFP fusion proteins and nucleofection increased the transfection efficiency so that over 95% cells were fluorescent after 24h. These cells were all subjected to LIF withdrawal for 2h and then collected and processed for FISH analysis. Cells expressing WT LAP2α show a movement of the *Rex1* locus from the nuclear interior to the periphery as expected, suggesting that transfection alone does not cause any abnormalities in the phenotype (Figure 28B and C). However, when the S66A/S67A (Figure 28D and E) and the S66A/S67A/S422A (Figure 28F and G) mutants are over expressed, the locus fails to reposition to the nuclear periphery suggesting that the phosphorylation is not required to maintain an internal location and rather suggests that phosphorylation switch is involved in its movement to the periphery.

Mechanistically, this opens many questions about the role of LAP2α in mediating the *Rex1* tether to the periphery. One possible explanation might be that the phosphorylation of these residues keeps the protein from binding to DNA under pluripotent conditions. During early stages of *in vitro* differentiation, the loss of phosphorylation may enable the DNA-protein interaction thereby stably anchoring the locus to the protein. The LAP2α-Lamin A nexus could then potentially act to stabilise the locus in the interior or chaperone it to the periphery.

4.5 Characterising the role of Lamin Binding Receptor phosphorylation in *Rex1* relocation

While hundreds of transmembrane proteins are embedded in the nuclear envelope, only a handful have been characterised thus far. Of these, the lamin binding receptor (LBR) features prominently in the studies looking at genome organisation. The accumulation of LBR to the inner nuclear membrane occurs through a diffusion-retention based mechanism where the protein is synthesised on the surface of the ER membrane, moves laterally to the outer nuclear membrane by diffusion and then through the nuclear pores to the inner nuclear membrane where it is retained due to interactions with other components like lamins and chromatin (Soullam and Worman 1995; Boni et al. 2015; Ungricht et al. 2015). LBR has

been implicated for its ability to mediate peripheral tethering of heterochromatin at early developmental stages, a function that is later taken over by Lamin A/C (Solovei et al. 2009). Interestingly, presence of LBR is also important for heterochromatin tethering in cycling cancer cells and a loss of this protein induces cellular senescence of these cells, characterised by heterochromatic foci seen in the nuclear interior (Lukášová et al. 2017). Both these observations are supported by the fact that chromatin pulled down with LBR is enriched in heterochromatic marks such as H3K9me3 and H3K29me3 and is devoid of euchromatic epigenetic marks (Makatsori et al. 2004). Moreover, LBR specifically associates with Xist long noncoding RNA to tether the X chromosome to the inner nuclear membrane and inactivate it during development in mammalian females (C.-K. Chen et al. 2016).

LBR has 8 transmembrane domains that enable its retention in the membrane with an N-terminal nucleoplasmic fragment that is about ~200 aa long and binds chromatin to support retention in the inner nuclear membrane. Two adjacent domains in the nucleoplasmic region, namely globular/TUDOR domain (amino acids 1-60) and RS domain (amino acids 61-89) mediate chromatin binding. Deletion of either domains leads to reduced binding to chromatin (Q. Ye and Worman 1994; Hirano et al. 2012). The phosphorylation of LBR is essential to its chromatin binding activity. At the beginning of NE breakdown during mitosis, CDK1 mediated hyperphosphorylation of LBR makes it highly mobile and leads to its dissociation from chromatin and dispersal in the ER. In the late anaphase, LBR is dephosphorylated by PP1/2A allowing the oligomerization of its RS domains and subsequent chromatin association once the NE is reassembled. The major target of CDK1 phosphorylation is Ser-71 followed by Ser-86 and the mitotic phosphorylation of both these residues inhibits its chromatin-binding activity (Takano et al. 2004; Tseng and Chen 2011). Using a GAL-4 fused LBR, Hirano et al demonstrated the peripheral association of the reporter plasmid led to transcriptional repression (Hirano et al. 2012), which is in line with the widely acknowledged rule of thumb for genome organisation suggesting that the nuclear periphery tends to be a repressive environment. Such repression is a consequence of chromatin compaction but also requires recruitment of transcriptional modulators like HP1, which binds to the second globular domain (amino acids 90-211 in human LBR) (Q. Ye and Worman 1994; Lechner et al. 2005), and MeCP2 that binds to both the TUDOR and RS domains (Guarda et al. 2009; Liokatis et al. 2012).

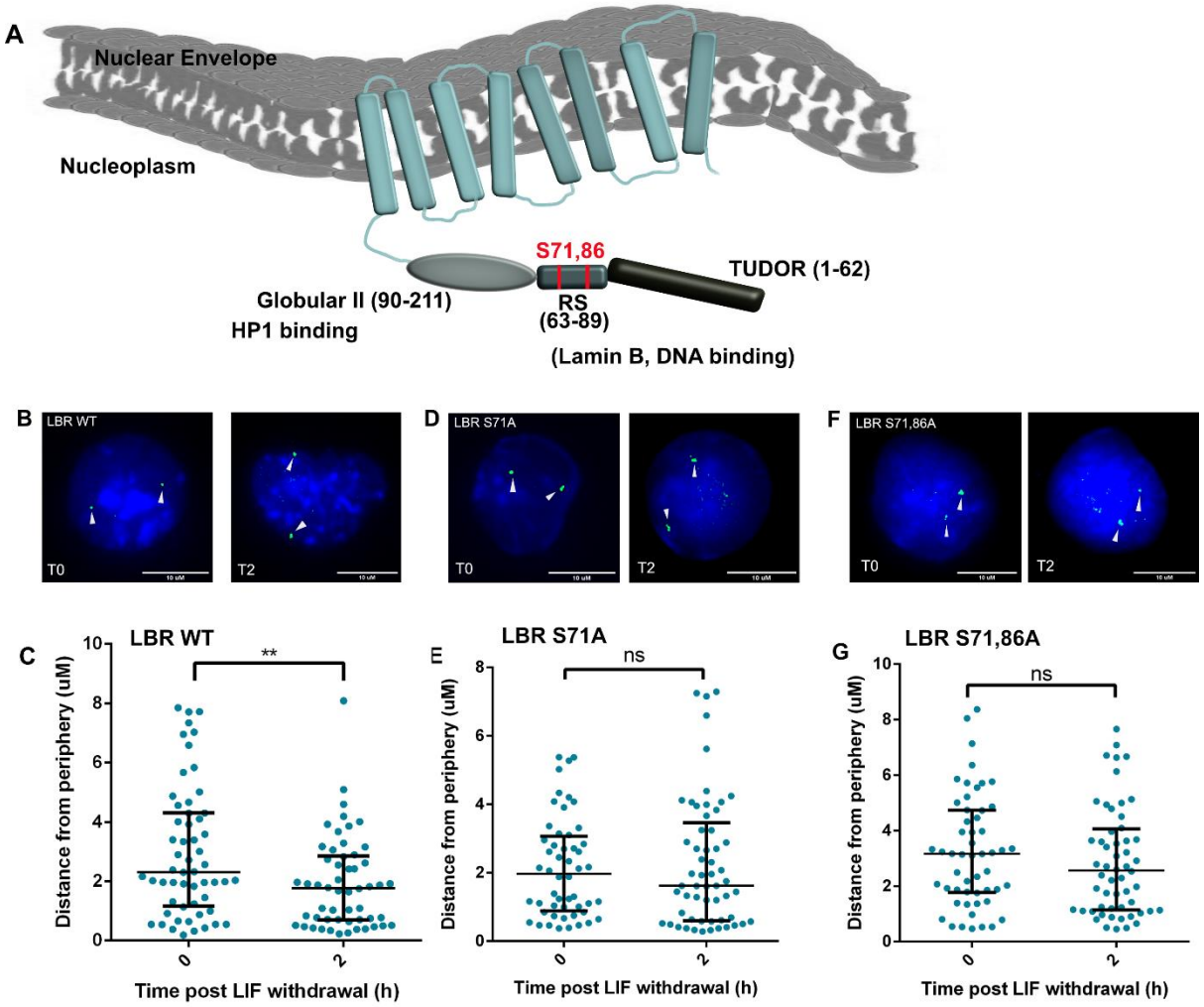

Figure 29: LBR phosphorylation status affects *Rex1* repositioning. A. Illustration showing the domain structure of LBR. The NET has 8 transmembrane domains and the nucleoplasmic domain contains the TUDOR, RS and Globular II domains. Both the TUDOR and RS domains can bind to DNA. The two serines mutated to alanine – 71 and 86 – are within the RS domain. B. Representative FISH images for *Rex1* (green) in cells transfected with LBR WT construct, subjected to 0 and 2h of LIF withdrawal. C. Distance from periphery quantitation for images in B. **p<0.01. D. Representative FISH images for *Rex1* (green) in cells transfected with LBR S71A mutant construct, subjected to 0 and 2h of LIF withdrawal. E. Distance from periphery quantitation for images in D. ns p>0.05. F. Representative FISH images for *Rex1* (green) in cells transfected with LBR S71,86A mutant construct, subjected to 0 and 2h of LIF withdrawal. G. Distance from periphery quantitation for images in F. ns p>p0.05. Scale bars represent 10µm.

The domain structure of LBR is shown in Figure 29A. The nucleoplasmic domain of LBR plays a crucial role in genome organisation by directly binding to chromatin and is responsible for its silencing by recruiting transcriptional modulators. The RS domain, which is only about 30aa in length, is particularly interesting as it interacts with both

chromatin and transcriptional modulators and its phosphorylation status seems to be critical in determining LBR-chromatin interaction. Therefore, to determine if phosphorylation of Ser-71 and Ser-86 within the RS domain could also have a role in tethering chromatin, particularly the *Rex1* locus, in interphase cells, I generated phospho-null mutants for both these residues, which were then overexpressed in ES cells. These cells were then subjected to LIF withdrawal followed by FISH where the *Rex1* locus was labelled. Cells expressing LBR WT show a relocation of the *Rex1* locus to the periphery upon LIF withdrawal, confirming that nucleofection or the over expression of LBR WT do not alter the phenotype (Figure 29 B and C). LBR S71A (Figure 29D and E) and S71,86A (Figure 29F and G) mutants fail to tether the locus to the nuclear periphery. The failure in tethering is more prominent in the double mutant where the locus is found at a median value of over 2µm from the nuclear periphery, similar to the control cells that have not been subjected to LIF withdrawal.

4.6 Discussion

The previous chapter characterised the rapid repositioning of a specific genomic locus, *Rex1*, during exit from pluripotency. Since this locus repositions from the nuclear interior to the periphery within merely two hours post LIF withdrawal in a reversible manner, the mechanistic details of such tethering make for an interesting study. However, the nuclear envelope of ES cells has never been assessed for its composition in detail. With a particular interest in identifying the components of the nuclear envelope that might have a role in tethering the *Rex1* locus to the periphery, I explained in this chapter the experimental design and samples used for a mass spectrometric analysis. The experiment is designed to look at which of the known NETs are expressed in ES cells and which of those then show changes in phosphorylation.

It is important to remember, however, that the size and abundance of some of these NETs and the potential domination of post-translational modifications in one state or the other might make the protein effectively invisible based on the initial mass spectrometry analysis for non-modified peptides. The distribution of the 67 unique NETs appearing during exit shows an increase in the ones that were exclusively found in blood in the earlier studies from the Schirmer lab (Figure 27B bottom panel). At such early stages of exit from

pluripotency stimulated merely by LIF withdrawal and no other defining exogenous factors directing lineage commitment, the cells might merely move to a poised state that starts to set the stage for impending cues that might direct their fate. Spontaneous differentiation due to LIF withdrawal has been shown previously to favour gene expression reflecting the endoderm and mesoderm states (Heo et al. 2005). During embryogenesis however, the first wave of erythropoiesis begins as early as the seventh day of embryonic life with the production of the primitive erythroid cells followed by an expanding population of definitive erythroid cells that predominate subsequently (Baron, Isern, and Fraser 2012). Blood is among the first tissues to be formed along with the development of a functional heart to sustain life as the embryo grows in size (Gritz and Hirschi 2016). Thus, the changes in NET composition could reflect the priming needed at the NE to prepare for the impending events leading to lineage commitment.

When comparing the phosphorylation status of these NETs, I found 6 NETs that show gain or loss of phosphorylation within the first two hours of exit form pluripotency. Of these, LBR and the LAP2 isoform LAP2α are of specific interest as they have been widely studied before and have been known to interact with chromatin. Phospho-null mutants were generated for both LBR and LAP2α using site directed mutagenesis, which were overexpressed in ES cells. From the results summarised above, it seems that mutating the phosphorylation sites on the LAP2α chromatin binding domain or the LBR RS domain have a similar effect in that the tethering of the *Rex1* locus to the periphery upon LIF withdrawal is prevented. This leads to further interesting questions about the mechanistic details of the tethering.

While LBR is an inner nuclear membrane protein, LAP2α lacks a transmembrane domain and is therefore nucleoplasmic. It is easier to interpret the effects of the LBR mutants, as it could be that LBR is a direct tether point for the *Rex1* locus and a loss of phosphorylation at Ser-71 and maybe Ser-86 enables DNA-protein interaction. It could also be that the interaction of the locus to LBR is indirectly mediated by LBR-HP1 binding or other epigenetic silencing marks on histones. However, this needs to be investigated further. While the mitotic kinase CDK1 phosphorylates serines 71 and 86 to initiate the detachment of LBR from chromatin and NE breakdown, The RS domain residues of LBR are also targets of SRPK1 (Papoutsopoulou, Nikolakaki, and Giannakouros 1999; Takano et al. 2004) and potential targets of CLK kinases (Giannakouros et al. 2011; Zhou and Fu 2013).

Interestingly, our mass spec data shows that 2h post LIF withdrawal there is a loss of phosphorylation on SRPK1 and a gain of phosphorylation on CDK1. Any of these kinases could mediate the phosphorylation of these residues during interphase. A loss of phosphorylation seen during exit stimulated by LIF withdrawal could consequently lead to LBR binding to the locus and tethering it at the nuclear periphery. A ChIP-seq experiment with LBR could reveal conclusive evidence of the binding between LBR and *Rex1*.

Why LAP2α mutants would prevent the tethering of the locus to the periphery is a trickier question to answer. It could be that LAP2α binds to the locus to anchor it and mediates its tethering at the NE due to its ability to bind to Lamin A. It is important to bear in mind while considering LAP2α functions in ES cells the controversy over whether or not Lamin A is actually present in ES cells. As mentioned in the introduction, Lamin A/C has often been used as a marker for differentiation due to its presumed absence in ES cells based on immunofluorescence assays. However, this absence could be a likely result of epitope masking. Western blotting revealed the presence of Lamin A/C in 9 independent ES cell lines and in ICM cells (Eckersley-Maslin et al. 2013). The detection of Lamin A, albeit low, with 15 peptides and 57 spectral counts in my proteomic dataset from intact nuclei in ES cells further corroborates this result. Furthermore, the localisation of Lamin A/C in ES cells depends on Lamin B1 (Guo et al. 2014). These results suggest that Lamin A/C is present in ES cells but its massive upregulation during differentiation makes it much more detectable in IF studies on differentiated cells. It is thus possible that LAP2α-Lamin A/C nexus is as important in maintaining chromatin states in the pluripotent cells as it is in differentiated cell types. Alternatively, could also be that LAP2α acts as an adaptor for the locus and mediates its translocation to the nuclear periphery where LBR then binds to it. However, either of these hypotheses will need further testing.

Chapter 5

Ectopic Expression of tissue specific NETs in ES cells leads to exit from pluripotency

Division of labour between the various component organ systems results in the functionally coherent system that is multicellular life. The various tissue types that form organs are themselves formed as a result of cellular differentiation and lineage commitment of either pluripotent or multipotent cells during the course of development. At the core of cellular differentiation are two things – the stimuli which initiate the process and the reorganisation of gene expression networks which facilitate it. The precise actions of genes are dependent on their tissue context and over time, several diseases have been identified to be the result of a disordered interplay of tissue and cell-lineage specific gene expression. Thus, the establishment and maintenance of tissue identity has been of interest for several decades. And at the core of it all lies the following broad question -

How is tissue specific gene expression achieved?

While this was first thought to be exclusively due to differential expression of distinct combinations of transcription factors, it has become clear over the years that there are a multitude of factors that contribute. From transcription and RNA processing to protein translation and post translational modifications, epigenetics and spatial genome organisation, any and all of these factors might work to control gene expression for a physiological state.

One approach to identifying factors involved in establishing the differentiated state of cells is human genetics as its disruption leads to human disease. A curious question in human genetics is what underlies tissue specific manifestations of hereditary diseases, considering these are caused by germline mutations that are present across the human body. Germline aberrations for over 1500 hereditary diseases can be retrieved from the OMIM database (Amberger et al. 2009) and yet the molecular mechanisms explaining their manifestations in specific tissues are largely unknown. A likely explanation for such selective vulnerability is a tissue specific gene expression profile so that the functionality of a mutated protein is compromised within the tissues in which it is expressed. Tissue specificity may arise not just from tissue specific expression of a certain protein but also through tissue specific protein-protein interactions. It is not entirely impossible that a widely expressed protein

could have tissue specific interactions with other proteins, either enabled by compartmentalisation or the specific expression profiles of the interacting partners or inhibitors of these interactions. Thus, the study of tissue specific interaction networks becomes necessary to better represent disease pathologies. Towards this end, in one of the first large-scale studies of protein networks revealed that over ~70% of the disease causing genes were significantly expressed in the tissues where the disease manifested (Magger et al. 2012). In another quantitative approach combining microarray and RNA-seq approaches to look at gene expression across various tissue types, a bi-modal distribution of expressed genes across tissues was revealed, with most genes showing either ubiquitous or tissue specific expression across tissues. Of these, it was shown that the disease-causing genes tend to have elevated transcript levels and increased number of tissue-specific protein-protein interactions in their disease tissues compared to unaffected tissues. For instance, a *muscle specific protein-protein interaction* between the widely expressed cell adhesion receptor dystroglycan 1 (DAG1) to its muscle specific ligand dystrophin (DMD) and to caveolin 3 (CAV3), which regulates DMD by preventing the DAG1-DMD interaction, could explain why mutations in these three genes give rise to various forms of muscular dystrophies (Barshir et al. 2014). However, this is not strictly true. Tissue specific mis-splicing and low-level expression can also cause diseases as in the case of familial dysautonomia (G. Lee et al. 2009). Following such findings, the need for studying the mechanisms underlying the tissue specific regulation of gene expression was highlighted. Interestingly, the interconnected approach also can lead back to the nuclear envelope, the focus of this thesis. For example, in a recent study identifying additional alleles of Emery-Dreifuss muscular dystrophy, the authors argue that the EDMD pathomechanism is a result of altered gene regulation and mechanotransduction due to connectivity of proteins from the nuclear envelope to the plasma membrane (Meinke et al. 2020).

3D genome organisation regulates gene expression by controlling the physical position of a certain genomic locus in the three-dimensional space of the nucleus, thus potentially restricting its access to transcriptional factors by tethering it to the nuclear envelope or making it more accessible by the formation of a loop in the nuclear interior such that the transcriptional factors might easily bind to it. This, in addition to epigenetic marks which affect DNA compaction and transcription factor binding itself, plays a huge role in the modulation of gene expression. The role of the nuclear envelope (NE) in orchestrating

genome organisation during development and differentiation has been widely studied and has been discussed in detail in Chapter 1. Interest in tissue-specific components of the NE arose from the discovery of tissue specific human diseases known as laminopathies which posed an interesting paradox – this class of diseases was linked to mutations in *LMNA*, the gene encoding the widely expressed A type lamins, when the pathology was seen to selectively manifest in striated muscle or cause restrictive dermopathy where the worst pathology occurs in skin, or peripheral neuropathy affecting nerves, or lipodystrophy affecting fat (Bonne and Quijano-Roy 2013; Howard J. Worman and Dauer 2014). These distinct pathologies might be explained by a disruption in tissue specific interactions between A type lamins and putative tissue specific binding partners. The Schirmer lab has made seminal contributions to the field of nuclear biology by discovering several tissue specific Nuclear Envelope Transmembrane Proteins (NETs) and demonstrating several of these to have the ability to affect genome organisation as well as linking them to disease. These proteins and their potential roles in establishing genome organisation will be discussed in some detail in the following sections of this chapter.

While tissue specific NETs might be a likely answer to how genome organisation is maintained in differentiated tissues, the absence of these proteins in embryonic stages leaves a gap in the understanding of how tissue specific genome organisation is defined in the first place. This chapter will look at the distribution of some of these NETs in early stages of mouse embryonic development, the effects of ectopic expression of these tissue specific NETs on ES cell pluripotency and explore the ability of such ectopic expression in altering genome organisation before ES cells go through the process of lineage commitment.

5.1 Expression of tissue specific NETs during early development and its significance

The NE is an impenetrable double-membrane barrier between the nucleus and the cytoplasm that is perforated by nuclear pore complexes that regulate directed nucleocytoplasmic transport. Both the outer and the inner nuclear membranes have unique sets NETs and the tissue specificity of these NE proteins is of primary interest in the Schirmer lab. As a first step to attempt to identify candidate proteins that mediate tissue-restricted disease pathologies, novel proteomic approaches were undertaken to isolate the nuclear envelopes and identify the NET proteomes in three different tissue types, namely

liver, muscle and blood. In the first study, the nuclear envelopes were isolated from resting and activated lymphocyte enriched peripheral blood leukocyte fractions. About 14% of the NETs were found to be uniquely expressed either in the resting or the activated state of the lymphocytes (Korfali et al. 2010). In another study, a similar comparison between the NET proteome identified in the three tissue types revealed that less than 20% of the NETs are shared between the three tissue types, suggesting that most NETs are tissue restricted in expression (Korfali et al. 2012). A total of 954 NETs were identified collectively from the three tissue types studied. Several of these were also shown by immunofluorescence to target to the nuclear envelope (Korfali et al. 2010; Malik et al. 2010b; Wilkie et al. 2011). Given the subcellular localisation of these proteins, many of these NETs were tested for their ability to affect genome organisation and indeed were found to do that (explained in more detail in the following sections). However, these NETs were all isolated from adult tissues and thus, their expression during early embryonic stages was not known.

I hypothesised that if a NET is functionally important in a certain stage of development, it would show higher expression level at that stage. Thus, NETs important for the maintenance of genome organisation in ES cells would preferentially be expressed in ES cells followed by their downregulation in later stages of development during which lineage commitment is already underway. While the mass spectrometry experiments described in the previous chapter reveal the composition of the NE in ES cells, the study was directed at looking at the changes in the NE composition during the first two hours of exit from pluripotency and not the later stages. Furthermore, we must bear in mind that the mass spectrometry was performed in a particular line of ES cells. To test the hypothesis of stage-specific expression of NETs yielding unique NET protein signatures during lineage commitment, expression data was downloaded from the BioGPS transcriptome database that compares the expression of the vast majority of protein-encoding human and mouse genes from a panel of 79 human and 61 mouse tissues on microarrays (Su et al. 2004; Wu et al. 2009). Importantly, the tissues tested include mouse embryos at early stages of development. In this chapter, I will be specifically focusing on the expression patterns of previously described *tissue-specific NETs* over early developmental stages. The median expression values for these NETs were obtained over the complete set of tissues and the normalised data over this median is shown for a subset of tissues, namely mouse ES cells

and early developmental stages from e6.5 to e10.5 stages of mouse development (Figure 30).

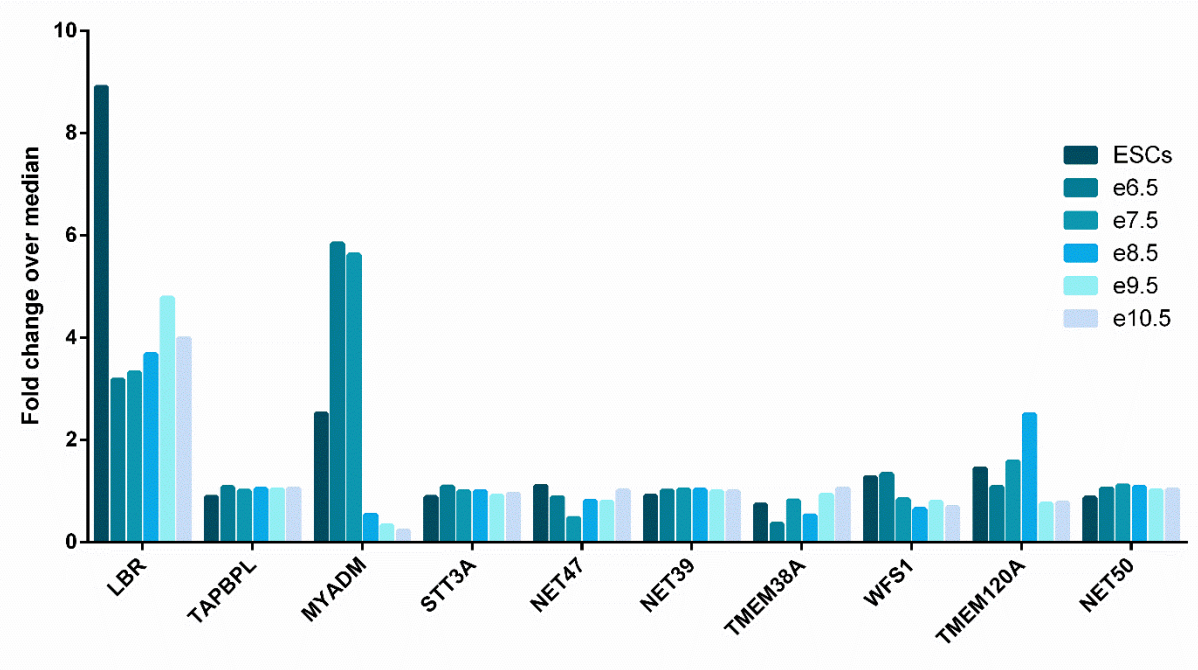

Figure 30: Microarray expression data for NETs in pluripotent ES cells (ESCs) and 5 early stages of embryogenesis (e 6.5 to e10.5). Data is shown as fold change over median values across the 61 mouse tissues used in the study (Wu et al., 2009). LBR expression drops more than 2-fold between ESCs and e6.5 but is still maintained at >3-fold over median value across the samples. Tissue-specific NETs like TAPBPL, STT3A, NET39 and NET50 are expressed consistently at median value in all the 6 stages of early development shown here.

The well-studied NET lamin binding receptor (LBR) serves as a good case in point for the above-mentioned hypothesis. LBR is an evolutionarily conserved and developmentally essential inner nuclear membrane protein, ubiquitous in vertebrates, *Drosophila* and yeast (H. J. Worman et al. 1988; Wagner 2004). As seen in Figure 30, it is highly expressed in ES cells followed by at least a two-fold reduction in expression levels in e6.5 to 10.5 stages of development. This, along with the change in its phosphorylation status during exit from pluripotency as described in Chapter 4, highlights its importance in the maintenance of genome organisation in pluripotent ES cells. Of medical significance, mutation of the LBR gene causes developmental abnormalities, reduced survival of homozygous embryos and hereditary diseases (Waterham et al. 2003; Shultz 2003). It has been previously argued that the downregulation of LBR at later developmental stages is functionally complemented by the upregulation in LaminA/C and that these two proteins control peripheral heterochromatin accumulation in a stage specific manner (Solovei et al. 2013).

Of specific interest is that fact that NETs like NET39, TAPBPL, NET50 and even TMEM120A are consistently expressed only around median value through all the early developmental stages shown here. Whereas in the specific tissues that they were originally identified from in the mass spectrometry studies in the Schirmer lab, their expression was over 8-fold over median value (data not shown), clearly highlighting their subsequent tissue restricted expression patterns. The low abundance or absence of tissue specific NETs in pluripotent cells and early stages of development might suggest no functional role for these proteins in the maintenance of pluripotency.

5.2 Transient Transfection of tissue specific NETs in ES cells leads to morphological changes similar to differentiation in presence of LIF

Several tissue specific NETs have been studied for their roles in genome organisation, cell cycle progression and cytoskeletal associations, with genome organisation being the primary interest in the Schirmer lab. The ability of these NETs to affect genome organisation was tested by inserting a lacO array into chromosomes 5 (typically found in the nuclear interior) and 13 (typically peripheral) in HT1080 cells and then transiently transfecting these cells with individual NETs. The position of the array with respect to the nuclear periphery was assayed using erosion analysis that divides the nuclear volume into five concentric voxels of equal volume, with 1 being the most peripheral and 5 being the most central rings. Upon transfection, if the lacO array shows greater probability of being in the first two rings, which are most peripheral, it confirms the ability of these NETs to tether that chromosome to the periphery. Using such screens, TAPBPL and STT3A, previously identified to be blood-specific NETs, and were shown to recruit Chr5 to the periphery (Korfali et al. 2010). Similarly, muscle specific NETs NET39 and TMEM38A and fat specific NET29/TMEM120A were also shown to reposition certain chromosomes using the array as a positioning marker (Zuleger et al. 2013). NET39, TMEM38A and TMEM120A were further studied for their specific roles in myogenesis and adipogenesis in the Schirmer lab. Using C2C12 differentiation system as a model, it was shown that NET39 and TMEM38A are expressed upon differentiation of myoblasts into myotubes. During this transition, chr8 is seen to relocate to the nuclear periphery and this relocation is facilitated by NET39, but this study also investigated specific genes under NET39 positional regulation and found roughly 70 myogenic genes such as *Nid1*, that reposition

to the periphery in a NET39 specific manner. Critically, the lack of muscle specific NETs NET39, TMEM38A and WFS1 resulted in impaired myotube formation, highlighting their role in myogenesis (Robson et al. 2016). Similarly, the role of fat specific NETs TMEM120A and B were characterised in adipogenesis using the *in vitro* differentiation model of 3T3L1 cells. These NETs were shown to be induced during adipocyte differentiation and necessary for adipogenesis (Batrakou et al. 2015). While it is clear that this screening approach identified tissue specific NETs that contribute to genome organisation in their relative tissues, it is important to bear in mind that the output of the screens was only certain chromosomes and only ~50 NETs have been tested in total for this function. Therefore, it is possible that other untested NETs could affect genome organisation and that the ones tested thus far could affect other genes and chromosome positions too.

Although the role of some of these NETs has been characterised during adipogenesis and myogenesis to some degree, both the *in vitro* differentiation systems namely 3T3L1 and C2C12 cells represent the differentiation of progenitor cells into their terminally differentiated lineages. The expression data obtained from BioGPS suggests that these NETs are expressed at low levels during pluripotency. Thus, it is of interest to ask the following two questions –

Can ectopic expression of tissue specific NETs affect the differentiation status of pluripotent cells?

If yes, is this because of their ability to alter genome organisation?

Answering these questions might reveal the importance of stage specific expression of NETs and will further indicate the importance of their absence in pluripotent cells.

To answer these questions, mouse ES cells grown in serum/LIF conditions were transfected with the following NETs fused with either GFP or RFP – NET39, TMEM38A, TMEM120A and TAPBPL to assess the effects of this ectopic expression on pluripotency. H2B-mRFP, being a pan nuclear protein was used as a control. NET50, being a NET with no known effects on genome organisation was also used as a control. It has been previously shown that the localisation of a NET at the NE is often cell-type specific (Malik et al. 2010b). Therefore, it is imperative to confirm the subcellular localisation of these NETs upon transfection in ES cells. All the NETs localise at the nuclear envelope showing a nice rim staining as expected (Figure 31A). However, transfection in mouse ES cells proved

especially challenging as these cells grow in tight clusters. Therefore, to study the effects of transfection of NETs on pluripotency, the cells were subjected to transfection, followed by FACS sorting to yield a population of transfected cells (Figure 31B). Using untransfected cells as controls, forward scatter plots were used to determine gates for the Alexa-fluor 488A (GFP) and PE-Texas Red-A channels (RFP) (Figure 31C). Using these gates, fluorescent transfected cells as seen in Figure 31D were sorted that could then be studied in culture for morphological changes. The sorted cells were then replated on gelatin coated plates (10k cells/well of a 24 well plate) in complete medium containing LIF overnight.

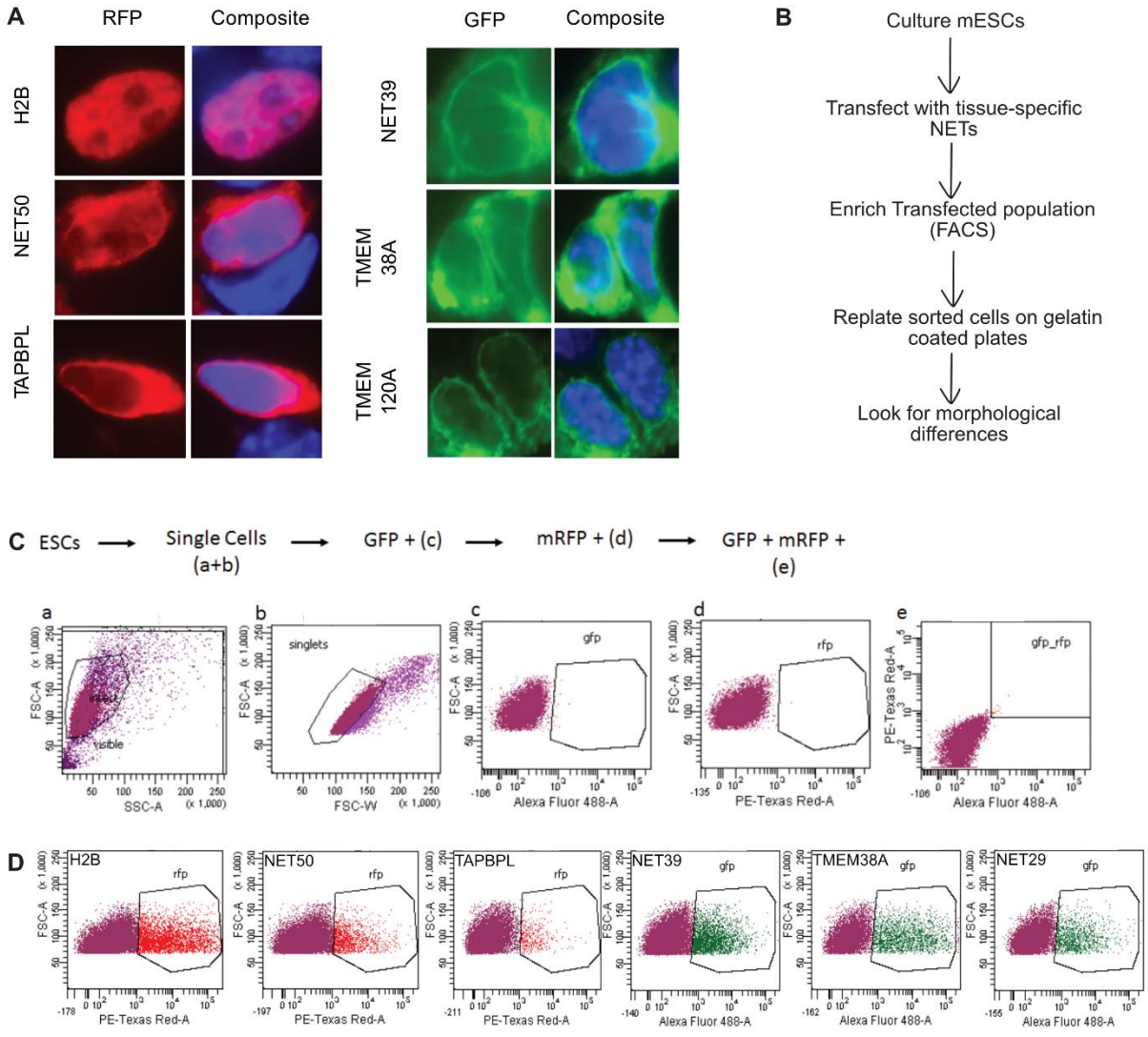

Figure 31: Tissue specific NETs localise at the NE in ES cells. A. Representative immunofluorescence images of ES cells transfected with histone H2B and other NETs as shown. All the NETs localise at the NE. B. Schematic showing the experimental design for enriching transfected ESCs expressing tissue specific NETs for experiments characterising their pluripotent state. C. Design and gating strategy for flow cytometry to sort the cells expressing GFP/RFP tagged NETs. Unstained cells were used to identify the population of single cells (a and b) which were subjected to gates in the Alexa-fluor 488-A and PE Texas Red-A channels. D. Representative FACS images showing the transfected cells expressing GFP or RFP tagged NETs in the corresponding channels that were sorted for further experiments.

The process of transfection followed by sorting itself causes about 7% of the untransfected cells to show fibroblast like morphologies highlighting the delicate nature of stem cells in culture and how easily prone these cells are to differentiation. Curiously, in spite of the presence of LIF in the culture medium, cells transfected with genome organising NETs like NET39, TAPBPL, TMEM120A, and TMEM38A showed a significantly higher percentage of fibroblast like flattened cells compared to those that were transfected with H2B or NET50 (Figure 32). For several days post sorting, these cells were maintained in culture in presence of LIF. The controls took well to the culture conditions forming well defined colonies of pluripotent cells while the cells transfected with the genome-organising NETs formed colonies but also had several spontaneously differentiated cells with the characteristic flattened morphology and extended processes (data not shown). Although these cells were sorted on the basis of them expressing the NETs, the expression of proteins following transient transfection often lasts only for ~48-72h post transfection. Whether the effects of transient expression of NETs are reversible upon loss of expression is a curious question and remains to be studied.

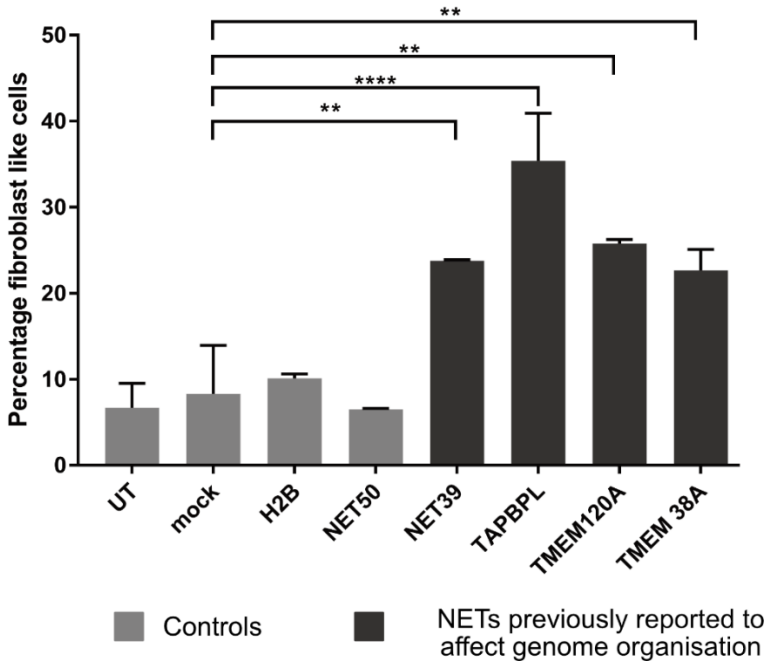

Figure 32: Cells expressing genome organising NETs show fibroblast like flattened morphologies. 10k transfected cells sorted using FACS were re-plated per well of a 24 well gelatin-coated plate in serum/LIF culture conditions overnight. Post adhesion of cells to the plate i.e. 24h post replating, the percentage of cells with fibroblast-like morphologies was scored in each sample. Cells expressing genome organised NETs show a higher percentage of fibroblast-like cells characteristic of spontaneous differentiation. Statistics were performed using Students t-test. **p<0.01,

Morphological changes are characteristic of differentiation and lineage commitment and is a result of cytoskeletal reorganisation and differential expression of adhesion proteins on the cellular surface. In fact, this property has been exploited in a study where different scaffold architectures were used to enhance osteogenic differentiation to aid in the development of novel scaffolds for bone regeneration (Guvendiren et al. 2017). Taken together, these results suggest that NETs with the ability to reorganise the genome have a greater effect on compromising stem cell pluripotency.

5.3 Cells co-transfected with tissue specific NETs show flatter colony morphology but no changes in surface markers

While the expression of single NETs alone showed morphological changes, it is of interest to see if the effects might be compounded when more than one NET is co-transfected. Additionally, in the previous set of experiments, morphological changes were observed several days post transfection and sorting since cells were sorted 48h post transfection and scored for morphology 24h post sorting. Additionally, the percentage of fibroblast like cells were counted manually under the microscope. To have a better understanding of the characteristic morphological changes in response to ectopic expression of the genome-organising NETs, it was imperative to image them over time. To this effect, ES cells were both single- and co-transfected with two of the genome-organising NETs that showed promising effects in the previous set of experiments – TAPBPL and NET39 – and NET50 and H2B transfections were again used as controls. The transfected cells were subjected to FACS sorting 48h post transfection and replated on gelatin coated plates in complete culture medium with LIF. However, in this instance, phase contrast images were taken of these cells every three hours over three days using Incucyte, which is a powerful live-cell analysis tool with an automated microscope set up inside the tissue culture chamber. As anticipated, untransfected cells and those transfected with H2B or NET50 continue to grow in well-defined colonies. However, when the cells are co-transfected with TAPBPL and NET39, the effects are compounded with a much higher proportion of the cells expressing flatter morphology, loss of well-defined colonies and various cells developing extended processes (Figure 33).

Mouse ES cells characteristically proliferate at an unusually rapid rate, with a doubling time of roughly 8-10h. These cells have a short G1 phase with a high proportion (~65%) of

cells in S phase. With the formation of the germ layers, cell cycle typically restructures so that the length of G1 phase is now longer, resulting in increased division times (Lawson, Meneses, and Pedersen 1991). There are several explanations as to why an extended G1 phase might facilitate differentiation. The transition from pluripotency to fate determination requires an extensive re-wiring of the transcriptional network that then results in a wide range of biological changes including changes in cell size and shape, acquisition of contact inhibition, expression of cell surface markers, etc and a longer G1 phase may allow for this re-wiring of the network. The G1 phase also provides a more permissive chromatin state to respond to extrinsic fate determinant signals so that the corresponding epigenetic changes, genome organisation changes and changes in transcriptional activity might occur. Several reports place Myc and the Rb-E2F pathway at the centre of the regulatory network that links the cell-cycle machinery with self-renewal of pluripotent cells (Singh and Dalton 2009). Interestingly, it was observed that cell cycle perturbations also change the timing and the course of differentiation in a model for muscle differentiation (V. C. Li and Kirschner 2014). Thus, the balance between proliferation and differentiation is achieved primarily through changes in cell cycle durations such that as cells begin to commit to a lineage, there is a marked decrease in the proliferation time due to an increase in G1 phase.

As the cells transfected with tissue-specific NETs showed morphological changes characteristic of differentiation, it was also of interest to study if these cells had a slower growth rate. The Incucyte system enables real-time automated cell proliferation assays in three ways – a label-free method depending on confluence, a label-free method depending on direct cell count and direct cell count based on fluorescent labelling. Although the cells were transfected with GFP/RFP tagged NETs, the basis on which they were sorted, the sorting itself was done 48h post transfection after which the cells were imaged for 3 days. Thus, fluorescence was not a reliable means to study the cell cycle as there would be a loss in fluorescence over time as it happens in transient transfections. Since these cells tend to grow in colonies often on top of each other and have no contact inhibition, it is difficult to mark cell boundaries accurately on the Incucyte. Therefore, growth rate using direct cell counts on the Incucyte could also provide false values. Thus, confluence was used to study growth rates. As anticipated, all the controls have similar growth curves. Cell transfected with single NETs show no significant deviation in growth curves either. It was expected

that at least the cells co-transfected with TAPBPL and NET39 would have slower proliferation rates and in agreement with that the growth curve itself shows a marked deviance from the curves for the controls (Figure 34). However, if cell sizes and shapes were similar, such marked deviance would reflect an increase in proliferation. In this case as the cells have a flatter morphology occupying larger areas on the plate, the *confluence* of the plate is higher. And as a direct count of cells is not easy to obtain for these cells, growth curves studied by confluence are not really reflective of proliferation rates of these cells.

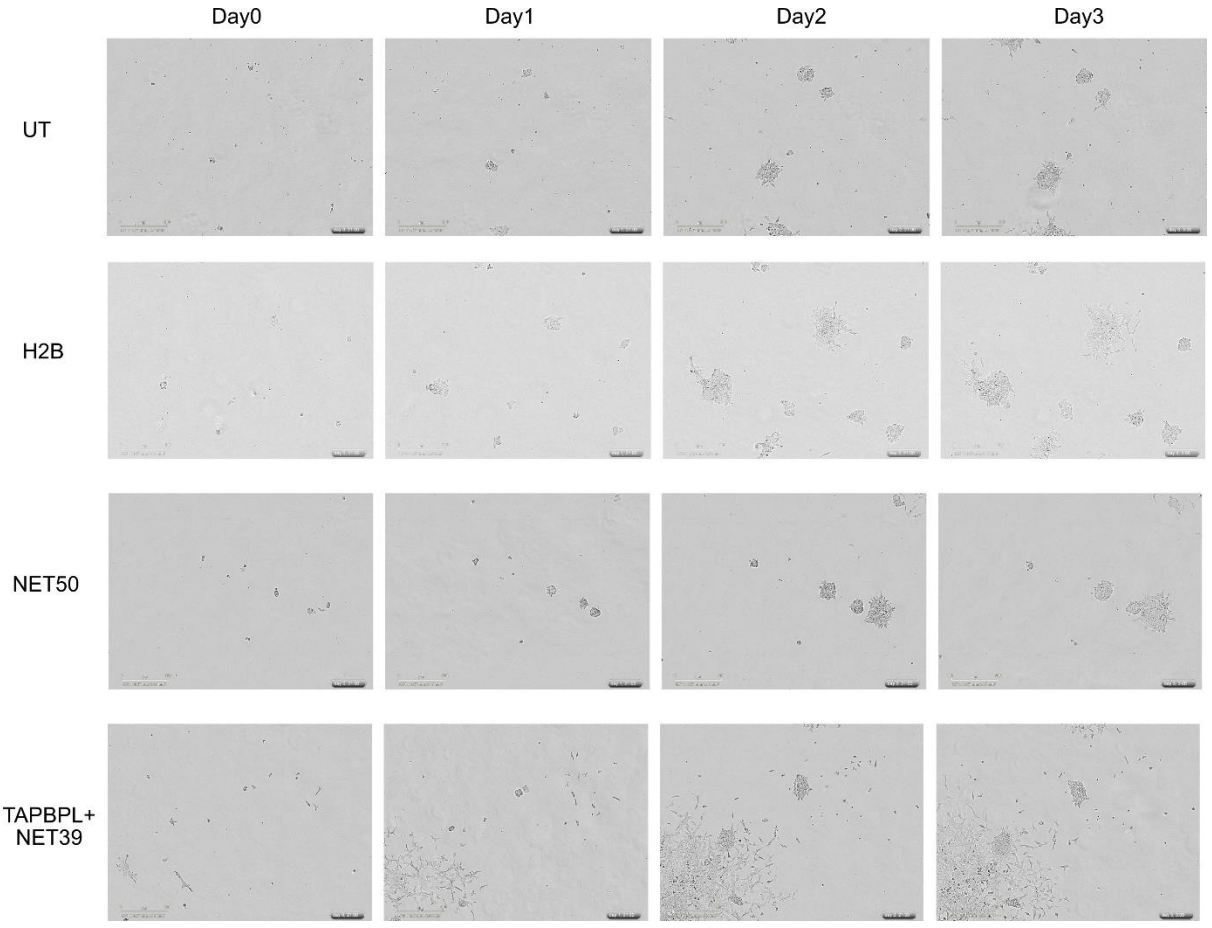

Figure 33: Representative phase contrast images of untransfected cells (UT) and cells transfected with histone H2B, NET50 and co-transfected with TAPBPL and NET39 taken on the Incucyte. Cells were sorted 48h post transfection, re-plated on gelatin coated plates overnight and imaged for three days following that.

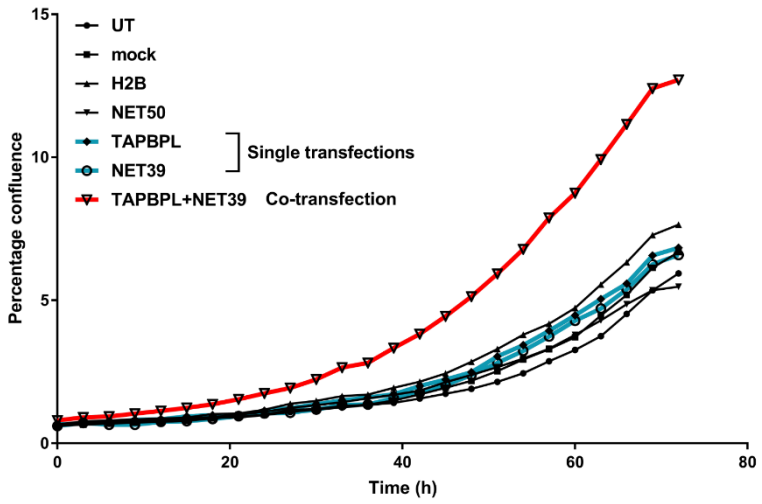

Figure 34: Growth curve plotted as percentage confluence. Untransfected cells (UT) and cells transfected with NETs were sorted and re-plated on gelatin-coated plates and imaged over 3 days. Cells transfected with NETs TAPBPL and NET39 individually show similar growth rates to untransfected cells and controls expressing H2B and NET50. But co-transfected cells show higher percentage confluence owing to the flattened morphologies.

Finally, in an attempt to study if the potential differentiation of these cells due to the expression of tissue-specific NETs might lead to a difference in cell surface markers, I used FACS to study the expression of SSEA-1 expression on these transfected cells. SSEA-1 is regarded as an excellent cell surface marker to monitor early stages of ES cell differentiation (Fox et al. 1981). An SSEA-1 antibody conjugated to Alexa647 was used for the FACS study and gated as shown in Figure 35A. Cells transfected with NETs TAPBPL, TMEM38A, NET39 and TMEM120A were analysed for SSEA-1 expression along with some cells co-transfected with NET39 and TAPBPL, which shows promising differences in colony morphologies. Additional co-transfections of TAPBPL with TMEM38A and TMEM120A were also analysed in this experiment. However, an overlay of the SSEA-1 expression histograms clarifies that none of the cells ectopically expressing NETs showed any difference in the expression of SSEA-1 72h post transfection (Figure 35B).

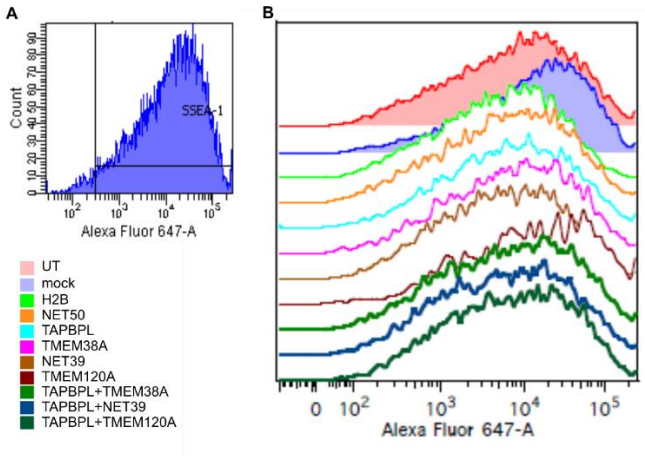

Figure 35: SSEA-1 expression in cells transfected with tissue specific NETs. A. Cells were stained with SSEA-1 conjugated to Alexa Fluor 647 and gate used to identify SSEA-1 positive population is shown. B. Overlay of SSEA-1 profiles for the indicated samples.

5.4 Continued induction of tissue specific NETs in ES cells leads to exit from pluripotency in presence of LIF

In addition to the low efficiency of transient transfection in ES cells, this method is not ideal for studying such problems as differentiation as it poses the following challenges.

1. As the transfected genetic material is not integrated in the host genome, the effects of transient transfection are time limited and are lost during ongoing cell division. Thus, one cannot study the long-term effects of protein expression using this method.
2. Some of these experiments, such as those on the Incucyte, have been done over the course of 5 days post transfection. Over this time, certain cells would lose expression while others might still retain it. Such heterogeneous loss of expression might present difficulties in assaying the phenotypes. It would also remain unclear if the effects of NET expression in these cells are reversible. If the effects are irreversible, the loss of protein expression over time may not affect the phenotypes observed but if the effects were reversible, one would not be able to account for the variability this might introduce in the results.
3. Although the transfected cells have been sorted for, these cells show a range of expression levels for the proteins they express. Thus, it is impossible to assay whether protein levels itself might affect the phenotype. It is entirely possible that low level expression of a NET in a particular cell might cause no phenotype but a higher expression level will s that the population levels measured for expression are

misleading. It is also possible that moderate expression level over time is important for these cells to undergo differentiation. Such intricate details cannot be studied using transient transfections.

In light of these challenges, there was a need to generate clonal cell lines stably expressing NETs so that the effects of ectopic expression of these NETs on pluripotency could be studied over longer durations. However, continuous expression of NETs with known roles in reorganising the genome might itself cause an irreversible exit from pluripotency. Therefore, Doxycycline-inducible stable cell lines were generated to express NET50, NET39 and TAPBPL (Figure 36A). The clones were carefully picked such that they neither express very low nor very high amounts of the protein upon induction (checked microscopically, data not shown) and the NETs all show predominant localisation at the NE as expected. These inducible stable cell lines were the ideal system to study the effects of prolonged expression of NETs on the pluripotent status of ES cells.

A classical marker of pluripotent cells is the expression of alkaline phosphatase (AP), which is lost upon their differentiation. It has been previously shown that spontaneous differentiation due to LIF withdrawal leads to morphological differences in cells indicating an exit from pluripotency followed by a loss of AP expression over several days (Trouillas et al. 2009). In an attempt to study if the prolonged induction of NETs might force a similar exit from pluripotency and a loss of AP expression, the tet-on stable cells lines were subjected to NET induction *in presence of LIF* for 6 days and stained for AP expression. AP positive colonies are seen as red colonies in this assay. As expected, LIF withdrawal leads to both, characteristic flattening of cells indicative of the onset of differentiation and a loss of AP expression. Similarly, the prolonged induction of TAPBPL and NET39 also leads to near complete loss of AP expression and distinct changes in colony morphology as observed in the experiments with transient transfections (Figure 36B). For the purpose of quantitation, only completely red colonies were counted as AP positive. By this estimate, only about 25% of the colonies were AP positive upon the prolonged expression of TAPBPL and NET39 (Figure 36C).

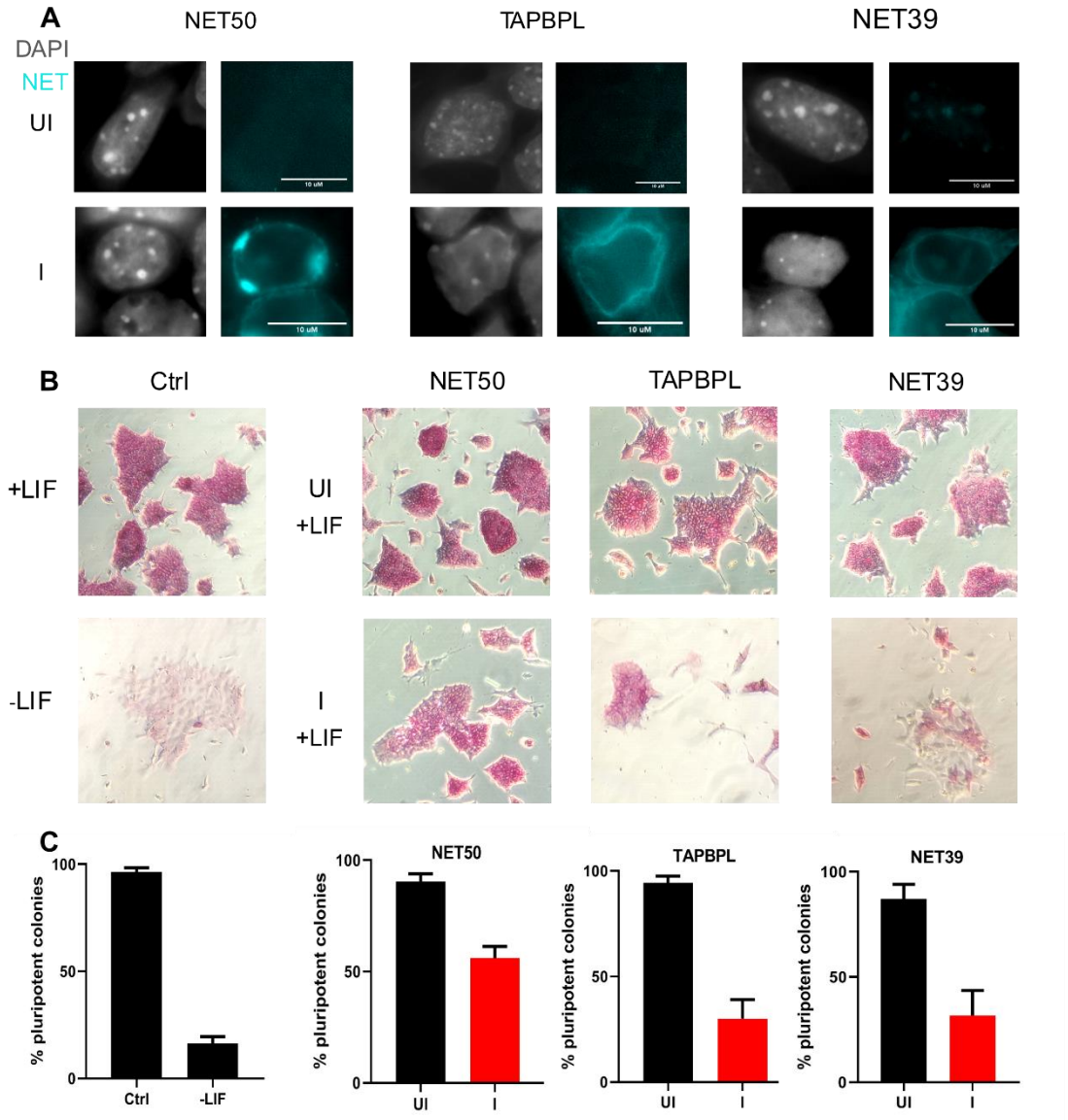

Figure 36: Prolonged expression of genome organising NETs leads to exit from pluripotency. A. Representative immunofluorescence images showing the expression of NET50, NET39 and TAPBPL when cells are induced with doxycycline. B. Alkaline phosphatase expression (red) in cell lines induced with doxycycline. Ctrl represents standard E14tg2a cells grown with and without LIF. The stable cell lines for TAPBPL, NET39 and NET50 are grown in presence of LIF. Both uninduced (UI) and induced (I) samples are cultured in presence of LIF. TAPBPL and NET39 expression leads to a loss of alkaline phosphatase expression even in pluripotent culture conditions. This is similar to the effects of LIF withdrawal for several days. C. Quantitation of alkaline phosphatase positive colonies for the samples shown in B. Uniformly red colonies were scored as AP positive in each condition.

5.5 Tissue specific NETs show specificity in the pluripotency loci they tether to the periphery

The loss of ES cell pluripotency upon over expression of tissue specific NETs like TAPBPL and NET39 emphasises the importance of regulating the expression of such proteins during

development. It is intriguing that while the prolonged expression of these proteins leads to an unequivocal loss of pluripotency, the effects of NET50 overexpression are milder. Since all the three NETs are tissue-specific, and the known difference between them is their ability to affect genome organisation, it is of interest to study whether their ability to affect genome organisation aids in the exit from pluripotency.

That tissue specific NETs alter genome organisation even in heterologous systems has been previously shown (de las Heras et al. 2017). Interestingly however, while NET39 expression regulates the radial position of about 20% of the myogenic genes during *in vitro* differentiation of myoblasts, only 3% of these seem to be affected in a heterologous system, suggesting that when a tissue specific NET is appropriately expressed in the context of other factors such as transcriptional regulators expressed during differentiation, its role in orchestrating genome organisation is much more significant. Furthermore, the correlation between repositioning genes and their expression status is weaker in a heterologous system (de las Heras et al. 2017). Thus, it would seem that the expression of a NET alone is not enough to affect gene expression networks and other factors such as the presence of transcriptional activators/repressors and chromatin remodelling proteins might also contribute to the differentiation association changes during physiological development. In the present context, I was interested in studying if the ectopic expression of tissue specific NETs could affect the radial position of certain pluripotency genes.

Chapter 3 elaborately describes the rapid repositioning of the *Rex1* locus within an hour of LIF withdrawal which leads to an exit from pluripotency. Considering how quickly this locus repositions from the nuclear interior to the nuclear periphery, it is perhaps one of the first changes to take place during exit. I have also shown that this repositioning is tightly related to exit and that resubstituting the medium with LIF quickly results in the locus returning to its position in the nuclear interior. Thus, *Rex1* repositioning is a reliable marker to confirm the exit of ES cells from naïve pluripotency. In the same chapter, I have also described the slight relocation of the *Nanog* locus towards the nuclear periphery during early stages of exit and that this is due to a distal tether established by a LAD near the *Nanog* locus which only appears following exit. Finally, I have also shown that the *Ptn* locus does not reposition during early stages of exit and is found at the nuclear periphery in ES cells. However, DamID data from previous studies (Peric-Hupkes et al. 2010) suggests that as ES cells commit to defined neural lineages, the *Ptn* locus is released from

the periphery. Thus, using these three loci, I studied the effects of overexpression of tissue specific NETs in ES cell genome organisation.

Tet-On stable cell lines for NET39, TAPBPL and NET50 overexpression were maintained in serum/LIF culture conditions. Doxycycline treatment was used to induce the expression of NETs *in pluripotent culture conditions*. Cells were harvested at 48h post induction for FISH on the three loci mentioned above. Of the three NETs, only NET39 expression caused *Rex1* repositioning from the nuclear interior to the nuclear periphery, as is seen during exit due to LIF withdrawal (Figure 37A). This is interesting because both NET39 and TAPBPL overexpression led to an exit from pluripotency, as detected by the loss of alkaline phosphatase expression (Figure 36 B and C). Yet, TAPBPL expression seems to not affect *Rex1* repositioning, which is one of the earliest changes seen during exit. Alkaline phosphatase expression was studied at 6 days post induction and so it is entirely possible that *Rex1* might reposition later during TAPBPL mediated differentiation of these cells. However, if this were the case, it would mean that the expression of TAPBPL by itself does not directly tether the *Rex1* locus to the periphery and that TAPBPL expression might bring about other changes, either by affecting the position and expression of other genes or through some other means, leading to exit and *Rex1* repositioning merely happens as a result of exit. On the other hand, while NET50 and NET39 do not affect *Nanog* gene position, TAPBPL seems to recruit it to the periphery more strongly than it is seen to be recruited during exit caused by LIF withdrawal. Similarly, TAPBPL expression also seems to mildly affect *Ptn* gene position and although statistically insignificant, the spread of data points suggests that the locus is released from the periphery (Figure 37B and C). Thus, while it is still not possible to absolutely determine a direct tethering function from these experiments, the specificity of effects on the three genes studies here due to NET overexpression strongly suggests a direct role for NET39 in the *Rex1* repositioning and for TAPBPL in *Nanog* repositioning.

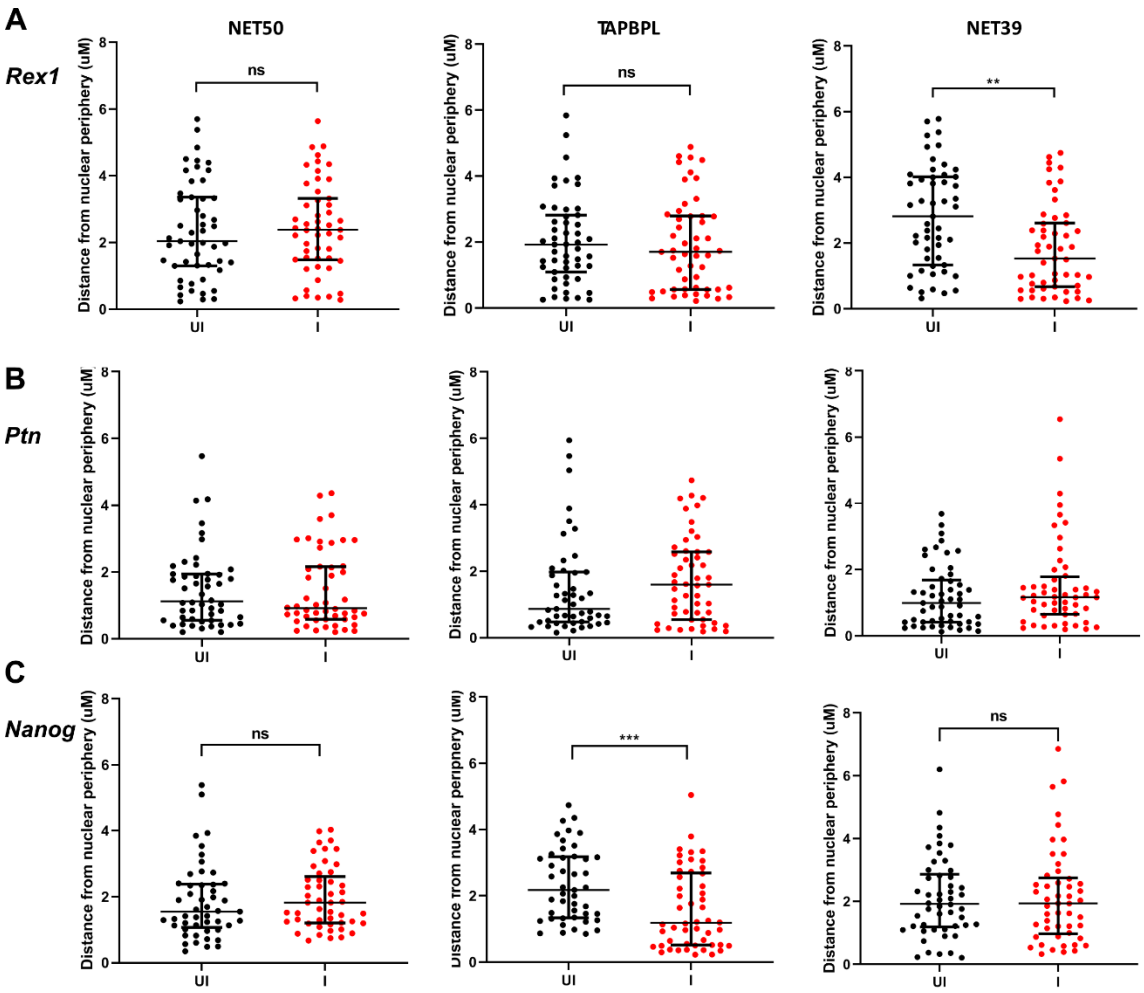

Figure 37: NETs tether pluripotency loci. Distance from periphery measurements for *Rex1* (A), *Ptn* (B) and *Nanog* (C) in uninduced cells (UI) and upon NET50, TAPBPL and NET39 expression induced by addition of doxycycline (I). NET39 tethers *Rex1* to the periphery while TAPBPL expression leads to recruitment of *Nanog* at the periphery. **p<0.01, ***p<0.001, ns p>0.05.

5.6 Discussion

In this chapter, I have studied the expression of various NETs during early embryonic stages of mouse development. I have shown that tissue specific NETs are expressed at very low levels during the earliest stages of development. Some NETs show a fluctuation in their expression. For instance, blood NET MYADM is expressed in e6.5 and e7.5 stages of embryonic development, during which the first erythroid precursors are observed. It is possible that the stage-specific expression of NETs is a mechanism by which their developmental roles are controlled. In keeping with this idea, other NETs such as NET39, TAPBPL, TMEM120A, etc, which have been previously studied in the Schirmer lab for their roles in differentiation and genome organisation, are expressed at low levels during

early embryogenesis suggesting that potentially, these NETs potentially have roles in lineage committed tissues.

Mouse development is the most tractable in mammalian species and thus, this has been studied with great interest over several decades. Stage e6.5 marks cell movements that characterise gastrulation which is followed by the increasing definition of the neural plate and the development of extra embryonic tissues at e7.5. Between e6.5 and e10.5, the development of the cardiovascular system, thymus, neural and muscular structures are seen (Kaufman and Bard 1999). The first two developmental needs of a growing embryo are blood, so that it may be able to obtain nutrition and oxygen through placental exchange, and a functional heart and cardiovascular system so that this blood may be pumped to the various parts of a growing foetus to support its increasing size and further development. Embryonic haematopoiesis in mice starts right after gastrulation when a subset of the specialised mesodermal precursor cells commits to becoming blood cells. Haematopoietic cells are observed as aggregates, termed blood islands, until yolk sac vasculatures are firmly formed and these aggregates are observed as early as embryonic day 7.5. Such yolk-sac derived blood cells constitute a transient wave of embryonic and foetal haematopoiesis (Mikkola and Orkin 2006; Yamane 2018). At the same stage of e7.5, the first cardiac precursors in the splanchnic mesoderm differentiate into cardiomyocytes and form a bilateral structure known as the cardiac crescent in the mouse. By complex morphogenetic processes, this cardiac crescent subsequently transforms into an early heart tube. Eventually, through a coordinated flow of calcium ions between different cardiomyocytes, the heart starts beating (Tyser et al. 2016; Ivanovitch, Temiño, and Torres 2017). Thus, broadly speaking, the first two tissue types to be defined during mouse development are blood and muscle (heart) as early as e7.5, followed by the development of other tissue types and organs. In line with this, MYADM, previously described to be blood-specific, shows a spike in expression at e6.5 followed by a stark downregulation. On the other hand, blood specific TAPBPL and STT3A show fairly consistent expression levels during the early stages of development with perhaps a marginal increase at e6.5 over ES cells. Analogously, while muscle specific NET WFS1 persists in expression until e6.5 followed by its downregulation, NET39 shows a fairly constant expression over these stages. If the above-mentioned hypothesis were true, such expression patterns might suggest a functional importance for MYADM during hematopoietic origins and for WFS1 in heart development,

as these are the earliest tissue types to form during mouse development. While the formation of the adipose niche is poorly understood during embryogenesis, perilipin^{+} or adiponectin^{+} proliferating preadipocytes are seen only at e16.5 (Hong et al. 2015). The fluctuations in levels of fat-specific TMEM120A during the early stages discussed here are curious and might draw relevance from other functions of the protein, for example, as an ion channel (Beaulieu-Laroche et al. 2020), but it could also reflect an early transient function in genome organisation.

Furthermore, I studied the effects of ectopic expression of tissue specific NETs such as NET39, TMEM120A, TAPBPL and NET50 in ES cells with the aim of understanding if such ectopic expression might lead to a forced exit from pluripotency in spite of maintaining these cells in culture conditions. I have shown that when these NETs are transiently expressed in ES cells, it is accompanied by morphological changes that signify the onset of differentiation. However, I did not detect any changes in SSEA1 levels. This could be due to various reasons. While morphological changes in these cells have been detected 48-72 post transfection, it is possible that SSEA-1 downregulation might only occur later. It is known that changes in morphology by visual observation are a faster indication of the onset of differentiation than the downregulation of markers such as Oct4, SSEA-1, or alkaline phosphatase (Berrill et al. 2004). It is also known that SSEA-1 expression is heterogeneous in an undifferentiated ES cell population while CD9 expression is more ubiquitous (Cui et al. 2004). Thus, assaying CD9 expression could have been a better indicator of the pluripotent status of these cells. In conclusion, studying SSEA-1 levels alone is by no means sufficient to comment on the differentiation status of these cells as that would require a more comprehensive study of the expression of a few different markers of pluripotency and differentiation.

Transient transfection poses several technical challenges in a study like this and to circumvent these, inducible stable cell lines for NET50, NET39 and TAPBPL expression were generated. Using these lines, I show that the prolonged expression of tissue specific NETs leads to a loss of alkaline phosphatase expression which is a classical marker of pluripotency. The effects of TAPBPL and NET39 expression are stronger than those mediated by NET50 in terms of loss of AP activity. However, prolonged expression of NET50 seems to also lead to loss of AP activity, suggesting that a certain population of cells exit pluripotency upon longer expressions of this NET. While NET50 was picked as

a control for a nuclear envelope protein with no known ability to alter genome organisation, it needs to be reiterated that these NETs were screened for their genome organisation effects with FISH for whole chromosomes and with LacO arrays inserted into chr5 and chr13 in a non-pluripotent cell line. Thus, the inability to reposition any of these arrays or chromosomes is merely indicative of its lack of genome organising abilities but not confirmative of it. It is possible that NET50 affects the position of other untested genomic regions. Furthermore, NET50, though unknown for its genome organising effects remains a tissue-specific NET and has been studied for its effects on nuclear size and a potential role in prostate cancer progression (Rizzotto et al. 2020). Thus, its prolonged expression could have other unknown effects in ES cells, one of which seems to be to promote an exit from pluripotency over time although the effects are not as strong as those mediated by TAPBPL or NET39 expression.

Taken together, the observations thus far suggest that the ectopic expression of tissue specific NETs in ES cells, either by transient transfection or a prolonged expression by doxycycline induction, leads to their exit from pluripotency in spite of the presence of LIF in the medium. As a proof of concept, this was shown in another study with the overexpression of NET5/SAMP1, an inner nuclear membrane protein typically expressed in muscles, in human iPS cells. Even under pluripotent culture conditions, the ectopic expression induced a rapid differentiation of iPS cells as measured by changes in colony morphology, increase in Lamin A/C expression and the gain of the neuronal marker βIII tubulin after about 6 days of SAMP1 expression (Bergqvist et al. 2017). However, the study only used transient transfections and did not look at the loss of any pluripotent markers to characterise the exit. Furthermore, they did not explore genome reorganisation as a potential cause of differentiation due to SAMP1 expression.

Curiously, in my experiments, while NET39 alone had the ability to reposition *Rex1*, TAPBPL affected *Nanog* and *Ptn* gene positions. These results reiterate what we know about NETs from other studies in the Schirmer lab, which is that they are very specific in the genes they target. The gene position changes seen for both *Nanog* and *Ptn* loci are stronger than those observed during exit and it suggests that TAPBPL overexpression reorganises the genome in ES cells but not in a manner similar to that during exit. This observation in particular highlights the importance of controlled and stage-specific expression of these NETs in their target tissues as their misexpression can cause a mis-

regulation of genome organisation which could lead to developmental defects. It would be interesting to study if the prolonged expression of these tissue specific NETs in combination with LIF withdrawal might in fact lead not just to exit from pluripotency but also affect cell fate determination.

Chapter 6

Discussion

The work discussed in this thesis is at the cusp of the broad fields of stem cell biology and genome organisation. Using a combination of fluorescence in situ hybridisation (FISH), mass spectrometry and other cell-based assays to study pluripotency, this study characterises changes in genome organisation taking place during the early hours of exit from pluripotency. It furthers our understanding of the composition of nuclear envelope in pluripotent cells and identifies the differences in the composition over the first couple of hours of *in vitro* differentiation when cells exit their pluripotent state. The study identifies two key proteins that play a role in the regulation of the tethering of the *Rex1* genomic locus either directly or indirectly. Finally, through a series of experiments involving ectopic expression of tissue-specific NETs in ES cells, the study highlights the importance of stage-specific expression of NETs during development.

6.1 Regulating the transition from naïve pluripotency

The developmental potential of cells, termed pluripotency, is an interesting state of cell identity. This state is maintained through a complex yet poorly understood interplay between 3D genome organisation, the epigenome and transcription factors. Through several studies conducted over the past decade, scientific jargon has now been reformed to define *exit from pluripotency* and *differentiation* as being related but non-identical.

The departure from naïve pluripotency is a multi-step process which progresses through a continuum of naive, formative and primed states. Each of these states has a distinct transcriptome. The departure from naïve pluripotency is enabled by the remodelling of transcriptional, epigenetic, signalling and metabolic networks through which cells enter an executive state where they gain responsiveness to specification cues. Transcription factors like Oct4, Sox2 and Nanog play a key role in the maintenance of pluripotency. In addition to the core factors, other transcription factors like TCF3, ETV5 and RBPJ have been characterised as ESC regulators. A triple knockout of these three factors liberates ES cells from their dependence on 2i or LIF for maintaining pluripotency (Kalkan et al. 2019). In other words, the absence of these three factors permanently locks the cells in their pluripotent states and prevents their exit even in the absence of 2i/LIF. Genes like p53,

Jarid2, TCF7l1 and Fbxw7 have also been identified in genome-wide CRISPR-KO studies as being important for promoting exit (T. Zhao and Xu 2010; Landeira et al. 2015; Yang et al. 2020). In fact, the transcriptional networks governing naïve and formative states have distinct target genes. During the early time points after induction of differentiation, transcriptional regulation mediated by naïve transcription factors dominates the transcriptional networks. The pivotal transformation where the formative transcriptional networks override the naïve networks occurs between 12-24h of *in vitro* differentiation (H. J. Kim et al. 2020). While the transcription factor network maintaining pluripotency has drawn considerable attention over the years, little is known about the changes in genome organisation during exit. Whether genome organisation changes would even occur in the small window lasting about a day when cells exit pluripotency was previously unknown. Most studies thus far addressing genome organisation changes focus on large scale changes in LAD and TAD organisation over later time points during the course of lineage commitment which takes several days *in vitro*. The first 24 hours of exit are interesting as the events in these hours set the stage for eventual lineage commitment and genome organisation changes have not been studied within this short window. The fundamental understanding of the events in this reversible state leading up to the formative state are key to understanding how these cells gain competence.

Studies from the Smith lab have been instrumental in identifying the malleability in the transition from naïve pluripotency. By tracking the transition using Rex1 promoter activity as a marker, they have described the initial stages of the transition as reversible and the loss of Rex1 promoter activity clearly delineates the boundary between naïve and formative stages (Kalkan et al. 2017; Mulas, Kalkan, and Smith 2017; A. Smith 2017). This same genomic locus was identified in a DamID experiment, performed a decade ago, to reposition from the nuclear interior in ES cells to the periphery in neuronal lineages (Peric-Hupkes et al. 2010). Together, these two pieces of information suggest that the transcriptional status of this locus somehow dictates the state of cells. Thus, I decided to look at the temporal dynamics of its repositioning and found that it moves from the nuclear interior to the periphery within the first hour of exit stimulated by LIF withdrawal. In addition to being rapid, this repositioning is also reversible during exit. While REX1 is dispensable to pluripotency in mouse ES cells (Masui et al. 2008), it is essential for maintaining their undifferentiated state in human ES cells (Son et al. 2013). Therefore, it

would be important to test if the genomic locus repositions in human ES cells at all during exit and if it does, it would be crucial in establishing the time course for its repositioning.
The drawback of experiments presented in this thesis is that it uses a DNA-FISH, which is a very low throughput technique. However, this study is the first of its kind to characterise genome organisation changes at such early stages of exit. Thus, it sets the stage and defines the need for further experimentation using high-throughput techniques like DamID and HiC to study LAD and TAD reorganisation during exit. The results from DamID would demonstrate if large scale repositioning events occur to and from the NE during exit. If they do, it would establish the NE as being extremely important in regulating genome organisation changes leading up to pluripotency exit. Looking at genomic regions repositioning at early hours of exit would additionally identify gene targets that are potentially positionally regulated for their expression. These gene targets would make for interesting candidates to study also for their role in establishing competence, which makes pluripotent cells responsive to differentiation cues. HiC at early stages of pluripotency exit establish changes in TAD organisation that might illustrate changes in the distal interactions between genes and regulatory elements. Perhaps the slight movement of the Nanog locus towards the periphery leads to a change in its interactions with regulatory elements. Such changes would be better understood by interpreting HiC data to see if the TAD organisation around this locus changes.
The lack of an immediate transcriptional shut down post repositioning suggested that the locus might contain regulatory elements which are perhaps positionally regulated. The recent discovery of a putative superenhancer (S. Zhang et al. 2019) bound by naïve transcription factors Oct4, Nanog and Sox2 upstream of the Rex1 promoter element along with the putative superenhancer that I discovered in my analysis make for at least two known enhancers within this genomic locus. The superenhancer discovered by Zhang et al interacts with the Rex1 promoter (S. Zhang et al. 2019). The superenhancer I discovered interacts with Triml2, a gene within the same locus. However, neither of these enhancers have been characterised yet for their interactions with any other gene targets. Additionally, positional regulation of the enhancer activity is yet to be confirmed with experiments. Towards that end, it would be interesting to do a 4C experiment in ES cells with the enhancers as the viewpoint to look at other genes that the enhancer interacts with.

Comparing 4C information from cells with and without LIF withdrawal, would add strength to the hypothesis that this enhancer is positionally regulated.

Superenhancers control gene expression programs by associating with promoters and modulating their transcriptional output. Promoter capture HiC has revealed that ES specific promoter-superenhancer interactions often span large distances over 800kb. These long-range interactions are rewired and replaced by associations over shorter distances in epiblast stem cells which are developmentally more advanced. This suggests that long-range superenhancer interactions are a hallmark of pluripotency (Novo et al. 2018). Studies from the Smith lab present evidence showcasing the importance of the *Rex1* promoter activity in marking the transition from naïve pluripotency. REX1 protein itself is dispensable to pluripotency (Masui et al. 2008). Crucially, the mouse knockout allele for Rex1 was generated by replacing the first 100bp of the open reading frame in exon4 with an EGFP gene cassette containing puromycin resistance gene. This would render the protein functionally null. However, 5'superenhancer identified by Zhang et al and the predicted enhancer in the intronic region identified in this study would both have been left undisturbed by this manipulation. Therefore, the results presented here would argue strongly for further studies exploring the role of this superenhancer in the maintenance of naïve pluripotency.

6.2 Genome organisation in ES cells

The NE anchors a large part of the genome thereby freeing up the nucleoplasmic space for DNA-DNA and DNA-protein interactions. The anchoring tether involves the DNA/chromatin and the NE proteins. Genomic regions often reposition to and from the periphery in a regulatory manner that influences their function, whether they are regulatory elements or protein coding genes. Chromatin tethers at the NE are likely established by multi-protein complexes involving NETs, lamins and chromatin remodellers. For instance, NETs like MAN1 and LAP2β can bind to DNA directly (Cai 2001; Caputo et al. 2006). In fibroblasts, the tethering of IgH and Cyp3a loci at the NE requires LAP2β together with its silencing partner HDAC3 and the transcriptional regulator cKrox (Zullo et al. 2012). One proposed hypothesis for how these tethers are stabilised at the NE is the nut-and-bolt model where the components of the multi-protein complex fan out under the membrane to better distribute the forces to withstand the pulling forces exerted by large chromosomal tethers (Czapiewski, Robson, and Schirmer 2016). Several studies in the Schirmer lab have

focused on addressing the roles of tissue specific NETs in genome organisation. However, we know little about the NETs or their complexes in ES cells that might play a role in orchestrating genome organisation.

Experiments from this thesis indicate a role for LBR and LAP2α in the repositioning of *Rex1* from the nuclear interior to the periphery upon differentiation. While these mutagenesis experiments indicate that their phosphorylation status affect the repositioning phenotype for the *Rex1* locus, they are not sufficiently informative about whether the phenotype is an effect of direct binding. Towards that end, it would be interesting to add a nucleolar localisation signal (NOLS) to the two proteins and test whether the locus repositions to the nucleolus, rather than to the NE. If it does, this would indicate that the binding of the chromatin to either/both of these proteins is direct. Furthermore, chromatin immunoprecipitation experiments designed to pull down LBR and LAP2α in cells subjected to LIF withdrawal, will yield more compelling results establishing the physical association of these proteins with the locus either directly or as part of a larger complex. These experiments open up the one important question that needs answering:

How do LBR and LAP2α control the repositioning dynamics of this locus?

The role of LBR in tethering the locus to the periphery is easier to imagine as it is a bona fide NET that provides essential chromatin docking sites at the NE. It binds to LaminB1 through the RS and TUDOR domains and selectively interacts with heterochromatin, inducing chromatin compaction and repressing transcription (reviewed by Castro-Obregón 2020). The phosphorylation of Ser-71 and Ser-86 has been previously shown to inhibit the chromatin binding activity of LBR (Takano et al. 2004; Tseng and Chen 2011). While the NMR structure of TUDOR domain in chicken LBR has been solved (Liokatis et al. 2012), we lack the structural knowledge of the RS domain that contains these two mutations and the experiments presented here argue for a need to study the RS domain carefully for its role in tethering chromatin during pluripotency and early differentiation.

LAP2α is predominantly nucleoplasmic as it lacks the C-terminal transmembrane domain anchoring 4 of the 6 isoforms to the NE (Dechat 1998). LAP2α also binds to Lamin A/C through its C-terminal domain (Dechat et al. 2000). While this interaction maintains a nucleoplasmic pool of LaminA/C, a subfraction of LAP2α might similarly associate closer to the NE. The interaction of LAP2α with chromatin depends on an N-terminal domain (Kazuhiro Furukawa, Fritze, and Gerace 1998) and a C-terminal α-specific domain (Vlcek

et al. 1999), which house the three phosphorylation sites mutated in this study, namely serines 66, 67 and 422.

LAP2α also binds to chromatin modifier HMGN5 and the proteins reciprocally alter their interaction with chromatin (S. Zhang et al. 2013). Besides binding to chromatin, LAP2α has been shown to be an important anchorage protein for the retinoblastoma protein, pRb. This is shown by two key observations in the study. Firstly, the forced aggregation of LAP2α and Lamin A/C by the expression of dominant negative lamin mutants leads to an accumulation of Rb within the aggregates. Secondly, cells with low LAP2α expression show the absence of hypophosphorylated Rb in the nucleus (Markiewicz et al. 2002). Thus, LAP2α stabilises pRb in the nucleoplasm by anchoring it to nucleoplasmic lamins. Furthermore, LAP2α also regulates cell cycle progression by binding to the C-terminal region of hypophosphorylated Rb and delaying its deactivation and maintaining E2F in a repressed state (Dorner et al. 2006). An additional role for LAP2α as a chaperone was reported recently. It was found that the acetylation of the transcription factor GLI1 regulates its binding to LAP2α and LAP2β. Acetylated GLI1 binds to LAP2β and is sequestered at the inner nuclear membrane for subsequent activation. In the nucleoplasm, active deacetylated GLI1 is bound by LAP2α that stabilises it on the chromatin (Mirza et al. 2019). Thus, the roles of LAP2α in the nucleoplasm are not singularly of the chromatin binding nature. It seems to be that the LAP2α-Lamin A/C complex acts as a docking site to stabilise other proteins as well.

The findings presented in this thesis imply that the phosphorylation status of serines 66,67 and 422 in the chromatin binding domains determine the role of LAP2α in regulating the *Rex1* repositioning dynamics. The experiments do not illuminate the exact molecular mechanisms of how LAP2α might exert its regulatory function on *Rex1* repositioning. However, some possibilities can be envisaged: first, it could be that when the serines are dephosphorylated during exit, it leads to LAP2α binding to and chaperoning the locus to the periphery where it is then bound by LBR. Such a role for LAP2α as a physical chaperone for chromatin repositioning events has never been studied before. However, given its binding to Lamin A/C, which is distributed in the nucleoplasm and also found at the nuclear periphery, LAP2α would make for an ideal candidate to mediate chromatin repositioning. By binding to the locus, LAP2α might bring it in proximity to BAF, which might lead to DNA compaction and heterochromatinization which is essential to the

transcriptional shut down of the locus. It could also be that the subfraction of LAP2α that is found closer to the periphery is the one that stabilises the locus at the periphery along with LBR. Second, it could be that the phosphorylation of these residues is somehow important for LAP2α-Lamin A/C complex formation. The LAP2α-Lamin A/C nexus might stabilise the naïve transcription factors that regulate Rex1 transcription in naïve cells. This can be tested by transfecting ES cells with the WT and phospho-null mutants of LAP2α and staining for nucleoplasmic Lamin A/C would reveal if the co-localisation of LAP2α-LaminA/C is perturbed by the expression of the mutants. Since ES cells express very low levels of LaminA/C it might prove difficult to test for this directly in ES cells using IF and might require testing this hypothesis in an alternate cell line.

Both LBR and LAP2α are developmentally important NETs. LBR predominantly tethers peripheral heterochromatin in early stages of development (Solovei et al. 2013). A knockdown of LBR facilitates cellular senescence (Arai et al. 2019; Castro-Obregón 2020) and the associated inversion in chromatin arrangement. A muscle specific knockout of LAP2α leads to an increase in myofiber associated stem cell pool but a delayed loss in satellite cell differentiation (Gotic et al. 2010). However, it has not been studied in context of maintenance of pluripotency. Investigating the mechanism by which the nucleoplasmic LAP2α and the NET LBR regulate the repositioning of the *Rex1* locus would reveal a functional connection between nucleosome-binding and lamin-binding proteins and suggest an additional link between the chromatin fibre and the nuclear lamin network.

Among the NETs that changed their phosphorylation status during exit, TMEM201 appeared to lose phosphorylation. This is interesting as TMEM201 is an alias for NET5/SAMP1, which is a muscle specific NET that induces exit from pluripotency when introduced ectopically in human iPSCs (Bergqvist et al. 2017). We attempted to study the importance of this NET in *Rex1* repositioning by generating phospho-null mutants but the high GC rich content in the sequence encoding the phospho-residues proved site directed mutagenesis tricky. However, both the presence and the change in phosphorylation status of TMEM201 during exit makes it an interesting NET to explore further for its role in genome organisation in ES cells. POM121 is another transmembrane nucleoporin that gains phosphorylation during exit. POM121 is an interesting nucleoporin as its soluble variant has been previously identified to interact with nucleoplasmic Nup98, a transcriptional regulator, at gene promoters to control transcription of its target genes (Franks et al. 2017).

Although its role in genome organisation has not been tested before, its potential role as a transcriptional regulator could imply its involvement in multi-protein complexes that function together in regulating gene position and expression. Thus, exploring the roles of these NETs in ES cells could shed further light into how genome organisation is maintained in the pluripotent and how these NETs might orchestrate the early changes in genome organisation and gene expression during exit.

6.3 Could the introduction of NETs enhance differentiation protocols?

Tissue specificity of the NE is key to establishing tissue specific genome organisation. With examples of muscle-, fat- and liver-specific NETs, studies from the Schirmer lab have shown how the presence of these NETs is crucial to genome organisation in these three systems (Korfali et al. 2010; Robson et al. 2016; 2017; Gatticchi et al. 2020). Further, tissue specific NETs are important for the terminal differentiation of lineage committed progenitor cells (Batrakou et al. 2015; Robson et al. 2016) and their absence in mice leads to metabolic imbalances(Gatticchi et al. 2020) and/or presents severe phenotypes resembling lipodystrophy (Czapiewski et al, unpublished data). These observations all insinuate crucial roles for NETs to ensure normal development. However, we lack studies presenting their importance in physiological development.

The finding that a protein might act as a NET only in certain tissues (Malik et al. 2010a) highlights that their expression in a certain tissue alone does not guarantee their role as a NET. Their localisation at the NE is equally important. However, when expressed exogenously in heterologous systems, several NETs not only localise at the NE but also alter genome organisation (Zuleger et al. 2013; de las Heras et al. 2017). This highlights the need for tissue restricted expression of these NETs as their mis-expression could lead to detrimental developmental phenotypes. But a direct study addressing the effects of their mis-expression has not been done before, except the one study from Hallberg group showing that the ectopic expression of muscle specific NET5/SAMP1 in human iPSCs leads to an exit from pluripotency (Bergqvist et al. 2017).

Ectopic expression of tissue specific NETs like TMEM120A, NET39 and TAPBPL in mouse ES cells leads to an exit from pluripotency characterised by the morphological flattening of colonies and a loss in alkaline phosphatase expression. This corroborates the proof of concept presented by the Hallberg group with SAMP1 ectopic expression. By generating inducible stable cell lines for the expression of NET39, TAPBPL and NET50,

we have generated valuable reagents that can be used to further study the effects of prolonged expression of these NETs on pluripotency.

While the loss of alkaline phosphatase is a good marker of exit from pluripotency, this study could have benefitted from a better characterisation of this exit. For instance, it would be interesting to induce the expression of NETs in ES cells and establish the time course for the downregulation of naïve transcription factors like Oct4, Nanog and Sox2 that maintain the pluripotent state. In the same conditions, it would be interesting to contrast the downregulation of naïve factors with the upregulation of differentiation markers. Specifically, using markers like Pax6 and Sox1 for ectoderm (Gajović et al. 1997, 6; Bylund et al. 2003, 1), Brachyury and Twist2 for mesoderm (Showell, Binder, and Conlon 2004; Barnes and Firulli 2009) and Gata4 and Gata6 for endoderm (Arceci et al. 1993, 4; Morrisey et al. 1996, 6) will allow a more careful tracing of lineage specification during differentiation.

Stem cells, especially iPSCs, have been touted as a novel autologous cell source from cell replacement therapy for several degenerative diseases. The fact that iPSCs can be generated from any individual and in theory, a massive range of clinically relevant phenotypic cells make them an interesting medical resource. Also, extracting patient cells for the generation of iPSCs involves none of the ethical or immune rejection concerns surrounding human ES cells. Thus, these cells are seen as an important resource for the so called "personalised medicine" approach in which each individual patient would receive a tailored treatment and this is gaining critical importance in medicine, pharmacology and toxicology to overcome possible adverse side effects. Of note among recent publications is the fact that on the first clinical trial with autologous iPSC-derived retinal epithelial cells, a patient with age-related macular degeneration showed long-term survival of transplanted cells for 25 months without immune suppression (Mandai et al. 2017). It is important to acknowledge that the use of iPSCs in regenerative medicine can only be guaranteed if we can successfully rule out their ability to cause a teratoma (Okita, Ichisaka, and Yamanaka 2007; Gunaseeli et al. 2010; Knoepfler 2012).

In vitro differentiation for the generation of lineage committed cells to study diseases is still limited in that we still do not have protocols to successfully differentiate ES cells into *every* cell type. Conventional differentiation protocols involve the generation of embryoid bodies followed by the introduction of culture conditions that stimulate directed

differentiation. Efforts over the years has led to the development of several differentiation protocols for each lineage commitment. For instance, there are over 6 defined culture conditions for the generation of mature astrocytes *in vitro* (Engel et al. 2016) and a major difference between the protocols is the maturation time from neural progenitor cells into astrocytes which ranges from 14 to a 100 days (Krencik et al. 2011; Shaltouki et al. 2013), depending on which protocol is being followed . Furthermore, most current differentiation protocols use growth factors aligned with reported in vivo development and there is limited reporting of the functional characteristics which prevents a reliable assessment of the maturity of the differentiated cells. Thus, improving current differentiation protocols with alternative strategies is crucial to enable the study of development and disease. The experiments presented here show withdrawal from pluripotency *in spite of maintaining serum/LIF in the media*, which is an interesting observation in itself. However, it then leads to the question of what might happen if the effects of LIF withdrawal were combined with NET expression. If the effects of NET expression combined with LIF withdrawal were synergistic, it would be interesting to explore if the introduction of tissue specific NETs could somehow enhance differentiation of ES cells into certain lineages. Perhaps expressing NET39 alone or with other muscle NETs like TMEM38A and WFS1 at specific points in a myogenic differentiation protocol could improve the efficiency with which differentiation occurs. Repeating some of these experiments in iPSCs and establishing the observed phenotypes in that model would be crucial. If iPSCs behave similar to ES cells, there would be the additional issue of heterogeneity in IPSC populations from different donors. Exploring the potential improvement in differentiation protocols with the introduction of NETs would enable us to better establish disease models in the lab.

Final Remarks

The work presented herein and recent advancements in the understanding of genome organisation in pluripotency and differentiation now allow me to propose the hypothesis that genome organisation changes are mediated at least in part, by stage-specific expression of NETs. While the ubiquitous NETs play a key role in maintaining peripheral chromatin organisation, it would seem that the tissue-specific NETs act on limited and specific genomic loci, thus fine tuning the configuration and promoting optimal gene expression in a given physiological state. Furthermore, the expression of these tissue-specific NETs themselves needs to be tightly controlled so that they are expressed at desired developmental stages and in the designated cells/tissues. A mis-regulation in the expression of NETs can lead to adverse effects on gene expression profiles and physiological development.

Changes in genome organisation in response to various stimuli have been studied in the past. While some changes occur over several hours or even days, other changes need to be more rapid. The rapid repositioning of the *Rex1* locus to the nuclear periphery within two hours of exit from pluripotency is surprising as the REX1 protein seems dispensable for maintaining pluripotency in mouse embryonic stem cells. Yet, somehow the relocation of this locus is dramatic and the Smith lab has shown that the loss of its promoter activity eventually marks an irreversible exit. This led to the hypothesis that there might be regulatory elements within this genomic locus that might be under positional regulation. The recent finding of the presence of a superenhancer within this genomic locus along with the putative enhancer found in our study present a strong case for further investigations into the role and importance of regulating the superenhancer activity in maintenance of pluripotency.

The potential role of ubiquitous proteins LBR and LAP2α in the repositioning of *Rex1* allude to a functional link between nucleoplasmic proteins and nuclear envelope transmembrane proteins in maintaining chromatin architecture. Several ubiquitous and tissue-specific NETs have been described before for their roles in establishing peripheral tethers for genomic loci. However, little is known about how these genomic loci are physically propelled to the nuclear periphery or maintained at specific 3D locations in the nuclear interior. For directed and rapid gene position changes, it can be envisioned that

there must be protein complexes that stabilise the locus in the nuclear interior and also those that steer it towards the nuclear periphery in a directed fashion. To date, we haven't been successful in testing this theory or identifying potential components of this machinery.

More generally, the work described herein adds to our current understanding of how cells exit pluripotency and characterises a fundamental and crucial event that occurs upstream of several other transcriptomic and proteomic changes that have been previously described to accompany lineage commitment. Furthermore, these observations present a strong case for the need to widen the study of spatial genome organisation beyond gene-centric questions. Regulatory elements are now being given their due for their roles in the regulation of gene expression. That their activity itself is a function of their steric accessibility is not inconceivable. By forming loops into the nuclear space, regulatory elements can become more accessible for their interactions with target genes and tethering them away at the NE is an effective way to make them less accessible. Therefore, the study of spatial genome organisation and the specific role of tissue-specific NETs in orchestrating the accessibility of regulatory elements in 3D space will have broad implications in our understanding of development and disease. Finally, establishing a functional link between nucleoplasmic and nuclear envelope proteins is crucial to the mechanistic understanding of how genome organisation changes are brought about. The lines of scientific enquiry presented here may prove to be a fundamental breakthrough in our understanding of pluripotency and the role of genome organisation in maintaining this state and in orchestrating the departure from this state.

Appendix

List of NETs detected in mass spectrometry of whole nuclear samples from E14tg2a cells subjected to 0 (T0) and 2h (T2) of LIF withdrawal. BLM.id refers to the parent tissues that the NETs were first identified in i.e. Blood (B), Liver (L) and Muscle (M) in previous studies in the Schirmer lab (Wilkie et al. 2011; Korfali et al. 2010; 2012).

Annotations			NETs	detection
UniProtKB.AC	GeneID	Gene.Name	BLM.id	presence
Q9D6J1	67260	Cers4	B	T0
E9Q236; E9Q467	239273	Abcc4	B	T0
Q3TWL2; E0CYE6; F8WHW3	219024	Pip4p1	B	T0
P60904; A2AUE1; G5E8T0	13002	Dnajc5	B	T0
Q9R0A0	56273	Pex14	B	T0
O35682	50918	Myadm	B	T0
Q8JZQ2; Q920A7	114896; 69597	Afg3l1; Afg3l2	B	T0
Q9WTN3; A0A0A0MQK3; F6XZS9	20787	Srebf1	L	T0
P03930	17706	Mtatp8	M	T0
E9PZQ0; A0A1L1SQG7; K3W4M2	20190	Ryr1	M	T0
O55022	53328	Pgrmc1	BL	T0
P19783	12857	Cox4i1	BM	T0
Q99L43; A2AMQ5; Q6PBC0	110911	Cds2	BLM	T0
Q80UU9	70804	Pgrmc2	BLM	T0
Q80UM7	57377	Mogs	BLM	T0
Q9D6U8	70186	Fam162a	B	T2
Q9CX30; A0A140LHN0; D3YY42; D3Z5F9	77254	Yif1b	B	T2
Q9EQ20	104776	Aldh6a1	B	T2
Q8R2Q8	69550	Bst2	B	T2
P35821	19246	Ptpn1	B	T2
Q9CWU2	243983	Zdhhc13	B	T2
Q9Z0X1; B1AU25	26926	Aifm1	B	T2
O08992; Q3TMX0	53378	Sdcbp	B	T2
O70472	56030	Tmem131	B	T2
Q8VE47	66663	Uba5	B	T2
Q9JHF5	27060	Tcirg1	B	T2
Q8CD26	270066	Slc35e1	B	T2
Q8K0C4	13121	Cyp51a1	B	T2
Q9QUJ7	50790	Acsl4	B	T2
Q5FWI3	83921	Cemip2	B	T2
A0A0A6YWX1	71472	Usp19	B	T2
P35762; A0A140LJL0	12520	Cd81	B	T2
P24668	17113	M6pr	B	T2

O55111	13511	Dsg2	L	T2
Q8R5M8; E0CY16; E9PYN1	54725	Cadm1	L	T2
Q99KK1; A0A1W2P8A8	28193	Reep3	L	T2
O54992; A0A0G2JDU2	17165	Mapkapk5; Gm4	L	T2
Q8BJM7	100929	Tyw1	L	T2
Q6JPI3; A0A0J9YUA8; E9QLJ3	76199	Med13l	L	T2
P57787	80879	Slc16a3	M	T2
Q6DYE8	209558	Enpp3	M	T2
P97449; A0A0U1RNS3	16790	Anpep	M	T2
O88738; S4R1L5; S4R2P8	12211	Birc6	BL	T2
P00405	17709	Mtco2	BL	T2
P37040	18984	Por	BL	T2
O35488; A2ANX6	26458	Slc27a2	BL	T2
Q9QYI4; Q8C4C9	56709	Dnajb12	BL	T2
O35704	268656	Sptlc1	BL	T2
Q8BXQ2; B7ZC19	78928	Pigt	BL	T2
Q8C0I1; A2AL49; A2AL50	228061	Agps	BL	T2
P55096; A0A0G2JDI9	19299	Abcd3	BL	T2
Q9CZW4; D3Z4I4	74205	Acsl3	BL	T2
P62342	69227	Selenot	LM	T2
P52875	21982	Tmem165	B	T2
Q8BHC4	68087	Dcakd	B	T2
Q9Z127	20539	Slc7a5	B	T2
P70227	16440	Itpr3	B	T2
P09055	16412	Itgb1	BM	T2
Q91VD9	227197	Ndufs1	BM	T2
Q69ZN7; A0A286YDF5; E9Q390	226101	Myof	BM	T2
P41216; D3Z041	14081	Acsl1	BLM	T2
Q91YQ5	103963	Rpn1	BLM	T2
Q9D0M3	66445	Cyc1	BLM	T2
O08547; E9Q6R3	20333	Sec22b	BLM	T2
P56395; G5E850	109672	Cyb5a	BLM	T2
O08579; I7HJS1	13726	Emd	BLM	T2
Q9D0F3	70361	Lman1	BLM	T2
O70252; D3YX62	15369	Hmox2	BLM	T2
Q8K273	236792	Mmgt1	BLM	T2
Q8BM55; D3Z6S1	68796	Tmem214	BLM	T2
Q9DC16	67458	Ergic1	BLM	T2
Q9CQX2	66427	Cyb5b	BLM	T2
Q8BJS4	223697	Sun2	BLM	T2
Q9WU40; D3YU56; E9QP59	380664	Lemd3	BLM	T2
Q99LR1	76192	Abhd12	BLM	T2
Q8VC65	106582	Nrm	BLM	T2

P70295; A0A0N4SVA6; Q3U3K9	11993	Aup1	BLM	T2
Q6PA06; E9QND8	56298	Atl2	BLM	T2
O35114	12492	Scarb2	BLM	T2
Q8VHE0	140740	Sec63	BLM	T2
Q3U7R1	23943	Esyt1	BLM	T2
P61620	53421	Sec61a1	BLM	T2
E9PYH6	233904	Setd1a	B	T0T2
Q921G6; H3BIX9; H3BLL3	231798	Lrch4	B	T0T2
Q07813; A0A1B0GS13; A0A1B0GT81; A0A1B0GTA4	12028	Bax	B	T0T2
Q9WVB0; T1ECW4	19663	Rbpms	L	T0T2
E9Q6J5	665775	Bod1l	L	T0T2
Q8R2U2	212547	Nepro	L	T0T2
Q922Q8	98238	Lrrc59	BLM	T0T2
A0A087WNW3; A0A087WP14; A0A087WP48; A0A0	16709	Ktn1	BLM	T0T2
Q80WJ7	67154	Mtdh	BLM	T0T2
O35465	14232	Fkbp8	BLM	T0T2
Q9DBG6	20014	Rpn2	BLM	T0T2
Q99P72	68585	Rtn4	BLM	T0T2
Q9WV55	30960	Vapa	BLM	T0T2
Q61033	21917	Tmpo	BLM	T0T2
Q8VCB1	72787	Ndc1	BLM	T0T2
Q8K3Z9	107939	Pom121	BLM	T0T2
A2A8U2	230917	Tmem201	BLM	T0T2
Q8BRG8	72649	Tmem209	BLM	T0T2
Q61029	21917	Tmpo	BLM	T0T2
Q6ZPR5	77626	Smpd4	BLM	T0T2
O55143	11938	Atp2a2	BLM	T0T2
Q9QY81; A0A0R4J1I6	54563	Nup210	BLM	T0T2
O54734	13200	Ddost	BLM	T0T2
Q9D0E1	76936	Hnrnpm	LM	T0T2
Q8R1B4	56347	Eif3c	LM	T0T2
Q9Z0H1	57315	Wdr46	LM	T0T2
Q3U9G9	98386	Lbr	BLM	T0T2
Q8VEM8; G5E902	18674	Slc25a3	BLM	T0T2
P35564	12330	Canx	BLM	T0T2
Q9D666	77053	Sun1	BLM	T0T2
Q9CQC7	100041273; 100	Ndufb4	BM	T0T2
Q8BMK4	216197	Ckap4	BM	T0T2
Q61543; F8WHM5	20340	Glg1	BM	T0T2
Q62351	22042	Tfrc	BM	T0T2
Q9DCW5	12861	Cox6a1	BL	T0T2
Q8VCM8; D3YU17	103425	Ncln	BL	T0T2
Q8VDN2	11928	Atp1a1	BL	T0T2
P14901	15368	Hmox1	BL	T0T2
Q99PL5; A2AVJ7	81910	Rrbp1	BL	T0T2
A2ALR9	170707	Usp48	BL	T0T2

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