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HUMAN ADIPOCYTES

PROTEOMIC APPROACHES

ÅSA JUFVAS

Division of Cell Biology
Department of Clinical and Experimental Medicine
Faculty of Medicine and Health Sciences
Linköping University, Sweden

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During the course of the research underlying this thesis, Åsa Jufvas was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden

SUPERVISOR

Peter Strålfors, Professor

Department of Clinical and Experimental Medicine

Faculty of Medicine and Health Sciences, Linköping University

SUPERVISOR

Alexander Vener, Professor

Department of Clinical and Experimental Medicine

Faculty of Medicine and Health Sciences, Linköping University

CO-SUPERVISOR

Maria Turkina, PhD

Department of Clinical and Experimental Medicine

Faculty of Medicine and Health Sciences, Linköping University

OPPONENT

Hans Jörnvall, Professor

Institution of Medical Biochemistry and Biophysics

Karolinska Institutet, Stockholm

ABSTRACT

Type 2 diabetes is characterized by increased levels of glucose in the blood originating from insulin resistance in insulin sensitive tissues and from reduced pancreatic insulin production. Around 400 million people in the world are diagnosed with type 2 diabetes and the correlation with obesity is strong. In addition to life style induction of obesity and type 2 diabetes, there are indications of genetic and epigenetic influences. This thesis has focused on the characterization of primary human adipocytes, who play a crucial role in the development of type 2 diabetes.

Histones are important proteins in chromatin dynamics and may be one of the factors behind epigenetic inheritance. In paper I, we characterized histone variants and post-translational modifications in human adipocytes. Several of the specific post-translational histone modifications we identified have been characterized in other cell types, but the majority was not previously known. Moreover, we identified a variant of histone H4 on protein level for the first time.

In paper II, we studied specific histone H3 methylations in the adipocytes. We found that overweight is correlated with a reduction of H3K4me2 while type 2 diabetes is associated with an increase of H3K4me3. This shows a genome-wide difference in important chromatin modifications that could help explain the epidemiologically shown association between epigenetics and metabolic health.

Caveolae is a plasma membrane structure involved in the initial and important steps of insulin signaling. In paper III we characterized the IQGAP1 interactome in human adipocytes and suggest that IQGAP1 is a link between caveolae and the cytoskeleton. Moreover, the amount of IQGAP1 is drastically lower in adipocytes from type 2 diabetic subjects compared with controls implying a potential role for IQGAP1 in insulin resistance.

In conclusion, this thesis provides new insights into the insulin signaling frameworks and the histone variants and modifications of human adipocytes.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Idag räknas ungefär en tredjedel av världens befolkning som feta vilket är dubbelt så många som antalet undernärda. Enligt världshälsoorganisationen beror ungefär 5 % av alla dödsfall på fetma och dess följsjukdomar, som till exempel kan vara hjärt- och kärlsjukdomar, stroke, cancer, njursvikt och typ 2-diabetes. Arbetet i denna avhandling har syftat till att bidra med en ökad förståelse för hur mänskliga fettceller fungerar, både i normalt tillstånd och vid övervikt och typ 2-diabetes.

Det enkla svaret på frågan varför någon blir fet är att den äter för mycket och rör på sig för lite. Men, verkligheten är sällan enkel och olika människor drabbas i olika grad av vår moderna livsstil med energirik kost, motorburen transport och stillasittande arbete. Det verkar som att arvet spelar en avgörande roll men vad gäller de precisa mekanismerna bakom ärftligheten så finns mycket kvar att utforska.

Fettcellerna har en central roll i utvecklingen av typ 2-diabetes. Denna sjukdom innebär en oförmåga att kontrollera blodsockret på grund av minskad känslighet för insulin i fettväv, muskler och lever samt otillräcklig produktion av insulin från bukspottkörteln. Det finns ett mycket starkt samband mellan fetma och typ 2-diabetes även fast långt ifrån alla feta är diabetiker.

Resultat från vårt arbete på mänskliga fettceller kan bidra till den molekylära förståelsen för epigenetisk ärftlighet av fetma och typ 2-diabetes. Epigenetiska faktorer anses kunna föra vidare ärftliga egenskaper utan att förändra själva DNA-sekvensen och studier tyder på att det finns ett samband mellan epigenetik och problematiken med fetma och typ 2-diabetes. En av dessa epigenetiska faktorer tros vara varianter och modifieringar av histoner. Histoner är proteiner som är inblandade i den avancerade packningen av DNA i cellkärnan. Vi har karakteriserat histonerna i mänskliga fettceller och identifierat ett stort antal modifieringar varav många aldrig tidigare påvisats. Dessutom identifierades en variant av histon H4, som tidigare inte upptäckts på proteinnivå. Vi visade även att övervikt korrelerar med minskad nivå av en specifik typ av histonmodifiering medan ökningen av en annan typ av modifiering är kopplat till typ 2-diabetes.

Vi har även funnit resultat som tyder på att proteinet IQGAP1 kan vara inblandat i de initiala stegen av insulinstimulerad cellsignalering i fettcellerna. För att signaleringen i cellerna ska fungera på ett effektivt och kontrollerat sätt används ofta så kallade scaffoldingproteiner. Dessa proteiner har förmågan att föra samman andra proteiner till större komplex, finjustera deras aktivitet och i vissa fall styra en cells hela öde. Scaffoldingproteinet IQGAP1 har i flera celltyper visat sig ha en koppling till cancer och typ 2-diabetes, vi studerade IQGAP1 i mänskliga fettceller då det aldrig tidigare gjorts. Vi såg att det finns betydligt mindre mängd IQGAP1 i celler från personer med typ 2-diabetes än från icke-diabetiska personer. Vi undersökte även vilka andra proteiner IQGAP1 interagerar med och dessa kunde vi dela upp i två huvudsakliga grupper; cytoskelett-proteiner och caveolae-associerade proteiner. Caveolae är en grotliknande inbuktning i ytan av fettcellen där bland annat insulinreceptorn finns. När insulin binder till receptorn startar insulinsignaleringen i fettcellen och ett viktigt steg är då caveolae snörps av och tas in i cellen tillsammans med receptorn. Både tillståndet då en caveolae befinner sig vid cellens yta och då den tas in i cellen är beroende av cytoskelettet. Cytoskelettet fungerar bland annat som ett inre stöd som ger cellen dess form men också som en tågräls för transport av olika komponenter inne i cellen och som ankare för olika strukturer. Våra resultat tyder på att IQGAP1 kan spela en viktig roll för kopplingen mellan caveolae och cytoskelettet och möjligen påverka processen då caveolae tas in i cellen tillsammans med den aktiverade insulinreceptorn.

Med den lavinartade utbredningen av fetma och dess följsjukdomar vi ser idag står vi inför en stor utmaning vad gäller världshälsa och livskvalitet för de drabbade. För att hitta effektiva och säkra behandlingar krävs att vi förstår hur cellerna fungerar och hur mekanismerna bakom sjukdomarna verkar. Detta arbete har bidragit till förståelsen gällande både ärftlighet och signaleringsmekanismerna i fettcellerna.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers which are referred to in the text by their roman numerals:

- I **Åsa Jufvas**, Peter Strålfors and Alexander V. Vener
Histone variants and their post-translational modifications in primary human fat cells
PLoS one, 2011 Jan 7;6(1):e15960

- II **Åsa Jufvas**, Simon Sjödin, Kim Lundqvist, Risul Amin, Alexander V. Vener and Peter Strålfors
Global differences in specific histone H3 methylation are associated with overweight and type 2 diabetes
Clinical Epigenetics, 2013 Sep 3;5(1):15

- III **Åsa Jufvas**, Meenu R. Rajan, Cecilia Jönsson, Peter Strålfors and Maria V. Turkina
IQGAP1 links caveolae to the cytoskeleton in primary human adipocytes
Manuscript

ABBREVIATIONS

BMI	body mass index
Cdc42	cell division cycle 42
CID	collision induced dissociation
CSD	caveolin-1 scaffolding domain
ERK	extracellular signal-regulated kinase
ESI	electrospray ionization
ETD	electron transfer dissociation
FDR	false discovery rate
FFA	free fatty acid
GLUT4	glucose transporter 4
HAT	histone acetyltransferase
HD	high density (caveolae)
HDAC	histone deacetylase
HDM	histone demethylase
HMT	histone methyl transferase
HPLC	high performance liquid chromatography
IR	insulin receptor
IRS1	insulin receptor substrate 1
IQGAP	IQ-motif containing GTPase activating protein
LC	liquid chromatography
LD	low density (caveolae)
MAPK	mitogen-activated protein kinase
me1/2/3	mono- /di- / trimethylation
MS	mass spectrometry
MS/MS	tandem mass spectrometry
mTORC	mammalian target of rapamycin complex
Myo1c	unconventional myosin 1c
m/z	mass over charge ratio
PKB	protein kinase B
PTM	post-translational modification
PTRF	polymerase 1 and transcript release factor
Rac1	Ras-related C3 botulinum toxin substrate 1
T2D	type 2 diabetes
TAG	triacylglycerol
VHD	very-high density (caveolae)
WHO	world health organization

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INTRODUCTION

According to a fresh study almost one third of the global population is obese or overweight today, the number is more than twice of those undernourished in the world [1]. The consequences of obesity are many, for one there is the psychological aspect of low self-esteem, it has for example been shown that obese teenage girls are less likely to attend higher education than their non-obese friends (this was not the case for boys so actually the issue lies in both obesity and inequality between sexes). Another important consequence is the mortality – 5 % of all deaths worldwide are attributed to high body-mass index (BMI) according to the World Health Organization (WHO) [2]. Being obese increases the risk of developing heart disease, kidney failure, stroke, cancer, blindness and type 2 diabetes. Obesity's impact on global economy is as large as smoking or the cost for the whole world's armed conflicts and terrorism. 20 % of all health care costs are spent on obesity associated diseases and a large portion of this money is spent on diabetes treatment [1]. About 400 million people are now diagnosed with type 2 diabetes (T2D) and almost as many have impaired glucose tolerance – a pre-stage of diabetes.

OBESITY

Obesity is traditionally defined as BMI higher than 30 kg/m². This measurement can be informative for many of us but a bodybuilder might not agree. To get a more fair assessment of body composition the waist to hip ratio [3] can be measured and a circumference ratio of 0.85 for women and 0.9 for men is defined as abdominal obesity according to WHO [4].

The simplest answer to why we become obese is imbalance between calorie intake and utilization – in our modern world many of us spend most of our days sedentarily and we eat too much food with high energy content. However, reality is never simple and it is believed that genetics and epigenetics play important roles in the development of obesity [5-7]. The correlation between obesity and metabolic disorder is quite clear, but

a significant fraction of obese individuals are metabolically healthy while some non-obese show metabolic dysfunction [8].

THE ADIPOSE TISSUE

The adipose tissue is distributed throughout the body where it performs various tasks such as energy storage and homeostasis, cushioning and insulation. The biggest bulk of the tissue is made up of adipocytes but other cell types such as connective tissue, inflammatory- and immune cells, preadipocytes and fibroblasts as well as vasculature and nerves are also present [9]. The largest adipose depots are the subcutaneous and visceral fats, subcutaneous fat is stored just under the skin, mostly around the belly, buttocks and thighs. Visceral fat resides deeper, wraps the inner organs and is often considered the most dangerous fat type regarding the risk of developing insulin resistance and T2D (**Figure 1**) [10,11]. However, subcutaneous fat located in the upper body has been shown to influence the development of insulin resistance in many studies and some even claim that impaired function of the subcutaneous fat in the torso contributes more to the overall metabolic dysfunctionality than the visceral depot [12-15], reviewed in [16].

Figure 1. The subcutaneous adipose tissue can be found just under the skin in both the upper and lower body while the visceral adipose tissue depot surrounds the inner organs.

When nutrient intake is in excess the mass of the adipose tissue needs to expand to meet the increased demand of energy storage. Hyperplasia means increasing the number of adipocytes while hypertrophy increases the size of the already present adipocytes. The development of an individual's adipose tissue begins during the second trimester of pregnancy [17] and the number of adipocytes in an individual seems to be determined during childhood and remain constant during the adult life [18]. Obese individuals who are not insulin resistant seem to have small adipocytes in large numbers (hyperplasia) while the insulin resistant ones have fewer but bigger cells (hypertrophy) [19] [20]. Large adipocytes are insulin resistant [21] and have altered gene expression [22] and cell size has been shown to be a good predictor of T2D development regardless of BMI or waist to hip ratio [23] [19,24].

Both absence and excess of adipose tissue can cause systemic insulin resistance. There are several explanations to this and most likely a combination of several factors is responsible. The adipose tissue is not only present as an energy reservoir but also secretes hundreds of different adipokines [25], important hormone-like substances that regulate metabolism of the whole body and can induce insulin resistance in other tissues like muscle and liver. Obesity and insulin resistance have been correlated to dysregulation of adipokine secretion [26-29]. Obesity is also correlated to adipose tissue inflammation with macrophage infiltration and secretion of pro-inflammatory agents, as these cytokines spread via the blood they could affect the systemic insulin sensitivity [30,31]. Another factor that could contribute to systemic insulin resistance is the ectopic fat deposition that occurs when the adipocytes cannot store more fat due to e.g. insulin resistance or defective adipocytes or just not enough adipocytes to cope with the energy intake. The effect is lipid accumulation in non-adipose tissues and it is believed that this induces insulin resistance in e.g. muscle and liver cells [32-34]. Hence, in the context of ectopic fat deposition, lipodystrophy (absence of fat cells) equals the situation of "non-storing" adipocytes in obesity.

PRIMARY HUMAN ADIPOCYTES

The human adipocyte is large enough to be seen with the naked eye and consists mostly of stored triacylglycerol (TAG). The cell size varies from 20 μm to more than 200 μm in diameter and the cytosol is only a thin film between the lipid-droplet and the plasma membrane (**Figure 2**). The single large lipid droplet is one of the features that distinguish the human adipocyte from cultured and *in vitro* differentiated cells and also from brown adipocytes, where the fat is stored in multiple, smaller lipid droplets.

Figure 2. The human adipocyte contains a large lipid droplet where TAG is stored, and a thin cytosol. The big lipid droplet makes the nucleus protrude from the cell.

One of the most important tasks of the adipocyte is to store and release energy in times of excess and shortage. Storage is implemented through lipogenesis where esterification between free fatty acids (FFAs) and glycerol form TAG that is stored in the lipid-droplet. In contrast, lipolysis is the process where the stored TAG is hydrolyzed to fatty acids and glycerol that can be used as an energy source in other parts of the body. The main regulator of lipolysis and lipogenesis is insulin.

INSULIN SIGNALING, INSULIN RESISTANCE AND TYPE 2 DIABETES

Insulin signaling

Insulin is released from the pancreatic β -cells in response to raised levels of blood glucose, for example after a meal. The hormone promotes e.g. glucose uptake in insulin

sensitive tissues (muscle, liver and adipose tissue) to maintain the blood glucose concentration at a suitable level.

When insulin reaches the adipocyte it binds to insulin receptors (IRs) that auto-phosphorylate and this phosphorylation triggers a cascade of signaling events in the cell. The insulin receptor is rapidly internalized and later recycled to the plasma membrane (**Figure 5**) [35,36]. One of the effects of insulin signaling is activation of lipoprotein lipase [37], which hydrolyzes TAG in circulating VLDL and chylomicron lipoproteins to glycerol and free fatty acids. Most of these FFAs enter the adipocytes where they are re-esterified to TAG and stored in the lipid-droplet. Glucose is essential to provide glycerolphosphate needed for the re-esterification process and insulin stimulates the translocation of glucose transporter 4 (GLUT4) from an intracellular vesicular pool to the plasma membrane where it permits glucose to enter the cell. Translocation of GLUT4 vesicles is mediated through stepwise activation by phosphorylation of several proteins, in particular the insulin receptor substrate-1 (IRS1), protein kinase B (PKB) and the Akt substrate of 160 kDa (AS160) [38,39] (**Figure 3**).

PKB is an important hub in insulin signaling and can activate mammalian target of rapamycin complex 1 (mTORC1) that conveys a feedback signal to IRS1 leading to increased phosphorylation of serine 307. The positive or negative effect of this feedback is debated (reviewed in [40]) but in human adipocytes phosphorylation of IRS1 at serine 307 is shown to be required for proper insulin signaling [41,42]. Cell growth is promoted by insulin via PKB and mTORC1 activation of the ribosomal protein S6 kinase (S6K) leading to increased translation of proteins (**Figure 3**). mTORC1 activation can also stimulate cell growth by increased nucleotide and lipid synthesis [43]. PKB activation of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) pathway controls the transcription of many genes which can also contribute to increased cell growth (**Figure 3**).

Insulin-dependent inhibition of lipolysis is mediated through PKB catalyzed phosphorylation and activation of cGMP-inhibited 3',5'-cyclic phosphodiesterase B (PDE3B) [44] and perhaps also through downregulation of lipolytic gene transcription via inhibition of forkhead box protein O1 (FoxO1) [45] (**Figure 3**).

Figure 3. A simplified schematic view of the insulin signaling pathways in human adipocytes. Insulin stimulation of the cell leads to e.g. increased glucose uptake, decreased lipolysis, increased protein translation and altered transcription. These processes are regulated through intricate networks of many proteins.

Insulin resistance and type 2 diabetes

In insulin resistant tissues the glucose uptake is less efficient and more insulin is produced to achieve glucose homeostasis. In time, the β -cells fail to compensate for the decreased insulin sensitivity and the concentration of glucose rises in the blood.

The exact mechanism of insulin resistance – which steps in the insulin signaling pathways become dysfunctional in the adipocytes – is not clear. To get an overview is not made easier by the fact that different groups report contradicting results and different model systems deviate from each other. However, work on internally consistent data from mature primary human adipocytes in combination with

mathematical modeling has revealed three steps in the insulin signaling pathways that can explain the insulin resistance in human adipocytes. First, lower concentrations of insulin receptor in adipocytes from T2D subjects compared to cells from non-diabetics results in reduced maximal phosphorylation and internalization of the receptor. Second, lower concentrations of GLUT4 in T2D cells leads to reduced maximal glucose uptake. Last and most important is an attenuated positive feedback from mTORC1 to the phosphorylation at serine 307 of IRS1 in T2D adipocytes. This attenuation of the feedback affects insulin signaling through IRS1, PKB, mTOR and ERK branches of the insulin signaling network [42].

Large adipocytes are linked with insulin resistance and one can hypothesize that the resistance is a defense mechanism that kicks in when the cell senses that it either has to stop growing or die. It has been shown that cell death is correlated to adipocyte size and that dead adipocytes attract macrophages [46] that mediate inflammation in the tissue, which can in turn cause local and systemic insulin resistance [31]. Moreover, endoplasmatic reticulum stress and hypoxia, both known to trigger cell death, attenuate mTOR activity which can affect the insulin signaling and sensitivity [47-50].

CAVEOLAE

Caveolae – platforms for signaling and metabolism in the plasma membrane

In adipocytes the insulin receptor is located in cave like invaginations of the plasma membrane called caveolae [51-53] (**Figure 4**).

Figure 4. Caveolae are cave like invaginations in the plasma membrane that contain important proteins like the insulin receptor.

Following insulin stimulation the insulin receptor is endocytosed in a caveolae-mediated process [35], and after insulin is released the receptor is recycled back to the plasma membrane [36,54,55] (**Figure 5**). Destruction of caveolae by cholesterol extraction using betacyclodextrin does not seem to affect the insulin receptor binding of insulin or the receptor autophosphorylation but downstream signaling has been shown to be impaired [56]. Moreover, IRS1 is localized to caveolae in human adipocytes strengthening the role of caveolae in insulin signaling [57]. The controversial relationship between caveolae and GLUT4 has been thoroughly reviewed [58,59] and the debate is still lively. It seems likely that GLUT4 mediated uptake of glucose can occur through both clathrin and caveolae governed processes [60].

Figure 5. Insulin binding and activation of the insulin receptor (IR) leads to internalization of IR via caveolae. When insulin dissociates IR is deactivated and recycled back to the plasma membrane.

Caveolae can be found in many cell types but are most numerous in adipocytes. Around one third of the adipocyte plasma membrane is constituted of caveolae [61]. The structural integrity of caveolae is dependent on both lipid composition, in particular cholesterol and sphingolipids [62], and on protein constituents like caveolin-1, cavin-1

(also known as polymerase 1 and transcript release factor, PTRF) and pascin 2 (reviewed in [63]).

There are different subclasses of caveolae and by density gradient centrifugation they can be separated into three classes called very-high (VHD), high (HD) and low density (LD) caveolae. These classes differ in cholesterol concentration and their content of specific proteins; the VHD caveolae with the lowest concentration of cholesterol seem to lack an extracellular opening, HD caveolae are sites for uptake of FFAs and synthesis of TAG and they are enriched in proteins like perilipin and hormone sensitive lipase, while LD caveolae have high cholesterol content and is believed to be involved in cholesterol metabolism. Both HD and LD caveolae are associated with IR and GLUT4 and can thus be involved in insulin signaling [62].

Caveolin-1

The protein caveolin-1 is often used as a marker for caveolae and is necessary for its formation [64,65]. Knocking out caveolin-1 in mice leads to a 90 % decrease of insulin receptor protein in adipocytes without any reduction in mRNA levels indicating that caveolin-1 and/or caveolae are essential for IR stability [66]. Caveolin-1 knockdown in 3T3-L1 cells resulted in a 95 % decrease of caveolae and a substantial decrease in IR and GLUT4 protein levels without any effects on mRNA and additional experiments showed that the reductions were due to shorter half-lives of the proteins [67]. The caveolin-1 knockout mouse phenotype showed signs of insulin resistance even on a normal diet, had a reduced amount of body fat and seemed to be protected against age related obesity [66]. 3T3-L1 knockdown cells also showed signs of insulin resistance by reduced insulin stimulated IR phosphorylation, GLUT4 translocation to the plasma membrane and glucose uptake [67].

The Cavines

There are three homologous cavine proteins in adipocytes and all of them were first discovered in cellular processes seemingly unrelated to caveolae. Cavin-1 is also known as PTRF and is involved with the RNA polymerases [68,69]. After insulin stimulation cavin-1 is translocated to the nucleus but also to the cytosol in company with hormone sensitive lipase where it is involved in the control of lipolysis and fat mobilization [70,71]. Cavin-2 is also known as the serum deprivation-response protein (SPDR) and

cavin-3 is known as both protein kinase C delta-binding protein (PRKCDBP) and serum deprivation response factor-related gene product that binds to C-kinase (SRBC).

The same effects as described for caveolin-1 depletion (almost no caveolae, reduced IR and GLUT4 levels and impaired insulin signaling) have also been shown for cavin-1 knockout mice [72,73]. Moreover, depletion of cavin-1 leads to reduced amounts of caveolin-1, and vice versa [67,72,74]. Cavin-1 is the only one of the cavin proteins necessary for caveolae formation but they all seem to be involved in the membrane-curvature formation [75,76]. It is suggested that the cavin proteins form polymerized complexes that line the cytosolic side of caveolae [76-79].

Caveolae are associated with both actin and microtubules [80], interconnected by filamentous networks [81] and can fuse with, fission from and travel just beneath the plasma membrane [82]. Cavin-3 promotes caveolae dynamics [79] and absence of cavin-3 leads to less trafficking of caveolae derived vesicles along microtubules [82]. On the contrary, the caveolae associated protein EH-domain containing protein 2 (EHD2) stabilizes caveolae at the plasma membrane by linking it to actin [83,84].

SCAFFOLDING PROTEINS

In efficient signaling there is often one or more scaffolding proteins involved to organize the different components into multiprotein complexes. A scaffolding protein has the ability to organize the signaling participants in a much more complex manner than for example adaptors and dockers that more or less tethers a couple of proteins without affecting them. In order to be classified as a scaffolding protein certain criteria of function should be fulfilled; the capacity to assemble more than two other proteins and organize them into a higher order structure, the ability to synchronize different macromolecular complexes in different locations of the cell and the ability to affect the assembled proteins and also be affected by them. These features allow the scaffolding proteins to fine tune the specificity and sensitivity of signaling in response to crosstalks, feedbacks and dose-dependent signals [85,86].

Scaffolding proteins are multidomain proteins with a combination of domains that are specific for their function and the pathway(s) they are involved in. The recruitment of a component can induce allosteric effects or conformational changes exposing or restricting the availability of other binding sites and domains delimiting the complex to a specific signaling pathway or response [85,87]. At the same time, some scaffolding protein can interact with other multimolecular complexes from other pathways to orchestrate multiple responses or form even larger complexes [85].

Post-translational modifications are important for the functionality of the scaffolding proteins. Modifications like phosphorylation and ubiquitination can regulate e.g. the localization, the conformation, the catalytic activity and the degradation of the protein. The amount of a specific scaffolding protein in relation to its interacting components is important for the cellular function. If the concentration of the scaffolding protein is too low the effect can be diminished, if it is too high the scaffolding protein may engage the components individually and prevent them from participating in larger complexes and in other important functions [88]. A malfunctioning or deregulated scaffolding protein could lead to serious diseases such as cancer, obesity, diabetes, cardiac- and neuronal diseases (reviewed in [85]).

SCAFFOLDING PROTEINS IN INSULIN SIGNALING

A typical example of a scaffolding protein in insulin signaling is IRS1 (reviewed in [89]). IRS1 is a multidomain protein which is controlled by multiple phosphorylation sites and is, in human adipocytes, located at the plasma membrane, more specifically in caveolae in close proximity or bound to the insulin receptor [57] via one of its domains [90,91]. Upon insulin stimulation IR phosphorylates IRS1 on tyrosine residues, which facilitates the recruitment of other signaling proteins that in turn are activated by binding to IRS1. Feedback from the downstream signaling is relayed back to IRS1 through further phosphorylation at serine residues and the signaling pathway is thus tightly regulated.

Caveolin-1 has a specific domain called the caveolin-1 scaffolding domain (CSD) [92] implying that the protein has the potential to act as a scaffolding protein. Indeed, mutation or downregulation of caveolin-1 affect many signaling pathways, for example insulin signaling [58,67]. Two CSD binding motifs have been found and several caveolae associated proteins, like the insulin receptor, contain one of these motifs [93]. However, Collins *et al.* have showed that in most cases the specific binding motif is not accessible in the tertiary structure of the protein, making an interaction with caveolin-1 via this motif unlikely [94]. Moreover, the binding motifs are present in about one third of all proteins and are not enriched among the cytoplasmic proteins, which have the possibility to interact with caveolin-1 in regard to their localization [94]. Liu *et al.* speculate that perhaps the lipid composition of caveolae acts as the actual scaffold and caveolin-1 is involved through its stabilizing effect on cholesterol levels in caveolae and not because of its ability to scaffold other proteins [95].

A number of scaffolding proteins have been identified in the MAPK-ERK pathway. The best characterized is the kinase suppressor of Ras 1 (KSR1) that is thought to translocate from the cytoplasm to the plasma membrane in growth factor-stimulated cells where it tunes the Raf/mitogen-activated protein kinase kinase (MEK)/ERK signaling (reviewed in [96]). Another scaffolding protein indicated in ERK-signaling is the IQ-motif containing GTPase activating protein (IQGAP) [97,98].

IQGAP1

IQGAP comes in three variants, IQGAP 1, 2 and 3. The isoforms are homologous in sequence and structure but have different tissue specificity and function. IQGAP1 is ubiquitously expressed while IQGAP2 is mostly expressed in liver and IQGAP3 can be found predominantly in brain [99]. Contrary to what the name implies IQGAPs do not promote GTPase activity but stabilize the active state of GTP-bound proteins like the cytoskeletal effector protein cell division cycle 42 (Cdc42) [100,101]. IQGAP1 is composed of several domains; a calponin homology domain (CHD), a poly-proline protein-protein domain (WW), 4 IQ-motifs, a rasGAP-related domain (GRD) and a RasGAP-C terminus domain (RGCT) (**Figure 6**).

IQGAP1 is mostly found close to the plasma membrane but has also been found in the nuclear fraction in cell cycle arrested cells [102] and is found to be removed from the cell cortex in response to elevated calcium levels [103]. In paper III we find IQGAP1 evenly distributed throughout the thin cytoplasm of the human adipocyte.

Figure 6. A Schematic view of IQGAP1 domains and examples of associated proteins. Shown on top are Calponin homology domain (CHD), coiled-coil region, poly-proline protein-protein domain (WW), 4 IQ-motifs (IQ), rasGAP-related domain (GRD) and RasGAP-C terminus domain (RGCT). Domains are indicated with amino acid numbers taken from the Pfam database. Shown at the bottom are associated proteins summarized from [99,104-106].

IQGAP1 is known to associate with more than a hundred different proteins and many of them are reviewed in [107], [104] and [105]. In regard of the diversity of the IQGAP1 associated proteins (**Figure 6**) it is not surprising that the roles of IQGAP1 are many. Cell

growth and proliferation, cytoskeletal regulation and cell migration, and scaffolding of different cell signaling pathways are just a few of the functions IQGAP1 is involved in [105,107].

Overexpression of IQGAP1 can induce tumors in mice [108] and many studies show that an upregulation of IQGAP1 is related to cancer [109-111] [112,113]. IQGAP1 knock out animals show no obvious developmental defects but exhibit gastric hyperplasia [114] and reduced tumorigenesis [115]. Silencing IQGAP1 in cell lines also seems to inhibit their cancerogenic behavior [109,116].

IQGAP1 and the cytoskeleton

Cytoskeletal reorganization is important for cell-cell interactions, cell movement and intracellular organization, trafficking and signaling etc. and IQGAP1 is emerging as an essential regulator of cytoskeletal dynamics (reviewed in e.g. [106,117,118]). IQGAP1 has been shown to interact with F-actin [119] and regulate its dynamic behavior by for example capping the barbed ends of actin filaments both *in vitro* [120] and in T cells [121]. Moreover, interaction between IQGAP1 and the myosin essential light chain (ELC) as well as myosin 1c (Myo1c) has been demonstrated [122,123]. There are also indications of a coordinating role for IQGAP1 between F-actin and the microtubule networks through Cdc42, Ras-related C3 botulinum toxin substrate 1 (Rac1) and the cytoplasmic linker protein Clip170 [124].

Studies on β -cells have revealed that IQGAP1 recruits the endocytic machinery to the plasma membrane together with GTP-bound Cdc42 [125] and that IQGAP1-exocyst-septin enhanced exocytosis is inhibited by Cdc42 [126]. In adherens junction fractions from rat liver Rac-Cdc42-IQGAP1 was shown to inhibit the clathrin-dependent (caveolae-independent) endocytosis of E-cadherin, probably through cross-linking of F-actin [127]. Taken together it seems likely that IQGAP1 could be involved in controlling endo- and exocytosis through cytoskeletal regulation which is potentially important for caveolae internalization and recycling.

IQGAP1 and caveolae

It has been suggested that IQGAP1 plays a role in the plasma membrane insertion of caveolin-1 containing vesicles via the integrin-linked kinase (ILK) and through

stabilization of microtubules [128]. Increased dynamics of both microtubule and caveolae was shown in IQGAP1 deficient keratinocytes and caveolin-1 was relocated from the cell periphery to the perinuclear region in those cells [128].

Both IQGAP1 and caveolin-1 are associated to cholesterol rich membrane ruffles in PC-3 cells [129]. Cholesterol treatment of the cells increased the association of both proteins with detergent-resistant membrane fractions without affecting their transcription, and caveolin-1 knock-down in the cells reduced the IQGAP1 recruitment to these cholesterol rich membrane structures [129].

IQGAP1 and caveolin-1 seems to cooperate to facilitate the phosphorylation and activation of ERK1/2 by protein kinase C (PKC). It has been shown by Vetterkind *et al.* that IQGAP1 tethers ERK1/2 to actin filaments, which appears to be crucial for the activation. Caveolin-1 on the other hand, is required for the actual phosphorylation of ERK1/2 [130]. A direct binding between caveolin-1 and IQGAP1 was not suggested due to negative immunoprecipitation results [130], but neither can it be ruled out.

In paper III we immunoprecipitated IQGAP1 and could identify caveolin-1 as well as the caveolae-associated proteins cavin1, 2 and 3, EHD2, hormone sensitive lipase and perilipin as IQGAP1 associated proteins. By proximity ligation assay (PLA) we could determine that IQGAP1 and caveolin-1 colocalize. However, since caveolae are detergent resistant structures it is difficult to determine if the association between caveolin-1 and IQGAP1 is direct or mediated through some intermediate caveolae associated protein.

IQGAP1 and type 2 diabetes

Osman *et al.* has called IQGAP1 “A molecular rheostat at the interface of cancer and diabetes” [99]. This statement is based on the regulatory control IQGAP1 is believed to exert on insulin secretion and cell size versus cell division and differentiation in β -cells. The fine tuning of these pathways is thought to be controlled via phosphorylation at serine 1443 that changes the conformation of IQGAP1 [126,131]. Dysregulation of IQGAP1 levels or its phosphorylation could tilt the balance in a direction that can lead to diabetes and/or cancer. It has been stated that that IQGAP1 mRNA is downregulated in the β -cells of a subpopulation of type 2 diabetics [99,132] and knock-down of IQGAP1

decreases the glucose stimulated secretion of insulin [126]. We have shown in paper III that IQGAP1 is clearly downregulated in adipocytes from T2D subjects compared with non-diabetic controls.

There are indications of a role for IQGAP1 in the cytoskeletal reorganization required for translocation of GLUT4 vesicles to the plasma membrane upon insulin stimulation via Rac1 in muscle cells and possibly in adipocytes, reviewed in [133]. IQGAP1 is found to associate with exocyst [126] and TC10 [134], who are involved in tethering GLUT4 vesicles to the plasma membrane [135]. Additionally, exocyst seems to be a part of the machinery that connect GLUT4 vesicles to actin filaments via Ral1 and the motor myosin Myo1c in adipocytes [123,136] and in paper III we show an association between IQGAP1 and Myo1c in primary human adipocytes. If IQGAP1 has a functional role in the insulin induced glucose uptake as implied, it's dysregulation could have a role in the reduced glucose uptake observed in adipocytes from type 2 diabetic subjects [137].

In September 2015, a poster abstract by Hedman *et al.* was published [138] stating that the insulin receptor directly binds to the IQ region of IQGAP1 and IRS1 to IQGAP1's C-terminal tail *in vitro*. Furthermore, they claim that decreased levels of IQGAP1 impaired the activating phosphorylations of IRS1, PKB and ERK *in vivo* [138]. These results strengthen the possibility that improper regulation of the concentration and/or malfunctioning control of IQGAP1 could be involved in the impaired insulin signaling via caveolae in insulin resistant adipocytes.

PROTEOMICS

Proteomics is the study of which proteins are present at a specific time under specific conditions, how these proteins are modified, where they are localized, how they interact with each other, what their functions are, etc. The proteome is constantly changing and is influenced by a large number of variables; hormonal stimuli, stress, cell cycle, age and disease are just a few. To study proteomics in a large scale is technically challenging and the development of advanced instrumentation over the last few years has allowed rapid progress in the field.

MASS SPECTROMETRY

Mass spectrometry (MS) is an indispensable proteomic tool that can provide both qualitative and quantitative information about the complex proteome. The principle of mass spectrometry is to determine the precise mass to charge ratio (m/z) of charged molecules (ions) in gas phase (**Figure 7**). Two different Nobel Prize awarded techniques are employed to get the molecules ionized and into the gas phase; matrix-assisted laser desorption-ionization (MALDI) and electrospray ionization (ESI). Molecules are separated according to their m/z in different types of mass analyzers. A time-of-flight (TOF) analyzer is based on the principle that a smaller ion flies faster than a larger ion with the same charge when accelerated by an electrical field. The behavior of an ion in an oscillating electrical field is another approach of separating the ions and can also be used as a mass filter, this technique is used in quadrupole mass analyzers and iontraps.

Bottom up, top down and middle down approaches

The analysis of proteins by mass spectrometry can be divided into three categories; bottom up, top down and middle down proteomics (**Figure 10**). The most commonly used approach is bottom up which means that the proteins are proteolytically digested before analysis, usually by trypsin that cleave the proteins after the basic amino acid residues lysine and arginine, which yields peptides of up to 20 amino acids (500-3000 Da).

Figure 7. Simplified schematic of a typical nanoLC/MS/MS workflow used in papers I and III. Preparation of protein and peptide samples is followed by nanoLC separation directly coupled to the injection into the mass spectrometer by electrospray ionization. Peptide ions with the highest intensities are selected for fragmentation. The detected peptides and their fragments are analyzed in search-engines and annotated, these results can further be studied by different bioinformatical approaches.

A drawback with bottom up proteomics is the loss of context. In top down proteomics the intact protein is analyzed but the size of the protein is for the time being restricted to ~50 kDa, the demand of a pure protein is high and the interpretation of the mass spectra is complicated. In paper I we employed limited proteolysis and a protease (ArgC) that yielded longer peptides in a middle down approach, thereby we gained in context compared to bottom up proteomics without having struggle with the restrictions and complexity of the top down approach.

Peptide mass fingerprinting and MS/MS

Interpretation of mass spectrometry data of proteins can be done by peptide mass fingerprinting (PMF) or by tandem mass spectrometry (MS/MS) ion analysis of the amino acid sequence. With PMF the m/z of all peptides in the sample are measured and the masses are compared with a database with theoretical m/z of digested proteins, the masses are combined and assigned to the most probable protein identification(s). If a peptide is modified by mutation or a post-translational modification (PTM) the difference in mass that the modification inflicts on the peptide enables its identification. In an MS/MS ion analysis workflow the peptides are further fragmented in the mass spectrometer. A precursor ion is selected in a quadrupole or iontrap and then fragmented. The fragments are then analyzed according to their m/z and compared to a database with theoretical MS and MS/MS data combined. With MS/MS ion analysis the primary amino acid sequence of a peptide can be determined by comparing the m/z of the fragments with information of the specific mass of each amino acid and modifications of the peptide can be assigned to the specific amino acid.

Fragmentation

The traditional fragmentation technique is collision induced dissociation (CID) where the precursor ion is collided with an inert gas like nitrogen, helium or argon. Kinetic energy in the collision is converted to internal energy in the ion leading to characteristic fragmentation of the peptide backbone resulting in primarily y- and b-ions (**Figure 8**). Many PTMs fall off the peptide at fragmentation but e.g. serine and threonine phosphorylation will fall off together with a piece of the amino acid allowing detection by the specific reduction of the amino acid mass (**Figure 8**).

Figure 8. The peptide backbone is fragmented between different atoms depending on the technique. ETD fragmentation leads to primarily C-terminal z-ions, N-terminal c-ions and PTMs intact on the amino acid. CID results in C-terminal y-ions, N-terminal b-ions and fragmentation of many PTMs. However, serine and threonine phosphorylation can be fragmented together with the oxygen of the amino acid resulting in a detectable reduction in mass.

Electron transfer dissociation (ETD) is a different type of fragmentation technique that leaves PTMs intact on the peptides. An odd electron is transferred from a carrier molecule, e.g. fluoranthene, to the peptide which is fragmented along the backbone resulting in mainly z- and c- but also some y-ions (**Figure 8**). ETD is also very useful for fragmenting large, multiply charged peptides. Alternation between CID and ETD fragmentation in the same experiment as we did in paper I can provide more confident assignments of sequences and give better sequence coverage (% of the sequence that is assigned by the analysis) of the identified proteins.

Bioinformatic analysis

To make sense of all the data generated from a modern mass spectrometry experiment the implementation of bioinformatics is crucial. Earlier, the throughput of the instruments was not very high and spectra were read manually, assigned to peptides and collected into lists of identified proteins. This was a tedious task of puzzling with amino acids and modifications but the control over the results was very good. Also, to finally be able to puzzle out a long sequence from a complicated mass spectrum was truly rewarding. Today we connect our mass spectrometers with nano-LCs running hour-long gradients with automated MS/MS methods and the amount of data is not only dazzling but also impossible to interpret manually. Algorithms covered in user friendly interfaces allow us to submit large amounts of data and retrieve a list of identified proteins and peptides. These search-engine algorithms compare data from the acquired spectra against a theoretical database predicted from either nucleic acid or amino acid sequences which means that the studied species has to have at least a sequenced genome. The matches are scored and ranked according to their degree of similarity with the theoretical data. In the results list, some identifications will always be false positive because in the large theoretical database matches will happen by chance. In a database search, the number of variable modifications and the number of allowed missed proteolytic cleavages can be included as criteria, which will increase the quantity of possible combinations that can lead to false positive identifications. To validate the results a false discovery rate (FDR) is calculated by using a decoy database presumed to contain no real peptide sequences and is usually a reversed, randomized or shuffled version of the target database. There are different approaches on how to use the decoy database. A target-decoy strategy assumes that the number of identifications in the decoy database reflects the number of false positive identifications from the target database [139]. A semi supervised learning strategy (Percolator) use identifications from the decoy database as “bad” examples and the highest scoring identifications from the real database as “good” examples and can correct an incorrect ranking of different sequences assigned to the same spectrum (this approach was used in paper III) [140].

The available algorithms used to compare experimental data with the theoretical databases are not adapted to a search including many different variable modifications and/or missed cleavages. A large dataset searched against a large database with many

variables allowed will need an unreasonable amount of time to complete the search and the FDR will be very high and result in many false positive and negative identifications. A solution is to use a limited database including only relevant proteins (as we did in paper I) but then FDR cannot be calculated and the validation has to be done manually. Stepwise approaches are now often included in the softwares to reduce the required search time. A dataset can first be searched without variables, the unassigned spectra will then be searched using a couple of variables and those still unassigned will be searched using a few more variables and so on.

Once a list of confidently assigned peptide identifications is produced the results can be overwhelming. The analysis of a complex sample can identify hundreds or thousands of proteins. There are many softwares available to make sense of the results and they put the proteins into a context of for example cellular compartmentalization, interactions, pathways or diseases. The softwares can be based on e.g. gene ontology, verified literature references, text mining, co-expression and/or high throughput experimental data.

PREFRACTIONATION OF SAMPLES

Analysis of a complex sample by mass spectrometry usually requires some pre-fractionation (**Figure 7**). Only the most abundant peptides at a given time will be identified meaning that low abundance proteins and modified peptides may not be detected. Depending on the question to be answered there are numerous alternatives to choose from. Chromatography methods based on protein and peptide hydrophobicity (reversed phase high performance liquid chromatography, rpHPLC) (paper I and III), hydrophilicity (hydrophilic interaction chromatography, HILIC) or charge (strong cation/anion exchange chromatography, SCX/SAX) can be combined in several steps in order to generate less complex fractions of the sample. The affinity of phospho-groups for positively charged metal ions is used to enrich for phosphorylated peptides which would otherwise not be detected due to their low abundance. An immunoprecipitation approach (as we have used in paper III) employs the affinity of a specific antibody for a protein to pull the protein out of the mixture and study it and its interaction-partners more closely. Proteins with a certain pI can be enriched by gel-based separations,

chromatography or by extracting the proteins from the cells with acid (paper I) or base. In-gel digestion is a frequently used method to separate proteins according to size in a polyacrylamide gel, either 1 dimensional (size) or 2 dimensional (pI + size), followed by digestion of the proteins and extraction of their peptides from bands or spots cut from the gel (paper I and III) [141]. Separated proteins are visualized by staining, coomassie, silver and SYPRO Ruby are most commonly used, and the sensitivity of the stains is nowadays exceeded by the sensitivity of the mass spectrometers so to get the most comprehensive picture of the studied proteome unstained areas of the gel should also be analyzed.

CHALLENGES OF A PROTEOMIC APPROACH

The sequencing of the human genome was considered complete around a decade ago, the human proteome is however still under construction – an almost overwhelming task considering the complexity of alternative splicing, truncations and post-translational modifications in different combinations. More than half of the data in a typical bottom up LC/MS/MS experiment (**Figure 7**) remains unassigned by standard search-engine approaches. Chick *et al.* reports that by complementing the normal search with an ultra-tolerant search a large portion of these spectra can be assigned to peptides modified by “unusual” modifications [142]. The need for this type of searches is obvious and as computational power continues to grow, so will the field of proteomics.

Reproducibility, even within the same laboratory environment, is challenging and small differences in a protocol can induce large differences in the outcome. In the workflow of sample preparation and mass spectrometry analysis (**Figure 7**) there are several critical steps that can introduce variation in the results. Almost every scientist has her/his own special tricks when it comes to e.g. proteolytically digesting the proteins, enriching the phosphopeptides and storing the samples. Moreover, different vendors and even different batches of enzymes and other chemicals can vary in efficiency and purity introducing potential disparities. The instruments like HPLCs and mass spectrometers can also show variation in performance depending on construction, contaminations and attrition of consumables.

HISTONES IN HUMAN ADIPOCYTES

There is a heritability of type 2 diabetes but only about 10 % can be explained by genetics [143]. Around 40 gene variants have been identified that correlate with T2D, most of those are related to β -cell function and very few to obesity and T2D [144]. A large part of the heritability is probably due to environmental factors as habits of eating and sedentary life style are often passed on through generations. However, attention has lately been paid to the epigenetic influence on inheritance and development of disease.

Epigenetics means “above genetics” and can be defined as heritable changes, both mitotically and meiotically, which does not involve changes in the DNA sequence [145]. Alteration of epigenetic patterns in response to stimuli can be rapid and often beneficial, for example a nutrient deprived fetus can adapt its phenotype to better cope with the intrauterine conditions. However, it seems like these effects can be stable even when the initiating stimulus is removed and affect the probability that the individual will develop diseases like obesity and type 2 diabetes later in its adult life. The correlation between transgenerational and in-utero/early life experiences with the risk of disease in adulthood is extensively studied both epidemiologically [146-151] and at the level of modifications in animal models [152,153] as well as in humans [154-156]. The number of reviews on the topic of linking epigenetic modifications to obesity and T2D seems convincingly huge, e.g. [157-167].

There are three mechanisms that are often suggested to be epigenetic: DNA-cytosine methylation, non-coding RNA-associated gene silencing and covalent post-translational modifications (PTMs) and variants of histones. Studies of DNA-methylation in relation to disease and inheritance are dominating but in this thesis I will focus on histones alone.

Histone variants and PTMs are believed to affect the accessibility of genes for transcription by altering the chromatin structure [168]. A human cell contains approximately 2 m of DNA which is stored in the cell nucleus with a diameter of around 3-4 micrometers, this amazing condensation is facilitated by chromatin condensation.

Chromatin is made up of nucleosomes and nucleosomes are essentially DNA wrapped around core histone octamers (**Figure 9**). There are four different core histones; H2A, H2B, H3 and H4 and a linker histone H1. The tertiary structure of all core histones is similar, they have a globular domain which is arranged in the nucleosome octamer and they have N-terminal tails that protrude from the nucleosomes (**Figure 9**). Histones can be subjected to reversible, covalent post-translational modifications and especially the N-terminal tails are heavily modified.

Figure 9. A nucleosome is DNA wrapped around a histone octamer. The N-terminal tails of the histones protrude from the nucleosome and are often heavily modified by PTMs.

Chromatin can be tightly packed silenced heterochromatin, or more loosely structured active euchromatin. There are distinct patterns of chromatin modifications associated with either hetero- or euchromatin. DNA-methylation generally suppresses gene transcription and is typically found in heterochromatin. The role of histone modifications is not as obvious since specific modifications at specific sites promote different responses, and the combination of many modifications could be what ultimately determines if transcription is activated or repressed [169-171]. Moreover, histones can be replaced in the nucleosome by highly homologous variants that are different enough to cause prominent effects [172,173]. Histone modifications and variants are believed to affect transcription 1) by altered charge of the modified amino acid that will change the DNA-histone interaction, 2) by specific recruitment of repressing or activating proteins and complexes, 3) by affecting the DNA-accessibility to transcription factors and 4) by altering the nucleosome-nucleosome interactions [174].

The idea of histone variants and PTMs as epigenetic, in the sense that they are heritable or convey a memory of something that happened a long time ago, is surprisingly uncontroversial. Interestingly, the half-life of histone PTMs are suggested to be as short

as minutes and days in cell culture (reviewed in [175]). Moreover, there is not much of hard evidence indicating a direct transfer of histone PTMs to daughter cells during mitosis and it seems even less is clear regarding meiosis. Models of inheritance are published (e.g in [176,177]) but the exact mechanisms are mostly unknown. Petruk *et al.* have shown that histone methylating enzymes can stay associated with DNA during replication while the methylated histones do not [178]. This could be a possible explanation for maintenance of histone PTMs over generations of cells but means that the enzymes, and not the histone PTMs, are the epigenetic factors. Ptashne, one of few tenacious opposing voices against the epigenetic quality of histone PTMs, ordain for example regulatory factors, self-perpetuation and possibly micro RNAs as the more likely epigenetic mechanisms [179,180]. I do not doubt the importance of histone variants and modifications regarding chromatin dynamics, neither do I question the epigenetic inheritance of certain traits, but the actual transmission of those traits via histone PTMs is to me a bit vague.

Regardless of heritability, histone variants and PTMs could be one of the factors facilitating the rapid adjustments of gene transcription to a changing environment. And regardless of whether they are the cause or the consequence, histone modifications have been associated with for example long term hyperglycemic memory after restoration of normoglycemia [181,182], and with altered transcription of adipokines after in-utero high fat diet exposure [183]. In paper II we showed that one specific histone modification is markedly downregulated in adipocytes from overweight individuals while another modification is increased in adipocytes from type 2 diabetic individuals, which indicates a relation between histone PTMs and metabolic state.

HISTONE VARIANTS AND MODIFICATIONS

Histone variants

Histones can be canonical (transcriptionally dependent on DNA-replication) or non-canonical (constitutively expressed). Both the canonical and non-canonical histones come in an array of variants and the differences between the variants are often small but with seemingly specific effects on transcriptional activity (reviewed in [177,184-188]). The linker histone H1 has the most diversity between its variants and histone

H2A the most variation among the core histones, while H2B and H3 variants differ in only a few amino acids. Histone H4 was considered to be expressed in only one isoform but alternative variants have been identified in soybean [189] and in paper I we identified a histone H4 variant in human adipocytes. Histone variants, as well as modifications, can be associated with disease. Over- or underexpression, mutations and defective splice variants of H2A variants and the variant H3.3 have been associated with many different human cancers and are potential biomarkers and therapeutic targets [190,191] and macroH2A1 knock-out can protect against diet-induced obesity in mice [192]. In paper I we identified several histone variants in mature, primary human adipocytes, some of these had never been identified on protein level before and some had been considered to be oocyte or testis specific.

Histone acetylation

Acetylation of histones was first described in 1963 [193] and in 1964 it was proposed that histone acetylation might affect RNA synthesis in a positive way *in vivo* [194]. The mechanism is believed to be neutralization of the positively charged lysine which leads to less interaction between histone and DNA [195]. There seems to be a specificity to the modification, certain lysines are mainly acetylated around transcription start sites while others are more common in promoters and in the transcribed regions of active genes [196]. Acetylation is catalyzed by histone acetyltransferases (HATs) that transfer an acetyl group from acetyl-CoA to the target lysine. The availability of acetyl-CoA varies with the metabolic state implicating acetylation as a way of controlling transcription in relation to metabolism [197]. Deacetylation is facilitated by different classes of histone deacetylases (HDACs). Class III HDACs are NAD⁺-dependent and thus also linked to the metabolism of the cell [198,199].

Malfunctioning HATs and HDACs or loss of these enzymes have been implicated in a range of diseases such as cancer, diabetes and neurodegenerative disorders. For example the class III HDAC Sirtuin 6 (SirT6) controls transcription of glycolytic genes depending on nutrient availability and can block tumor initiation and growth by regulating metabolism [200]. In several human cancers SirT6 is downregulated and could be used as both a prognostic marker and a therapeutic target [201]. Another example is the suppression of *glut4* transcription in adult muscle tissue after perinatal

nutrient restriction that may be mediated by deacetylation of the *glut4* promoter by HDAC1 (class I) and HDAC4 (class II) [152].

Histone methylation

Methylation of lysine and arginine does not alter the charge of the amino acid but increases the hydrophobicity and the basicity of the histone tail, affecting the affinity towards specific interactors. The location of the methylation and if the specific amino acid is mono-, di- or trimethylated (me1, me2, me3) is crucial for which effect the methylation will exert on transcription. Histone methyl transferases (HMTs) catalyze the addition of methyl groups and histone demethylases (HDMs) remove them. HMTs and HDMs are quite specific both regarding the amino acid and the number of methyl groups.

Methylation of lysine 4 in histone H3 (H3K4me) is usually associated with active transcription and different multiplicity of the methyl groups is associated with different parts of the transcribed gene [196,202,203]. Trimethylation is believed to be most concentrated to the beginning of active genes while di- and monomethylation are more dispersed throughout the gene and towards the end respectively [203]. Of course there are exceptions to the rule and there have also been reports on association between H3K4me3 and silent genes [204]. Other H3 methylations are also strongly correlated to active transcription, for example K36 and K79 [205-207]. Proper control over H3K4 methylation could be important for adipogenesis as H3K4-HMT knockout mice have less adipose tissue and a more favorable metabolic profile compared to wild type [208]. In paper II we show that the global level of H3K4me2 is clearly downregulated in adipocytes from overweight individuals, while H3K4me3 is markedly upregulated in adipocytes from type 2 diabetic individuals.

One of the most studied histone methylations associated with gene repression and heterochromatin is Lysine 9 methylation in histone H3 (H3K9me) [209,210]. The deacetylation of *glut4* in response to perinatal nutrient restriction discussed above is followed by H3K9me2 leading to heterochromatin formation and recruitment of a corepressor complex that inhibits transcription of *glut4* [152]. A correlation between deficiency of the H3K9me1/me2 specific demethylase 3A (JHDM2A) and obesity has been reported and it has been shown that this HDM is important for regulation of genes

involved in energy expenditure and fat storage [211-213]. In paper II we did not detect any global differences in H3K9me2 levels in adipocytes from normal weight, overweight or overweight T2D individuals, which of-course does not exclude gene-specific differences.

Other histone modifications

Although focus is often directed towards acetylation and methylation, the histones can be modified by many different PTMs. Phosphorylation, ubiquitination, sumoylation, formylation, crotonylation, ADP-ribosylation, glycosylation, hydroxylation, and citrullination are examples other post-translational modifications of histones.

Histone H1 is thought to have cell cycle-dependent phosphorylated sequences that leads to increased transcription [214] while serine and threonine phosphorylation of the core histones correlate to various events such as chromatin condensation, transcriptional activation, DNA-repair and apoptosis [215].

When a histone is mono-ubiquitinated its mass will increase with more than 50%, however, the effect of ubiquitin does not seem to depend on its bulky size but rather on the same mechanisms as the smaller modifications; by acting as a binding site for activating/repressing proteins and complexes, by blocking binding sites and by changing the chromatin conformation [216].

The histone code

The histones are often heavily modified and it is clear that the combination of PTMs is often much more important than a single modification. Strahl and Allis suggested that a “histone code” could be read by regulatory proteins and complexes [217] and many have studied the code since then [170,171,218-226]. Strahl and Allis also speculated that histone PTMs could influence the modification status of other sites on the same histone or in the same nucleosome by attracting or blocking specific enzymes and it did not take long until this was shown – H2BK123-ubiquitination is for example necessary for H3K4-methylation [227] and blocking H3K14-acetylation by mutation will prevent trimethylation of H3K4 [228].

Mass spectrometry is a very useful tool in the quest to decipher the histone code but there are limitations to the methodology (discussed in greater detail in the proteomics section). Since the histones are most often digested into peptides before analysis in a bottom-up approach there is no way of knowing if PTMs on different peptides are present in the same histone or if the peptides originate from different histones or even from different histone variants (**Figure 10**).

Figure 10. Schematic representation of bottom up, middle down and top down proteomic approaches for post-translational modification analysis. A bottom up approach does not allow comprehensive characterization of differentially modified proteins. Analysis of intact proteins (top down proteomics) or generation of larger peptides (middle down proteomics), are useful approaches when studying heavily modified proteins, e.g. histones.

To overcome this problem a top-down approach is preferable, however this is technically demanding both in regard to mass spectrometry instrumentation and purity of the histone sample. The compromise of a middle-down approach, as we used in paper I, yields longer peptides and thus more information about the combination of histone PTMs than a top-down approach (**Figure 10**). Another major problem is that the occurrence of many PTMs at the same peptide, which is often the case with histone N-terminal peptides, is not optimal for the algorithms of the search engines used to analyze the results, which can lead to an increase of both false positive and false negative results.

MAJOR FINDINGS AND CONCLUDING REMARKS

This work started with an attempt to characterize histone variants and post translational modifications in mature primary human adipocytes on a global (as opposed to gene specific) level. To make that possible we had to develop a method of histone extraction since the conventional approach did not work on the adipocytes. This resulted in a “direct acid extraction” without the traditional preceding enrichment of nuclei. The complexity and the variation of the histones between different subjects turned out to be huge. In **paper I** we managed to confidently identify several PTMs by nanoLC/MS/MS not previously identified. We could also identify four histone variants (HIST1H2BN, H2A (fragment), protein similar to H2AFY, and HIST2H4B) on protein level for the first time.

One of the most studied histones regarding different diseases is histone H3. However, in **paper I** we could not sequence the heavily post translationally modified N-terminal of histone H3, probably due to the high abundance of cleavage sites for the proteases trypsin and ArgC as well as the complexity of the PTMs. Therefore we decided to study histone H3 methylations by an immunochemical approach in **paper II**. We discovered a global reduction of lysine 4 dimethylation in overweight and type 2 diabetic subjects compared to normal weight controls and an increase in lysine 4 trimethylation in subjects with T2D compared with normal weight and overweight non-diabetics. These findings demonstrate that extensive changes in important chromatin modifications are associated with overweight and T2D.

The characterization of intact histones and their modifications by using a top-down approach is very appealing. The histones are relatively small and could theoretically be sequenced by mass spectrometry without proteolytic cleavage. However, only a few laboratories in the world master the approach and the complexity of histone modifications and variants in primary cells would be overwhelming for even the most chastened sequencer. Histones from primary human adipocytes turned out to be impossible to separate into the different classes (H1, H2A, H2B, H3 and H4) and the amount extracted from adipocytes from a single person was too low for the analysis.

In **paper III** we analyzed the interactome of IQGAP1 by immunoprecipitation and nanoLC/MS/MS and found several known but also many previously unknown IQGAP1 associated proteins. These proteins belonged mainly to the cytoskeleton and to caveolae. We also found that the abundance of IQGAP1 is reduced by approximately 40 % in adipocytes from T2D subjects compared with non-diabetic controls. IQGAP1 is believed to regulate cytoskeletal dynamics and caveolae is highly dependent on the cytoskeleton for both tethering to the plasma membrane and translocation within the cytosol. Our results lead us to believe that IQGAP1 could be a link between caveolae and the cytoskeleton and possibly a regulator of caveolae dynamics, and thus influence one of the important parts of insulin signaling in human adipocytes. Indeed, proximity ligation assay confirms the colocalization of caveolin-1 and IQGAP1.

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Ten years ago I didn't have any idea what it meant to be a PhD-student, I figured they worked as lab-instructors in my university courses and had some vague connection to research (well, my grandparents had a neighbor who had a son who did some kind of research on snails and got a doctor's degree...). During the past years I have learned the hard (and the fun) way and I would like to thank the people who helped me in one way or another during this time.

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