Metabolic influence on chromatin organization in adipose stem cells

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List of publications

Paper I

T. Rønningen, A. Shah, A.H. Reiner, P. Collas and J.Ø. Moskaug. Epigenetic priming of inflammatory response genes by high glucose in adipose progenitor cells. *Biochemical and Biophysical Research Communications*, 2015, *in press*

Paper II

T. Rønningen*, A. Shah*, A.R. Oldenburg, K. Vekterud, E. Delbarre, J.Ø. Moskaug and P. Collas. Pre-patterning of differentiation-driven nuclear lamin A/C-interacting chromatin domains by GlcNAcylated H2B, *Genome Research*, 2015, *in press*

* shared first authorship

List of abbreviations

AMPK	adenine monophosphate activated protein kinase
ASC	adipose stem cell
bp	base pair
CEBP	CCAAT/enhancer binding protein
cLAD	constitutive LAD
CTCF	CCCTC binding factor
ChIP	chromatin immunoprecipitation
ChIP-chip	ChIP combined with microarray hybridization
ChIP-seq	ChIP combined with high throughput sequencing
CspA	cyclosporin A
CXCL5	chemokine (C-X-C motif) ligand 5
Dam	DNA adenine methyltransferase
EDD	enriched domain detector
EGF	epidermal growth factor
ESC	embryonic stem cell
EZH2	Enhancer of zeste homologue 2
bFGF	basic fibroblast growth factor
FPLD	familial partial lipodystrophy
GAD	H2BGlcNAc associated domain
O-GlcNAc	*O*-linked β-N-acetylglucosamine
GROα	growth related oncogene alpha
H	histone
HAT	histone acetyl transferase
HBP	hexosamine biosynthetic pathway
HDAC	histone deacetylase
HDM	histone demethylase
HIF1α	hypoxia induced factor 1, isoform alpha
HMM	hidden Markov modelling
HMT	histone methyl transferase
hPTM	histone post translational modification
IBMX	3-isobutyl-1-methylxanthine
IL1β	interleukin 1 beta
IL8	interleukin 8
IR	inflammatory response
INM	inner nuclear membrane
K	lysine
LAD	lamin associated domain
LAP	lamina associated peptide
LBR	lamin B receptor
MCP1	monocyte chemotactic protein 1
MNase	micrococcal nuclease

MSC	mesenchymal stem cell
NE	nuclear envelope
NL	nuclear lamina
NFκB	nuclear factor kappa-light-chain of activated B cells
OGA	*O*-GlcNAcase
OGT	*O*-GlcNAc transferase
ONM	outer nuclear membrane
PDH	pyruvate dehydrogenase
PPARγ	peroxisome proliferator activated receptor gamma
PTMs	post translational modifications
qPCR	quantitative PCR
RNA-seq	high throughput RNA sequencing
ROS	reactive oxygen species
S	serine
T	threonine
TET	ten-eleven translocase
TNFα	tumor necrosis factor alpha
TPR	tetratricopeptide repeat
SVF	stromal vascular fraction
UCP1	uncoupling protein 1
vLAD	variable LAD

1. Introduction

1.1 Adipose tissue biology and metabolism

1.1.1 Adipose tissue and disease

Dysfunctions of adipose tissue constitute the fundament of the occurrence of obesity and associated increased risk for diabetes mellitus type II and cardiovascular disease. Adipose tissue plays important roles in energy homeostasis by storing excess nutrients in the form of intracellular lipids. Upon starvation, adipose tissue is stimulated to release fatty acids and triglycerides into circulation, providing energy to peripheral tissues. In addition to its role in intermediary metabolism, adipose tissue is an endocrine organ, secreting various proteins called adipokines into circulation, affecting various tissues and organs. Adipokines include hormones such as leptin and adiponectin, with important roles in appetite regulation [1, 2], and inflammatory mediators such as tumor necrosis factor α (TNFα), interleukin 6 (IL6) and monocyte chemotactic protein 1 (MCP1) [2]. Adipose tissue of obese individuals secretes increased levels of pro-inflammatory cytokines that are believed to contribute to disease development [3, 4]. This chronic low grade systemic inflammation is believed to be involved in development of insulin resistance in peripheral tissues, partly by nuclear factor κ-light-chain-enhancer of activated B cells (NFκB)-mediated impairment of insulin signaling [5].

Adipose tissue is distributed into distinct depots and can be divided into visceral and subcutaneous adipose tissue (**Fig. 1A**). The different adipose depots have different properties, and the location of fat accumulation is correlated with disease risk [6]. Accumulation of visceral and deep abdominal subcutaneous adipose tissue is associated with increased risk of diabetes type II, cardiovascular disease and morbidity [7, 8]. In contrast, accumulation of gluteofemoral (lower body) subcutaneous fat is protective against diabetes [9]. The difference between these fat depots is presumably due to differences in their metabolic and secretory profiles [9].

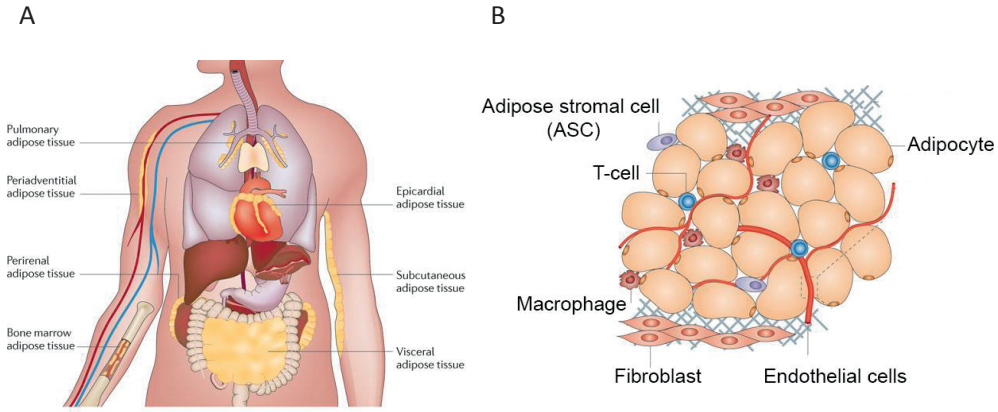

Figure 1 Adipose tissue depots and cellular composition. **(A)** Adipose tissue is mainly found in subcutaneous and visceral depots. Under conditions of obesity, adipose tissue expands in these and other depots throughout the body. Differential adipokine secretion by various adipose tissue depots may selectively affect organ function and systemic metabolism. From [2] **(B)** Cellular composition of adipose tissue. The main cell type in adipose tissue is the adipocytes, but adipose tissue also contain other cell types including immune cells (T-cells, macrophages), fibroblasts, progenitor cells (ASCs), fibroblasts, and endothelial cells covering blood vessel walls. All these cell types are important for adipose tissue function. Modified from [2].

The major cell type of adipose tissue is the adipocyte, which stores lipids in the cytoplasm (**Fig. 1B**). Adipocytes can be classified into white and brown adipocytes. White adipocytes are the classical adipocytes of white color due to high lipid contents. Brown adipocytes are also lipid-filled, but have a brown color due to high mitochondria content. Brown adipocytes have a high metabolic rate and express uncoupling protein 1 (UCP1), which plays a key role in thermogenesis [10]. In small rodents, brown adipocytes are responsible for maintaining body temperature. The existence of brown adipocytes in humans has been debated, and until recently it was believed that these cells were only present in infants. It has however been shown that adults have metabolically active adipose tissue expressing UCP1 [11, 12]. White and brown adipocytes are believed to derive from distinct developmental origins [13]. White adipocytes originate from adipose progenitor cells whereas brown adipocytes are derived from myogenic precursors, due to expression of the myogenic factor MYF5 [14]. Another class of adipocytes was recently discovered in mice models in which white adipocytes can acquire a brown phenotype *in vivo*. These so-called beige cells can express high levels of UCP1 upon cold exposure or activation of cyclic AMP (cAMP)-dependent pathways [15, 16].

Adipose tissue harbors a large fraction of immune cells including neutrophils, macrophages and T cells (**Fig. 1B**). In line with the increased inflammatory state of

adipose tissue, adipose tissue of obese individuals contains an elevated number of immune cells compared to lean individuals; this includes local accumulations of macrophages in crown-like structures surrounding necrotic adipocytes [17]. These macrophages secrete high amounts of pro-inflammatory mediators, including the central inflammatory regulators TNFα and interleukin 1 beta (IL1β) [18] and are believed to contribute in large extent to increased levels of circulating inflammatory molecules in obese individuals [19].

1.1.2 Adipose stem cells

Adipose tissue also contains progenitor cells/stem cells. A stem cell is an unspecialized cell with the capacity to self-renew and differentiate [20]. Stem cells can be classified based on their differentiation potential. Pluripotent stem cells, such as embryonic stem cells (ESCs) derived from the inner cell mass of the blastocyst [21], can differentiate into cells of all three germ layers (endoderm, ectoderm, mesoderm). Multipotent cells harbor a more restricted differentiation capacity; these include tissue-specific progenitor cells such as neural progenitor cells (NPCs), hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs).

MSCs are stromal cells found in various stromal tissues but were first identified among cells isolated from bone marrow [22]. MSCs have also been identified in adipose tissue and can be isolated from the stromal vascular fraction (SVF) of liposuction aspirates [23, 24]. Adipose tissue MSCs are also commonly referred to as adipocyte progenitor cells, adipose stromal cells or adipose stem cells, these terms are used somewhat arbitrarily in the literature. In this thesis, these cells are referred to as adipose stem cells (ASCs). Isolation of ASCs from adipose tissue involves enzymatic digestion of the tissue to break down the extracellular matrix, sedimentation of SVF, and removal of leukocytes and endothelial cells by negative selection against CD45 (a hematopoietic cell surface marker) and CD31 (an endothelial cell surface marker). The remaining cells are seeded and expanded in culture [25]. Of note, while the $CD45^{-}CD31^{-}$ plated SVF contains ASCs [24], plating of the SVF leads to a 'positive selection' of ASCs because standard ASC culture conditions do not support attachment of hematopoietic and endothelial progenitors. Thus within 1-2 days, remaining cells have gene and surface marker expression profiles virtually undistinguishable from $CD45^{-}$ and $CD31^{-}$ sorted cultured cells (Boquest, Collas, unpublished). ASCs used in our work were derived from cultured SVF. ASCs share many common properties with MSCs from bone marrow [26]. A

definition of MSCs has been proposed by the International Society for Cellular Therapy [27]: (i) MSCs have potential for differentiation toward the mesodermal lineages including adipogenic, osteogenic and chondrogenic lineages [28]; (ii) cells must grow adherent in culture; (iii) cells must express a defined set of surface markers including CD105, CD73, CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLADR [27].

1.1.3 Immunomodulatory properties of MSCs

MSCs show unique immunomodulatory properties [29]. MSCs are immuno-privileged due to low expression of MHC class II [30], which may be linked to their beneficial effect dampening a graft vs. host disease response [31] and in treating type I diabetes [32]. MSCs modulate the function of a wide range of immune cells *in vitro* [33]. MSCs inhibit proliferation of T lymphocytes and natural killer cells, impair antibody production by B cells, inhibit maturation and function of dendritic cells and affect macrophage polarization [33]. These effects are mediated partly through interaction with immune cells, and through secretion of soluble immunomodulatory factors such as indoleamine 2,3-dioxygenase (IDO), tumor necrosis factor-induced protein 6 (TSG6), prostaglandin E2 (PGE2), IL6 and nitric oxide (NO) [34].

MSCs act both in a pro- and anti-inflammatory manner depending on the microenvironment [35]. Upon stimulation of toll-like receptor (TLR) 2 or 4 by bacterial products or other stimuli, MSCs act pro-inflammatory, attracting and promoting differentiation of monocytes into the pro-inflammatory M1 phenotype [36]. MSCs can also secrete chemokines promoting neutrophil infiltration [37]. On the other hand, co-culture of native MSCs with monocytes promotes differentiation of monocytes to the anti-inflammatory M2 subset of macrophages [38]. Unlike classically activated M1-macrophages, M2 macrophages are anti-inflammatory, promoting tissue repair [38, 39].

MSCs secrete chemokines, which attracts immune cells and facilitates MSC-immune cell interactions [40]. Infiltration of innate immune cells, including monocytes and neutrophils, into adipose tissue may contribute to adipose tissue inflammation [41]. Chemokines relevant to the work presented in this thesis are the monocyte attracting proteins chemerin (encoded by the *RARRES2* gene) and MCP1 (encoded by the *CCL2* gene). Chemerin attracts monocytes and dendritic cells, and its serum levels increase in obese individuals [42, 43]. Chemerin also plays roles in adipogenic differentiation [44]. MCP1 is involved in monocyte attraction and in increased monocyte infiltration

associated with obesity [45, 46]. MCP1 also promotes monocyte proliferation within adipose tissue, exacerbating the inflammatory state [47]. The neutrophil attracting chemokines interleukin 8 (IL8), growth regulated oncogene alpha (GROα; encoded by *CXCL1*) and chemokine (C-X-C motif) ligand 5 (CXCL5) are involved in neutrophil attraction and angiogenesis, and generally act in a pro-inflammatory manner. MSCs promote neutrophil infiltration in the initial stages of inflammation [48]. IL1β is a central initiator of inflammation that acts by activating NFκB in target cells, leading to expression of pro-inflammatory mediators [49]. IL1β is involved in induction of insulin resistance in adipocytes [50].

1.1.4 Stem cell metabolism

Adipose tissue is a metabolically active organ, and it is likely that ASCs are influenced by the surrounding metabolic state. Metabolism plays important roles in determining stem cell fate. Similar to cancer cells [51], proliferating stem cells rely highly on anaerobic glycolysis for energy, rather than glycolysis linked to the Krebs cycle and oxidative phosphorylation [52]. ESCs show high levels of glycolytic metabolism [53]. Similarly when somatic cells are reprogrammed into induced pluripotent stem cells, a shift from oxidative to glycolytic metabolism is observed [54]. Multipotent adult stem cells rely on different combinations of glycolysis and oxidative phosphorylation in their proliferative state [55].

It has been hypothesized that stem cells use glycolysis even in the presence of oxygen to prevent oxidative damage, which may accelerate cell senescence [55]. Also, many stem cells reside in a hypoxic environment *in vivo*, thus glycolysis may be an adaptive mechanism to this environment. Differentiation of MSCs is affected by cellular metabolic states. Chondrogenic differentiation of MSCs typically increases glycolytic metabolism, while osteogenic and adipogenic differentiation is accompanied by increased mitochondrial metabolism [56-58]. Increased mitochondrial metabolism during adipogenic differentiation is partly due to the increased demand for energy for synthesis of fatty acids, and reactive oxygen species (ROS) produced during oxidative phosphorylation is indeed required for activation of the adipogenic transcription program [57].

A major regulator of the glycolytic pathway is the transcription factor hypoxia induced factor 1, isoform alpha (HIF1α). Protein stability of HIF1α is regulated by oxygen tension and HIF1α is rapidly degraded under normoxic conditions. In a hypoxic

environment, HIF1α upregulates genes encoding glycolytic enzymes [59]. HIF1α also increases expression of pyruvate dehydrogenase kinases PDK2 and PDK4, which phosphorylate pyruvate dehydrogenase (PDH), lowering the amount of pyruvate entering the Krebs cycle, and hence reducing mitochondrial metabolism [60]. HIF1α activity is also responsive to inflammatory stimulation by TNFα [61]. In **Paper II**, we have investigated gene expression changes associated with glycolytic metabolism and the HIF1α pathway during adipogenic differentiation.

1.2. Chromatin changes in adipogenesis

1.2.1 Adipogenic differentiation

Adipose tissue expansion observed in weight gain is caused by both an increase in adipocyte volume and number [6]. Approximately 10% of fat mass in adult humans is renewed annually, presumably by differentiation of adipose progenitor cells [62]. Most of our knowledge on the mechanisms of adipogenic differentiation emanates from *in vitro* studies. In our studies, ASCs are expanded in medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [63]. Proliferating cells are seeded confluent in medium without EGF and FGF to induce cell cycle arrest. Cell confluency induces changes in cellular shape and intracellular architecture, favoring adipogenic differentiation [64]. Differentiation is elicited by an adipogenic cocktail containing dexamethasone, indomethacin, 3-isobutyl-1-methylxanthine (IBMX) and insulin (**Fig. 2A**). These drugs collectively induce an adipogenic differentiation program which includes changes in cytoskeletal organization and in the appearance of lipid droplets (**Fig. 2B**). In addition, accumulating cytoplasmic lipids compress the nucleus against the cellular periphery and causes it to shrink, most likely affecting chromatin organization [65]. **Paper II** examines large-scale chromatin organization changes elicited during the early adipogenic process, indeed revealing major changes.

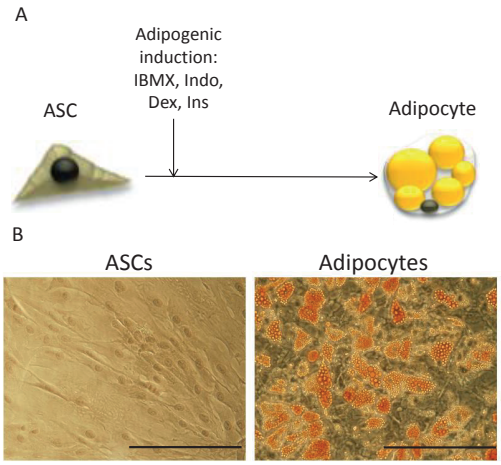

Figure 2 Adipogenic differentiation of ASCs. (**A)** Adipogenesis is induced in ASCs after incubation with IMBX, indomethacine, dexamethasone and insulin [66] (**B)** Undifferentiated and differentiated ASCs (day 21) stained with Oil Red-O to visualize lipid droplets. Scale bars: 200 μm.

1.2.2 Transcriptional changes in adipogenic differentiation

Adipogenic induction is accompanied by changes in gene expression [66] facilitated by chromatin remodeling and induction of transcription factor networks [67]. The transcription factors peroxisome proliferator-activated receptor γ (PPARγ), and CCAAT/enhancer binding proteins (CEBP) CEBPα, CEBPβ and CEBPδ are central in this process. PPARγ is a master regulator of adipogenesis necessary and sufficient for adipogenic differentiation *in vivo* and *in vitro* [68, 69]. In its active form, PPARγ forms a dimer with retinoic acid X receptor (RXR) to activate target genes [64]. PPARγ colocalizes with CEBPα and both cooperate to elicit adipogenesis [70]. Remarkably, ~60% of genes induced during differentiation have PPARγ or CEBPα bound at the promoter [71]. Induction of adipogenic differentiation involves expression of transcription factors in a regulated sequential manner (**Fig. 3A**) [72]. The first wave of expression entails upregulation of CEBPβ, CEBPδ and Kruppel like factors (KLFs), and downregulation of repressors of adipogenesis, including GATA2 and KLF2. These transcription factors activate transcription of genes of the second transcriptional wave, in particular PPARγ and CEBPα, which further activate transcription of genes associated with adipocyte formation and lipid metabolism [72].

Waves of gene expression are also detected at a genome-wide level. Our laboratory has characterized transcriptional changes at 4 time points of differentiation using high-throughput RNA sequencing (RNA-seq). Time points examined were ‘day-2’

(D-2), that is, undifferentiated ASCs cultured under proliferation conditions (with EGF and bFGF; see above); day 0 (D0), the time point at which ASCs are confluent; this is also the time point of addition of the adipogenic cocktail; day 3 (D3), immature adipocytes, 3 days after adipogenic induction; and day 9 (D9), differentiated adipocytes [66, 73]. Transcriptomic data identify several gene clusters with distinct expression patterns (**Fig. 3B)**. While some clusters are sequentially up- or downregulated, others are only transiently up- or downregulated. In particular, one cluster includes genes expressed in undifferentiated ASCs that are upregulated upon adipogenic induction (cluster 8; **Fig. 3B**); this cluster includes many genes associated with lipid metabolism and adipocyte formation [66]. Adipogenesis therefore involves coordinated gene expression modules or waves of expression, leading to expression of genes associated with lipid metabolism and adipocyte formation. This thesis explores chromatin changes that accompany this process.

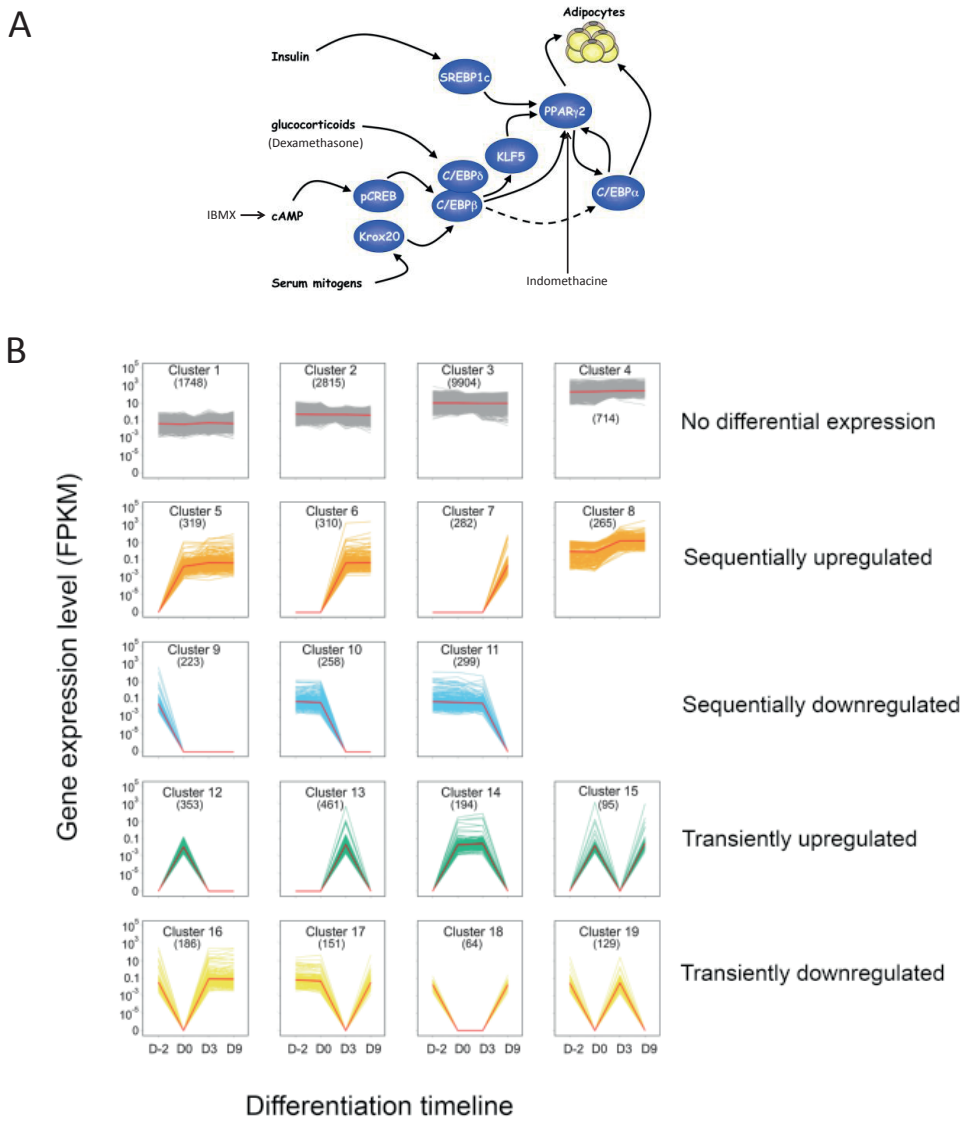

Figure 3 Transcriptional changes in differentiation of ASCs into adipocytes. (**A)** Adipogenic stimulation of ASCs with Insulin, IBMX, dexamethasone and indomethacin induces activity of transcription factor networks, ultimately leading to PPARγ activation and expression of adipocyte specific genes. Modified from [74] (**B)** Hierarchial clustering of genes into 19 clusters based on expression patters throughout adipogenic differentiation of ASCs (Day -2, 0, 3 and 9). Modified from [66].

1.2.3 Changes in histone modifications and chromatin states during adipogenic differentiation

Post-translational histone modifications

Cells of multicellular organisms in principal share the same genome, but show highly diverse phenotypes. These differences are mainly regulated by epigenetic mechanisms. *Epi*-genetics means *on top of*' genetics and is defined as heritable biochemical changes of DNA or chromatin that result in changes in gene expression without affecting the DNA sequence [75]. Epigenetics involves covalent modifications of DNA including methylation on CpG nucleotides, post-translational modifications (PTMs) of histones and action of non-coding RNAs. Chromatin refers to the complex of DNA and associated proteins in the eukaryotic nucleus. Generally, chromatin is divided into open, accessible, transcriptionally permissive euchromatin, and transcriptionally silenced heterochromatin. The basic unit of chromatin is the nucleosome which consists of DNA wrapped 1.65 times around a histone octamer consisting of two dimers of histone H2A and H2B and one tetramer with two copies of each of histone H3 and H4 [76]. The nucleosomes are separated by the linker histone H1, which is involved in regulating nucleosome density [77]. Histones have N-terminal tails that protrude from the core of the nucleosome. Both the globular domain and the tail of histones are common targets for PTMs that affect chromatin structure and function [78, 79]. These modifications include acetylation, methylation, phosphorylation, ubiquitylation, ADP-ribosylation, sumoylation and *O*-GlcNAcylation [80]. Combinations of histone modifications affect transcription and chromatin organization by affecting chromatin accessibility or facilitating binding of transcription factors or other chromatin associated proteins. Transcriptional changes occurring during adipogenic differentiation are accompanied by epigenetic changes, including large scale chromatin remodeling and changes in patterns of histone modifications [66, 67, 81].

Histone acetylation is mainly associated with transcription and transcriptionally permissive chromatin. Acetylation by histone acetyl transferases (HATs) of positively charged histones reduces the global charge of histones and their binding to negatively charged DNA, thus promoting a more open chromatin configuration [82]. Histone deacetylases (HDACs) catalyze removal of acetyl residues and chromatin condensation. Typically in active gene promoters, histone H3 is acetylated on lysine (K) 9 (H3K9ac) [83], while active enhancer regions are enriched in H3K27ac [84, 85].

Histone methylation is associated with transcriptional activity of silencing depending on which residue and to what extent it is methylated (mono, di or trimethylation). Histone methyltransferases (HMTs) catalyze methylation of lysine and arginine residues, while histone demethylases (HDMs) remove methyl groups [86]. These enzymes are highly specific. In particular, methylation on lysines (K) on H3 has been shown to be related to transcriptional outcome. Methylation of H3K4 is typically associated with transcriptional activity. Mono- and dimethylation of H3K4 (H3K4me1/2) is associated with enhancer regions [85], while actively transcribed genes typically contain H3K4me3 in promoter regions. However, H3K4me3 is not always associated with transcriptional activity, as it is also present on silenced genes, e.g. on bivalently marked promoters (described below). H3K36me3 has also been associated with gene expression and is typically enriched in gene bodies. In contrast, methylation on H3K9 and H3K27 is associated with transcriptional repression. H3K9me2/3 is enriched in heterochromatin [87], and acts by recruiting heterochromatin protein 1 (HP1/CBX3), which facilitates chromatin compaction [88, 89]. H3K9me3 can also be present in euchromatic regions, and H3K9me3 in promoters is typically associated with long term gene repression [90]. H3K27me3 on promoter regions is also associated with gene repression, however, these promoters have potential to become rapidly induced [91]. H3K27me3 have important roles in development, and is catalyzed by EZH2, a subunit of the polycomb repressor complex PRC2. Additional PTMs also provide biochemical and functional properties to histones [80]; however these are not discussed in further detail.

The distribution of histone PTMs (hPTMs) throughout the genome can be assessed by chromatin immunoprecipitation (ChIP) [92]. In the ChIP assay, proteins and DNA are cross-linked with formaldehyde and chromatin is sonicated to generate 200-500 base pair fragments (bp). The protein of interest is immunoprecipitated with a specific antibody, and associated DNA is purified and analyzed by quantitative PCR (qPCR), hybridization to DNA microarrays or whole genome sequencing. In the case of ChIP-sequencing (ChIP-seq), the DNA fragments are mapped to the genome, and their enrichment (or number of sequence reads) in the immunoprecipitated fraction is normalized to the number of reads obtained from sequencing the 'input' chromatin. Enrichment profiles can be viewed in a genome browser (e.g. the integrated genomic browser; IGV; [93]) (**Fig. 4A**). Regions enriched in the protein of interest can be determined by peak calling algorithms [94]. It is not the purpose of this introduction to describe peaks callers used in analysis of ChIP-seq data. Suffice is to mention that the

width of the enriched domains detected is a key factor for the choice of the algorithm. As described below, nuclear lamins (structural proteins of the nuclear periphery), associate with chromatin in the form of large domains; this has necessitated development in our laboratory of a new algorithm (enriched domain detector, or EDD) [94], to identify significant interactions. EDD has been used in the present work to identify lamin-interaction domains and domains of GlcNAcylated histone H2B (Paper II).

Dynamic chromatin states during cell differentiation

Epigenetic modifications act in a combinatorial manner to regulate gene expression and chromatin function. Histone modifications occur in combinations, resulting in 'chromatin states'. Promoters of developmentally regulated genes have been described in ESCs to be marked by a combination of H3K4me3 and H3K27me3, a so-called 'bivalent' state [91, 95]. H3K27me3 at promoter regions is associated with transcriptional repression even in the presence of H3K4me3. These genes are 'poised', meaning that they retain the potential for expression. Adipogenic promoters are also marked by H3K4me3/H3K27me3 in ASCs, and differentiation elicits a reduction in H3K27me3 consistent with transcriptional activation, while H3K4me3 is retained [96].

Combinations of chromatin marks (histone PTMs and other chromatin-bound proteins) and their dynamics over time can be examined in a comprehensive manner using Hidden Markov Modeling (HMM) techniques. ChromHMM [97] is a machine learning algorithm well suited for the modeling discovery of chromatin states, that is, recurrent combinations of chromatin marks throughout the genome (**Fig. 4A,B**). We have used ChromHMM in Papers I and II. In Paper I, we describe the chromatin environment of inflammatory response genes and how the prevalence of chromatin states is altered between expressed and non-expressed genes. In Paper II, ChromHMM has been used to describe the chromatin environment of H2BGlcNAc and lamin A/C (see below), and determine how it evolves during adipogenic differentiation.

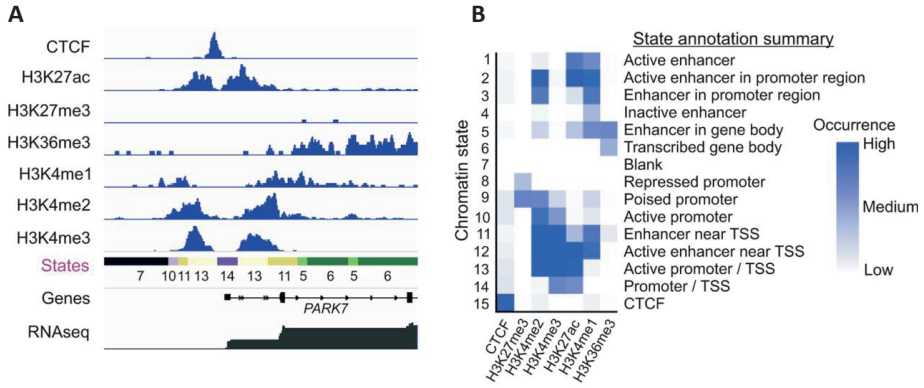

Figure 4 Chromatin states analyzed by ChromHMM. (**A)** Enrichment of histone modifications and CTCF binding sites on a 6 kb region of Chromosome 1. States are indicated and colored based on the classification of states in B. (**B)** ChromHMM emission parameters and functional element annotations from the 15 states. Color intensity indicates the level of enrichment of the indicated histone mark. From [66].

Using ChromHMM, our laboratory has recently reported dynamic changes in chromatin states during adipogenic differentiation of ASCs [66]. This study reveals that the number of states within the promoter and gene body largely depends on the expression *level* of the associated genes, rather than *changes* in expression. Highly expressed genes generally display the highest number of (i.e. variation in) chromatin states, while lowly expressed genes are occupied by few chromatin states. During differentiation, clusters containing highly expressed genes are also the most dynamic, meaning that chromatin states change the most at these loci between differentiation time points. In particular, genes expressed before differentiation and upregulated after adipogenic induction, are the most dynamic. This cluster notably includes genes associated with adipocyte differentiation and metabolism. The most dynamic state is the bivalently marked promoters, consistent with a role of bivalency in regulating adipogenesis [66]. Collectively, this work shows that the chromatin landscape is extensively remodeled in the early stages of adipogenic differentiation.

1.3. Metabolic influence on epigenetics

1.3.1 Metabolic regulation of epigenetics

Obesity and adipose tissue expansion is associated with increased risk of developing insulin resistance and diabetes type II, conditions that causes somatic cells to continuously be exposed to elevated glucose levels. Hyperglycemia has been associated with epigenetic changes in promoters of inflammatory genes [98]. These changes have in turn been linked to a 'metabolic memory' concept associated with diabetes, where diabetic patients experience complications even after treatment and restoration of normal blood glucose levels [99, 100]. NFκB is considered a central player in the pathology of diabetes [101]. Exposure to transient hyperglycemia increases transcription of *RELA* encoding P65, an NFκB subunit, and elevated *RELA* transcript levels persist even after reduction of glucose levels to normal levels [102]. These changes have been ascribed to changes in promoter histone methylation patterns. Transient exposure of endothelial cells to hyperglycemic conditions increases occupancy of H3K4me1 on the *RELA* promoter. This effect has been ascribed to increased activity of the HMT SET7 [102, 103]. The *RELA* promoter also shows reduced H3K9me2/3 after exposure to high glucose, which correlates with increased binding of the HDM LSD1 and reduced levels of the HMT SUV39H1 [103, 104]. Diabetic complications may also be epigenetically regulated by increased levels of *O*-GlcNAcylated proteins affecting transcriptional outcome [105]. Interestingly, GlcNAcylation of NFκB upon exposure to elevated glucose increases its activity [106]. GlcNAcylation and its role in transcription are discussed in the next section.

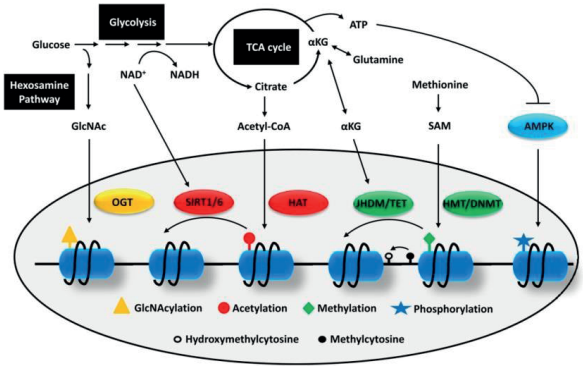

Figure 5. Crosstalk between intermediates in metabolism and DNA and histone modifying enzymes. From [107].

Nutrients may affect the epigenome in several ways (**Fig. 5**). Intracellular concentrations of specific metabolites may directly act as substrates for histone modifying enzymes, so the level of some histone modifications may be directly influenced by intracellular nutrient levels. These metabolites include acetyl CoA, UDP-GlcNAc and S-Adenosyl-methionine (SAM), which serve as donors for histone acetyltransferases, *O*-GlcNAc transferase and DNA or histone methyl transferases, respectively. Other metabolites act as co-factors for chromatin modifying enzymes. These include NAD^+, a co-factor for HDACs SIRT1 and SIRT6, α-ketoglutarate, a co-factor for the Jumonji domain class of HDMs and ten-eleven translocation (TET) enzymes involved in DNA demethylation [107]. Additionally, phosphorylation of histones can be directly regulated by cellular metabolic state through adenosine monophosphate activated protein kinase (AMPK) activated in response to increasing intracellular AMP levels [108]. There is also extensive crosstalk between these pathways; for instance, AMPK can phosphorylate the *O*-GlcNAc transferase (OGT), the enzyme responsible for *O*-GlcNAc modification, affecting its nuclear localization and indirectly, GlcNAcylation of histones [109].

1.3.2 *O*-GlcNAc modifications of cytoplasmic and nucleoplasmic proteins

Two to five percent of glucose taken up by cells is routed from glycolysis to the hexosamine biosynthetic pathway (HBP). The HBP integrates signals from metabolism of several nutrients including glucose, fatty acids, amino acid and nucleotides (**Fig. 6A**). The end-product of the HBP is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), a substrate for complex extracellular *N*-linked glycosylation and for *O*-linked glycosylation (*O*-GlcNAc) of ~3000 nuclear and cytoplasmic proteins [110]. Unlike extracellular glycosylation, *O*-GlcNAcylation involves addition of a single GlcNAc moiety to serine (S) or threonine (T) residues on target proteins. Protein GlcNAcylation is reversible and plays important functions in cellular signaling. Additionally, as the proportion of intracellular glucose directed to the HBP is relatively constant, activity of the HBP is directly proportional to extracellular glucose uptake. Consequently, UDP-GlcNAc levels fluctuate in proportion to intracellular glucose levels and thus can be considered a glucose sensor [111].

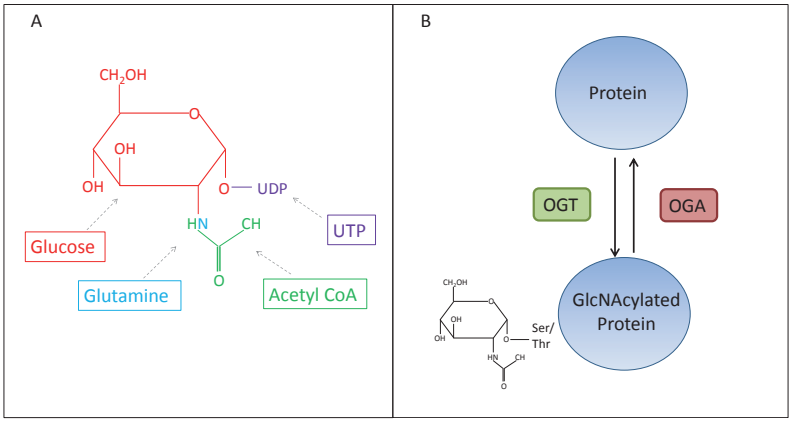

Figure 6 (A) Chemical composition of UDP-GlcNAc. UDP-GlcNAc integrates signals from both carbohydrate (glucose), lipid (acetyl coA), amino acid (glutamine) and nucleotide (UTP) metabolism. **(B)** PTM of proteins with *O*-GlcNAc is catalyzed by the enzyme *O*-GlcNAc transferase (OGT), while hydrolysis is catalyzed by *O*-GlcNAcase (OGA).

Aberrant GlcNAc cycling has been associated with disease including various cancers and diabetes [112]. Exposure of cells to hyperglycemia increases the global levels of GlcNAc modified proteins [112]. Increased GlcNAc levels were also shown to induce insulin resistance in adipocytes [111]. Consistently, knocking down GFAT (the enzyme catalyzing the committed step in HBP) reverse glucose induce insulin resistance. Thus, the HBP has a causal role in diabetes development

O-GlcNAc transferase (OGT) catalyzes the GlcNAcylation of serine or threonine residues on target proteins (**Fig. 6B**). OGT is encoded by a single gene in humans, located on the X chromosome [113]. It is essential for mammalian development, and OGT knockout mice die during early embryonic development [113]. In *C. elegans*, OGT knockout is not lethal; however OGT knockout mutants show severe metabolic defects, suggesting a role for *O*-GlcNAc in metabolic regulation [114]. Three different OGT splice variants have been identified in humans; these mainly differ in their sub-cellular localization and the length of their N-terminal tetratricopeptide repeat region (TPR) domain [115]. Nucleocytoplasmic (ncOGT) and short OGT (sOGT) are both present in the nucleus and the cytoplasm, differing only in the length of their TPR domains (12 and 2.5 repeats, respectively). The TPR region is important for substrate recognition by OGT [116]. Moreover, a mitochondrial form of OGT (mOGT) contains a mitochondrial location signal. We have in the course of this work found that in ASCs, sOGT is the most abundant isoform while ncOGT is the dominant form in other cells like HeLa cells (Rønningen and Moskaug, unpublished data). Functional differences between these

isoforms remain largely unknown; however it is possible that intracellular OGT isoform distribution or concentration may regulate the substrate specificity of OGT.

Removal of GlcNAc residues from target proteins is catalyzed by *O*-GlcNAcase (**Fig. 6B**). GlcNAc turnover is rapid for many proteins and relies on OGA activity. OGA conditional knockout mice show severe metabolic and transcriptional defects, suggesting that *O*-GlcNAc cycling is important for metabolic regulation [117]. Expression level and activity of OGT and OGA are tightly coupled and evidence suggests that these two enzymes mutually regulate their activity, maintaining an intracellular 'GlcNAc balance'. Intriguingly, OGA expression is reduced or abolished following OGT knock-down or knock-out [118] (Rønningen, Moskaug, unpublished results). How this down-regulation occurs is unknown, but one possibility is that OGA is post-translationally modified by OGT; indeed, both OGT and OGA have been shown to be GlcNAcylated [116].

Many proteins are GlcNAcylated. GlcNAcylation may (i) affect protein folding, in turn modulating protein-interactions or stability, (ii) compete or act synergistically with phosphorylation, or (iii) act as recruitment signals for other proteins [110]. Consistent with the essential role of OGT in development [113], OGT and *O*-GlcNAc modifications are associated with metabolism, cell cycle regulation, transcription and translation. GlcNAc also has important roles in stem cell differentiation [119]. Accordingly, adipocyte differentiation is influenced by GlcNAc cycling. In 3T3L1 pre-adipocytes, protein GlcNAcylation increases during adipogenic differentiation. Further, inhibition of the HBP inhibits adipogenic differentiation [120]. Of note, CEBPα, CEBPβ [121] and PPARγ [122] are modified by *O*-GlcNAcylation, although the effect of these modifications on differentiation remains unclear. Increasing GlcNAcylation on CEBPβ or PPARγ by inhibiting OGA inhibits adipogenic differentiation [121, 122]; however this effect is not necessarily due to these specific modifications, as OGA inhibition affects GlcNAcylation of many proteins. More studies are needed to elucidate the mechanisms by which GlcNAc is involved in regulating adipogenic differentiation.

Various nuclear proteins are *O*-GlcNAcylated, including transcription factors [106, 123, 124], histone modifying enzymes [125, 126], nuclear pore complex proteins [127] and proteins associated with the nuclear lamina [128]. OGT is involved in several aspects of transcriptional regulation. Supporting a role of OGT in transcriptional activation, OGT has been identified as a component of the transcriptional pre-initiation complex and RNA polymerase II has been shown to be GlcNAcylated on serine 5 and 7 [129]. Furthermore, OGT knockdown reduces the rate of transcriptional initiation [129,

130]. OGT is also found in association with enzymes linked to transcriptionally active chromatin, including TET2/3 enzymes implicated in DNA demethylation [126, 131], and SET1/COMPASS which is responsible for H3K4me3 [126]. GlcNAcylation is also associated with increased activity of transcription factors including NFκB [106]. In contrast, OGT can also be linked to transcriptional repression. In *Drosophila*, OGT is a Polycomb Group protein essential for Polycomb-mediated gene repression during development [132, 133]. Also, *O*-GlcNAcylation of EZH2 regulates EZH2 protein stability, and H3K27me3 is partly dependent on OGT expression [125]. Additionally, GlcNAcylation of the SIN3A subunit of HDAC1 has repressive effects on gene expression [134]. OGT thus harbors multiple and complex functions in transcriptional regulation.

O-GlcNAc sites have been identified on all four core histones; these include T101 on H2A, S36 and S112 on H2B, S10 and T32 on H3 and S47 on H4 [135-138]. The function of these PTMs remains largely unclear. GlcNAcylation of H3S10 (H3S10GlcNAc) has been associated with mitosis regulation and transcriptional repression [136]. Interestingly, this modification may affect modifications on the neighboring H3K9 favoring methylation rather that acetylation [136]. GlcNAcylation of H2AT101 is involved in destabilization of nucleosomes *in vitro*, providing a chromatin state permissive for transcription [139].

GlcNAcylation of H2BS112 (H2BS112GlcNAc; abbreviated in Paper II as 'H2BGlcNAc') has been associated with transcriptional activation in HeLa cells [138]. Indeed, Fujiki and colleagues report a correlation between H2BS112GlcNAc and monoubiquitylation on H2BK120 (H2BK120ub1), a PTM associated with transcriptional elongation [140, 141]. Fujiki et. al. also show that OGT is necessary for H2BK120Ub1, as mutating S112 on H2B reduces levels of H2BK120ub1[138]. The authors propose that the S112-GlcNAc residue on H2B serves as anchor for the ubiquitin ligase BRE1A, which facilitates ubiquitylation and subsequent gene activation [138]. In ESCs, TET2 is necessary for recruitment of OGT to for modification of H2BS112 [131]. H2BS112GlcNAc has also been shown to be negatively regulated by AMPK [109], supporting the role of this modification as a metabolic sensor. In Paper II, we have mapped by ChIP-seq the distribution of H2BS112GlcNAc during adipogenic differentiation, and propose that domains of H2BS112GlcNAc serve as platforms for *de novo* association of chromatin with nuclear lamins.

1.4. Interactions of chromatin with the nuclear lamina

1.4.1 The nuclear lamina

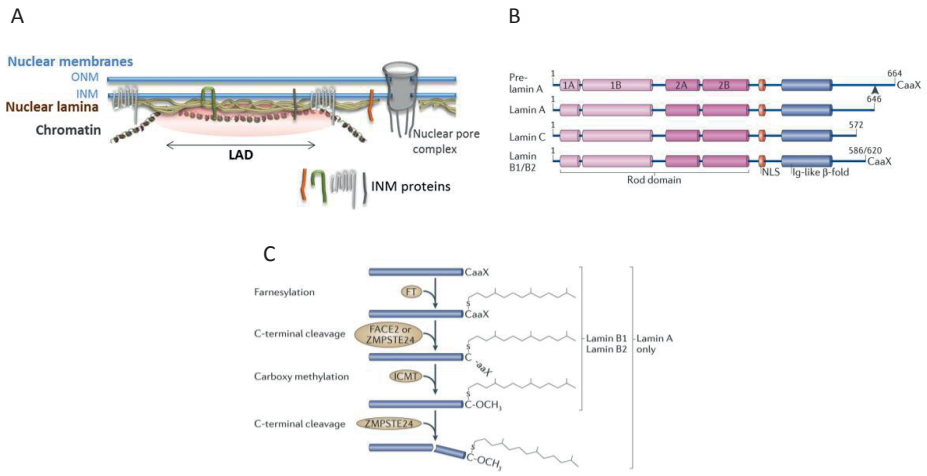

Figure 7 Lamins and the nuclear lamina. (**A)** Schematic overview of the nuclear envelope, with the outer nuclear membrane (ONM), inner nuclear membrane (INM) and perinuclear space. Nuclear pore complexes span the nuclear envelope, facilitating transport of proteins. The nuclear lamina covers the inside of the INM, and is associated with inner nuclear membrane proteins and chromatin in regions called lamina associated domains or LADs. Modified from [142]. (**B**) A- and B-type lamins have similar structure, containing a Rod domain, a nuclear localization signal and a Ig-fold. Lamins mainly differ in the post translational modifications and length of their C-terminal tail. From [143]. (**C)** Lamin A and lamin B are farnesylated on the cysteine residue of their CaaX domain followed by cleavage of the aaX motif and methylation of the cysteine residue. Lamin A, but not lamin B is cleaved by the protease ZMPSTE24, removing the farnesyl group. From [143].

We have earlier described how progenitor cell differentiation entails chromatin and gene expression changes. Differentiation also involves large-scale changes in the spatial distribution of chromatin, including re-organization of chromatin at the nuclear lamina. In eukaryotic cells, the genome is physically separated from cytoplasm by the nuclear envelope. The nuclear envelope consists of a double membrane where the outer nuclear membrane is continuous with the endoplasmatic reticulum. Nuclear pore complexes perforate the nuclear envelope to facilitate transport of macromolecules in and out of the nucleus. The nucleoplasmic side of the inner nuclear membrane (INM) is adjacent to a network of filamentous proteins called the nuclear lamina (**Fig. 7A**). The nuclear lamina is biochemically defined as the fibrous component of the nucleus which is resistant to nucleases, non-ionic detergents and high salt [144]. The nuclear lamina plays important functions in the organization and activities of the nucleus. It mediates contact between microtubules, actin and intermediate filaments in the cytoplasm and the nuclear lamina

through interaction with proteins of the linker of the nucleoskeleton and cytoskeleton (LINC) complex [143, 145]. The LINC complex spans the inner nuclear membrane and interacts with emerin (in the inner nuclear membrane) and nuclear lamins, providing a mechanism for signaling from the cytoplasm to the nuclear interior [146]. The nuclear lamina also provides mechanical strength (mechano-resistance) to the nucleus and contributes to maintaining nuclear shape [147, 148]. The lamina also plays important roles in chromatin organization, tethering transcription factors and signaling molecules [149].

A major component of the nuclear lamina is the nuclear lamins, which are type V intermediate filament proteins. Nuclear lamins are composed of A- and B- type lamins based on their structural, functional and biochemical properties. B-type lamins (lamins B1 and B2, encoded by the *LMNB1* and *LMNB2* genes respectively) are ubiquitously expressed. B-type lamins harbor a C-terminal CaaX motif which is post-translationally modified by farnesylation (**Fig. 7B, C**). The farnesyl group is hydrophobic and enables insertion of B-type lamins into the lipid bilayer of the inner nuclear membrane at the nuclear envelope. Due to their C-terminal farnesylation, B-type lamins remain membrane-anchored during the entire cell cycle, even during mitosis where they remain associated with the endoplasmic reticulum [150].

A-type lamins, namely lamins A and C (also often referred to as lamin A/C) are splice variants of the *LMNA* gene. As in B-type lamins, the C-terminal CaaX motif of lamin A is farnesylated. However, unlike for B-type lamins, the metalloprotease ZMPSTE24 cleaves the farnesyl group from lamin A after incorporation into the nuclear lamina (**Fig. 7C**). Lamin C is a shorter *LMNA* splice variant which does not contain a CaaX motif and is not farnesylated. Accordingly, A-type lamins are not inserted into the INM. They polymerize into the nuclear lamina at the nuclear periphery, but also exist as a detergent-soluble nucleoplasmic pool [151]. While the functions of nucleoplasmic A-type lamins remain unknown, evidence implicates the nucleoplasmic protein lamina-associated polypeptide LAP2α, which directly binds lamin A/C, in the regulation of the nucleoplasmic vs. peripheral pools of lamin A/C [152, 153].

Unlike B-type lamins, A-type lamins are developmentally regulated and show variable expression level between cell types [154] and during stem cell differentiation [155]. Lamin A/C is not or weakly expressed in pluripotent ESCs, and is expressed to a variable extent in lineage committed progenitor cells and differentiated cells [154, 156, 157]. Lamin A/C play a role in differentiation of tissue-specific stem cells [153, 158, 159].

The role of A-type lamins in development is unclear, but may at least in part be linked to changes in chromatin organization.

Interestingly, the nucleoplasmic vs peripheral distribution of A-type lamins has been shown to be important in the differentiation of somatic progenitor cells [160]. Nucleoplasmic lamin A/C and LAP2α both affect retinoblastoma protein function [161] and promote cell cycle arrest in somatic progenitor cells [153, 158, 159, 162]. Moreover, LAP2α overexpression in mouse pre-adipocytes promotes adipogenic differentiation [163], arguing that the maintenance of an intranuclear pool of A-type lamins is important for adipogenesis. Furthermore, our laboratory has shown that in ASCs, lamin A/C knockdown impairs differentiation into adipocytes [164]. Thus, nucleoplasmic lamin A/C seem to be critical for differentiation of tissue progenitor cells and conceivably play important roles regulating chromatin organization and gene expression in the nuclear interior [160, 165].

Other proteins are associated with the nuclear lamina, including proteins anchored in the INM. These include the lamina associated polypeptides (LAPs) LAP1 and LAP2β, emerin and lamin B receptor (LBR). These proteins have important role in anchoring lamins to the INM, and some of these have been also shown to bind DNA, and presumably also have roles in chromatin organization [166].

Point mutations in the *LMNA* gene are associated with disease. To date, ~400 mutations have been mapped to the *LMNA* gene [167] and are linked to ~15 classes of diseases collectively called laminopathies. The symptoms are diverse, including myopathies affecting skeletal and cardiac muscle, progeria (premature aging) which affects all tissues except the brain, and partial lipodystrophies leading to abnormal body fat distribution [167, 168]. Several lamin A point mutations notably cause familial partial lipodystrophy of Dunningan-type (FPLD2), a disease associated with adipogenic and myogenic differentiation defects [169]. FPLD2 patients present metabolic syndromes including glucose intolerance and insulin resistance, and are prone to type 2 diabetes [170].

A key question is how lamin A mutations can cause such a wide range of diseases. Given the developmental regulation of lamin A/C, it is likely that these mutations affect properties of stem cell differentiation. Additionally, some laminopathies are associated with defects in heterochromatin organization at the nuclear periphery [171], thus changes in genome organization during differentiation may be affected. The role of *LMNA*

mutations in FPLD2 makes the study of nuclear lamin-genome interactions in an adipogenic context particularly relevant; this is the topic of Paper II.

1.4.2 Lamin–chromatin interactions

Nuclear lamins interact with chromatin in part by direct interaction with histones and DNA [172]. Interactions between lamins and chromatin have been shown to occur within large, spatially defined regions of chromatin entitled lamina-associated domains (LADs), ranging from 0.1-10 megabases (Mb) [173]. LADs were initially identified by mapping associations of lamin B1 with chromatin genome-wide using DamID. [173, 174]. DamID involves the fusion of a protein of interest (in this case, lamin B1) with the DNA adenine methyltransferase (Dam) of *E. coli*. The Dam enzyme methylates adenines in GATC sequences that lie in close proximity (within 5 kb) of the protein of interest. The methylated regions can be isolated, amplified and hybridized to DNA microarrays for identification of interacting genomic sites, or sequenced and mapped back to the genome [174]. Recently, LADs have also been identified using ChIP [65, 94, 175], with results showing high similarity to DamID LADs [94, 176].

In general, LADs are associated with transcriptionally inactive regions, they are overall gene-poor, and are typically enriched in repressive H3K9me2/me3 [173]. LADs are spatially restricted by distinct borders, typically marked with H3K9me2/me3, H3K27me3, the transcription factor yin-yang 1 (YY1) and the insulator protein CCCTC-binding factor (CTCF) [173, 177]. LAD borders also often harbor transcriptionally active genes oriented away from the LADs. These regions typically contain H3K4me2 [173]. Changes in LAD borders are believed to be important in the dynamics and differences of LADs between cell types [177]. Although LADs are generally transcriptionally silent, recent studies show that LADs can be associated also with other chromatin environments, and some genes in LADs may also be highly transcribed [65, 178]. While our work was ongoing, results from our laboratory showed that in ASCs ~5% of the genes associated with lamin A/C harbor H3K4me3 in the promoter, suggesting a potential for transcriptional activation [65]. Accordingly, a fraction of lamin A/C-associated gene are expressed [65].

DamID combined with immunofluorescence microscopy with a 6-methyladenine M_6A tracer has recently shown that lamin A LADs, in contrast to lamin B LADs, localize not only at the nuclear periphery but also in perinucleolar regions in the nuclear interior [179]. This is in line with the reported nucleoplasmic pool of lamin A/C [160].

Furthermore, supporting a role for lamin A/C in nucleoplasmic and perinuclear compartments, our laboratory has shown that cross-linked chromatin fragmented by sonication or with micrococcal nuclease (MNase) leads to identification of distinct lamin A/C LAD patterns [176]. ChIP with MNase-digested chromatin presumably preferentially targets more 'open' (nuclease-accessible) chromatin, and these LADs probably reflect euchromatin regions in the nuclear interior. In contrast to sonicated LADs, MNase LADs are identified in gene-rich regions; the latter are however enriched in H3K9me3 and H3K27me3 and are predominantly repressed. LADs specific for sonicated chromatin are in contrast gene-poor and devoid of histone modifications, presumably reflecting more peripheral regions. In line with this possibility, sonication lamin A LADs display strong overlap with previously described lamin B DamID LADs [176]. Thus, genes in LADs are mostly but not always repressed, and can be present in different chromatin environments, mainly at the nuclear periphery, as well as in the nucleoplasm. The functional relevance of lamin A LADs detected in transcriptionally active or repressed chromatin contexts remains to be explored.

Lamin-chromatin interactions are believed to play important parts in development. Lamin B1 LADs are largely conserved between cell types [180, 181]. LADs conserved between cell types are entitled constitutive LADs (cLADs), and are even conserved between species. cLADs are believed to play a part in basic chromatin architecture [180]. Over 90% of LADs identified by DamID are conserved throughout differentiation of ESCs into neural progenitor cells [181]. Nevertheless, rearrangements also occur in LADs during differentiation. LADs that are not conserved between cell types are commonly named variable LADs (vLADs) [177]. These regions typically contain genes associated with lineage commitment or pluripotency. For instance, upon differentiation of ESCs into neural progenitors, key pluripotency genes gain lamin association and are likely targeted to the nuclear lamina for repression. Genes associated with commitment to the neurogenic lineage lose lamin association and gain an increased potential for expression [181]. There is however strikingly no overall correlation between gain or loss of lamin occupancy and gene expression. A striking example is the overall absence of changes in gene expression (although exceptions do occur) after siRNA-mediated known-down of lamin A/C in ASCs [65]. Moreover, a fraction of the genes that lose lamin association upon ESC or ASC differentiation remain silenced [65, 181]. Release of these genes from the nuclear lamina has been proposed to constitute a 'priming' mechanism for transcriptional activation before differentiation into more specialized cell types [65, 142, 181].

The mutations in the *LMNA* gene leading to FPLD suggest that lamin A/C may have important roles in adipogenic differentiation, which may partly be mediated by regulation of chromatin structure. Lamin A/C occupancy in gene promoter regions in ASCs has recently been studied using ChIP combined with microarray hybridization (ChIP-chip) [65]. Adipogenic differentiation of ASCs into adipocytes causes a large scale remodeling of lamin A/C-promoter interactions. Approximately 4000 promoters lose lamin A/C interaction, while ~2000 gain interaction and ~800 retain interaction. Adipogenic promoters such as the *PPARG2*, *FABP4*, *FABP8* and *FABP9* promoters lose lamin A/C during differentiation, while genes associated with differentiation into other lineages (e.g. endodermal- and ectodermal-specific genes) remain bound to lamin A/C [65]. These results suggest an overall change in chromatin configuration upon adipogenic differentiation [65]. This study corroborates findings by Oldenburg and colleagues in our laboratory, showing impaired adipogenic differentiation in ASCs expressing a lamin A mutant or knocked-down for lamin A/C [164]. Although the Lund et al. (2013) study provides information about promoter-lamin interactions, genome-wide changes that accompany adipogenic differentiation remain unknown. In Paper II, we report and characterize genome-wide profiles of lamin A/C LADs in the course of *in vitro* adipogenic differentiation.

In light of the information provided in this Introduction, we address several fundamental issues on ASC biology. These are: (1) what chromatin configuration characterizes inflammation response genes in ASCs; (2) does exposure to elevated glucose impact expression – or expression potential – of inflammation response genes in ASCs; (3) how does adipogenic differentiation affect associations of chromatin with the nuclear lamina and how do changes in these associations affect gene expression; and (4) is there an epigenetic state favoring nuclear lamin-genome interactions, which would help rationalize and understand the significance of developmentally-regulated lamina-chromatin interactions. The papers presented here provide evidence for an epigenetic 'priming' of inflammation response genes in ASCs by high-glucose exposure, conceivably accounting for their immunological properties (Paper I; Rønningen et al., submitted), and for domains of GlcNAcylated H2B (which we name GlcNAcylated domains or GADs) constituting an epigenetic platform for the *de novo* formation of lamin A/C LADs during adipogenic induction (Paper II; Rønningen, Shah et al., Genome Res., in press). The latter findings suggest a link between the spatial arrangement of the genome (through lamin A/C-chromatin interactions) and the metabolic state of ASCs.

2. Aims of the study

Adipose stem cells (ASCs) play a central role in the regulation of immune functions and in adipose tissue development through differentiation into adipocytes. Relatively little is known on how chromatin-associated processes regulate these functions. In this thesis work, I have examined how gene expression and chromatin organization in human ASCs are affected by metabolic cues, with emphasis on dynamic changes in histone post-translational modifications and spatial chromatin organization. The aims of the study were therefore to:

- Determine the effect of elevated extracellular glucose on expression and epigenetic regulation of inflammatory genes in ASCs and adipocytes, at single-gene and genome-wide levels
- Determine whether adipogenic induction affects genome-wide distribution of the nutrient responsive S112GlcNAc on histone H2B, and characterize regions enriched in H2BS112GlcNAc
- Identify changes in the genome-wide associations of chromatin with nuclear lamin A/C during adipogenic differentiation
- Assess the relationship between changes in lamin A/C-chromatin interactions and H2BS112GlcNAc driven by adipogenic differentiation

3. Summary of publications

Paper I: Epigenetic priming of inflammatory response genes by high glucose in adipose progenitor cells (Rønningen et al., *Biochemical and Biophysical Research Communications*, *in press*)

Cellular metabolism confers wide-spread epigenetic modifications required for the regulation of transcriptional networks that determine cellular states. Mesenchymal stromal cells (MSCs) are responsive to metabolic cues including circulating glucose levels, and modulate inflammatory responses. We show here that long-term exposure of undifferentiated human primary adipose stem cells (ASCs, MSCs from adipose tissue) to high glucose upregulates a subset of inflammation response (IR) genes and alter their promoter histone methylation patterns in a manner revealing transcriptional de-repression. Modeling of chromatin states from recurrent combinations of seven chromatin modifications in nearly 500 IR genes unveil three overarching chromatin configurations reflecting repressive, active, and potentially active states in promoter and enhancer elements. We show that a high fraction of the non-expressed IR genes contain potential for expression, enriched in the H3K4me1 mark. Accordingly, we further show that adipogenic differentiation in high glucose predominantly upregulates IR genes, when compared to differentiation in a control glucose level. Our results indicate that elevated extracellular glucose levels sensitize in ASCs an IR gene expression program which is exacerbated in adipocytes. We propose that high glucose exposure conveys an epigenetic 'priming' of IR genes, favoring a transcriptional inflammatory response upon adipogenic stimulation. Chromatin alterations at IR genes by high glucose exposure may play a role in the etiology of metabolic diseases.

Paper II: Pre-patterning of differentiation-driven nuclear lamin A/C-interacting chromatin domains by GlcNAcylated H2B (Rønningen, Shah et al. *Genome Research*, 2015, *in press*)

Dynamic interactions of nuclear lamins with chromatin through lamin-associated domains (LADs) contribute to the spatial organization of the genome. Here, we provide evidence for pre-patterning of differentiation-driven formation of lamin A/C LADs by domains of histone H2B modified on S112 by the nutrient sensor *O*-linked N-acetylglucosamine (H2BS112GlcNAc), which we term GADs. We reveal a two-step process of lamin A/C LAD formation during *in vitro* adipogenesis, involving spreading of lamin A/C-chromatin interactions in the transition from progenitor cell proliferation to cell cycle arrest, and genome-scale redistribution of these interactions through a process of LAD exchange within hours of adipogenic induction. Lamin A/C LADs are found both in active and repressive chromatin contexts that can be influenced by differentiation status. We show that *de novo* formation of adipogenic lamin A/C LADs occurs non-randomly on GADs, which consist of megabase-size intergenic and repressive chromatin domains. Accordingly, whereas pre-differentiation lamin A/C LADs are gene-rich, post-differentiation LADs harbor repressive features reminiscent of lamin B1 LADs identified in other cell types. We find that release of lamin A/C from genes directly involved in glycolysis concurs with their transcriptional upregulation after adipogenic induction, and with downstream elevations in H2BS112GlcNAc levels and *O*-GlcNAc cycling. Our results unveil an epigenetic pre-patterning of adipogenic LADs by GADs, suggesting a coupling of developmentally regulated lamin A/C-genome interactions to a metabolically-sensitive histone modification.

4. Discussion

4.1 Epigenetic priming of gene expression in high glucose conditions

Given the central role of adipose tissue inflammation in disease, expression of inflammatory factors in adipose stem cells (ASCs) may contribute to disease development. In **Paper I**, we show that long-term exposure to elevated extracellular glucose (an approximation of an *in vitro* 'diabetic' condition) induces epigenetic changes in a panel of inflammatory response (IR) gene promoters in human ASCs. These changes include a reduction in H3K9me3 and H3K27me3 on promoters, suggestive of a transcriptional de-repression mechanism. This is associated with modest upregulation of expression of some but not all the examined genes in our qPCR analysis, suggesting that these epigenetic changes are necessary but not sufficient for transcriptional activation. Rather, they may act as a 'priming' mechanism enabling transcriptional upregulation following stimulation, e.g. after adipogenic or inflammatory induction. This is in line with previous studies showing effects of hyperglycemia on H3K9 and H3K27 methylation and on activity of the respective H3K9 and H3K27 HMTs and HDMs in endothelial cells, cardiomyocytes and monocytes [103, 104, 182, 183]. The epigenetic changes observed are likely to be due to altered activity of these enzymes also in ASCs. It would be interesting in future studies to examine the impact of sustained or acute high glucose exposure on HMT/HDM levels and activity in an adipose context.

We further show, by high-throughput bioinformatic analyses of IR genes in ASCs, that most non-expressed IR genes harbor chromatin states compatible with potential for expression. These chromatin states include marking of enhancers (or putative enhancers) by H3K4me1, and marking of promoters by H3K27me3, with no detection of the transcription elongation mark H3K36me3; these epigenetic marks are consistent with a repressed transcriptional state. Interestingly, the detection of H3K4me1 in these regulatory regions suggests an additional priming mechanism for IR gene expression. H3K4me1 marking of enhancer elements suggests that either these enhancers are in use (i.e., they regulate distant cognate promoters), or that they will be used at a later stage, e.g. after an adipogenic or an inflammatory signal.

Supporting the view of 'priming' of gene activation by H3K4me1 enhancer marking is the discovery of a set of enhancers mono-methylated on H3K4 prior to, or in early stages of, adipogenic differentiation, and in any case prior to transcriptional activation of their cognate genes [66]. H3K4me1 has also been described to act as a

priming mechanism prior to subsequent activation of enhancers upon acquisition of the H3K27ac mark [66, 184, 185]. Priming IR gene activation by H3K4me1 upon cell culture in high glucose may therefore determine the inflammatory potential of ASCs after adipogenic induction. Interestingly, hyperglycemia is associated with increased activity of SET7, the H3K4me1 methyltransferase, resulting in increased levels of H3K4me1 on inflammatory promoters [103, 186], suggesting that this priming mechanism may be more evident in cells exposed to elevated glucose.

In support of this possibility, we show that adipogenic differentiation under high glucose conditions promotes an overall upregulation of IR genes to a larger extent than in undifferentiated cells. An implication may be that adipocytes differentiated under hyperglycemic conditions *in vivo* (e.g. in a context of insulin resistance) secrete elevated levels of inflammatory mediators, contributing to a systemic low-grade inflammatory state and disease development. Additionally, epigenetic changes mediated by hyperglycemia may influence ASCs' immunomodulatory properties following injury. We hypothesize from our observations that culture in high glucose induces changes in chromatin organization which may facilitate or poise IR genes for increased expression following additional input from other pathways.

4.2 Metabolic regulation of early adipogenic differentiation?

Transcriptional changes occur rapidly, within hours of adipogenic induction and involve extensive chromatin remodeling. These chromatin changes entail both transient and more stable 'opening' of chromatin in vicinity of lineage-specific genes, facilitating transcription [67]. In **Paper II**, we report massive transcriptional changes associated with induction of adipogenic differentiation (from D0 to D1), corroborating the D0 – D3 gene expression changes reported by Shah et al. [66]. Interestingly, a large fraction of the genes upregulated on D1 is associated with cellular metabolism. Upregulated genes include *HIF1A*, genes encoding several glycolytic enzymes (e.g. *GAPDH, PGK1, TPI1, ENO1, LDHA, LDHB, PGAM1*) and genes associated with repression of oxidative phosphorylation (e.g. *PDK4*) (**Paper II**, Supplemental Fig. 4). Remarkably, increased expression of most of these genes is transient, returning to undifferentiated ASC expression level by D3 and D9, suggesting a 'boost' of glycolytic metabolism following adipogenic induction. Previously published studies have claimed that oxidative metabolism is a pre-requisite for adipogenic differentiation, and that differentiation is accompanied by a boost of oxidative phosphorylation [56, 57]. However, these studies

have examined later time-points in adipogenesis, and oxidative phosphorylation may be more important at these stages. Since the metabolic changes associated with adipocyte formation is dependent on PPARγ [72], which is upregulated at a later stage of adipogenic differentiation (day 2-6), the early stage of adipogenic differentiation presumably does not involve lipid synthesis to a large extent, which rely on oxidative metabolism. Thus, glycolysis may play a role in adipogenic induction. We are initiating studies to further investigate metabolic changes associated with initiation of adipogenic differentiation of ASCs. Interestingly, the boost in glycolytic gene expression is consistent with a transient increase in H2BGlcNAc and associated proteins (OGA, OGT and overall GlcNAc modifications) – suggesting an increase in O-GlcNAc cycling – and a reorganization of chromatin at the nuclear lamina on D1 of adipogenic differentiation. Our data suggest a functional link between cellular metabolic state, histone modifications and chromatin architecture.

4.3 LAD dynamics in adipogenic differentiation

Lamin A/C LADs are associated with actively transcribed regions in undifferentiated ASCs

LADs are typically associated with a repressive chromatin environment [65, 94, 173, 175, 177, 187]. LADs are largely conserved between cell types [180] and these cLADs are believed to play roles in the fundamental organization of chromatin architecture in the nuclear space [172]. This is contrast to the vLADs which may perhaps play a more regulatory function (although this is not strongly substantiated yet). In **Paper II**, we show that lamin A/C LADs are enriched also in active genes and regulatory elements in undifferentiated ASCs. However, after differentiation, LADs gain repressive chromatin features that are more typical of LADs. As lamin A/C is found both at the nuclear periphery and in the nuclear interior [160, 165], the heterochromatic vs. euchromatic nature of lamin A/C-chromatin interactions evidenced in our study is likely influenced by its dual localization. Peripheral LADs are enriched in heterochromatin [173, 188] while intranuclear lamin A/C LADs are likely in a more euchromatic environment, as expressed genes tend to localize in the nuclear interior. Supporting a role for lamin A/C also in euchromatin organization, work in our laboratory has shown that a fraction of lamin A/C-bound promoters is marked by H3K4me3 and/or is expressed [65]. Our results therefore indicate that lamin A/C LADs can be found both in active and repressive chromatin contexts, the balance of which can be influenced by cell differentiation status.

De novo LAD formation after adipogenic induction – how does LAD remodeling occur?

In **Paper II**, we observe a major shift in lamin A/C LADs after adipogenic induction, with essentially no sequence overlap between D0 and D1 LADs. The question emerges then, of whether nuclear lamins are relocalized or translocated within the nuclear interior during adipogenic induction. The existence of an intranuclear pool of lamin A/C complicates the interpretation of our results. ChIP-seq data provide information on the sequence associated with lamin A/C, but does not provide indications on the nuclear localization of the LADs. The intranuclear lamin A/C pool is regulated by the lamin binding protein LAP2α [158]. LAP2α knock-out causes redistribution of nucleoplasmic lamin A/C to the nuclear periphery, and also inhibits differentiation [158, 159]. This suggests that undifferentiated progenitor cells require a pool of intranuclear lamin A/C to be able to differentiate. This is consistent with our findings (**Paper II**) showing that a large fraction of lamin A/C LADs are enriched in genes and regulatory elements, some of which are active, which tend to be localized in the nuclear interior (in a euchromatic environment, as discussed above). The *de novo* LADs formed on D1 of adipogenic induction show some properties suggesting positioning at the nuclear periphery. Indeed, post-adipogenic induction lamin A/C LADs become more intergenic (reduced gene density and reduced association with regulatory elements) and thus are more similar to lamin B1 LADs identified by DamID in other cell types [173, 180]. Given that lamin B1 is restricted to the nuclear periphery (by virtue of its anchoring in the inner nuclear membrane), lamin B1 LADs are thus associated (presumably) exclusively with the nuclear periphery; our findings therefore suggest that *de novo* lamin A/C LADs form predominantly at the nuclear periphery.

Based on our results, we propose the following model (**Fig. 8**). In undifferentiated cells, lamin A/C is associated with a gene-rich, euchromatic environment, mainly in the nuclear interior; these associations may involve LAP2α, which binds directly to lamin A/C and chromatin. Nevertheless, lamin A/C also likely interacts with chromatin at the nuclear periphery. Upon differentiation, LADs are globally reorganized, resulting in increased amount of LADs at the nuclear lamina. The LADs formed on D1 lose their overall euchromatic properties and are more heteroch romatic, similarly to lamin B1 LADs.

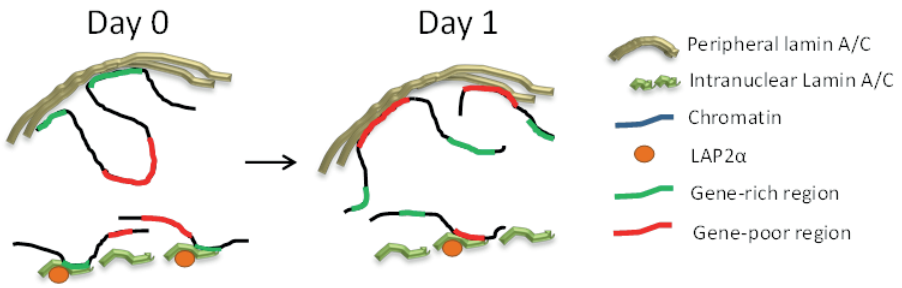

Figure 8 Model of shift in lamin A/C LAD organization upon adipogenic induction. On day 0, lamin A/C LADs are mainly associated with gene-rich, actively transcribed regions; a significant fraction of LADs is present in the nuclear interior, presumably bound to LAP2α. Upon adipogenic induction, there is a global rearrangement of LADs, and LADs become overall gene-poor and transcriptionally repressed. This probably involves re-shuffling of LADs both at the periphery and in the nucleoplasmic pool, and likely also between the different compartments. The increased overlap of lamin A/C LADs with lamin B1 LADs on D1 suggests an increased amount of peripheral vs. internal LADs after differentiation.

Are LADs stable in differentiated cells?

Another important point that remains to be elucidated is whether lamin A/C and associated LADs translocate at a later stage of adipogenic differentiation. We report in **Paper II** that lamin A/C LADs are overall maintained between D1 and D9. This suggests that the radial positioning of lamin A/C-genome interactions is relatively stable in the nuclear space. This however does not exclude the possibility that lamin A/C-chromatin complexes affecting specific genomic regions translocate within the nucleus: in this scenario, the genomic sequence underlining these LADs would remain the same, but their position would change. We have currently no direct evidence that the *de novo* lamin A/C LADs harbor a more repressive configuration than D0 LADs but we show a reduction of lamin A/C LAD-associated gene expression on D1 and D3 (**Paper II**, Fig. 1G).

Another important issue is that the ChIP-seq results are combined from millions of cells. When investigating LADs at the a single cell level, only approximately 30% of the LADs are found at the nuclear periphery [178]. 3D fluorescence *in situ* hybridization (3D-FISH) combined with lamin A/C immunostaining (3D immuno-FISH) corroborates these findings for single loci within LADs [65]. So clearly, not (or very seldom) all alleles of a locus interacting with nuclear lamins are at the nuclear periphery. The LADs identified to date using high-throughput genomic approaches (ChIP, DamID) represent the ensemble of lamin A/C-chromatin interactions in the entire cell population. It may be that changes in lamin-chromatin association occur in single cells between the different time-points

examined in our study, although the overall LAD sequence is, at the cell population level, the same.

Interestingly, a recent study reports the genome-wide mapping of LADs by DamID-seq in an array of 400 single cells [189]. The data reveal a core organization involving constitutive gene-poor LADs between individual cells, which may play a fundamental architectural role. Variable contacts in contrast form the basis of cell-specificity of LADs. In support of our findings in **Paper II**, the variable LADs tend to be more gene-rich and associated with transcriptionally active regions [189]. A single-cell analysis of lamin A/C LADs in the context of our adipogenic differentiation system would provide invaluable information on the dynamics of lamin-genome interactions during the D0-D1 transition, as data from individual cells may be interpreted as time-point transitions between D0 and D1.

4.4 Is H2BS112GlcNAc enriched in genic or intergenic regions?

We report also in **Paper II** that H2BS112GlcNAc is present in large repressed regions in ASCs. The genomic context of H2BGlcNAc however differs from previous reports [131, 138]. We have shown that in ASCs and adipocytes, H2BGlcNAc is primarily localized in gene-poor or intergenic regions, and that genes found in H2BGlcNAc domains (GADs) are generally repressed. In contrast, Fujiki and colleagues have reported that, H2BGlcNAc enrichment is predominantly found in genes that are transcriptionally active [138] and in euchromatic areas co-enriched with H3K4me2 and H2BK120ub1. Several factors may explain this apparent key discrepancy.

One important factor is the difference in antibodies used in the studies. Whereas Fujiki and colleagues used a custom made rabbit polyclonal antibody, we used in our work the only commercially available antibody at the time to our knowledge, namely a rabbit polyclonal antibody to H2BS112GlcNAc from Abcam (ab130951). Unfortunately we were not able to access the ’Fujiki antibody’ at the time we undertook our studies (and to date). Therefore, we have extensively characterized the Abcam antibody (**Paper II**, Fig. 2, Supplemental Fig. 2**)**. Our conclusion is that the Abcam antibody is specific to the GlcNAcylated form of H2B, although it has some non-specificity to unmodified H2B. In particular, H2BGlcNAc enrichment shows very little overlap with pan-H2B enrichment in ASCs, suggesting that the antibody is specific enough to ensure robustness of our ChIP data. It would be interesting to determine H2BGlcNAc genomic enrichment profiles using the ‘Fujiki antibody‘ in ASCs; however this seems currently of reach.

Another factor explaining the discrepancy is the cell types examined. Whereas Fujiki et al. have examined HeLa cells [138] – a transformed aneuploidy cell line, we have used primary, normal diploid ASCs [190]. To address whether there are cell type-specificity of H2BGlcNAc genomic profiles, we have recently initiated a comparative study (unpublished results). We have mapped by ChIP-seq in the human HepG2 hepatoblast cell line lamin A/C LADs and H2BGlcNAc, using the same antibodies as in Paper II. In addition, to provide an idea of how dynamic lamin A/C LADs would be in relation to GADs, we also treated HepG2 cells with 10 μM cyclosporin A (CspA) for 72 h to induce a "steatotic"-like state, i.e. stimulate an adipogenic phenotype (A. Sørensen and P. Collas; data not shown). The rationale for this was to provide an adipogenic system complementary to our ASC differentiation system and to determine the relationship between lamin A/C LAD and GAD enrichment after CspA treatment. Using the EDD algorithm [94] for domain detection, we found that lamin A/C LADs cover 350 and 255 Mb of the genome in control and CspA-stimulated HepG2 cells, respectively, making up 11 and 8% of the genome (**Fig. 9A**). Thus, LAD coverage under these conditions appears to be similar or slightly reduced. However, gene density increases after CspA treatment, from 2.1 genes/Mb to 6.4 genes/Mb, respectively (**Fig. 9B**), indicating a major redistribution of lamin A/C LADs after stimulation, and a change in the genomic properties of these LADs. Changes in LAD patterns are manifested by either loss of LADs, gain of LADs (de novo LADs) or extension of already existing LADs (spreading; **Fig. 9C**). These results indicate thus far that, as in ASCs (**Paper II**), lamin A/C LADs are remodeled during adipogenic differentiation, yet in this system, lamin A/C LADs tend to become gene-rich after stimulation.

We next examined H2BGlcNAc distribution through the HepG2 genome before and after CspA stimulation. We show that domains of H2BGlcNAc (GADs) cover 14 and 11% of the genome in control and treated cells, respectively (454 and 351 Mb) through 360 to 310 domains (**Fig. 9A**). Overlap between GADs is highly conserved between control and CspA-treated cells, as determined by Jaccard index calculation (**Fig. 10**). GADs show high gene density, both before and after treatment, at 14 vs.12 genes/Mb (**Fig. 9B**). Importantly, we note that gene density of GADs in HepG2 cells is clearly distinct from that in ASCs in which GADs are consistently gene poor (~2 genes/Mb; **Paper II**). Since we used the same antibody in both studies, this suggests that the genomic context of H2BGlcNAc varies between cell types. This is either cell type-dependent, or may represent a difference between primary cells (ASCs) and cell lines

(HepG2). The latter is supported by the fact that H2BGlcNAc has been reported to be enriched in genes in the HeLa cell line [138]; however, more work is clearly needed to validate this possibility, notably by ChIPing H2BGlcNAc from HeLa cells. This work is currently ongoing in our laboratory.

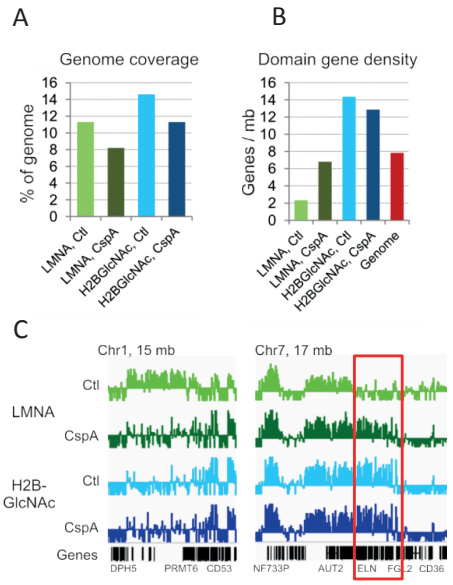

Figure 9 Characteristics of LADs and GADs in control and CspA-stimulated HepG2 cells. (**A**) Percent of the human genome covered by LADs and GADs. (**B**) Gene density in LADs and GADs. (**C**) IGV browser view of LMNA and H2BGlcNAc enrichment in a region of chromosomes 1 and 7 in control (Ctl) and CspA-stimulated HepG2 cells. Red frame highlights a region of de novo lamin A/C enrichment in CspA-treated cells, in a H2BGlcNAc-rich domain.

During adipogenic differentiation of ASCs, we report in **Paper II** *de novo* LADs form almost exclusively on pre-existing domains of H2BS112GlcNAc, which we called GADs, suggesting that GADs pre-pattern *de novo* LAD formation and that patterns of LAD formation may be dependent on the cellular metabolic state. We examined whether this held true for HepG2 cells, given the remodeling of LADs after CspA treatment we reported above. Our preliminary data show that a proportion of *de novo* lamin A/C LADs form on GADs (**Fig. 9C**, frame). This is quantified by calculating the Jaccard index of overlap between LADs and GADs before and after CspA treatment (**Fig. 10**). This shows that while LADs and GADs essentially do not overlap in control cells (Ji = 0.1), overlap is highly significant (Ji = 0.5) in stimulated cells. This suggests that, as in ASCs, *de novo* lamin A/C LAD formation upon adipogenic induction in HepG2 cells may be pre-patterned by GADs.

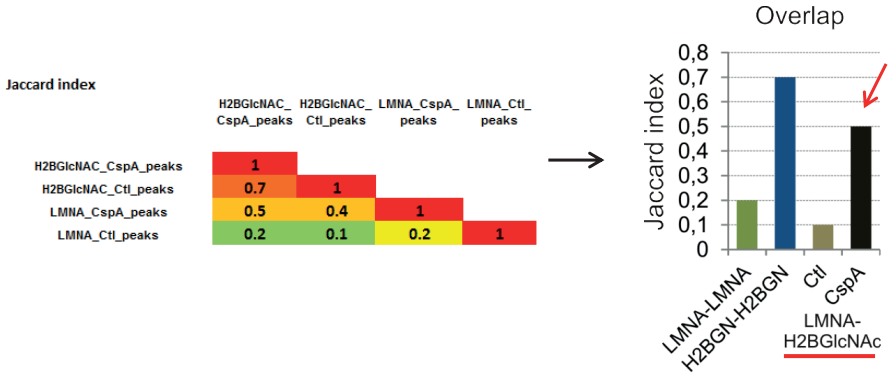

Figure 10 Jaccard index determination of the overlap between LADs and GADs. Note the increase in overlap after CspA treatment (red arrow), supporting, in a different cell type, our findings in **Paper II**.

A recent review also questions the quality of the H2BS112GlcNAc ChIP-seq data published by Fujiki et al (2011) [191]. Genomic profiles of promoters claimed to be enriched in H2BGlcNAc (**Fig. 11**) reveal, in at least 5 out of the top 20 genes supposedly enriched in H2BS112GlcNAc, very low if any enrichment in H2BS112GlcNAc in promoter regions relative to overall background level. This suggests that the conclusions drawn in the earlier study [138] may be overstated. It will be important to resolve the issue of genomic enrichment in H2BS112GlcNAc in a variety of primary cells and cell lines, as we have initiated in our laboratory.

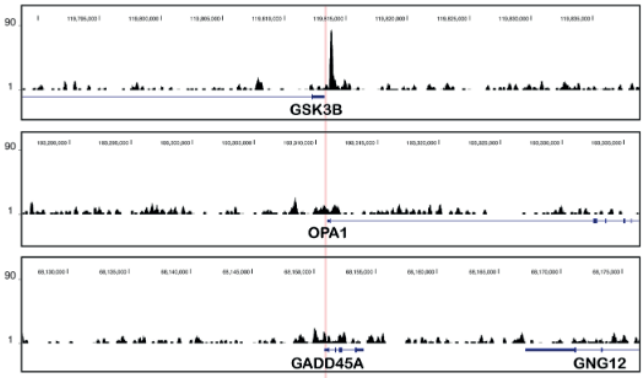

Figure 11 Re-analyzed H2BS112GlcNAc ChIP-seq profiles from selected 'highly enriched in H2BGlcNAc' loci in the Fujiki et al. (2011) data. Modified from [191]

OGT has multiple roles in chromatin regulation, and the mechanism by which OGT identifies its substrate in different contexts is incompletely understood. OGT substrate specificity may be regulated by several means including isoform distribution, oligomerization, UDP-GlcNAc concentration, PTMs and through interactions with other

proteins [192]. Several studies have shown that GlcNAcylation of H2B by OGT is dependent on TET enzymes for recruitment onto chromatin [126, 131, 193]. RNA-seq analysis shows however that TET enzymes are not expressed in ASCs [66]; therefore, other mechanisms may be involved in bringing OGT to chromatin in this cell type. The difference in regulation may also explain the discrepancy in genomic localization of H2BGlcNAc in ASCs compared to previous studies [138].

4.5 Is there a functional relationship between H2BGlcNAc and LADs?

We show in **Paper II** that *de novo* lamin A/C LADs form on previously enriched H2BGlcNAcylated domains on D1 of adipogenic differentiation. To our knowledge, this is the first study describing a relationship between lamin A/C and GlcNAcylated proteins. This connection is however not surprising, as OGT modifies several proteins at the nuclear envelope, including nuclear pore complexes [127] and the inner nuclear membrane protein emerin [128]. Indeed we have shown enrichment of O-GlcNAc modified proteins in the nuclear periphery in ASCs and HeLa cells (**Fig. 12**). Here, we provide additional evidence for a role of GlcNAcylation at the nuclear lamina, with H2BGlcNAc acting as a pre-patterning factor for positioning of genes at the nuclear lamina upon adipogenic induction.

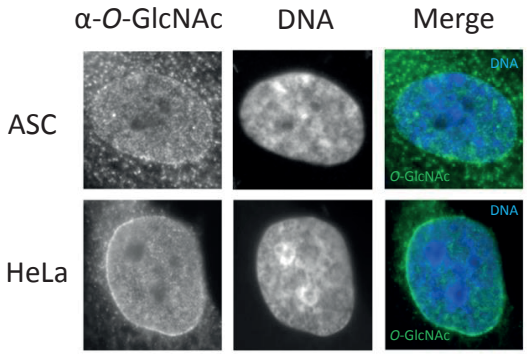

Figure 12 O-GlcNAc modifications are located at the nuclear periphery. Immunofluorescence images of human ASCs and HeLa cells stained with RL2 antibodies which recognize pan-O-GlcNAc modifications. DNA is stained with DAPI.

We have, however, not yet been able to show a clear functional link between H2BGlcNAc and the establishment of lamin A/C LADs. We have shown using HeLa cell lysates that lamin A/C co-immunoprecipitates with H2BGlcNAc and *vice versa*, and that this interaction is not direct, but rather mediated through DNA (A. Oldenburg,

unpublished results). H2B and lamin A/C have been shown to interact through the N-terminal part of H2B [194]. Due to the partial affinity of the anti-H2BS112GlcNAc antibody used in our work to unmodified H2B (see above), further studies are needed to validate this interaction. Nonetheless, the role of H2BGlcNAc in pre-patterning of lamin A/C-chromatin interactions does not necessarily require direct protein-protein interaction, and it is possible or even likely that lamin A/C, H2BGlcNAc and associated chromatin are part of larger protein complexes.

Establishing a causal relationship between LADs and GADs would require showing that removal of H2BGlcNAc prevents or somehow affects *de novo* LAD formation. This is however challenging. Methods for studying specific *O*-GlcNAc modifications typically involve manipulations of overall *O*-GlcNAc levels, not specific modifications on specific proteins. Typical approaches involve knock-down of OGT, inhibition of central enzymes in the HBP, or altering availability of metabolites in the HBP (glucose, glutamine, glucosamine, etc.). These approaches will however affect various GlcNAcylated proteins and a wide range of cellular processes, and have a large degree of non-specificity. Additionally, since both OGT and OGA are essential proteins for cell survival [113, 195], perturbing these enzymes may cause several off-target effects including stress responses, defects in cell cycle regulation or transcription. Another strategy to more specifically manipulate H2BGlcNAcylation would consist in introducing a mutant form of H2B. For antibody validation in **Paper II**, we expressed H2B fused to EGFP or Ty1 tags, and introduced a substitution from a serine to an alanine on residue 112 (H2BS112S→A mutation). We have attempted using these constructs for functional analysis; however under the conditions tested so far, expression levels of exogenous epitope-tagged H2B remains presumably too low (estimated from Western blots to less than 10% of total H2B; our unpublished data), it may not cause an overall change in genome organization. Indeed, using these constructs, we show by ChIP-qPCR no clear effect on the association of specific loci with lamin A/C in ASCs on D1 of differentiation (our unpublished results). An approach to study the functional effect of H2BGlcNAc would be to stably express H2BS112A in ASCs, assess H2BS112GlcNAc levels, and determine the effect of this substitution on *de novo* lamin A/C LAD formation and adipogenic differentiation.

4.6 Conclusions and perspectives

Overall, this thesis provides insight in how the epigenome and chromatin organization is potentially affected by the cellular metabolic state. We show that:

i. High glucose concentration affects epigenetic priming of inflammatory response gene promoters in ASCs, priming these for enhanced expression upon subsequent adipogenic stimulation.
ii. Adipogenic induction of ASCs induces up-regulation of metabolic genes, and a major reorganization of lamin A/C LADs.
iii. Metabolically-linked H2B GlcNAcylation acts as pre-patterning factor for differentiation-driven lamin A/C reorganization.

Our results speculatively suggest a coupling between cellular metabolic state and the spatial organization of genome involving a nutrient-sensitive histone modification. Nonetheless, several questions remain to be answered. In particular, the mechanism by which chromatin movement occurs in the D0/D1 transition, i.e. immediately after application of the adipogenic stimulus, needs to be examined in more detail. Does this involve shuffling of large chromatin domains from the nuclear interior to the nuclear periphery and *vice versa*? Or does it involve local reorganization of lamins in the respective compartments? FISH analysis would provide answers on any changes in the radial positioning of LADs and inter-LAD regions during the early stages of adipogenic induction. Live-cell locus tracking using for example CRISPR/Cas9-EGFP tagging of loci [196] within LADs would provide a dynamic real-time component to this analysis of chromatin dynamic. What is the role of the intranuclear lamin A/C and chromatin anchor LAP2α in this context? In addition, studying changes in LADs in three dimensional space using chromosome conformation capture (3C) techniques would provide useful information for understanding these mechanisms. It would also be important to establish a functional role of H2BGlcNAc in LAD formation; to this end, functional studies using a H2BS112 mutant that cannot be GlcNAcylated will be crucial. The work presented in this thesis provides novel information on chromatin organization in adipogenic differentiation, and how this may be affected by metabolic cues.

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Errata

Candidate:Torunn Rønningen

Title of thesis: Metabolic influence on chromatin organization in adipose stem cells

Page	Line	Original text	Corrected text
20	29	(Dechat et al. 2000; Naetar et al. 2009)	[152,153]
21	6	(Johnson et al 2004)	[161]
21	6-7	(Gotic et al. 2010a; Gotic et al. 2010b; Naetar et al. 2008; Naetar et al. 2009)	[153,158,159,162]
21	8	(Dorner et al. 2006)	[163]
21	14	(Dechat et al. 2010; Gesson et al. 2014)	[160,165]

The following references were added to the reference list.

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