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Nuclear Envelope Proteins Alter Chromosome Positioning With Corresponding Changes In Gene Expression During Differentiation

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Omnis enim res quae dando non deficit,
dum habetur et non datur,
nondum habetur quomodo habenda est.

Augustinus von Hippo

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INTRODUCTION

The nuclear envelope

The structure of the nuclear envelope

The nuclear envelope (NE) was confirmed to be a lipid bilayer by electron microscopy on amphibian oocytes over 50 years ago (Callan and Tomlin, 1950). It is composed of two concentric membranes, the outer nuclear membrane (ONM), which is continuous with the rough endoplasmic reticulum, and the inner nuclear membrane (INM). These two membranes are connected at the nuclear pore complexes (NPC) (see Figure 1A). Beneath the INM lies a polymer of intermediate filament lamins together with associated membrane proteins that collectively are termed the nuclear lamina.

The nuclear pore complex is a huge protein assembly consisting of multiple copies of approximately 30 nucleoporins (Rout et al., 2000) with an estimated overall mass of between 60 and 125 MDa (Cronshaw and Matunis, 2003; Reichelt et al., 1990). NPCs are the only site where nucleocytoplasmic transport takes place (Görlich and Kutay, 1999; Wente et al., 2000).

Being continuous with the rough endoplasmic reticulum the ONM shares a similar subset of proteins with it (Gerace and Burke, 1988; Newport and Forbes, 1987). However, recent studies demonstrate that the ONM has a unique set of proteins that function in connecting the nucleoskeleton with the cytoskeleton (Crisp et al., 2006; Starr and Han, 2002).

Similar to the outer nuclear membrane the inner nuclear membrane contains a specific set of integral proteins, nuclear envelope transmembrane proteins (NETs) and is anchored to the nuclear lamina through several NETs which bind lamin (Gruenbaum et al., 2003) (see Figure 1A). The luminal space between the INM and ONM is barely explored, but studies suggest that

some proteins reach across the lumen and contribute to its symmetric spacing (Crisp et al., 2006).

Lamins were the very first NE proteins to be identified and characterized over 30 years ago. As intermediate filament proteins their solubility characteristics and abundance enabled an easy enrichment (Aaronson and Blobel. 1975; Gerace et al., 1978). An average nucleus contains about 3 million lamin molecules (Gerace and Burke, 1988). There are different subtypes of lamins. B-type lamins are expressed and play an important role during development, A-type lamins on the other hand are expressed later in differentiated cells (Hutchison et al., 2002; Stuurman et al.,1998).

Recent studies have shown that the nuclear lamina does not only play a role in morphology and stability of the nucleus (Gruenbaum et al., 2003; Hoffmann et al., 2002; Liu et al., 2000; Schirmer et al., 2001) but also contributes to the regulation of essential processes such as transcription (Ellis et al., 1997; Moir et al., 2000; Nili et al., 2001; Spann et al., 1997), DNA replication (Kennedy et al., 2000; Martins et al., 2003), anchoring of the nucleus and its migration within the cell (Malone et al., 1999) and various signalling cascades (Markiewicz et al., 2006; Steen et al., 2000).

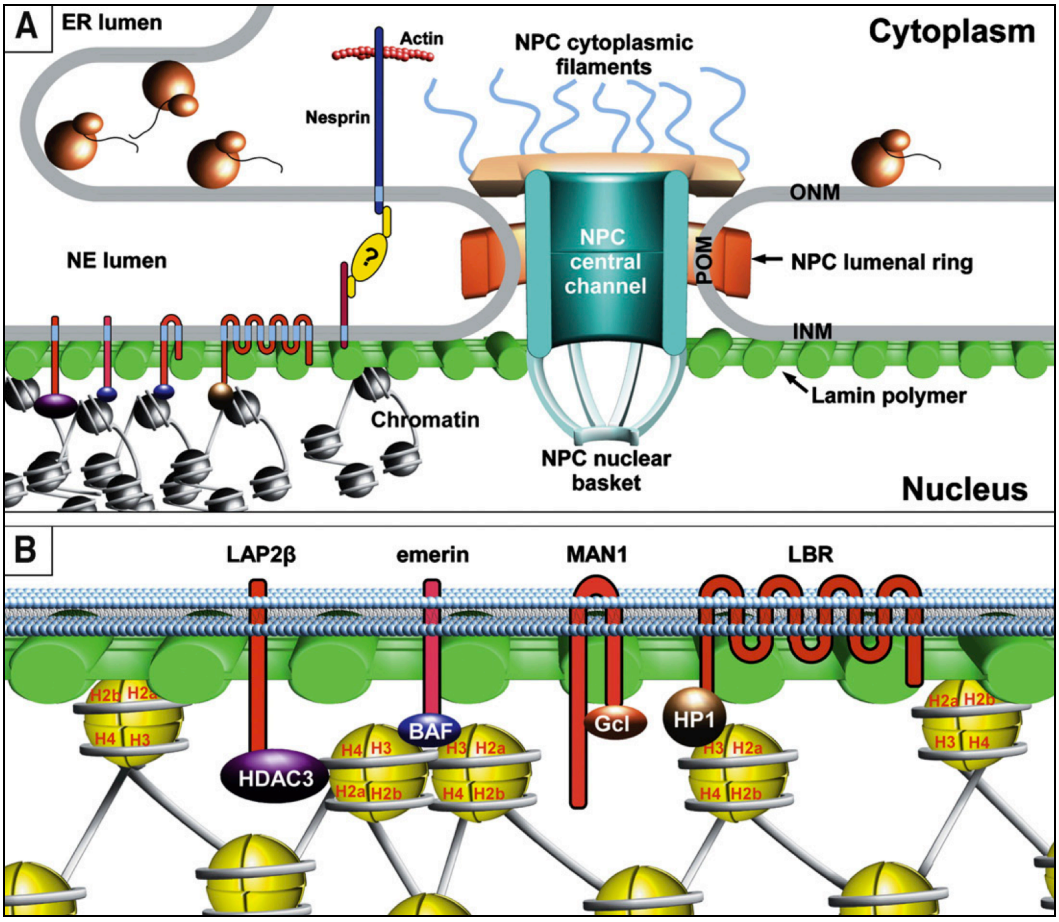

Figure 1: The nuclear envelope (Batrakou et al., 2009)
(A) The outer nuclear membrane (ONM) is continuous with the rough endoplasmic reticulum (ER) and connected with the inner nuclear membrane (INM) at the pore membrane. The INM contains many unique integral proteins, which are associated to the intermediate filament lamin polymer. The pore membrane apposed to the nuclear pore complexes (NPCs) contains specific integral proteins involved in membrane tethering of NPCs. (B) Many transmembrane proteins of the INM directly interact with the lamin polymer and/or chromatin proteins, though only a small number of the first identified proteins have been tested for such characteristics.

Nowadays it is well established that the NE is a dynamic structure. While some components are stably associated with proteins of the NE others are able to bind and dissociate quickly (Daigle et al., 2001; Griffis et al., 2003; Rabut et al., 2004). Some interactions between NE proteins and other proteins only occur in certain stages of the cell cycle. Nucleoporins, the nuclear pore complex proteins, interact with kinetochores and the spindle during mitosis via mitotic checkpoint proteins (Belgareh et al., 2001; Campbell et al., 2001; Iouk et al., 2002; Joseph et al., 2004; Loïodice et al., 2004; Salina et al., 2003).

Initial biochemical and traditional MS approaches identified roughly a dozen NETs, with lamin B receptor being the first to be described (Worman et al., 1988). The initial proteins were picked up in these studies due to their high abundance.

A more recent study using MudPIT resulted in a five-fold increase of the number of NETs (Schirmer et al., 2003). In this proteomics study 67 novel putative NETs were identified, using a subtractive approach in which proteins identified in isolated microsomal membranes (which contaminate NE preparations and contain most of the ER proteins but are free of inner and outer nuclear membrane) were subtracted from proteins identified in isolated NE fractions. Over 30 of these proteins have now been confirmed to be NETs and many are cell-type specific (Brachner et al., 2005; Chen et al., 2006; Malik et al., 2010; Wilhelmsen et al., 2005).

Many mutations in NE proteins result in pathologies (Worman and Bonne, 2007), yet little is known about the molecular mechanisms that lie beneath. Further characterization of the NE is highly important for the understanding of these diseases.

Interaction with chromatin

The dynamic nature of the NE is very obvious during cell division in metazoa. Disassembly of the membrane and the dispersal of nucleoporins, lamins and INM proteins are crucial for the progression of mitosis. According some models the NE membranes break down into vesicles different from the intact mitotic ER network, while others propose that the NE membranes and their integral proteins are absorbed into the ER during mitosis. NE assembly has been investigated for years and many studies use extracts from *Xenopus laevis* eggs as a model system (Vigers and Lohka, 1991). In *Xenopus*, two membrane fractions have been identified. One binds to chromatin, the other one associates with the chromatin-bound fraction (Sasagawa et al., 1999). It was further shown that two distinct vesicle types are necessary for the assembly of the NE. The recruitment of distinct vesicles to chromatin is an ordered one and NEP-B78 is involved in the earliest steps of the reassembly

in the *Xenopus* system and may be required for targeting the vesicles to the chromatin (Drummond et al., 1999). Another protein, which is a vertebrate homologue of MEL-28 (maternal effect lethal), a NE protein in *Caenorhabditis elegans*, interacts with the Nup107-160 complex, an important component of the NPC. MEL-28 is essential for the recruitment of the Nup107-160 complex to chromatin; this suggests that MEL-28 acts as a seeding point for NPC assembly (Franz et al., 2007).

Many other NE proteins have possible chromatin binding roles and might contribute to the rebuilding of the NE at the end of mitosis (Anderson et al., 2009; Ulbert et al., 2006). Chromatin proteins are known to bind to certain NE proteins (Mattout-Drubezki and Gruenbaum, 2003), including specific markers of silent chromatin (Ye and Worman, 1996), which could influence general association of chromatin at the nuclear periphery. So far several NE proteins have been shown to have a tendency to interact with heterochromatin (Brown et al., 2008; Capelson et al., 2010; Kalverda et al., 2010; Makatsori et al., 2004; Pickersgill et al., 2006) (see Figure 1B).

Specific chromosomes, chromosome regions and chromatin domains have a defined position in the nucleus. Gene rich regions tend to be in the nuclear interior whereas gene poor regions tend to accumulate at the nuclear periphery (Bolzer et al., 2005; Boyle et al., 2001; Croft et al., 1999; Guelen et al., 2008; Wiblin et al., 2005). Moreover, this genome organization seems to be both cell-type and tissue specific. In murine liver cells chromosome 5 tends to localize to the nuclear interior, in lung cells it localizes to the nuclear periphery (Parada et al., 2004).

Recent studies show that chromosomes can be relocated to the nuclear periphery by an affinity mechanism. In each of these studies a lac operator (LacO) was inserted into a chromosome locus that tended to be in the interior. Cells were then transfected with lac repressor (LacI) fused to a reporter and a NE protein. After passage through mitosis the LacO array and the bound chromosome was relocated to the nuclear periphery. These studies showed that a high affinity interaction between a NE protein and small region of a chromosome is sufficient to relocate and retain the whole chromosome the nuclear periphery (Finlan et al., 2008; Kumaran and Spector, 2008; Reddy et al., 2008).

Not many endogenous proteins have been shown to be able to relocate chromatin to the periphery. Ku and NPC proteins are able to direct telomeres to the periphery in yeast (Galy et al., 2000; Laroche et al., 1998; Scherthan et al., 2000): the NET SUN2 plays an important role in the localization of telomeres at the nuclear periphery in mammalian cells (Schmitt et al., 2007). The confinement of certain chromosomes at the nuclear periphery is influenced by Lamin B1 (Malhas et al., 2007); interactions cannot explain a tissue specific chromosome distribution.

In a study in the Schirmer lab 22 of the previously mentioned novel identified NETs were transfected into two cell lines (Chubb et al., 2002) containing LacO array insertions in different human chromosomes. The position of the array was then measured with respect to the NE as an indicator of chromosome repositioning. These NETs were not fused to the lac repressor; therefore the repositioning of the chromosomes in the array was due to effects of the NET overexpression only. The study showed chromosome specific effects of certain NETs. Four NETs substantially altered chromosome positioning in one cell line, yet only two of them showed the same effects in the second cell line. The NETs with effects are upregulated in liver and not expressed in kidney. Correspondingly, chromosome 5 was shown to be more peripheral in liver cells than in kidney cells and depletion of the liver NETs in HepG2 (liver derived) cells leads to less peripheral localization of chromosome 5. This suggests that the tissue-specific chromosome distribution is influenced by the expression of specific NETs (N. Zuleger, S. Boyle, D. A. Kelly, J. de las Heras, D. G. Batrakou, V. Lazou, G. R. Otti, D. J. Harrison, W. A. Bickmore and E. C. Schirmer, in revision).

Microarray expression analysis of these transfected cells suggests that the specific chromosome distribution influences both positive and negative gene regulation. Particularly, various developmental pathways are involved with many of the down-regulated genes, which suggests that the NE may be able to repress developmental pathways by influencing chromosome organization (N. Zuleger, S. Boyle, D. A. Kelly, J. de las Heras, D. G. Batrakou, V. Lazou, G. R. Otti, D. J. Harrison, W. A. Bickmore and E. C. Schirmer, in revision).

Adipogenesis

Adipose tissue

In the last 20 years obesity has become a worldwide problem. Changes towards a more sedentary lifestyle and more high calorie food have lead to increasing health problems associated with being overweight. In the United States about 65% of the population are classified as overweight and 30% as obese (Mokdad et al., 2001). Adipocytes store energy in the form of triacylglycerol when there is more input of energy than expenditure and break down the lipid to free fatty acids when energy is needed. Adipocytes also secrete paracrine and endocrine hormones and play a role in the control of metabolism.

There are two types of mammalian adipocytes, white and brown adipocytes. Brown adipocytes store less lipid, have more mitochondria and are able to dissipate energy as heat without the generation of ATP. They express almost all genes that are expressed in white adipocytes but also some distinct genes and brown adipogenesis is in general similar to white adipogenesis. In infancy, humans have large amounts of brown adipose tissue, but only small amounts persist in adults.

The adipose tissue, together with muscle and bone, develop from the mesenchymal stem cells derived from the mesodermal layer of the embryo. Multipotent precursor cells in the vascular stroma of adipose tissue become restricted to the adipocyte lineage without actually expressing any markers of terminal expression. Multiple transcription factors are activated and the cells differentiate. This process is initiated by factors secreted by cells in the vascular stroma and/or adipocytes undergoing hypertrophy.

The 3T3-L1 cell line is a well established murine preadipocyte cell line that has already been committed to the adipocyte lineage. Pre-adipocytes can morphologically not be distinguished from their precursor cells, but can easily be induced to terminally differentiate to adipocytes and have not been shown to differentiate into any other cell types. 3T3-L1 cells offer a homogeneous cell population and a stable pre-differentiated state. The 3T3-L1 cells are a

sublineage of the murine fibroblast 3T3 cells and were selected for their ability to accumulate cytoplasmic triacylglycerol while in a resting state (Green and Kehinde, 1974; Green and Kehinde, 1975; Green and Kehinde, 1976). 3T3-L1 cells undergo one round of cell division prior to differentiation. Whether this mitotic clonal expansion is required for differentiation is not entirely clear.

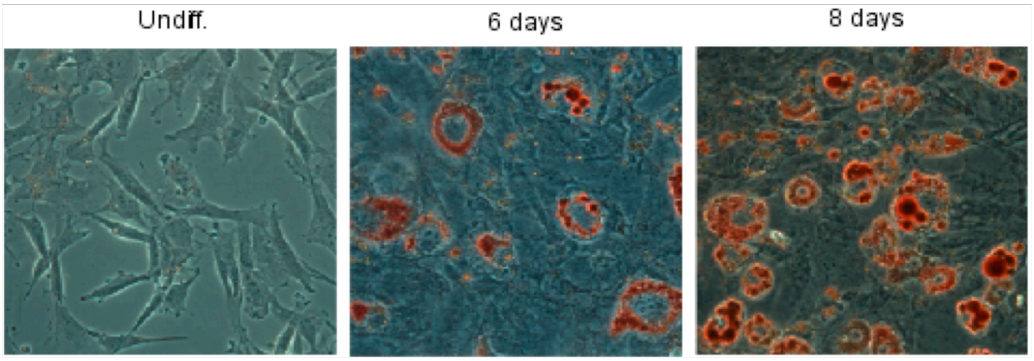

Figure 2: Differentiation of 3T3-L1 cells
3T3-L1 cells accumulate lipid droplets over the cause of differentiation. Lipid droplets were stained with Oil red.

The adipogenic transcriptional cascade

The transcriptional networks in adipogenesis are highly regulated and involve many different factors, of which a part is still unknown.

Different members of the C/EBP (CCAAT enhancer binding protein) family are temporally expressed during adipocyte differentiation. The early expression of C/EBPβ and C/EBPδ leads to induction of C/EBPα. C/EBPα is directly involved in the induction of many adipocyte genes and *in vivo* studies suggest an important role in the development of adipose tissue as *C/ebpa*$^{-/-}$ mice are almost completely lacking white-adipose tissue (Chen et al., 2000). Despite the important role of C/EBPs in adipocyte differentiation these factors are not able to function without PPARγ, peroxisome proliferator-activated receptor γ, which is indicated by different studies (Rosen et al., 2002; Zuo et al., 2006).

PPARγ is both necessary and sufficient for adipogenesis. It belongs to the nuclear-receptor superfamily and is able to induce adipocyte differentiation in

fibroblasts if overexpressed (Tontonoz et al., 1994). Until now no factors have been identified which are able to promote adipogenesis in the absence of PPARγ. Also, the function of the most pro-adipogenic factors seems to be at least partially to activate PPARγ expression. There are two protein-isoforms, PPARγ1 and PPARγ2, which are produced by alternative splicing and promoter usage. Both isoforms are induced during adipogenesis and PPARγ1 is also expressed in other cell types. The exact roles of both isoforms remain to be elucidated as different studies show contradicting results (Mueller et al., 2002; Ren et al., 2002). Nevertheless PPARγ2 appears not to be absolutely required for adipogenesis (Medina-Gomez et al., 2005; Zhang et al., 2004).

So far no endogenous PPARγ ligand has been identified. In 3T3-L1 cells cyclic AMP (cAMP) dependent ligand activity was shown but decreased after the first two days of differentiation (Tzameli et al., 2004). This suggests that ligand binding may be required to induce adipogenesis but is not necessary to maintain the differentiated state. PPARγ is crucial to maintain adipocytes differentiated. Dominant-negative PPARγ was introduced into differentiated 3T3-L1 adipocytes and caused de-differentiation with corresponding loss of lipid accumulations and reduced expression of adipogenic markers (Tamori et al., 2002).

Further, Krüppel-like factors (KLFs), a family of C2H2 zinc finger proteins, are produced in adipose tissue with varying expression patterns throughout differentiation. KLF15 was the first family member to be shown to promote adipocyte differentiation (Mori et al., 2005) and to induce expression of GLUT4, an insulin sensitive glucose transporter (Gray et al., 2002). KLF5, another member of this family, is induced by C/EBPβ and C/EBPδ and binds and activates the *Pparg2* promoter (Oishi et al., 2005). However, KLF2 and KLF7 are shown to function anti-adipogenic by repressing the *Pparg2* promoter (Banerjee et al., 2003; Kanazawa et al., 2005; Wu et al., 2005).

SREBP1c induces PPARγ and also apparently an as yet unknown PPARγ ligand (Kim et al., 1998; Kim and Spiegelman, 1996). It also influences the induction of lipid biosynthesis by insulin in adipocytes (Kim et al., 1998).

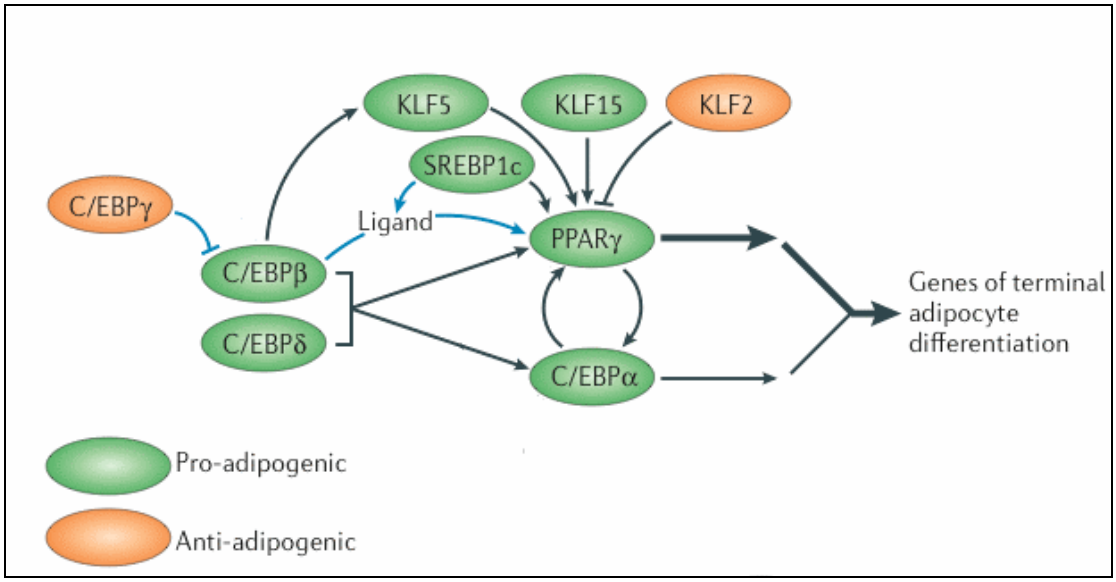

Figure 3: The transcriptional cascade regulating adipogenesis (adapted from Rosen and MacDougald, 2006)
PPARγ is the master regulator of adipogenesis and lies at the core of the signalling cascade. Its expression is regulated by pro- and anti-adipogenic factors and PPARγ is activated by a yet unknown ligand.

The role of PPARγ, C/EBPs and KLFs in adipogenesis is now well defined, but there are over a hundred other factors expressed in adipocytes which all have a role in the differentiation process. Many transcription factors repress adipogenesis by promoting alternative cell fates and are downregulated in adipocytes, which suggests that repression of these genes is one of the main functions of pro-adipogenic factors. The tight regulation of both positive and negative gene expression is crucial to effective adipogenesis and it is very likely that there are other mechanisms in place to regulate it even more detailed. While lots is known about the adipogenic transcription cascade, there remains much unknown about the detailed regulation of these cascades. Constantly more factors are found that participate in this process, which indicates that there are clearly more processes in adipogenesis still to be described.

Signalling cascades in adipogenesis

Different extracellular and intracellular factors activate different signalling pathways that in their turn activate downstream transcription factors that induce the expression of genes responsible for adipogenesis.

A highly conserved pathway that inhibits adipogenesis is the hedgehog pathway that regulates gene expression by a complex signalling pathway through members of the GLI family (Hooper and Scott, 2005). Addition of sonic hedgehog or its activated receptor prevents 3T3-L1 cells from differentiating whereas a dominant-negative GLI promotes adipogenesis (Spinella-Jaegle et al., 2001; Suh et al., 2006; Zehentner et al., 2000).

Insulin has noticeable effects on adipogenesis. In the early stages it functions through insulin growth factor-1 (IGF1) receptor signalling, as pre-adipocytes express many more IGF1 receptors than insulin receptors. This ratio changes as differentiation progresses (Smith et al., 1988). The downstream components of insulin/IGF1 signalling are also important for adipogenesis, which is inhibited by the loss of insulin-receptor substrate (IRS) proteins (Laustsen et al., 2002). Insulin signals are transmitted to the adipogenic cascade over various points of intersections (Klemm et al., 2001; Nakae et al., 2008; Tseng et al., 2005; Wolfrum et al., 2003), although more are very likely to exist.

Recent studies also indicate a positive regulation of adipogenesis by fibroblast growth factors (FGFs). FGF2 can induce development of adipose tissue (Kawaguchi et al., 1998) and FGF1 has pro-adipogenic activity on pre-adipocytes. Neutralization of FGF1 in 3T3-L1 cells reduces their ability to differentiate (Hutley et al., 2004).

The Wnt signalling pathway is highly conserved and functions through secreted glycoproteins to influence cell fate and development. Wnt signalling was shown to inhibit adipocyte differentiation by blocking the expression of PPARγ and C/EBPα (Bennett et al., 2002; Moldes et al., 2003; Ross et al., 2002; Ross et al., 2000).

Regulation of adipogenesis is also influenced by members of the TGFβ (transforming growth factor β) superfamily, which bind to serine/threonine kinases and function through SMAD-dependent and SMAD-independent

mechanisms. Adipocytes express TGFβ and its signalling components, on the other hand TGFβ inhibits differentiation of pre-adipocytes *in vitro* whereas inhibiting endogenous TGFβ signalling increases adipogenesis (Choy and Derynck, 2003; Choy et al., 2000; Rahimi et al., 1998) and overexpression of TGFβ inhibits the development of adipose tissue (Clouthier et al., 1997). Recent studies show that proteins of the INM affect SMAD signalling. The NET MAN1 binds to receptor-regulated SMADs and reduces signalling by TGFβ, activin and bone morphogenic protein. The phosphorylation of SMADs also seem to be regulated by Lamin A and C (Pan et al., 2005; Van Berlo et al., 2005). This shows that proteins within and associated with the inner nuclear membrane influence signal transduction pathways regulating adipogenic differentiation.

It was shown that the TGFβ and Wnt signalling cascades intersect with NE pathways, as emerin is able to regulate the activity of β-catenin by restricting its accumulation in the nucleus (Markiewicz et al., 2006)

Adipogenesis is influenced by almost every important signalling pathway, some pathways exercise both positive and negative regulation. These pathways meet in a tightly regulated and very complex cascade of transcriptional events that is only now better explored. Despite extensive research there are many unresolved issues due to the large set of proteins involved and the high complexity of different regulatory cascades.

The nuclear envelope and disease

Mutations of NE proteins have been shown to be responsible for at least 15 heritable human diseases - the nuclear envelopathies. Several mutations of Emerin, a protein in the INM, are responsible for X-linked Emery Dreifuss muscular dystrophy (Bione et al., 1994). A range of diseases with distinct tissue-specific pathologies is caused by mutations in NE proteins. These include lipodystrophies (Cao and Hegele, 2000; Shackleton et al., 2000),

neuropathy (De Sandre-Giovannoli et al., 2002), dermopathy (Navarro et al., 2004), dystonia (Naismith et al., 2004; Ozelius et al., 1997) and premature aging syndromes (Chen et al., 2003; De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003).

Disease	Synonym	Mutated NE Protein	Inheritance	Phenotype
Emery-Dreifuss muscular dystrophy, type 1/2/3	EDMD1 EDMD2 EDMD3	Emerin Lamin A/C Lamin A/C	X-linked AD AR	Early contractures (stiff/fixed joints) of elbows, Achilles tendon, neck and spine. Progressive weakness in upper arms and lower limbs. Dilated cardiomyopathy with conduction abnormalities.
Limb girdle muscular dystrophy, type 1B	LGMD1B	Lamin A/C	AD	Progressive weakness of shoulder, upper arm, hip and upper leg muscles with later development of dilated cardiomyopathy with conduction abnormalities.
Dilated cardiomyopathy with conduction defect	CMD1A	Lamin A/C Lamina-Associated Polypeptide 2a	AD	Ventricular dilation and impaired cystolic function. Sudden death due to cardiac pump failure may occur after conduction abnormalities. No skeletal muscles affected.
Dunnigan-type familial partial lipodystrophy	FPLD2	Lamin A/C	AD	Loss of subcutaneous fat from limbs and trunk with simultaneous accumulation in face and neck. Insulin resistance and diabetes mellitus.
Seip syndrome	BSCL2	Lamin A/C	Unknown	Adipose tissue absent from early infancy. Hypertriglyceridemia, hyperglycemia, diabetes mellitus, mild mental retardation, cardiomyopathy and dark, rough skin patches.
Lipoatrophy with diabetes, hepatic steatosis, hypertrophic cardiomyopathy and leukomelanodermic papules	LDHCP	Lamin A/C	Unknown	Generalised lipoatrophy with acute accumulation of fat in liver. Accompanied by skin pigment abnormalities and cardiomyopathy.
Mandibuloacral dysplasia, type A/B	MADA MADB	Lamin A/C ZMPSTE24	AD AR	Postnatal growth retardation, craniofacial anomalies (especially crowding or loss of teeth), skeletal malformations, mottled skin pigmentation, stiff joints and autoimmume hair loss. Partial lipodystrophy, insulin resistance and diabetes.
Restrictive dermopathy	RD	Lamin A/C ZMPSTE24	AR	Rigid and translucent skin, joint contractures and pulmonary hypoplasia. Impaired fetal body movements lead to deformity. Early neonatal death due to respiratory insufficiency.

Disease	Synonym	Mutated NE Protein	Inheritance	Phenotype
Charcot-Marie-Tooth disorder, type 2B1	CMT2B1	Lamin A/C	AR	Progressive deterioration of motor and sensory nerves leading to atrophy of limb muscles and numbness/sensory problems. Nerve conduction velocities not affected.
Pelger-Huet anomaly	PHA	Lamin B receptor	AD	Neutrophil nuclei in heterozygotes have fewer segments and course chromatin, with no effects on normal health. Homozygotes are also prone to epilepsy and skeletal abnormalities, *e.g.* polydactyly and metacarpal shortening.
Greenberg / HEM skeletal dysplasia	GSD/ HEM	Lamin B receptor	AR	Widespread tissue edema in fetus. Disorganised bone structrure, short limbs and conversion of cartilage to bone. Early *in utero* lethality.
Buschke-Ollendorff syndrome	BOS	MAN1 / LEMD3	AD	Skeletal defects include multiple spots of increased bone density (osteopoikilosis) and bands of sclerosis in a flowing pattern (melorheostosis). Sometimes accompanied by joint contractures, skin lesions, muscle atrophy, hemangiomas, and lymphedema.
Hutchison-Gilford progeria syndrome	HGPS	Lamin A/C	*De novo* / AD	Childhood onset of premature ageing including growth retardation, baldness, facial hypoplasia, delayed tooth formation, aged skin, osteoporosis, atherosclerosis, arthritis. Teenage mortality due to cardiovascular disease.
Atypical Werner syndrome	AWS	Lamin A/C	AD	Adult onset of premature ageing. Hard, tight skin, cataracts, subcutaneous calcification, premature arterioslcerosis, diabetes mellitus, premature ageing of face.
Torsion dystonia	DYT1	TorsinA	AD, 30-40% penetrance	Prolonged, involuntary muscle contractions induce abnormal posture and twisting or repetitive movements in arms and legs. Caused by CNS dysfunction rather than neurodegeneration.

Table 1: Inherited diseases associated with the nuclear envelope (adapted from Wilkie and Schirmer, 2006)
The disease is caused by mutations in the protein shown, the mutations are associated with the disease. AD: autosomal dominant, AR: autosomal recessive, CNS: central nervous system, HEM: hydrops-ectopic calcification-moth-eaten

Specifically familial partial lipodystrophy of the Dunnigan type (FPLD) is characterized by a lack of adipose tissue in the limbs, buttocks and trunk with fat accumulation in the neck and face. It is caused by heterozygous missense mutation of *LMNA* (Shackleton et al., 2000) possibly by disrupting the differentiation of adipocytes. This is supported by the interaction between the sterol response element binding protein 1 (SREBP1), an adipocyte differentiation factor and lamin A, which is noticeably reduced by FPLD mutations (Lloyd et al., 2002). It was also shown that overexpression of lamin A is an inhibitor of differentiation in 3T3-L1 cells by inhibiting the expression of PPARγ and GLUT4. Murine embryonic fibroblasts derived from lamin A knockout mice accumulate more lipids and synthesize more triglycerides compared to wild-type fibroblasts (Boguslavsky et al., 2006).

Several NETs interact with lamins and may even form complexes with lamins and SREBP. Therefore NETs that are preferentially expressed in adipocytes may also have a role in adipocyte differentiation by interfacing with differentiation factors in a similar way either directly or through a complex with lamin A or other proteins.

MATERIALS AND METHODS

Standard chemicals, if not explicitly mentioned, were purchased from Roche, Sigma Aldrich and Merck. Buffer formulations are listed below followed by bacterial strains, cell lines, plasmids, media, DNA-standards, enzymes and kits listed in tables.

Buffers and Solutions

Bacterial RbCl transformation buffer I pH 5.8
30 mM KAc
100 mM $RbCl_2$
10 mM $CaCl_2$
50 mM $MnCl_2$
15% Glycerol

Bacterial RbCl transformation buffer II pH 6.5
10 mM MOPS
75 mM $CaCl_2$
10 mM $RbCl_2$
15% Glycerol

10x SDS - PAGE running buffer
250 mM Tris base pH 8.8
1.9 M Glycine
1% SDS

Western blot transfer buffer
1x Running buffer
20% MeOH

1x TAE DNA running buffer
40 mM Tris-Acetate
1 mM EDTA pH 8.5

5x Protein sample buffer
125 mM Tris HCl pH 6.8
900 mM Glycine
1 mM EDTA
6% SDS
6 M Urea
10% β-Mercaptoethanol
0.01% Bromphenol Blue

Western blot blocking buffer
1x PBS
0.1% Tween20
5% non fat milk powder

10x Xylene Cyanol/Bromophenol Blue DNA loading buffer
Dissolve in 6.25 ml of H_2O
0.025 g Xylene cyanol
0.025 g Bromophenol Blue
1.25 ml 10% SDS
12.5 ml Glycerol

DNA Plasmid prep resuspension buffer
50 mM TrisCl pH 8.0
10 mM EDTA
100 µg/ml RNase A

DNA Plasmid prep lysis buffer

0.2 M NaOH

1% SDS

DNA Plasmid prep neutralization buffer

3M KaAc pH 6.5

20x Saline Sodium Citrate buffer pH 7.0

3 M NaCl

300 mM $Na_3C_6H_5O_7$

Luria Broth medium

1 % Tryptone

0.5 % Yeast Extract

0.5 % NaCl

Separating gel 12% (5 ml)

1.6 ml	ddH_2O
2.0 ml	30% Acrylamide/Bis Acrylamide Stock (Severn Biotech Ltd)
1.3 ml	1.5 M TRIS pH 8.8
0.05 ml	10% SDS
0.05 ml	10% APS
2 µl	TEMED

Stacking gel (1 ml)

0.55 ml	ddH_2O
0.17 ml	30% Acrylamide/Bis Acrylamide Stock (Severn Biotech Ltd)
0.26 ml	0.5 M Tris pH 6.8
0.01 ml	10% SDS
0.01 ml	10% APS
1 µl	TEMED

Cloning of Plasmid DNA

Bacterial strains used for cloning

Strain	Escherichia coli DH5α
Features	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17

Cell lines

Cell line	3T3-L1
Origin	Murine preadipose cell line with fibroblast morphology

Plasmids used for overexpression and cloning

Plasmid	**Features**
Net29-pEGFP-N2	KanR; GFP C-terminal human Net29
Net33-pEGFP-N2	KanR; GFP C-terminal human Net33
Emerin-pEGFP-N2	KanR; GFP C-terminal human Emerin
pEGFP-N2	KanR; GFP C-terminal GFP
p-CMV Tag 2A	KanR; FLAG Tag N-terminal
pSC-B amp/kan	AmpR; KanR;

Protein and DNA standards

Standard	**Provider**
PageRuler™ Prestained Protein Ladder	Fermentas
1 Kb^+ DNA Ladder	Invitrogen

Enzymes

Enzyme	Concentration	Provider
BamHI	10 U/µl	Fermentas
EcoRI	10 U/µl	Fermentas
Phusion Polymerase	2 U/µl	Finnzymes
T4 DNA Ligase	400 U/µl	New England Biolabs
Klenow Polymerase	4 U/µl	New England Biolabs
Ribonuclease A	10 U/µl	Sigma
Desoxyribonuclease I	10 U/µl	Sigma
ThermoScript™ Reverse Transcriptase	15 U/µl	Invitrogen

Kits

Kit	Provider
Zyppy™ Plasmid Miniprep Kit	Zymo Research
Zymoclean™ Gel DNA Recovery Kit	Zymo Research
BAC Miniprep Kit	Biomiga
Cell Line Nucleofector® Kit V	Lonza

Preparation of competent cells

DH5α were inoculated into 5 ml LB medium over night. The culture was diluted into 1000 ml LB medium and incubated shaking at 37°C until it reached OD_{600} 0.5. The culture was then transferred into two 500 ml centrifuge bottles and chilled on ice for 10 min. Cells were pelleted for 5 min by centrifugation at 4°C at 5000 rpm. After the supernatant was decanted cells were resuspended in 200 ml of ice cold bacterial RbCl transformation

buffer I per tube and incubated on ice for 5 min. Cells were pelleted at 3000 rpm for 10 min and the supernatant was decanted. The pellet was resuspended in 16 ml of bacterial RbCl transformation buffer II and incubated on ice for 15 min. 50 µl was aliquoted to each pre-cooled 1.5 ml microfuge tube using repeat pipetter and immediately frozen on dry ice. The competent cells where then stored at -80°C.

Bacterial transformation

Competent DH5α were thawed on ice. Plasmid DNA was added, mixed gently and incubated on ice for 30 min. Cells were heat shocked at 42°C for 45 sec. 0.5 ml LB medium was added and cells were then incubated shaking at 37°C for 1 hr. Cells were then centrifuged for 3 min at 3000 rpm at RT. Supernatant was decanted and cells were resuspended in the remaining supernatant. Cells were then plated on a pre-warmed LB plate with antibiotics corresponding to plasmid selection markers.

DNA plasmid purification

To obtain DNA for cloning, 1.5 ml of bacterial culture were transferred to an Eppendorf tube and segmented at 14000 rpm for 1 min at RT. Supernatant was decanted and the pellet was resuspended in 100 µl of resuspension buffer (50 mM TrisCl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). 100 µl of lysis buffer 0.2 M NaOH, 1% SDS) were added and tubes were inverted. After adding 200 µl of neutralization buffer (3 M KOAc pH 6.5) cells were pelleted at 14000 rpm for 10 min at RT. The supernatant was taken off carefully and transferred to a new Eppendorf tube where 600 µl of isopropanol were added. The DNA was precipitated by centrifugation at RT at 14000 rpm for 10 min. Supernatant was decanted and 500 µl of 70% ethanol were added and centrifuged at 14000 rpm for 1 min. The ethanol was decanted carefully and the DNA was allowed to dry at RT. The DNA pellet was then resuspended in 20 µl of resuspension buffer.

Plasmid DNA for transfection was purified with Zymo Research Zyppy™ Plasmid Miniprep Kit according to manufacturer's guidelines.

The DNA concentration was determined using the NanoDrop photometer according to the manufacturer's manual.

Restriction enzyme digests

Restriction was set up with Fermentas Fast Digest Enzymes for 2 hrs at 37°C.

Reaction setup according to manufacturer's guidelines:

2 µl	DNA (500 ng/µl)
1.5 µl	10x Fast Digest Buffer
0.5 µl	Enzyme
11 µl	ddH_2O

Ligation

Inserts digested with restriction enzymes were integrated into the appropriate vectors. The reaction resulted in a closed circular plasmid with the respective insert and was catalysed by the T4 DNA ligase. The ligations were incubated over night at 16°C.

Reaction setup according to manufacturer's guidelines:

1 µl	10x T4 Ligase Buffer
1 µl	Vector (150 ng/µl)
7.5 µl	Insert (50 - 100 ng/µl)
0.5 µl	T4 Ligase

Site-directed mutagenesis

Site-directed mutagenesis was performed using Phusion® Site-Directed Mutagenesis Kit from NEB following manufacturers protocol, except some modifications. Non-phosphorylated primers (Table 2) were used in PCR reaction with Phusion® High-Fidelity DNA Polymerase (F-530S, NEB), PCR product was gel purified using ZymocleanTM Gel DNA Recovery Kit according to manufacturers protocol, followed by simultaneous phosphorylation and ligation by T4 Polynucleotide Kinase (EK0031, Fermentas) and T4 DNA Ligase (M0202S, NEB) in T4 DNA Ligase buffer at room temperature for 2 hours. Prepared in house chemically competent DH5α cells were transformed with resulting ligation mix. Selected clones were verified by Sanger dideoxy sequencing.

Mutant	Mutagenic primers (5'-3')
Phospho-mimetic mutant	F: CGAGGAGGAGAAGTTCAAGCTCTACCTCAC R: TCCTTCTCGGCAAACTTAGCCTGCTTGC
Phospho-null mutant	F: CGAGTTCGAGAAGTTCAAGCTCTACCTCAC R: TCCTTGAAGGCAAACTTAGCCTGCTTGC

Table 2: Mutagenic primers (mutations are in red)

Molecular Biology Methods

Polymerase chain reaction

To amplify DNA for cloning and to check BAC clones PCR was performed. Annealing temperatures and cycles were modified according to the primers. Reaction setup according to manufacturer's guidelines:

13.6 µl ddH_2O
4 µl 5x Phusion GC Buffer
1 µl DNA
0.2 µl dNTPs
1 µl Primer Mix (10 µM)
0.2 µl Phusion Polymerase

Standard PCR Program

Temperature (°C)	Hold	Cycles
Initial denaturation		
98	30 sec	1
Amplification		
98	7 sec	5
Variable	10 sec	
7	15 sec/kb	
Amplification		
98	7 sec	25
Variable	15 sec/kb	
72	1 min	1x

Table 3: Standard PCR Program

Target Gene	Primers (5'-3')
ADIPOQ	F: GAGTGGAACAAGCAGAGGAAC R: AGTAACGTCATCATCTTCGGCATG
SLC2A 4	F: GTAACTTCATTGTCGGCATGG R: TGCTCTAAAAGGGAAGGTGTC
LEP	F: GTGCCTATCCAGAAAGTCCAG R: GACCTGTTGATAGACTGCCAG
PPARG	F: GCTGCTGGAGAGGCTGGAGAG R: CCGAAGCCTCACGGCAGAGG

Table 4: PCR Primers

Agarose gel

All DNA samples were analyzed by 1% or 2% agarose gels. DNA was stained with ethidium bromide and visualized by UV light.

Agarose gel purification of DNA fragments

DNA fragments of interest were excised and DNA was extracted according to manufacturer's guidelines using Zymo Research Zymoclean™ Gel DNA Recovery Kit.

Sequencing

The Sequencing Reaction was set up according to manufacturer's guidelines:

2.5 µl BigDye® Terminator v3.1 Cycle Sequencing RR-100 (AB)

2 µl BigDye® Terminator v3.1 5x buffer (Applied Biosystems)

0.5 µl Sequencing Primer

4 µl DNA (0.5 µg/µl)

1 µl ddH_2O

Temperature (°C)	Hold	Cycles
Pre-incubation		
96	2 min	1
Sequencing		
96	30 sec	28
50	20 sec	
60	3.5 min	

Table 5: Sequencing Program

The resulting DNA was analyzed by the SBSSS (School of Biological Sciences Sequencing Service, King's Buildings, Edinburgh) and sequences were analyzed by GENtle.

RNA isolation

Plates were washed with 1x PBS and 1 ml of TRIzol® (Invitrogen) was added directly to the plate. 1 ml of TRIzol® was transferred to a 1.5 ml Eppendorf tube and incubated at RT for 5 min. After the addition of 200 µl chloroform tubes were shaken vigorously by hand for 1 min and incubated at RT for 10 min. Phases were separated by subjecting tubes to centrifugation at 12 000 rpm for 15 min at 4°C. 200 – 300 µl of the upper aqueous phase were transferred into a new tube and mixed with 1 ml isopropanol. RNA was precipitated by centrifugation at 12 000 rpm at 4°C for 10 min. The pellet was washed with 0.5 ml 70% ethanol and centrifuged for 7500 rpm for 5 min at 4°C. After air-drying the pellet the RNA was resuspended in 20 µl AccuGENE® Molecular Biology Grade Water. RNA was incubated at 60°C for 10 min to dissolve, measured with NanoDrop and then stored at -80°C.

cDNA synthesis

5 µg of RNA were used for cDNA synthesis. 1 µl of the reverse primers mix (7.5 µM each) and 2 µl of 5x Buffer were mixed with the RNA and incubated for 5 min at 70°C for denaturation. After cooling down on ice 1 µl RNAsin, 0.25 µl 0.1 M DTT, 0.25 µl 25 mM dNTPs and 0.5 µl Thermoscript Reverse Transcriptase were added. The mix was incubated at 51°C for 2 h and then treated with 1 µg RNase A for 1 h at RT. 90 µl of ddH_2O was added and transferred to a 1.5 ml Eppendorf tube where another 400 µl ddH_2O were added. cDNA was then stored at -80°C.

Gene	Primers (5'-3')
TMEM53	F: AAGAACAGACAAGGTGGGAAG R: FACGCGAAGTGAAGGGATG
TMEM120a	F: GAAAACCAGATGAAAGAGCGC R: GTGAAGGAGATGACGATGAGG
SCARA 5	F: AGCTTCAAGGGACTTTCTGG R: TCAAGATGGAGCCGTTGTC
ADIPOQ	F: TGTCTGTACGATTGTCAGTGG R: AGTAACGTCATCATCTTCGGCATG
SLC2A 4	F: GTAACTTCATTGTCGGCATGG R: TGCTCTAAAAGGGAAGGTGTC
PPARG	F: TCACAAGAGCTGACCCAATG R: ATGCTTTATCCCCACAGACTC
CEBPA	F: GAGAACTCTAACTCCCCCATG R: GTCTCGTGCTCGCAGATG
FABP4	F: CACCGAGATTTCCTTCAAACTG R: CACGCCTTTCATAACACATTC
LEP	F: GTGCCTATCCAGAAAGTCCAG R: GACCTGTTGATAGACTGCCAG

C14orf1	F: TCTACGAGAAGCTCTACACTGG R: TGGATGTCAATGGCACAGAG
SREBF1	F: GAACCTGACCCTACGAAGTG R: TTTCATGCCCTCCATAGACAC
FASN	F: CTCAAGATGAAGGTGGCAGAG R: GGTCGGTGGCTGTGTATTC
INSR	F: GGAAGCTACATCTGATTCGAGG R: TGAGTGATGGTGAGGTTGTG
CEBPD	F: AGAACGAGAAGCTGCATCAG R: GGTCGTTCAGAGTCTCAAAGG
LMNA	F: CTTATGCTCCAGTGTCCACAG R: GGCAGGTCCCAGATTACATG

Table 6: Primers for cDNA synthesis

Quantitative real time PCR

Quantitative Real Time PCR reaction was set up on ice:

10 µl 2x SYBR Green I Mix (Roche)

1.6 µl Primer Mix (5 µM each)

8.4 µl cDNA

The PCR was run on LightCycler® 480 (Roche Applied Sciences).

Standard Program Setup

Target (°C)	Hold	Ramp Rate (°C/s)	Cycles
Pre-incubation			
95	5 min	4.40	1
Amplification			
95	10 sec	4.40	42
56	1 sec	2.20	
51	15 sec	1.00	
72	21 sec	4.40	
Melting curve			
95	5 sec	4.40	1
51	1 min	2.20	
97		0.19	
Cooling			

Table 7: qRT PCR Program

SDS page and western blot

All gels were run in a BioRad aperture.

The transfer from the gel to the membrane ran at 80 V for about 1.5 h. After the transfer poncean staining was done, the membrane was blocked in blocking buffer (5% non-fat milk powder in 1x PBS) for 60 min at RT and washed 3x5 min with 1x PBS-0,1%Tween20. Thereafter the membrane was incubated with primary antibodies over night at 4°C or 1 h at RT. The membrane was washed 3x5 min with 1x PBS-0,1%Tween20 and incubated for one hour with LICOR Odyssey® donkey anti mouse IRDye® 680 (1:5000) and LICOR Odyssey® donkey anti mouse IRDye® 800 (1:5000) conjugated secondary antibody. Finally the membranes were washed with 1x PBS

0,1%Tween20 for 3x5 min. Detection was performed by LICOR Odyssey® Infrared Imaging System.

Tissue Culture Methods

Cell culture and transfection

3T3-L1 cells were cultured in Dulbecco's Modified Eagle Medium Dulbecco's Modified Eagle Medium (Lonza), 1% Penicillin/Streptomycin, 0.1 M non essential amino acids, 0.2 M L-Glutamine, 1 mM Sodium Pyruvate. Cells were incubated in an atmosphere of 5% CO_2 at 37°C and split before a confluency of 80 - 90% was reached.

Cells were transfected with protein expression plasmids using FuGene 6 (Roche) transfection reagent according to the manufacturer's guidelines. Medium was changed on day 1 after transfection.

Pharmacological differentiation

3T3-L1 differentiation to adipocytes was induced 2 days after the cells had reached confluency. Dulbecco's Modified Eagle Medium Dulbecco's Modified Eagle Medium (Lonza) containing 1% penicillin/streptomycin, 0.1 M non essential amino acids, 0.2 M L-glutamine, 1 mM sodium pyruvate, 1 µg/ml Insulin, 0.5 µM isobutylmethylxanthine (IBMX), 0.25 µM dexamethasone was added to the cells, renewed on day 2 and normal culturing medium was added again on day 4 after induction. After 8 days full differentiation was reached.

Nile Red staining

A 1 mg/ml stock solution of nile red (Sigma) in acetone was prepared and stored at 4°C protected from light. Staining was carried out on PFA-fixed cells on coverslips. Cells were covered with 1x PBS. The dye was then added directly to the preparation to effect a 1:100 dilution and the preparation was incubated for a minimum of 5 min. Excess dye was removed by brief rinsing in 1x PBS and coverslips were mounted to slides with Southern Biotech Fluoromount.

Fluorescence In Situ Hybridisation

Probe labelling

BAC clone	Gene of interest
BMQ-297H24	PPARG
BMQ-223G20	ADIPOQ
BMQ-403N15	SLC2A 4
BMQ-375G9	LEP

Table 8: BAC Clones

BAC clones were purchased from Children's Hospital Oakland Research Institute. The bacteria were grown on LB plates with chloramphenicol and then single colonies were inoculated in 2 ml LB with chloramphenicol and grown over night at 37°C.

BACs were purified using Biomiga BAC Miniprep Kit according to manufacturer's guidelines. DNA amount was measured by using NanoDrop. BAC clones were checked by PCR.

BACs were labelled with Biotin-16-dUTPs (Roche) by the following reaction:

37 µl	ddH_2O
5 µl	10x NEB2 buffer
2 µl	dNTPs (1 mM)
1 µl	dUTPs (1 mM)
2 µl	DNA (5 µg/µl)
2 µl	DNAse (0.001 U/µl)
1 µl	Klenow Polymerase

The reaction was incubated at 37°C for 1 h and terminated by incubation at 90°C for 5 min. 20 µl of the reaction mix were run on a 2% agarose gel to test if label incorporation was sufficient. The labelled probes were stored at -20°C.

Staining

For whole chromosome painting Cambio StarFISH© Mouse Chromosome Specific Probes in biotin-labelled format were used. For staining of specific genes probes were prepared as described above.

12 µl of hybridization buffer were added to precipitated 10 µg mouse Cot1 and 5 µg salmon sperm DNA. After incubation at RT for 2 h. 3 µl of probe were added and incubated at RT for 1 h. Probes then were used for hybridization.

Cells plated on 13mm coverslips were washed with 1x PBS, fixed for 10 min at RT with freshly made 4% paraformaldehyde and then rinsed in 1x PBS. Cells were then allowed to age in 1x PBS at 4°C for 2 – 21 days. After aging, cells were permeabilized in 0.1% saponin / 0.1% triton in 1x PBS for 10 min at RT and washed with 1x PBS. Cells were blocked with 1% BSA in 1x PBS w/o Tween20 for 20 min and washed with 1x PBS. After 1 hr incubation with α-GFP rabbit IgG fraction (Invitrogen™ Molecular Probes®) in 1% BSA coverslips were washed with 1x PBS and fixed with 4% PFA in 1x PBS for 5 min at RT. Cells were washed with 1x PBS and incubated with 0.1 M Tris pH 7.4 for 10 min at RT. 20% glycerol in 1x PBS was added on the coverslips for

20 min. Cells were then dehydrated by freezing and thawing in liquid nitrogen and 20% glycerol in 1x PBS, respectively. Afterwards cells were rinsed in 1x PBS and then incubated in 0.1 M HCl for 10 min at RT. Cells were incubated in 0.5% saponin / 0.5% triton in 1x PBS were added to the coverslips for 10 min and then rinsed in 1x PBS. Coverslips were incubated on the 80°C heating block in 70% formamide / 2x SSC for 15 min, then 50% formamide / 2x SSC was added for 1 min. The prepared probes were incubated on 80°C for 5 min and then moved to 42°C for 3 min to preanneal. 7.5 µl of the probes were added on the prewarmed slide, coverslips were added and sealed with Marabu Fixo Gum Rubber Cement. The hybridization was incubated at 37°C over night.

Coverslips then were washed 4 x 3 min in 2x SSC at 45°C and then 4 x 3 min in 2x SSC at 60°C. Coverslips were transferred to 4x SSC / 0.1% Tween20 for 1 min and then incubated with blocking buffer (2x SSC / 0.1% Tween20) for 5 min at RT. Cells were then incubated for 1 h at RT with Alexa Fluor® 488 goat α-rabbit IgG H+L (Invitrogen™ Molecular Probes®) against the α-GFP antibody and streptavidin Alexa Fluor® 594 conjugate (Invitrogen™ Molecular Probes®) to visualize the chromosome staining. Coverslips were washed 3 x 2 min with 4x SSC / 0.1% Tween20 and then mounted microscope slides with Southern Biotech Fluoromount G.

Software

GENtle 1.9.4
ImageJ 1.42
Metamorph
GIMP 2.6
Adobe Photoshop CS4 11.0
GeneDoc 2.7.000

Online Resources

ENSEMBL	http://www.ensembl.org/Multi/blastview
BioGPS	http://biogps.gnf.org/
NCBI Clone Finder	http://www.ncbi.nlm.nih.gov/projects/mapview/mvhome/mvclone.cgi?taxid=10090
Finnzymes Tm Calculator	https://www.finnzymes.fi/tm_determination.html
NetPhos	http://www.cbs.dtu.dk/services/NetPhos/
NetPhosK	http://www.cbs.dtu.dk/services/NetPhosK/

RESULTS

Variation of nuclear envelope components among different tissues

Using a subtractive proteomic approach the Schirmer lab identified 907 putative novel NETs between analysis of liver, muscle and blood leukocyte nuclear envelopes. Bioinformatic analysis of the proteomic results revealed that among tissues there is a high degree of variation in the NE proteome and only 10% of the total proteins identified were shared between the three tissues. Of these 90 novel proteins were cloned and expressed as GFP or RFP tagged proteins and transfected into different cell lines to check whether they localize to the NE. Of the tested 90 NETs 67 were confirmed as definite novel NETs. Of those 67 NETs many remain proteins of unknown function or are uncharacterized hypothetical ORFs (Schirmer et al., 2003; Korfali et al., 2010; Malik et al., 2010).

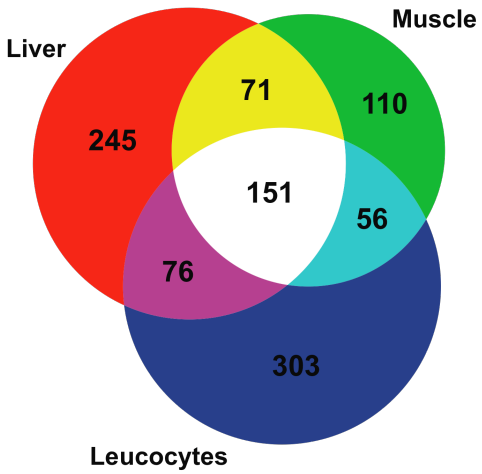

Figure 4: Tissue variation of NETs
Of 907 identified putative novel nets, only 10% are shared between muscle, liver and blood.

Expression levels of NETs in different tissues were compared using data from the transcriptome database of *Mus musculus* at biogps.gnf.org (Su et al., 2002; Wu et al., 2009). The median expression value over all 84 tissues analyzed was determined and the fold-expression over this value calculated for the individual tissues shown in Figure 5. Many NETs are highly expressed in certain tissues, but not in others. NET33 for example is highly expressed in adipose and epidermal tissue and moderately expressed in heart, but it is absent in any other tissue. NET29 on the other hand is highly expressed in white and brown adipose tissue and the small intestine while in other tissues expression levels are very low. This suggested that these NETs have a tissue specific function and we postulated that they might play a role in differentiation.

There are many changes that occur in cells during differentiation. Because NET29 and NET33 are nuclear proteins that are highly upregulated in adipose tissue, we predicted that they might affect gene expression during differentiation. qRT-PCR was performed to test NET upregulation during adipogenesis and a possible correlation with the upregulation of adipogenic markers. As NETs have previously been shown to recruit particular chromosomes to the periphery with consequences for gene expression (N. Zuleger, S. Boyle, D. A. Kelly, J. de las Heras, D. G. Batrakou, V. Lazou, G. R. Otti, D. J. Harrison, W. A. Bickmore and E. C. Schirmer, in revision), we tested the effect of these adipocyte NETs on chromosomes carrying the most well-known adipogenic markers by whole chromosome painting.

For our experiments we used the well established and characterized 3T3-L1 model system to test the function of NET29 and NET33 in adipogenesis.

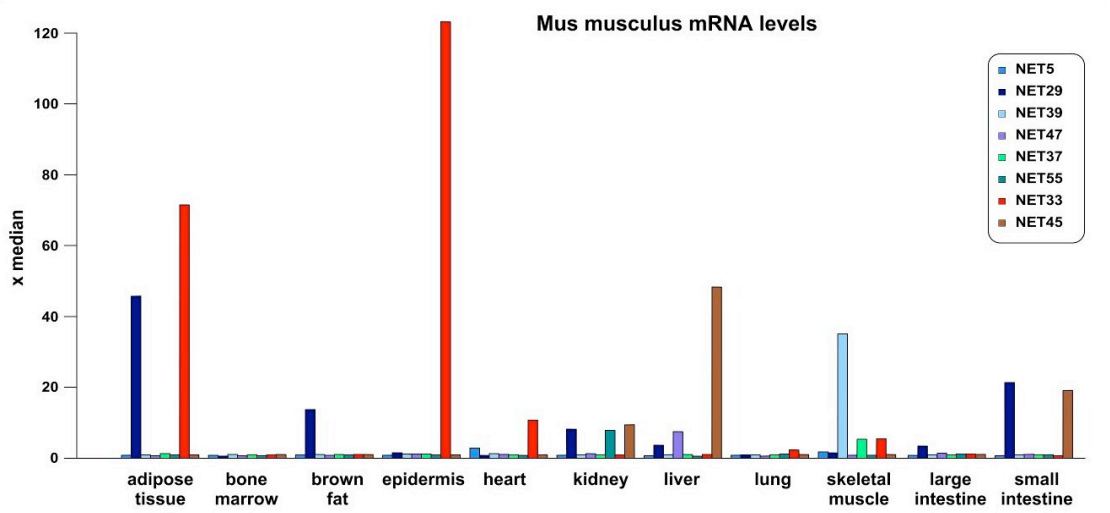

Figure 5: Tissue specificity of NETs
mRNA levels of different tissues show a high variation of NET expression in *mus musculus*. NET29 (blue) is highly upregulated in adipose tissue and the small intestine whereas NET33 expression is high in adipose tissue and epidermal cells.

Induction of differentiation in the 3T3-L1 model adipogenesis system

3T3-L1 cells were pharmacologically induced 2 days after reaching confluency and incubated for another 8 days at 37°C. Differentiation was confirmed by using Nile Red staining. Lipid droplets stained with the Nile Red have accumulated and are clearly visible in the cytoplasm (see **Figure 6**).

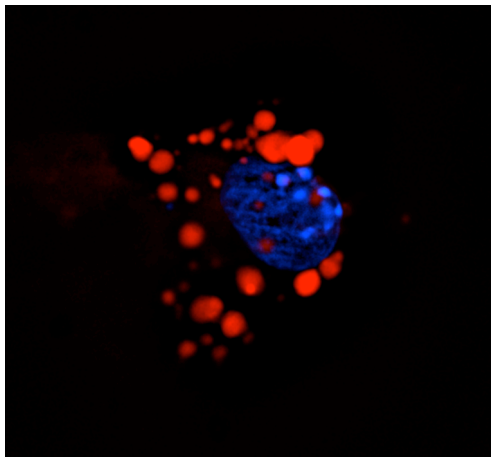

Figure 6: Visualization of lipid droplets
Lipid droplets (red) have accumulated in the cytoplasm around the nucleus (blue).

Upregulation of NET29 and NET33 during adipogenic differentiation

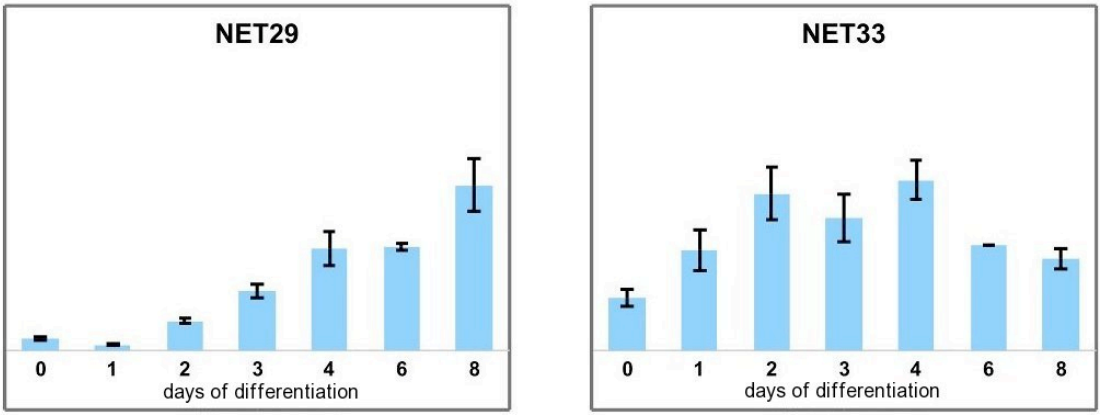

Figure 7: NET29 and NET33 expression during adipogenesis (with D. G. Batrakou)
mRNA levels of NET 29 and NET33 increase over the course of adipogenesis. NET29 increases steadily whereas NET33 starts to decline after day 5.

To investigate the levels of NET29 and NET33 during the differentiation process cells were induced and RNA was isolated at different time points during induction. qRT-PCR shows steadily increasing levels of NET29 over 8 days compared to Lamin A levels. NET33 levels rise from day 1 on, but start decreasing after day 4. NET29 appearing early in adipogenic differentiation suggests that the protein plays a role in early adipogenesis and the consistent high upregulation during the later states might indicate a function in the upkeep of the differentiated state of 3T3-L1 cells. NET33 seems to be involved in early processes during differentiation, but not to be required in differentiated cells.

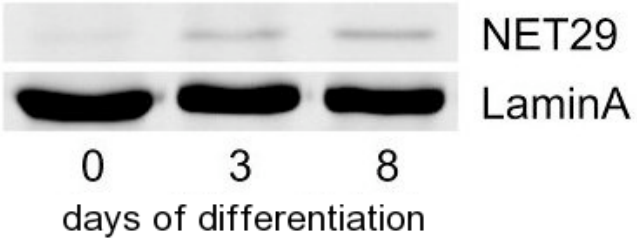

Figure 8: Protein levels of NET29 during adipogenesis
NET29 protein levels increase over the course of differentiation. Lamin A was used as a loading control.

Western Blot confirmed the upregulation of NET29 during adipogenesis. 3T3-L1 cells were induced and protein was isolated at different time points during pharmacological differentiation. Lamin A was used as a control due to its ubiquitous expression. As shown in Figure 7 NET29 protein levels steadily increase from day 0 to day 8.

Adipogenic markers during adipogenic differentiation

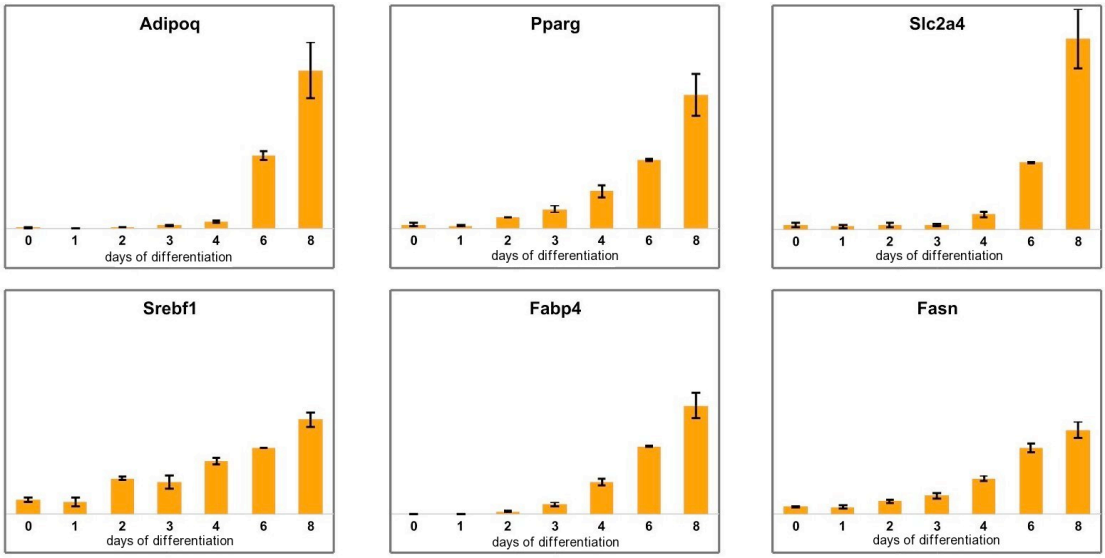

Figure 9: mRNA levels of adipogenic markers during adipogenesis (with D.G. Batrakou) mRNA levels of Adipoq, Pparg, Slc2a4, Srebf1, Fabp4 and Fasn increase notably during the differentiation process.

To confirm the differentiation process qRT-PCR for adipogenic markers was performed. As Figure 9 shows mRNA levels of Adipoq, Pparg, Slc2a4, Srebf1, Fabp4 and Fasn increase over the course of differentiation.

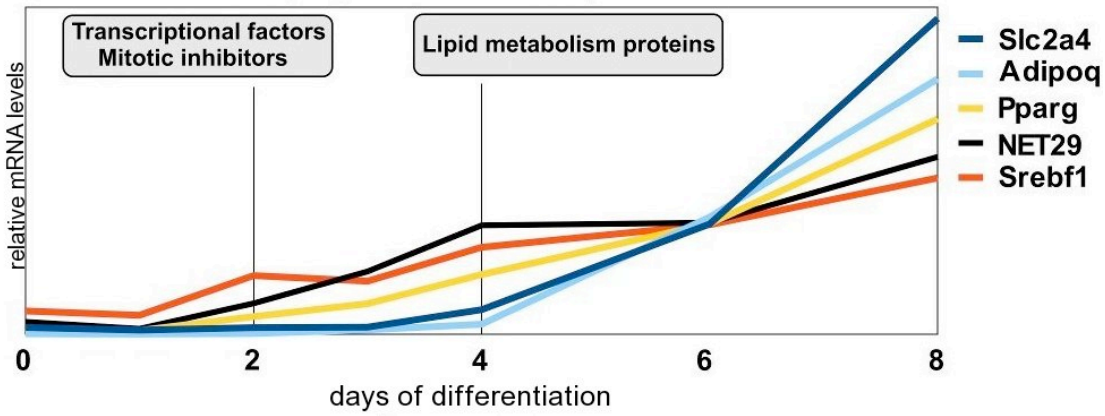

Figure 10: Relative mRNA levels of adipogenic markers and NET29
Adipogenic markers Slc2a4, Adipoq, Pparg and Srebf1 rise with the increase of NET29 levels. Relative mRNA levels were normalized by taking the mRNA level of any gene as 1 on day 6, lamin A mRNA was used as a reference.

The correlation of NET29 and Adipoq, Slc2a4, Pparg and Srebf1 is shown by comparison of the relative rates of increase of mRNA levels during differentiation by qRT-PCR. After the increase of mRNA for NET29 on day 2 mRNA levels of adipogenic markers rise as well. However, this does not prove any causal relation between NET29 expression and the adipogenic markers and needs to be further investigated by testing the effect of a NET29 knockdown on their expression.

Chromosome 6 relocation during adipogenic differentiation

In a previous study it was shown that chromosome positioning correlated with gene expression changes (N. Zuleger, S. Boyle, D. A. Kelly, J. de las Heras, D. G. Batrakou, V. Lazou, G. R. Otti, D. J. Harrison, W. A. Bickmore and E. C. Schirmer, in revision). We wanted to investigate if the chromosome position played a role in the changes in gene expression in adipogenesis and therefore first tested if the chromosomes encoding these important genes changed in position during pharmacological differentiation

3T3-L1 cells were plated at 50% confluency and pharmacologically differentiated 2 days after reaching confluency. Whole chromosome painting

was performed on undifferentiated and differentiated cells for chromosome 6, which carries the gene for PPARγ, one of the master regulators of adipogenesis.

The chromosome position relative to the nuclear periphery was quantified using a script that erodes nuclear area, as defined by DAPI staining, into five concentric shells of equal area from the outside (shell 1) to the inside (shell 5) (algorithm originally developed in Croft et al., 1999, adapted at the Wellcome Trust Centre for Cell Biology by Dr. David Kelly) (Figure 11). To avoid errors from the unequal resolution between the xy and the z directions inherent to light microscopy, only cells where the array could be visualized at a focus point where the nuclear diameter was at its widest were considered. However this underestimates peripheral incidence as there might be chromosomes on top and thus at the periphery that are counted as being internal or simply not counted at all.

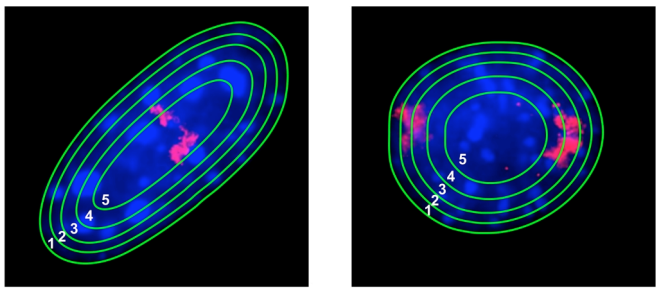

Figure 11: Erosion script
The nucleus is divided into 5 shells with equal area, from the interior (shell 5) to the periphery (shell 1).

After summing up the fluorescence intensity in the 2 central and the 2 peripheral shells it became clear that in undifferentiated cells chromosome 6 is mainly located at the interior, but has a higher tendency to be located at the periphery in differentiated cells.

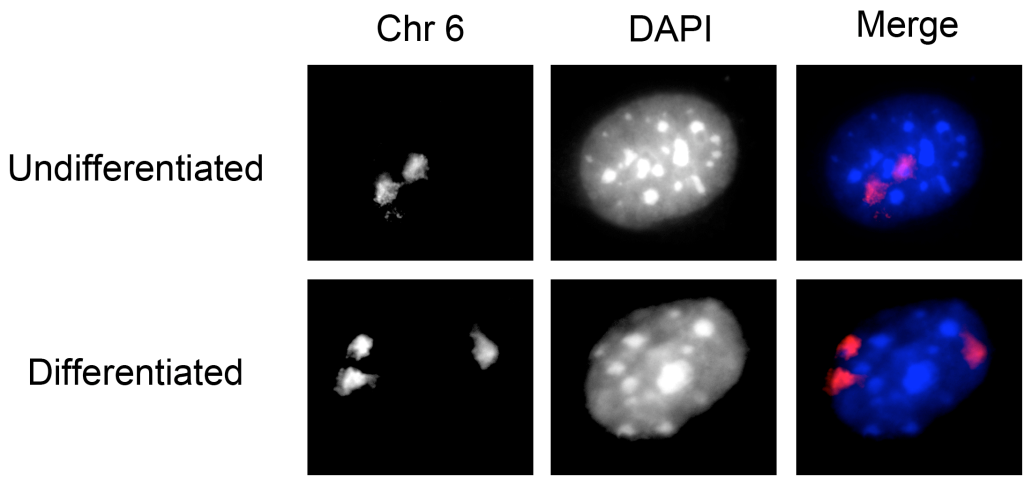

Figure 12: Localization of chromosome 6 in undifferentiated and differentiated cells
Whole chromosome painting revealed the different positioning of chromosome 6 in undifferentiated and differentiated 3T3-L1 cells. In the undifferentiated state, chromosome 6 localizes at the nuclear interior, in differentiated cells it tends to be at the nuclear periphery.

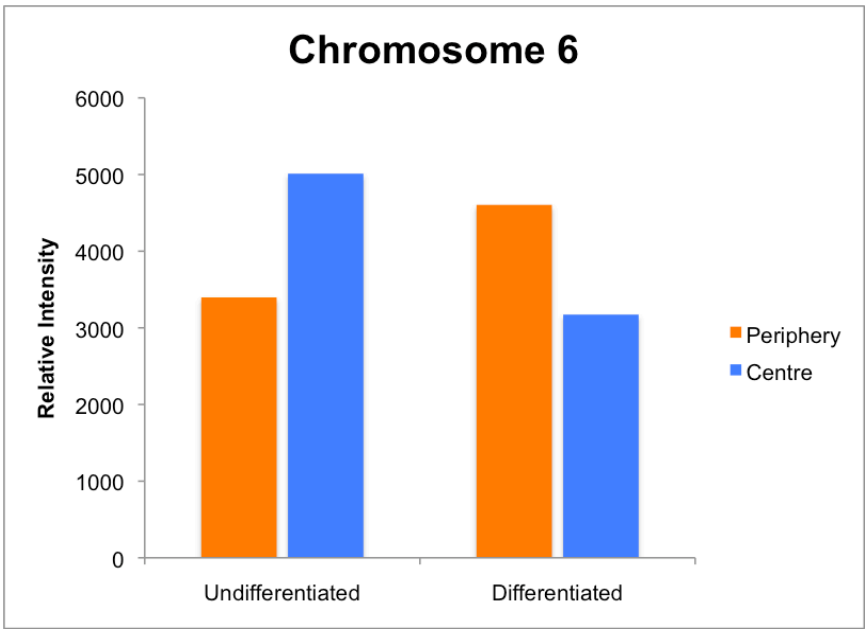

Figure 13: Chromosome positioning in undifferentiated and differentiated cells
Chromosome 6 is mainly located at the nuclear interior in undifferentiated cells but moves towards the nuclear periphery in differentiated cells.

Overexpression of NETs and chromosome relocation

As mentioned in the introduction, various studies showed that proteins in the NE are able to tether chromosomes and relocate them to the nuclear periphery (Finlan et al., 2008; Kumaran and Spector, 2008; Reddy et al., 2008). NET29 was able to relocate chromosomes 5 and 13 in HT1080 cells and since it is upregulated in adipogenesis we wanted to investigate if it relocates chromosome 6, which normally changes position during adipogenesis.

3T3-L1 cells were transfected with GFP and Emerin-GFP constructs as controls. NET29-GFP and NET33-GFP constructs were separately transfected to investigate the effect of these proteins on chromosome movement. Cells were transfected at 10 – 15% confluency and fixed after 3 days when they reached 80 – 90% confluency. This allowed the 3T3-L1 cells to divide at least twice and thus gave the chromosome a chance to reposition since this only occurs during mitosis (Finlan et al., 2008; Kumaran and Spector, 2008; Reddy et al., 2008). The cells were stained for chromosome 6, 7 and 16. Chromosome 6 carries the gene for PPARγ while Adipoq is localized on chromosome 16. Chromosome 7 shows 70% homology to the human chromosome 19, which was shown to be localized at the nuclear interior (Croft et al., 1999) and to be unaffected by NET29 in HT1080 cells (N. Zuleger, S. Boyle, D. A. Kelly, J. de las Heras, D. G. Batrakou, V. Lazou, G. R. Otti, D. J. Harrison, W. A. Bickmore and E. C. Schirmer, in revision). 100 transfected cells were imaged and macro analysis was carried out to quantify the chromosome distribution.

Chromosomes 6, 7 and 16 are mainly located in the nuclear interior without transfection and with the control GFP transfection. The chromosome positioning for chromosome 7 and chromosome 16 among all different transfections did not shift drastically. In contrast, chromosome 6 relocated from the interior to the periphery in cells transfected with NET29-GFP, while being unaffected in cells transfected with Emerin-GFP and NET33-GFP.

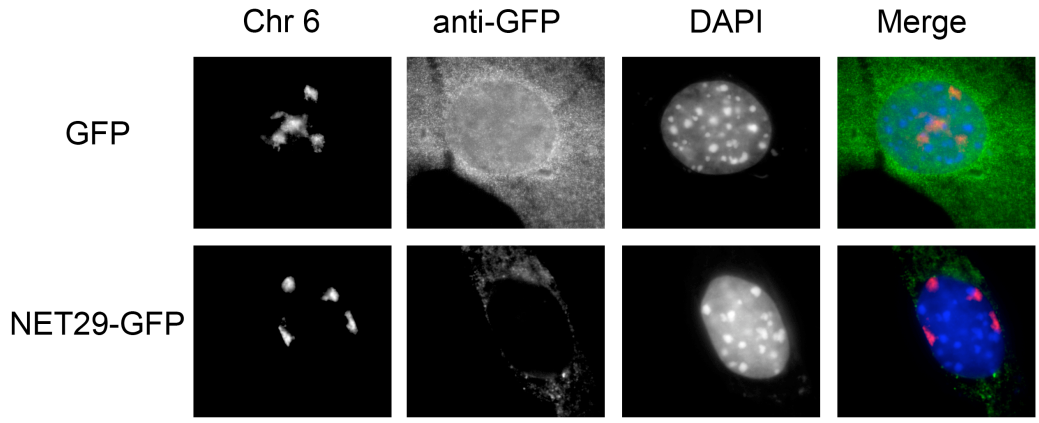

Figure 14: Localization of chromosome 6 in cells transfected with GFP and NET29-GFP
GFP transfected cells did not show an effect in chromosome repositioning, whereas in cells transfected with NET29-GFP chromosome 6 positioning at the periphery tends to be higher.

Quantification revealed that chromosomes 6, 7 and 16 were mainly positioned in the nuclear interior. Results were confirmed by quantification (see Figure 14).

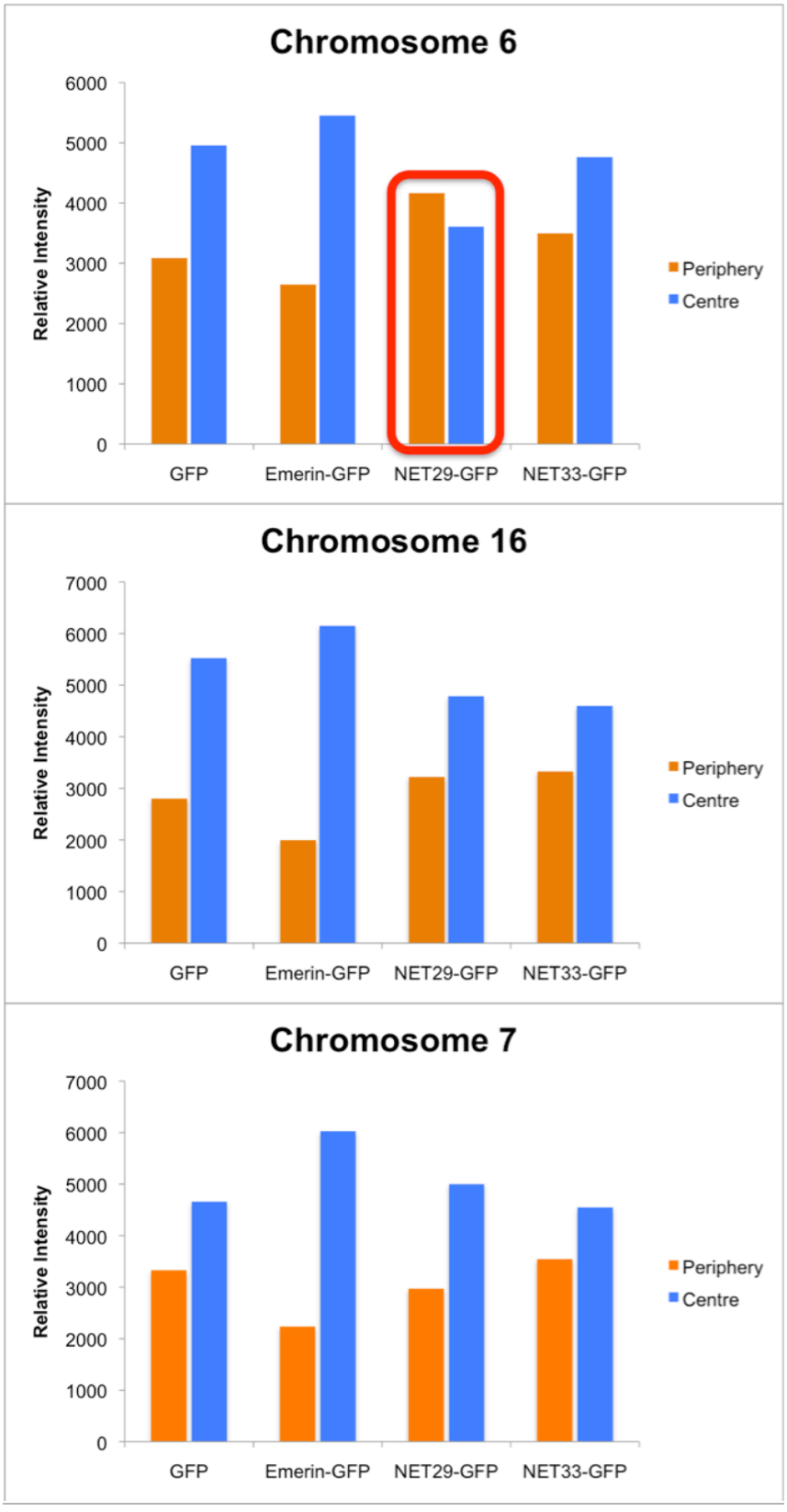

Figure 15: Chromosome positioning in cells overexpressing NETs
Movement of chromosomes 7 and 16 is not influenced by the overexpression of Emerin-GFP, NET29-GFP and NET33-GFP. Chromosome 6 is moving towards the nuclear periphery when NET29-GFP is overexpressed. No other protein shows this effect.

Evolutionary conservation of NET29 suggests sites for post-translational modification

A clustal analysis was performed comparing the NET29 sequences of *D. melanogaster, C. elegans, D. rerio, X. laevis, G. gallus, M. musculus, R. norvegicus* and *H. sapiens.* Before the first predicted putative transmembrane domain, a highly conserved putative phosphorylation site was found using http://www.cbs.dtu.dk/services/NetPhos/ (see Figure 16). The online database http://www.cbs.dtu.dk/services/NetPhosK/ suggested insulin receptor kinase as a possible kinase to phosphorylate this site. Insulin receptor kinase plays an important role in adipogenesis signalling and was shown to be localized in the nucleus (Smith and Jarett, 1987).

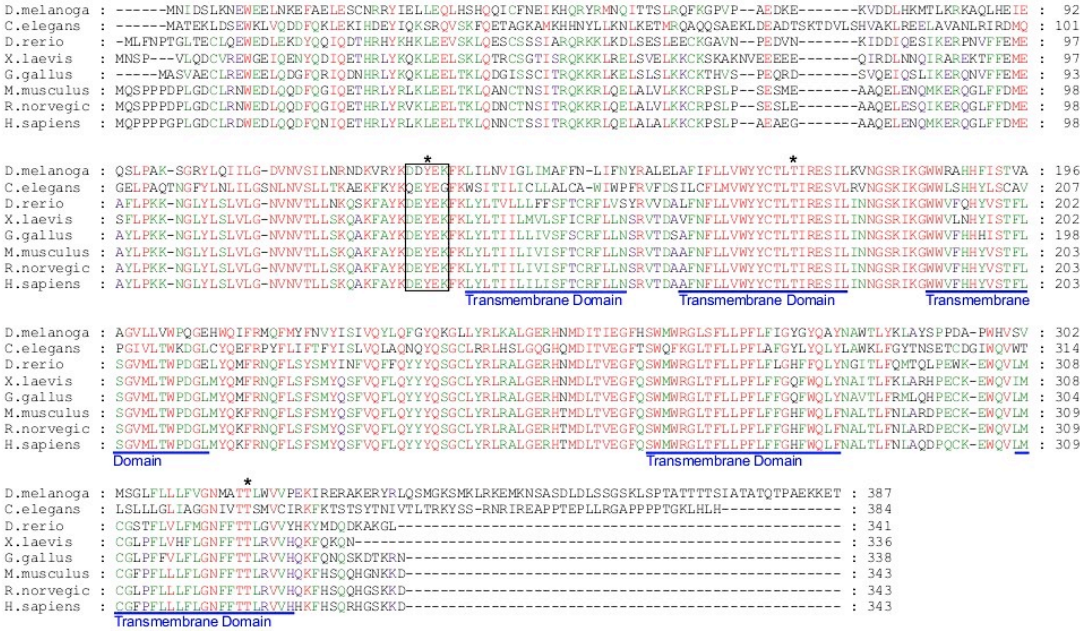

Figure 16: Clustal analysis of NET29
Highly conserved areas are in red, less conserved areas in green. The predicted transmembrane proteins show high grades of conservation. A highly conserved putative phosphorylation site is marked.

Phosphonull and phosphomimetic mutants show different chromosome 6 localization

In order to assess whether this conserved phosphorylation site influences the role of NET29 in chromosome repositioning this site was mutated to phosphomimetic Y129E Y133E and phosphonull Y129F Y133F sites, using site directed mutagenesis. Mutagenesis was confirmed by sequencing. The negative charge of the glutamic acid is intended to mimic phosphorylation at the phosphorylation site in the phosphomimetic mutant. Most phosphonull mutations are alanine, in this case we used phenylalanine due to structural similarities to tyrosine.

Whole chromosome painting was performed on cells transfected with NET29-PhM (Y129E Y133E phosphomimetic double mutant) and NET29-Ph0 (Y129F Y133F phosphonull double mutant) and the quantification of 20 imaged cells showed a much higher localization of chromosome 6 at the nuclear interior in cells overexpressing NET29-Ph0. The position of chromosome 6 in NET29-PhM expressing cells is only slightly more peripheral than in cells overexpressing wild-type NET29-GFP, which suggests that NET29 is usually maintained in a phosphorylated state in 3T3-L1.

Both sites were tested together to determine if there was an effect, however in future the effect of each mutation on chromosome positioning should be investigated independently, since combined mutations are more likely to alter protein structure.

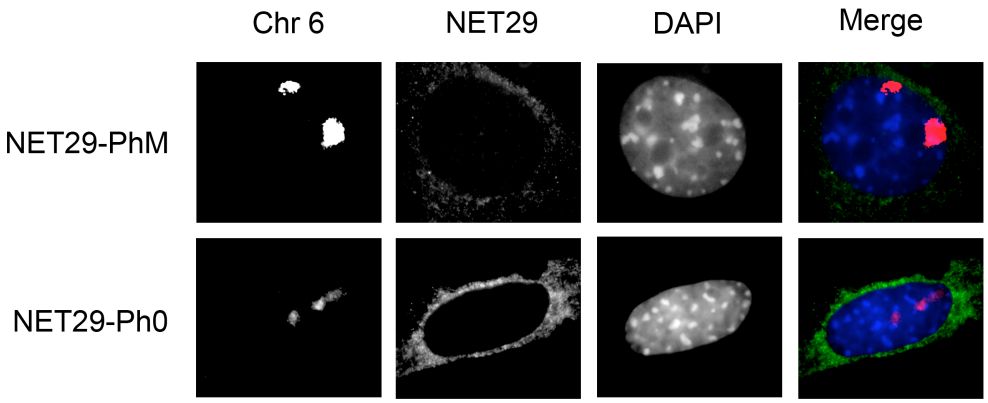

Figure 17: Localization of chromosome 6 in NET29-Ph0 and NET29-PhM cells
In cells transfected with NET29-PhM chromosome 6 tends to move towards the periphery whereas in cells transfected with NET29-Ph0 chromosome 6 localization in the interior tends to be much higher.

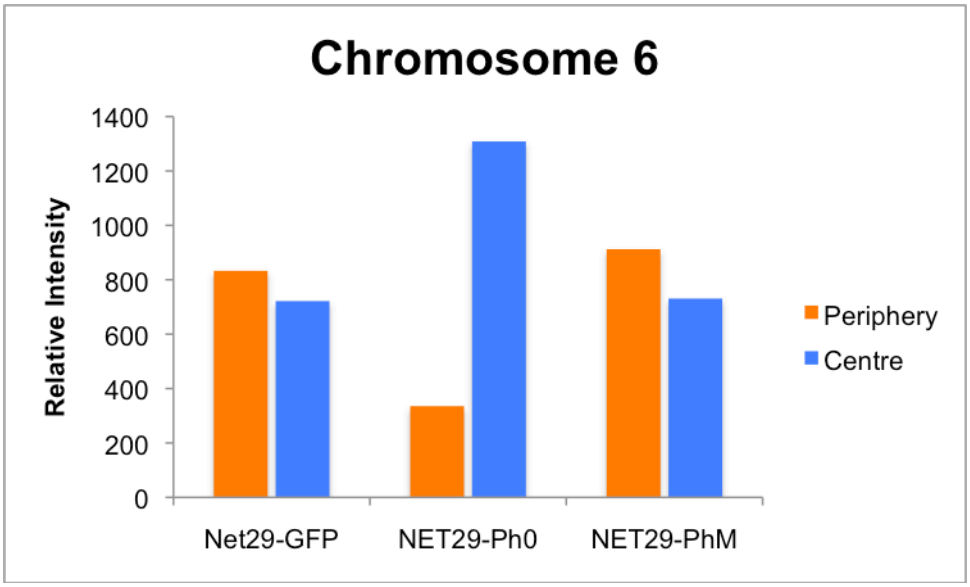

Figure 18: Chromosome positioning in NET29Ph0 and NET29PhM cells
NET29-GFP and NET29-PhM show the same chromosome distribution. NET29-Ph0 shows a much higher location of chromosome 6 at the nuclear interior.

DISCUSSION

The aim of this diploma thesis is to seek a deeper understanding of the role of the NE in cell differentiation and its influence on gene expression. As particular patterns of chromosome positioning have been observed in different tissues (Parada et al., 2004), we postulated that more tissue-specific NETs might contribute to this chromosome positioning. Thus the work focused on NET29 and NET33 that are highly expressed in adipogenic differentiation in 3T3-L1 cells.

NET29 and NET33 in adipogenic differentiation

NET29 and NET33 expression increases over the course of adipogenic differentiation. NET33 is upregulated early in adipogenesis and decreases towards the end of the differentiation process. NET33 expression increasing before the upregulation of adipogenic markers suggests that NET33 plays an early role in the differentiation process. NET29 upregulation is steady and starts later in the adipogenic process. It might be upregulated due to the influence of early adipogenic genes and necessary for the preservation of the differentiated state of 3T3-L1 cells.

To further investigate the role of NET29 and NET33 in adipogenesis we wanted to test the effects of expressing NET29 on expression of other adipogenic markers, however we could not get a clear result for this kind of population study due to low transfection efficiencies. In future it might be possible to address this problem by generating stable inducible cell lines, but because of the importance of keeping 3T3-L1 passage number low for efficient differentiation making of the stable cell lines might change the differentiation potential of these cells.

To gain a deeper insight on how crucial the role of NET29 and NET33 is in differentiation, a knockdown should be performed for NET29 and NET33, as

well as a combined knockdown. Cells should then be differentiated pharmacologically and this would indicate whether any of the two NETs is essential for adipogenesis or if both are necessary. Also it would be important to assess by qRT-PCR if the expression of the previously mentioned adipogenic markers during the differentiation process is influenced by the absence of NET29, NET33 or both.

NET 29 and chromosome repositioning

Chromosome painting revealed that overexpression of NET29-GFP in 3T3-L1 cells has an effect on the positioning of chromosome 6. When NET29 was overexpressed chromosome 6 was localized at the nuclear periphery in transfected cells compared to the nuclear interior in control and untransfected cells.

Tissue specific NETs are able to recruit certain chromosomes to the periphery with potential consequences to gene expression. It has been shown that when chromosomes move to the periphery gene expression is affected (Meaburn and Misteli, 2007). Thus NET29 could play a similar role. In liver where certain NETs are highly upregulated chromosomes 5 and 13 tend to be more localized at the periphery than in other tissues, overexpression of these NETs in HT1080 cells showed recruitment of the chromosomes to the periphery (N. Zuleger, S. Boyle, D. A. Kelly, J. de las Heras, D. G. Batrakou, V. Lazou, G. R. Otti, D. J. Harrison, W. A. Bickmore and E. C. Schirmer, in revision).

Whole chromosome painting on fully differentiated 3T3-L1 cells showed that chromosome 6 is more likely to be localized at the periphery than in undifferentiated ones. Vesicles are assembling on mitotic chromosomes during NE reassembly (Prunuske and Ullman, 2006) and we presume that on this vesicles NET29 binds with high affinity to chromosome 6. This suggests that due to this high affinity interaction it remains at the periphery while chromosomes with less tethers localize at the nuclear interior.

It is well established that each chromosome is confined to a discrete region, referred to as a chromosome territory. This spatial organization is

seen as an important aspect of gene regulation and genome stability (Meaburn and Misteli, 2007). Interactions of the NE and chromatin have previously been described, but show a greater tendency towards heterochromatin (Brown et al., 2008; Capelson et al., 2010; Kalverda et al., 2010; Makatsori et al., 2004; Pickersgill et al., 2006). These interactions are not specific for certain chromosomes, whereas NET29 recruits specifically chromosome 6.

So far the only NE protein shown to have a specific effect on chromosome repositioning is lamin B1, which releases chromosome 18 from the periphery when mutated (Malhas et al., 2007). However, it is still unclear how lamin B1 is able to distinguish chromosome 18 from other chromosomes since it is ubiquitously expressed and is known to bind to core histones (Goldberg et al., 1999). It is possible that lamin B1 reflects an indirect effect of a NET that actually requires lamin B1 for targeting or influencing a certain positioning pattern.

In contrast to ubiquitously expressed lamins the tissue-specific NET29 could have high-affinity interactions with transcriptional regulators tightly bound to particular genes that are involved in adipogenic differentiation. By binding to a NET by transcriptional repressors tightly bound to a gene, the recruitment of the chromosome to the nuclear periphery can lead to stronger gene silencing due to its repressive environment. This might be necessary if a certain threshold of transcriptional repressors is needed which only can be found at the nuclear periphery due to histone deacetylation by LAP2β and further recruitment of HDAC3 (Somech et al., 2005). This might represent a mechanism for long lasting gene silencing in differentiated cells.

Further association of NETs with enzymes that add silencing marks have also been shown (Somech et al., 2005), which provide a general mechanism to recruit heterochromatin to the periphery. Though these interactions are unspecific, other NET interactions are specific for certain transcriptional repressors or activators (Holaska et al., 2006; Nili et al., 2001). Thus it is possible that by recruiting chromosome 6 to the periphery some genes are silenced due to the repressive environment at the nuclear periphery. Transcription factors have the ability to build a high-affinity interaction between specific chromosome regions and the NE. The interactions described

showed the transcriptional regulators localized at the NE away from the genome to keep them inactive until they are needed (Markiewicz et al., 2006; Worman et al., 1988). It is likely that certain transcriptional regulators that are associated with a gene on chromosome 6 interact with NET29 and therefore cause the chromosome to localize at the nuclear periphery during adipogenic differentiation when gene expression changes.

Generation of a particular pattern of chromosome positioning in different tissues might reflect combined impact of several NETs and their possible binding partners. To further investigate the correlation between NET29 overexpression and altered gene expression a microarray analysis should be carried out on cells transfected with NET29-GFP, as control GFP transfected and untransfected preadipocytes should be used. This analysis will identify genes that are specifically upregulated or downregulated by NET29.

Potential phosphorylation of NET29 and its importance in chromosome 6 repositioning

In cells transfected with NET29-PhM-GFP whole chromosome painting showed the same tendency of chromosome 6 to localize at the nuclear periphery as in cells overexpressing NET29-GFP. However, in cells overexpressing the NET29-Ph0-GFP mutant chromosome 6 seems to be left in the interior.

Phosphorylation might increase the affinity of NET29 for a chromatin protein or for a transcriptional regulator bound to a gene in adipocyte differentiation. Insulin receptor signalling from outside the cell might influence differentiation by increasing phosphorylation and therefore stronger chromosome repositioning.

The putative phosphorylation site of NET29 is predicted to be phosphorylated by insulin receptor kinase. Insulin receptor kinase is activated by insulin and was previously shown to enter the nucleoplasm and function there. We hypothesized that with a lot of insulin output the insulin receptor goes to the nucleoplasm and there phosphorylates NET29 which in turn recruits chromosome 6 to the periphery. Thus NET29 might be

phosphorylated in the natural state in preadipocytes and this will need to be directly tested in future. Phosphorylation may only be required for interactions associated with insulin signalling so that NET29 could influence chromosome repositioning in an unphosphorylated state in other cell types. Alternatively NET29 phosphorylation could be used in another cell type that expresses it in order to release a chromosome from the periphery to become activated in the interior. To gain further knowledge it could be investigated if the phosphorylation is also required for NET29 function on HT1080 chromosome 5, which is also localized at the nuclear periphery when HT1080 cells are overexpressing NET29 (N. Zuleger, S. Boyle, D. A. Kelly, J. de las Heras, D. G. Batrakou, V. Lazou, G. R. Otti, D. J. Harrison, W. A. Bickmore and E. C. Schirmer, in revision).

Further the actual phosphorylation state in murine fibroblasts, 3T3-L1 cells and in differentiated adipocytes should be determined, as well as in HT1080 cells.

Future directions

This study shows the NE and NETs are potential important players in adipocyte differentiation. The mechanism by which they might be exerting their effect is by chromosome repositioning.

Although this study showed that NET29 mRNA and protein levels are unregulated during adipocyte differentiation the antibody against NET29 is of poor quality. Therefore, an antibody against NET29 should be generated. Additionally, NET29 protein levels should be assessed from different tissue lysates to confirm if NET29 is also expressed at higher levels in adipose tissues compared to others. Further qRT PCR for adipogenic markers should be repeated and fat depositions should be investigated by Nile Red staining. However, transfection efficiency should be improved and cells should be sorted by FACS prior to RNA extractions. This latter could be useful as in those cells the non-transfected cells could as well be isolated and could serve as the internal controls. Alternatively, stable cell lines overexpressing NET29-GFP and GFP should be made. Also it would be useful to make stable knock

out cell lines. Further experiments would include the overexpression of NET29 in other differentiation paradigms where it is not upregulated originally to clarify if NET29 can repress other differentiation processes.

In order to further investigate the importance of the NE and its role in adipogenic differentiation it is important to perform microarray analysis to test the effect of NET overexpression and knockout on gene regulation. Again, for overexpression should be optimised prior to this assay.

Whole chromosome painting so far has been done generally for just two chromosomes at a time, mostly in cell lines. A general mapping of all chromosomes in all tissues would support future work in this area. In order to confirm the role of chromosome repositioning on differentiation whole chromosome painting of chromosome 6 should be performed on cryosections of different tissues to show whether chromosome 6 is naturally localized at the periphery in adipose tissue. Also it is important to assess chromosome repositioning in the absence of NET29. This will prove very important if the knockdown on NET29 shows an effect on adipocyte differentiation.

The preliminary results regarding NET29 phosphorylation proved to be very interesting, NET29 phosphorylation seems to be influencing chromosome 6 repositioning. It would be also interesting to test if NET29 can cause chromosome repositioning in a tissue which originally does not express NET29. This will give further conclusion about the role of chromosome localization in differentiation and gene expression. In these cell lines NET29-PhM mutant could be used and would shed more insight on the role of NET29 phosphorylation on chromosome relocation.

Identifying binding partners by pulldown followed by MS is certainly an important experiment that should be conducted. The identification of a possible known binding partner could elucidate if NET29 binds directly to a unique chromatin protein or recruits an enzymatic activity to modify another protein that is involved in the direct tether.

To investigate if NET29 has a direct interaction with chromosome 6 the use of DamID would be advantageous. By DamID it is possible to identify DNA loci that interact in vivo with specific nuclear proteins in eukaryotes. By fusing E. coli DNA adenine methyltransferase (Dam) to NET29 DNA will be methylated and can be recovered. Sequences in contact with NET29 at the

periphery can then be determined using methylation-specific restriction enzymes or antibodies (van Steensel and Henikoff, 2000).

Final remarks

It is likely that a combination of certain NETs during different stages of adipogenic differentiation is needed to influence the position of chromosomes. Though we only have worked on NET29, it can be anticipated that there are other tissue-specific NETs in adipocytes. A proteomic analysis should be performed on adipocytes to identify other factors that work synergistically with NET29 to cause its effects. The peripheral localization of chromosomes is possibly involved in changes in gene expression by helping to induce genes associated with adipose tissues and repressing alternative pathways. However much further work is required to elucidate the details of the role of NETs in differentiation pathways and it is too early in the data collection process to make any clear links between NET29 and chromosome positioning and human disease.

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APPENDIX

Abstract

Summary

The nuclear envelope (NE) is a double membrane system separating the nucleus from the cytoplasm. Once only seen as a physical barrier, studies within the last few decades have proven that it serves a variety of functions such as DNA replication, transcription, various signalling cascades and differentiation. The NE interacts with chromatin and this interaction is one of the established mechanisms on how the NE might influence cell signalling and our new data indicate it also plays a role in cell differentiation. Inner nuclear envelope transmembrane proteins (NETs) are also able to interact with chromatin and thus may also be involved in a variety o f functions.

It has been shown that the NE and NETs affect chromatin positioning by pulling chromatin loci towards or away from the periphery. Chromatin movement might be one of the mechanisms whereby the NE plays a role in cell differentiation.

This study focuses on the two tissue preferential NETs, NET29 and NET33, which are highly up-regulated in adipose mouse tissue, and the role they might play in chromosome positioning and its link to adipogenic differentiation. To address this question we used the 3T3-L1 model system. 3T3-L1 cells were pharmacologically induced to differentiate to adipocytes and qRT-PCR confirmed upregulation of NET29 and NET33 during adipogenesis. Whole chromosome painting on chromosomes 6, 7 and 16 showed a statistically significant shift in the localization of chromosome 6 to the nuclear periphery, chromosome 6 interestingly carries several genes important for adipogenesis. Furthermore, when 3T3-L1 cells were transfected with several NETs, chromosome 6 was clearly localized at the nuclear

periphery of undifferentiated cells overexpressing NET29-GFP, while chromosome 7 and 16 were not similarly affected. No other NET showed the same effect.

Clustal analysis revealed a putative phosphorylation site on NET29, a potential target for the insuline receptor kinase, which is an important player in adipogenic signalling. Using site directed mutagenesis a serine-amino acid-aspartic acid and a serine-amino acid- alanine mutant were produced. Whole chromosome painting on cells overexpressing NET29-Ph0 showed a strong shift of chromosome 6 to the nuclear interior.

The results may indicate a function of NETs in cell differentiation by changing chromosome positioning and further influencing gene expression either directly or through complex signalling pathways. This yet remains to be elucidated.

Zusammenfassung

Die Kernmembran ist ein Doppelmembransystem, das den Kern vom Cytoplasma trennt. Früher nur als Barriere gesehen, so zeigten Studien der letzten Jahrzehnte dass die Kernmembran neben der Kompartimenttrennung Funktionen in mehreren Prozessen, so z. B. DNA Replikation, Transkription, in verschiedenen Signalkaskaden und in der Zelldifferenzierung hat. Die Kernmembran interagiert mit Chromatin, ein Mechanismus, durch den die Differenzierung von Zellen möglicherweise beeinflusst wird. Transmembranproteine der inneren Kernmembran (NETs) interagieren auch mit Chromatin, daher könnten auch sie an diesen Prozessen beteiligt sein.

Die Kernmembran und NETs können die Lokalisation vom Chromosomen hin zur Kernperipherie oder ins Kerninnere beeinflussen, dies wäre ein möglicher Mechansimus um auf die Differenzierung von Zellen Einfluss zu nehmen.

Diese Studie untersucht die gewebespezifischen NETs NET29 und NET33, welche im Fettgewebe der Maus hochreguliert sind, und ihre potenzielle Rolle in der Adipogenese und bei der Repositionierung von Chromosomen. Maus-Präadipozyten (3T3-L1) wurden pharmakologisch zur Differenzierung zu Fettzellen induziert, mittels qRT-PCR und Western Blot wurden gesteigerte mRNA- und Proteinlevels nachgewiesen. Chromosome Painting der Chromosomen 6, 7, und 16 zeigte eine signifikante Verschiebung der Lokalisation von Chromosom 6 zur Kernperipherie. Weiters wurden 3T3-L1 Zellen mit verschiedenen NETs transfiziert, hier zeigte sich bei der Transfektion mit NET29-GFP eine verstärkte Lokalisation von Chromosom 6 an der Peripherie, während kein anderes Protein denselben Effekt hatte.

Sequenzanalyse zeigte eine mögliche Phosphorylierungsstelle an NET29, ein potentielles Target der Insulin Rezeptor Kinase. Mittels gezielter Mutagenese wurden ein Phosphomimetic und ein Phosphonull Mutant hergestellt. Chromosome Painting an Zellen, welche den jeweiligen Mutanten überexprimierten, zeigte eine stärkere Lokalisation im Kerninneren bei Überexpression des Phosphonull Mutanten.

Diese Ergebnisse könnten auf eine Rolle von NETs in der Zelldifferenzierung hinweisen, möglicherweise durch das Beeinflussen der Chromosomenlokalisation und darauf folgend der Genexpression.

Abbreviations

AmpR	ampicillin resistance
APS	ammoniumpersulfate
ATP	adenosintriphosphate
BAC	bacterial artificial chromsome
C	Celsius
cAMP	cyclic adenosinmonophosphate
cDNA	complementary DNA
DAPI	4',6-diamidino-2-phenylindole
dd	double distilled
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dUTP	deoxyribouridine triphosphate
EDTA	ethylenediaminetetraacetate.
eGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FGF	fibroblast growth factor
FISH	fluorescence in situ hybridization
FPLD	Dunnigan-type familial partial lipodystrophy
h	hour
IBMX	3-isobutyl-1-methylxanthine
IGF	insulin growth factor
INM	inner nuclear membrane
KanR	kanamycine resistance
LB	Luria Broth
min	minute
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
MS	mass spectroscopy
MudPIT	multidimensional protein identification technology
NE	nuclear envelope

NEB	New England Biolabs
NET	nuclear envelope transmembrane protein
NPC	nuclear pore complexes
OD	optical density
ONM	outer nuclear membrane
ORF	open reading frame
PBS	phosphate buffered saline
Ph0	phosphonull
PhM	phosphomimetic
RFP	red fluorescent protein
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
sec	second
SSC	saline sodium citrate
TAE	Tris-Acetate-EDTA
TEMED	Tetramethylethylenediamine
TGF	tumor growth factor
Tris	tris(hydroxymethyl)aminomethane
U	unit
UV	ultraviolet

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