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DIABETES, OBESITY AND EXERCISE IN SKELETAL MUSCLE: EFFECTS ON GENE EXPRESSION AND DNA METHYLATION

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DIABETES, OBESITY AND EXERCISE IN SKELETAL MUSCLE: EFFECTS ON GENE EXPRESSION AND DNA METHYLATION

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my Family:

Claudia, Etienne, Myriam, Mélanie, Valentine, Chloé, Margot.

"It always seems impossible until it's done."

Nelson Mandela

ABSTRACT

Type 2 diabetes, obesity and depression are growing concerns for human health. Physical exercise is a known protective factor against these disorders, although the underlying mechanisms are incompletely understood. The studies in this thesis aim to increase the understanding of mechanisms controlling gene expression and DNA methylation in the context of type 2 diabetes, obesity and exercise.

TWIST1 and TWIST2 proteins play an important role in embryonic muscle development, inflammation and tumor metabolism. We demonstrated that *Twist1* or *Twist2* overexpression in mature skeletal muscle favors glycolysis and increases the expression of pro-inflammatory cytokines. Gene expression of *TWIST1* and *TWIST2* is unaltered by obesity, type 2 diabetes or exercise training.

Decreased circulating kynurenine levels are associated with resistance to depression. Kynurenine is transformed into kynurenic acid by kynurenine aminotransferases (KATs). Exercise training and PGC1α induce expression of *KATs* in skeletal muscle. We report that a single bout of exercise acutely decreased plasma kynurenine, while concomitantly increasing kynurenic acid in both type 2 diabetic and healthy subjects. Exercise-induced changes in kynurenine metabolism were independent of mRNA expression of the *KATs*. Kynurenine levels correlated with body mass index, suggesting kynurenine metabolism may link obesity and depression.

Exercise and diet affect skeletal muscle insulin sensitivity and DNA methylation. Using genome-wide approaches, we unraveled the effect of exercise on the skeletal muscle methylome. Training and high-fat diet, but not *in vitro* contraction, lead to epigenetic changes in the promoter of Sprouty RTK Signaling Antagonist 1 (*Spry1*), a gene involved in muscle stem cell quiescence. We found DNA methylation of *Spry1* increased binding of nuclear proteins to the promoter.

Insulin is a metabolic and growth promoting hormone. Using genome-wide approaches, we unraveled the effect of insulin on the skeletal muscle methylome. We observed that insulin treatment of skeletal muscle *in vitro* increased DNA methylation of the death-associated protein Kinase 3 (*DAPK3*). Conversely, *DAPK3* DNA methylation was reduced in type 2 diabetic subjects compared to controls. A glucose challenge further decreased DAPK3 methylation suggesting that additional factors in the systemic milieu may affect DAPK3 DNA methylation.

Collectively, our results indicate that TWIST proteins affect skeletal muscle metabolism and inflammation. We provide a potential mechanism for the anti-depressive effects of exercise and shed new light on the complex interplay between metabolic conditions, skeletal muscle and DNA methylation. We provide a new insight in the protective effect of exercise or the pathophysiology of type 2 diabetes and obesity, opening opportunities for improvements in the management and treatment of metabolic diseases.

LIST OF SCIENTIFIC PAPERS

I. **Mudry JM**, Massart J, Szekeres FL, Krook A. *TWIST1 and TWIST2 regulate glycogen storage and inflammatory genes in skeletal muscle.* J Endocrinol. 2015 Mar; 224(3):303-13. doi: 10.1530/JOE-14-0474.

II. **Mudry JM**, Alm PS, Erhardt S, Goiny M, Fritz T, Caidahl K, Zierath JR, Krook A, Wallberg-Henriksson H. *Direct effects of exercise on kynurenine metabolism in people with normal glucose tolerance or type 2 diabetes.* Diabetes Metab Res Rev. 2016 Mar 4. doi: 10.1002/dmrr.2798.

III. **Mudry JM**, Kirchner H, Chibalin AV, Krook A and Zierath JR. *Changes in skeletal muscle DNA methylation in rats following endurance training and high-fat diet.* In manuscript.

IV. **Mudry JM**, Lassiter DG, Nylén C, García-Calzón S, Näslund E, Krook A, Zierath JR. *Insulin and glucose alter death-associated protein kinase 3 (DAPK3) DNA methylation in human skeletal muscle.* Manuscript under revision.

SCIENTIFIC PAPERS NOT INCLUDED

I. de Castro Barbosa T, Ingerslev LR, Alm PS, Versteyhe S, Massart J, Rasmussen M, Donkin I, Sjögren R, **Mudry JM**, Vetterli L, Gupta S, Krook A, Zierath JR, Barrès R. High-fat diet reprograms the epigenome of rat spermatozoa and transgenerationally affects metabolism of the offspring. Mol Metab. 2015 Dec 25;5(3):184-97. doi: 10.1016/j.molmet.2015.12.002.

II. Lund J, Arild CR, Løvsletten NG, **Mudry JM**, Langleite TM, Feng YZ, Stensrud C, Brubak MG, Drevon CA, Birkeland KI, Kolnes KJ, Johansen EI, Tangen DS, Stadheim HK, Gulseth HL, Krook A, Kase ET, Jensen J, Thoresen GH. *Exercise in vivo marks human myotubes in vitro: Training-induced increase in lipid metabolism and insulin receptor substrate 1 (IRS1) first exon DNA methylation.* In manuscript.

CONTENTS

LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
Akt	Protein kinase B
BMI	Body mass index
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CpG	cytosine-guanine dinucleotide
DALY	Disability-Adjusted Life Year
DAVID	Database for Annotation, Visualization and Integrated Discovery
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferases
DMEM	Dulbecco modified eagle medium
EGR1	Early Growth Response 1
EMSA	Electrophoretic mobility shift assay
GLUT	Glucose transporter
HbA1c	Glycosylated hemoglobin
HDAC	Histone Deacetylase
HK	Hexokinase
HOMA-IR	Homeostasis model assessment – estimated insulin resistance
IGT	Impaired glucose tolerance
IFG	Impaired fasting glucose
IL	Interleukin
KAT	Kynurenine aminotransferase
KEGG	Kyoto encyclopedia of genes and genomes
MeDIP	Methylated DNA immunoprecipitation sequencing
mRNA	Messenger RNA
MYH	myosin heavy chain
MyoD	Myogenic differentiation 1
NGT	Normal glucose tolerant
PAX	Paired-box

NRF1	Nuclear Respiratory Factor 1
PDK	Pyruvate Dehydrogenase Kinase
PGC1α	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
qPCR	Real-time quantitative polymerase chain reaction
RRBS	Reduced representation bisulfite sequencing
RNA	Ribonucleic acid
SEM	Standard error of the mean
SPRY	Sprouty RTK signaling antagonist
TA	*Tibialis anterior*
T2D	Type 2 diabetes
TNFα	Tumor necrosis factor α
TWIST	TWIST Homolog Of Drosophila
VO2max	Maximal oxygen uptake
WHO	World Health Organisation

1 INTRODUCTION

The word metabolism comes from “metabolē”, the Grek word for “change”. Humans, like all living creature, “change” nutrients into energy for their survival, growth and activity. All transforming processes are part of metabolism. The three principal sources of energy in humans are glucose, fat and protein.

Sufficient glucose availability is critical for certain functions and organs, especially the brain. Besides being an energy source, glucose is also important for nucleotide and non-essential amino acid synthesis. When glucose intake exceeds expenditure, glucose can be stored as glycogen in skeletal muscle and liver or transformed and stored as fatty acids in adipose tissue. In case of insufficient glucose intake, amino acids can be used by the liver to produce glucose. This is termed “hepatic gluconeogenesis”.

Blood glucose levels are controlled by a feedback system between two peptide hormones produced in the pancreas: glucagon and insulin. Glucagon is secreted by the alpha-cells in response to low blood glucose concentration, while insulin is secreted by the beta-cells in response to elevated blood glucose concentration. Insulin decreases blood glucose levels by triggering glucose uptake in insulin-sensitive tissues such as adipose tissue and skeletal muscle, while inhibiting hepatic gluconeogenesis. If these tissues become insulin-resistant, blood glucose levels rises and represents a first step towards type 2 diabetes.

1.1 DIABETES MELLITUS

Diabetes mellitus is usually referred simply as Diabetes. The name was given by the Greek physician Aretaeus of Cappadocia (1st century CE). In ancient Greek, diabetes means "a passer through” while mellitus comes from Latin and means “honey-sweet”. It refers to a characteristic symptom of an untreated person with diabetes: the patient drinks a lot and releases abundant quantities of urine containing sugar (sweet water passing through the body). Two main types of diabetes mellitus are considered: type 1 and type 2. Type 1 refers to a disease caused by the loss of the insulin-secreting beta-cells of the pancreas. If exogenous insulin is not provided rapidly, type 1 diabetes is fatal. Type 2 diabetes has a more complex origin and is the topic of the present thesis.

1.1.1 Type 2 Diabetes

Type 2 diabetes is a complex non-communicable disease defined by chronic elevated levels of blood glucose (**Table 1**). Type 2 diabetes is characterized by a state of insulin resistance in metabolically important tissues including skeletal muscle, adipose tissue and liver, often in conjunction with impaired insulin secretion by the beta-cells of the pancreas. Type 2 diabetes progresses from the somewhat reversible impaired fasting glucose (IFG) and impaired glucose

tolerance (IGT) states followed by full blown diabetic state (Saltiel and Kahn 2001). There are two main diagnostic tests for type 2 diabetes: the measurement of fasting plasma glucose and the measurement of venous plasma glucose two hours after ingestion of a 75g oral glucose load, the so called Oral Glucose Tolerance Test (OGTT).

Classification	Fasting plasma glucose		2–h plasma glucose
Impaired fasting glucose	6.1 to 6.9mmol/l (110mg/dl to 125mg/dl)	AND	<7.8mmol/l (140mg/dl)
Impaired glucose tolerance	<7.0mmol/l (126mg/dl)	AND	≥7.8 and <11.1mmol/l (140mg/dl and 200mg/dl)
Diabetes	≥7.0mmol/l (126mg/dl)	OR	≥11.1mmol/l (200mg/dl)

Table 1: Diabetes classification (adapted from WHO 2006).

Over time, high blood glucose leads to inflammation and systemic tissue damage, especially of the blood vessels (Giugliano, Ceriello et al. 2008). Microvascular damage leads to nephropathy, retinopathy and neuropathy, ultimately resulting in kidney failure, loss of vision and ulceration of the foot. Macrovascular damages increase the risk of cardiovascular disease such as myocardial infarction and stroke. Additionally, type 2 diabetes increases the risk of major depression (Anderson, Freedland et al. 2001; Ali, Jyotsna et al. 2013).

Acutely, type 2 diabetes is not life threatening. Nevertheless, type 2 diabetes markedly increases the risk of fatal coronary heart disease (Huxley, Barzi et al. 2006). Diabetes reduces life expectancy dramatically, even in developed countries: In 2003 in the USA "*if an individual is diagnosed [with type 2 diabetes] at age 40 years, men will lose 11.6 life-years and 18.6 quality-adjusted life-years and women will lose 14.3 life-years and 22.0 quality-adjusted life-years*." (Narayan, Boyle et al. 2003). In 2004, an estimated 3.4 million people died from consequences of high blood glucose ((WHO) 2016).

Type 2 diabetes is a pandemic with an estimated 285 million people suffering from this disease in 2010 with a projected rise to 438 million by 2030 (Whiting, Guariguata et al. 2011). Of note, type 2 diabetes is not only an occidental problem, but a global concern as prevalence of diabetes in rural areas quintuples over twenty-five years in low- and middle-income countries from 1.8% in 1985–1989, to 8.6% for 2005–2010 (Hwang, Han et al. 2012) making type 2 diabetes a global concern and a heavy burden on society.

The financial impact of diabetes on the healthcare system is significant. In 2010, around 12% of the healthcare spending around the world was attributed to diabetes (Zhang, Zhang et al. 2010). In the USA, a diabetic patient spends 2.5 times more on medical care than a person without the disease (Association 2008). Most statistics refer to "diabetes", and do not

differentiate between type 1 and type 2 diabetes. Type 2 diabetes represents 90-95% of the cases (Narayan, Boyle et al. 2003; Boehme, Buechele et al. 2015).

Risk factors for developing type 2 diabetes as listed by the International Diabetes Foundation include, but are not limited to: family history of diabetes, overweight, unhealthy diet, physical inactivity, increasing age, high blood pressure, ethnicity, history of gestational diabetes and poor nutrition during pregnancy (Federation 2016). Although the pathophysiology of type 2 diabetes is not fully understood, any reduction of these risk factors through engagement in prevention programs is effective (Aziz, Absetz et al. 2015). Treatment of type 2 diabetes is based on lifestyle intervention focused on physical activity and diet modification, pharmacotherapy and the treatment of incidental complications.

1.2 OBESITY

Energy metabolism is a tightly regulated balance between food intake and energy expenditure. The combination of multiple biological feedbacks through hormonal secretion allow for a tight control of hunger and satiety in humans. Nevertheless, an imbalance can occur. Caloric intake comes from digestion and absorption of food. Caloric expenditure encompasses basal metabolism (the energy necessary to maintain body functions), the thermic effect of food, non-exercise physical activity and exercise (**Figure 1**). In humans, most of the energy storage is in the form of fatty acids in adipose tissue. If intake surpasses expenditure, fat will start accumulating.

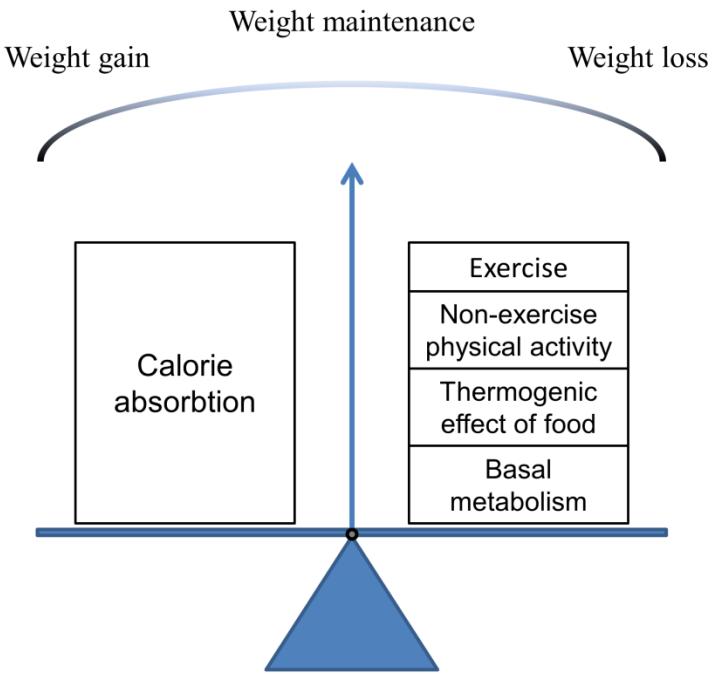

Figure 1: Graphical Representation of Energy Balance.

According to WHO "*Abnormal or excessive fat accumulation that may impair health*" is the definition of obesity ((WHO) 2016). Obesity is commonly quantified using body mass index (BMI). BMI is calculated by dividing a person's weight in kilograms by the square of his height in meters (kg/m^2). Limits for overweight and sub-groups of obesity are presented in **Table 2**. BMI is a simple and useful tool to assess weight to length ratio but is not a measure of body composition and can be misleading in some cases. Thus, a more accurate measurement of "*abnormal or excessive fat accumulation that may impair health*" requires other tools such as waist to hip ratio, skinfold-thickness measurements or dual-energy X-ray absorptiometry which directly measures body composition.

Classification	**BMI (kg/m^2)**
Underweight	<18.50
Normal range	18.50 - 25
Overweight	≥25.00

Overweight sub-classification	**BMI (kg/m^2)**
Pre-obese	25.00 - 29.99
Obese	**≥30.00**
Obese class I	30.00 - 34.99
Obese class II	35.00 - 39.99
Obese class III	≥40.00

Table 2: BMI Classification (adapted from WHO 2004).

The world prevalence of obesity, as assessed by BMI, has more than doubled between 1980 and 2014. Overall, 13% of the population on the planet was obese in 2014 (Finucane, Stevens et al. 2011; (WHO) 2016). Forecasts worldwide predict a prevalence of obesity between 20 and 38% of the population by 2030 (Kelly, Yang et al. 2008), thus research and enforcement of preventive measures and treatment of obesity and related comorbidities should be a public health priority.

Overweight and obesity are known risk factors for many medical conditions such as cardiovascular diseases (e.g. stroke), cancer (e.g. colon cancer), musculoskeletal disorders (e.g. osteoarthritis), type 2 diabetes, and many more (Guh, Zhang et al. 2009). Every year, 2.8 million people die prematurely as a consequence of being overweight. An estimated 35.8 million (2.3%) of global Disability-Adjusted Life Year (DALYs, years of healthy life lost) are

attributed to overweight or obesity (Organization 2009). Direct and indirect costs of overweight and obesity are heavy on healthcare systems and are most likely going to increase further with the rise in BMI worldwide (Dee, Kearns et al. 2014).

The development of obesity has an environmental and a genetic component (O'Rahilly and Farooqi 2000). The recent rise of overweight and obesity prevalence world-wide is mainly attributed to changes in lifestyle, including physical inactivity and excessive consumption of calorie-dense food ((WHO) 2016). Recent findings in twins reared apart estimate the heritability of BMI at 77%, outweighing the contribution of the environment (Wardle, Carnell et al. 2008). This suggests that although lifestyle changes trigger the increase in prevalence, genetic predisposition plays an important role in the development of obesity. A small portion of obesity cases are monogenic as in the case of leptin deficiency (Hamann and Matthaei 1996). Genome-wide association studies have identified several loci associated with BMI (Waalen 2014) such as the *FTO* (Fat mass and obesity-associated protein) gene variant (Loos and Yeo 2014). However, *FTO,* like other variants, is thought to account for only a limited increase in BMI (Frayling, Timpson et al. 2007). Epigenetic modifications may also contribute to the risk of obesity and type 2 diabetes, including intrauterine conditions of both under- and over-nourishment (El Hajj, Schneider et al. 2014). Epigenetic changes could provide a link between genetic and environmental influences. .Although several gene loci have been identified and different mechanisms proposed, further efforts are necessary to further our understanding of the development of obesity.

Although obesity is a complex, it is also a preventable disease. Preventive measures include increased physical activity, a healthy nutrition and social measures. A combination of the possible interventions in one coordinated effort (multilevel and multicomponent interventions) is being developed and appears to be effective (Ewart-Pierce, Mejia Ruiz et al. 2016). When obesity is already established, effective treatments are scarce and expensive, not without adverse effects for the patients and often insufficient to fully reverse the condition. Nevertheless, they often provide significant improvements in metabolic markers and outcomes (Douketis, Macie et al. 2005; Pi-Sunyer, Astrup et al. 2015). The management of obesity includes improving lifestyle through dietary recommendations and encouragement of physical activity together with behavior therapy. An effective and safe pharmacotherapy is not available today but extreme cases of obesity can be improved by bariatric surgery.

1.3 PHYSICAL ACTIVITY & DIET

With modern technology and comfort, lifestyles have changed drastically and physical activity has become a choice rather than a necessity. Being active and exercising reduces the risk of diabetes, cardiovascular disease and cancer (Fox 1999; Garber, Blissmer et al. 2011; Reiner, Niermann et al. 2013). The lack of physical activity is now the fourth greatest risk factor for global mortality. *"Approximately 3.2 million deaths and 32.1 million DALYs (representing*

about 2.1% of global DALYs) each year are attributable to insufficient physical activity" reports the WHO (Organization 2009). Thus, exercise is recommended for diabetic, obese and depressed patients, as well as healthy people throughout lifetime (Wei, Gibbons et al. 2000). Currently, WHO advocates for 150 minutes of vigorous physical activity each week ((WHO) 2016; (WHO) 2016).

While the level of physical activity is decreasing, energy intake is on the rise (Austin, Ogden et al. 2011). Energy-dense food has been blamed for the global obesity epidemic (Drewnowski and Darmon 2005). WHO estimates that 1.7 million of deaths and 16 million of DALYS are attributable to low fruit and vegetable consumption each year (Organization 2009). Consumption of a sufficient amount of fruits and vegetables has been shown to reduce the risk for cardiovascular diseases, stomach cancer and colorectal cancer (Bazzano, Serdula et al. 2003; Riboli and Norat 2003).

1.4 DEPRESSION

Depression, as described in the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10, 2016) is characterized by a lowered mood, reduction of energy and loss of pleasurable feelings.

Diabetes, obesity and lack of physical activity are linked to depression. Diabetes increases the risk of major depression (Anderson, Freedland et al. 2001; Ali, Jyotsna et al. 2013) while individuals with depression have an increased risk of diabetes (Nouwen, Winkley et al. 2010). Obesity and depression are also associated (Mannan, Mamun et al. 2016; Muhlig, Antel et al. 2016), while physical activity is an effective treatment for depression (Rethorst, Wipfli et al. 2009). Skeletal muscle, through kynurenine metabolism, has been postulated to play a role in exercise-induced depression resilience (Agudelo, Femenia et al. 2014).

Psychological, psychosocial and pharmacological treatments have been proven effective in the management of depression, but less than 10% of the 350 million people affected receive treatment due to lack of resources, lack of trained personal or social stigma associated with mental disorders ((WHO) 2016). Exercise could be promoted as a simple and inexpensive measure to improve depression symptoms and consequences.

1.5 SKELETAL MUSCLE AND ROLE IN METABOLISM

Skeletal muscle represents over 40% of total body mass in humans. Skeletal muscle allows us to move and interact with our environment. At rest, it consumes 30% of the calories necessary to keep the body at steady state, representing a large proportion of basal metabolism (Zurlo, Larson et al. 1990). Skeletal muscle is metabolically flexible meaning that it can utilize various

fuel sources depending on supply availability and demand (Storlien, Oakes et al. 2004). Skeletal muscle is a highly plastic tissue; it is responsive to stimuli from the environment, able to grow (hypertrophy as in bodybuilders) or shrink (atrophy, as during immobilization or in cachexia). Energy consumption and power output of skeletal muscle can increase to a large extent, as seen during intense physical activity, where a large amount of watts and heat are produced.

Skeletal muscle is the principal tissue responsible for insulin-stimulated glucose disposal and the tissue where the reduction in glucose utilization by diabetic subjects is the most striking (DeFronzo, Jacot et al. 1981). In insulin-sensitive tissues, insulin binding to cell surface membrane receptors induces a chain reaction leading to gene transcription, enhanced glucose uptake, storage and oxidation. A key component of the increased rate of glucose uptake after insulin stimulation in skeletal muscle is the translocation of the glucose transport (GLUT4) proteins to the cellular membrane allowing for an increased amount of facilitated glucose transport (Lund, Holman et al. 1997). Type 2 diabetic patients have reduced glucose transport following insulin stimulation despite similar amount of GLUT4 transporters within the muscle fibers (Handberg, Vaag et al. 1990). In this state, commonly referred as “insulin resistance”, insulin signaling is blunted. Impairments in lipid metabolism, as well as inflammation, play roles in the development of insulin resistance, but the exact mechanisms of insulin resistance are not completely understood (Samuel and Shulman 2012).

Physical activity and muscle contraction improve insulin sensitivity in skeletal muscle and activate several signaling pathways that result in changes in gene expression (Egan and Zierath 2013). A bout of exercise will trigger or repress the transcription of a wide variety of genes. Changes in gene expression occur mainly a few hours after the acute bout of exercise, but also up to several days afterwards (Neubauer, Sabapathy et al. 2014). The burst in gene expression after exercise is usually followed a few hours later by an increase in protein translation. Over the course of training, both protein and mRNA changes lead to muscle remodeling with respect to metabolic capacity and physical performance (Perry, Lally et al. 2010).

Skeletal muscle is organized in multinucleated muscle fibers (**Figure 2**), and further subdivided in myofibrils. Each myofibril is subdivided in contractile units called sarcomeres. Each sarcomere is delimited by a Z line. Within a sarcomere are actin and myosin filaments. During contraction, actin is pulled along myosin toward the center of the sarcomere. Maximal shortening of the muscle is achieved when actin and myosin filaments are completely overlapped.

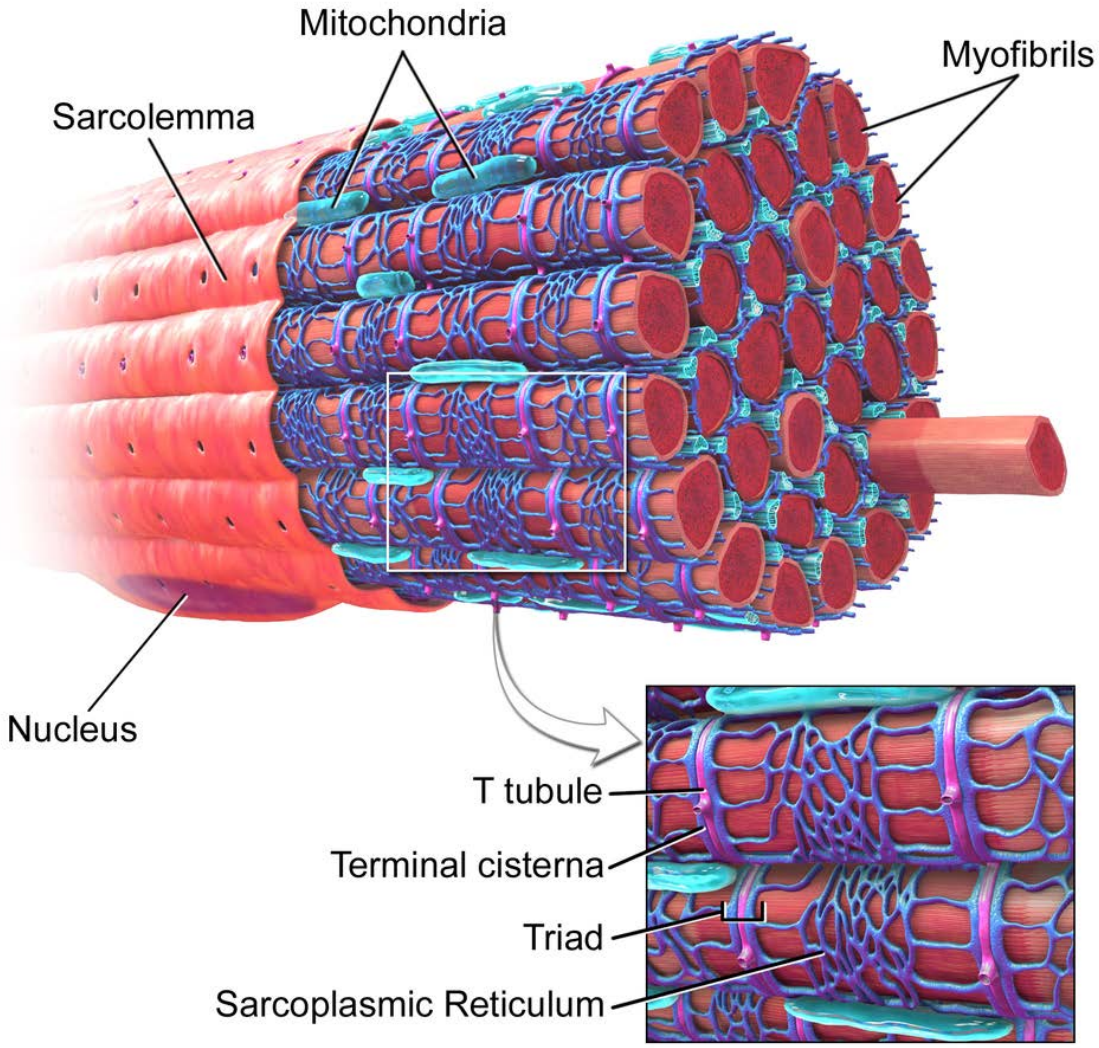

Figure 2: Graphical representation of a skeletal muscle fiber. Multiple myofibrils are represented. Sarcomere in greater detail (inset). (CC BY 3.0 license. Blausen gallery 2014". Wikiversity Journal of Medicine. DOI:10.15347/wjm/2014.010. ISSN 20018762).

To activate skeletal muscle, an electrical signal is sent by the central nervous system to the neuro-muscular junction. There, acetylcholine neurotransmitter induces the opening of sodium ion channels resulting in depolarization of the fiber. This depolarization causes the release of calcium by the sarcoplasmic reticulum. Calcium binds to troponin allowing the myosin to pull on actin filaments. This last step consumes ATP and is repeated to induce further shortening of the muscle (Field and Society 1983).

The term myosin encompasses a large family of proteins sharing the ability to bind actin, hydrolyze ATP and generate force. In skeletal muscle, the most abundant isoforms are the root of the fiber type classification. *MYH7* is the gene encoding for the myosin heavy chain-β, the main isoform in the slow-twitch type 1 fibers. *MYH2* is the gene coding for the fast-twitch and

fatigue resistant type 2A fibers while fast-twitch and fatigable type 2X myosin mainly express *MYH1*. Other myosin heavy chain genes are expressed in skeletal muscle such as *MYH7b*, *MYH15* and *MYH16 (Schiaffino and Reggiani 2011)*.

1.6 SATELLITE CELLS

In adult skeletal muscle, muscle-specific stem cells are located between the basal lamina and sarcolemma of the fibers. These cells are called satellite cells. In adult muscle, satellite cells are in a quiescent state (not dividing) (Schultz, Gibson et al. 1978). In response to mitogenic stimuli, satellite cells will activate transcription factors and start proliferating. Specific markers of satellite cells are transcription factors PAX3 and PAX7 (Relaix, Rocancourt et al. 2005). PAX7 levels will decrease, while MyoD and Myogenin will be expressed. Proliferation and asymmetric division of the satellite cells provide myonuclei to repair or support growth of muscle fibers (Schultz 1996). Receptor tyrosine kinases are key elements of growth signaling. The Sprouty RTK signaling antagonist genes (*Spry1-4*) are negative regulators of receptor tyrosine kinases. In satellite cells, SPRY1 controls quiescence. SPRY1 is expressed in intact muscles while it is downregulated in injured muscle to allow for proliferation and growth of the satellite cells (Shea, Xiang et al. 2010) (**Figure 3**).

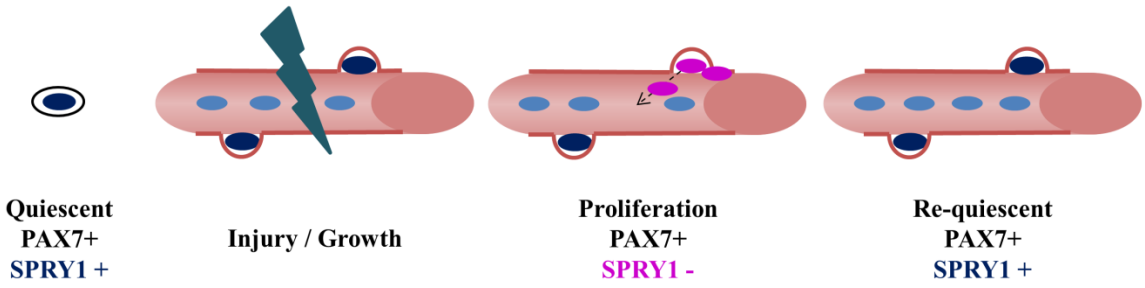

Figure 3: Satellite cells: After injury, quiescent PAX7+/SPRY1+ cells turn off *SPRY1* and enter cell cycling. After several rounds of division, a subset of cells turns on *SPRY1* again and returns to quiescence. Redrawn and adapted from Abou-Khalil and Brack 2010.

1.7 REGULATION OF GENE EXPRESSION

Nuclear DNA is the genetic code containing all the necessary information for one totipotent cell to give rise to a whole multicellular organism containing plenty of different cell types, each expressing a very specific set of genes (Mitalipov and Wolf 2009). 75% of the code is transcribed into RNA (Djebali, Davis et al. 2012), but the 20,500 protein-coding genes (Clamp, Fry et al. 2007) only account for ~2% of the DNA (Dinger, Pang et al. 2008). Gene expression is the medium to go from DNA to the actual macromolecular machinery for a functional cell.

Genes are transcribed into RNA to then produce proteins or regulate cell function. Gene transcription rate is controlled by proteins called transcription factors as they promote or block the transcriptional activity of RNA polymerase enzymes (Latchman 1997; Lee and Young 2000). A defining feature of transcription factors is the DNA binding domain, a motif that has affinity to DNA and which recognizes DNA sequences (Mitchell and Tjian 1989). Transcription factors can be classified according to their mechanism of action, regulatory function, or structural similarities. In addition to transcription factors, proteins such as coactivators, corepressors, chromatin remodelers, and methylases are crucial modulators of transcription (Naar, Lemon et al. 2001; Narlikar, Fan et al. 2002). Input signals from the extracellular environment alter expression of transcription factors and coregulators (Brivanlou and Darnell 2002) allowing the cell to adapt to different stimuli. In skeletal muscle, exercise induces transcription changes in a wide range of genes (Raue, Trappe et al. 2012; Lindholm, Marabita et al. 2014) while myogenesis induces a very different group of genes (Bentzinger, Wang et al. 2012).

1.7.1 Myogenesis and the TWIST Proteins

MyoD, MYF5, Myogenin and MRF4 are the four major transcription factors driving gene expression in skeletal muscle differentiation (myogenesis) (Hawke and Garry 2001). Termed myogenic regulatory factors (MRFs), all are basic helix-loop-helix proteins (bHLH). Basic helix-loop-helix transcription act in homo- or hetero-dimers formed with various other bHLH to control gene expression. MyoD was discovered in 1987 as the first case where ectopic expression could convert cells such as fibroblasts into skeletal muscle cells (Davis, Weintraub et al. 1987), opening the field of cellular reprogramming. The MRFs are also subject to regulation. Notably, TWIST1 and TWIST2 proteins, also bHLH, block myogenesis through inhibition of MyoD transactivation (Hamamori, Wu et al. 1997) and HDAC recruitment (Qiu, Ritchie et al. 2006). The mechanism of TWIST transcriptional activity, as for other bHLH, varies depending on post-transcriptional modifications, partner choice and cellular context, making the characterization of their modes of action a complex task (Castanon, Von Stetina et al. 2001; Laursen, Mielke et al. 2007; Sharabi, Aldrich et al. 2008). Numerous biological roles have been attributed to the TWIST proteins (Franco, Casasnovas et al. 2011). In addition to repressing MyoD, TWIST proteins also play a role in metabolism and inflammation, at least in adipose tissue, where *TWIST1* silencing reduces fatty acid oxidation and modulate pro-inflammatory cytokine expression and secretion (Pettersson, Laurencikiene et al. 2010; Pettersson, Mejhert et al. 2011).

1.7.2 PGC1α

Another protein of importance for skeletal muscle gene expression regulation is the Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α, gene name PPARGC1). PGC1α is not a transcription factor, but function as a “master regulator” by binding to, and modulating the activity of a number of different transcription factors. PGC1α stimulates

mitochondrial biogenesis and promotes fatty acid oxidation (Wu, Puigserver et al. 1999). Clinically, activation of PGC1α has been implicated in protective effects against type 2 diabetes, obesity, sarcopenia (Wenz, Rossi et al. 2009) and stress-induced depression (Agudelo, Femenia et al. 2014). A number of gene expression responses to resistance and endurance exercise are mediated by different PGC1α isoforms (Martinez-Redondo, Jannig et al. 2016).

1.7.3 Kynurenine Aminotransferases

Kynurenine aminotransferases (KATs) are four enzymes regulated by PGC1α. KATs have been suggested to be involved in the protective effect exercise on depression by acting on the tryptophan/kynurenine pathway (Agudelo, Femenia et al. 2014). Tryptophan is an essential amio-acid and is also the source of neurotransmitters and neuroactive compounds such as serotonin and kynurenine. Kynurenine itself can be transformed either into kynurenic acid (through the KATs) or into quinolinic acid (through kynurenine 3-monooxygenase, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase) (**Figure 4**). Mainly studied in the brain, kynurenic acid and quinilonic acid are two different neuroactive metabolites with opposing effects. Quinilonic acid is an N-methyl-D-aspartic acid receptor agonist and is formed in microglial cells of the brain. Conversely, kynurenic acid antagonizes the NMDA-receptor, blocks the cholinergic α7 nicotinic receptor and is produced in astrocytes (Perkins and Stone 1982). Kynurenine, in contrast to kynurenic acid and quinilonic acid is able to cross the blood brain barrier. Kynurenine flux from the periphery into to the brain is associated with an increased manifestation of psychiatric disorders such as schizophrenia or depression (Campbell, Charych et al. 2014). Intriguingly, increased circulating kynurenic acid is associated with resistance to depression (Myint, Kim et al. 2007). A possible mechanism is that peripheral tissues, especially skeletal muscle, convert kynurenine into kynurenic acid, preventing kynurenine from reaching the brain (Agudelo, Femenia et al. 2014).

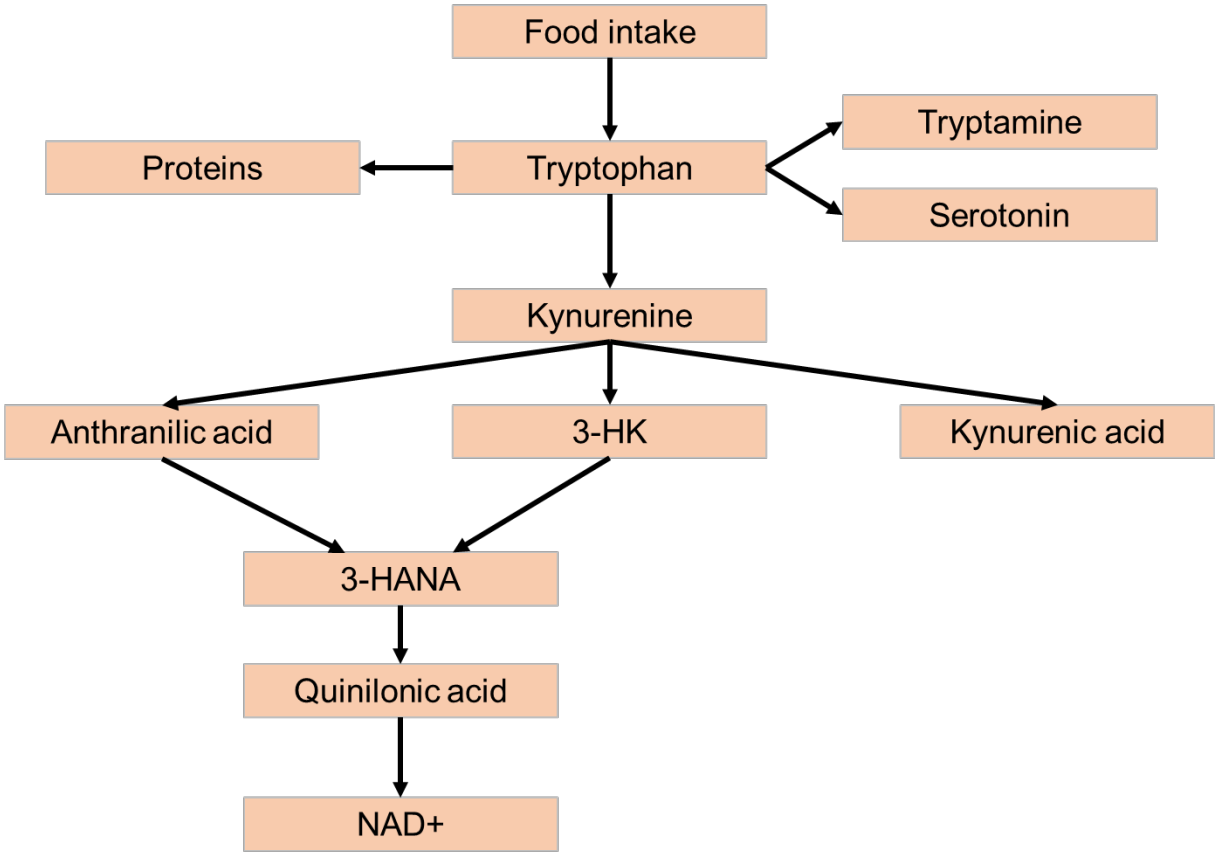

Figure 4: Schematic Representation of the Kynurenine Pathway.

1.8 EPIGENETICS

1.8.1 Defining Epigenetics

Epigenetics is the contraction of the Greek prefix "epi-", meaning "on top", and genetics. "Epigenetics" has emerged following the word "epigenesis", the process by which cells differentiate and form organs. In 1942 the word "epigenetics" became a field of research with Conrad Waddington writing "*the task is to discover the causal mechanisms at work, [to transition from a genotype to phenotype] and to relate them as far as possible to what experimental embryology has already revealed of the mechanics of development. We might use the name 'epigenetics' for such studies*." (Waddington 2012). Controversy about the exact meaning of "epigenetics" was started and continues today (Ledford 2008). Inheritance is the main point of controversy. In 1996, Riggs and colleagues describe epigenetics as "*the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence*" (Russo, Martienssen et al. 1996) expressing that epigenetics exclude changes in the genome, but without clarifying what phenomenon the work epigenetics encompasses. In 2007 Adrian Bird proposed a new definition for the word epigenetics: "*the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states*". Adding the following note "*It focuses on chromosomes and genes, implicitly*

excluding potential three-dimensional architectural templating of membrane systems and prions, except when these impinge on chromosome function" (Bird 2007). In this thesis, I will refer to epigenetics as in this last citation. With that definition, epigenetics encompasses cell programming, the fact that despite a similar genetic code, cell types in the body have a very diverse gene expression program and exhibit diverse physical and biological properties.

1.8.2 Epigenetic Regulation

Two well characterized epigenetic processes are imprinting and X-inactivation. In imprinting, a gene is silenced depending on his parent-of-origin. *IGF2* imprinting is an example of maternal imprinting (silencing of the maternal copy of the gene) (DeChiara, Robertson et al. 1991). X-inactivation is the inactivation of one of the two X chromosomes in females (Ng, Pullirsch et al. 2007). Imprinting and X-inactivation, as all other epigenetic processes, result from multiple levels of epigenetic regulation, such as histone modifications, nucleosome positioning, non-coding RNA and DNA methylation.

The 2 meter long human DNA needs to be organized and compacted to fit into a cell. For this reason, DNA is wrapped more or less tightly around nucleosomes composed of octamers of proteins called histones. Each histone presents a tail that can be post-translationally modified to alter the configuration of the DNA and thus modulate transcription (Huang, Sabari et al. 2014). Several environmental challenges modify the histone tails, including exercise (McGee, Fairlie et al. 2009). Advances in genomic technologies have revealed that the position on the DNA sequence of the nucleosome formed by histones also impacts DNA transcription (Jiang and Pugh 2009).

Recent advances in sequencing technologies have revealed the existence of several classes of non-coding RNAs in addition to previously appreciated species such as ribosomes and transfer RNAs. Among these new species are micro-RNAs, long non-coding RNAs and piwi-interacting RNAs. Novel non-coding RNAs have been identified in a wide range of sizes, and many have not been investigated for their function so far. Some novel non-coding RNAs have been implicated in the maintenance of the DNA methylation profile (Di Ruscio, Ebralidze et al. 2013), connecting these two epigenetic mechanisms.

1.8.3 DNA Methylation

DNA is methylated by the addition of a methyl group (CH_3) to the fifth carbon atom of cytosine. Methylation of the cytosine is catalyzed by DNA methyltransferases (DNMTs). DNMT1 is responsible for the maintenance of the methyl marks following DNA replication, as in proliferating cells. DNMT3A, and DNMT3B establish "*de novo*" methylation. The addition or removal of methyl groups alters the binding of regulatory elements and thus the transcription of the genetic code. In adult mammal cells, cytosine methylation happens essentially in the context of a cytosine-guanine dinucleotide (CpG) (Lister, Pelizzola et al. 2009). Cytosine's methylation within another dinucleotide (CpA, CpT,CpC) is termed non-CpG or CpH

methylation. 98 % of CpG methylation is symmetrical on both DNA strands, while this occurs for only 10% of non-CpG methylation. This is probably linked to the fact that CpA dinucleotides are paired with CpT dinucleotides and vice versa on the complimentary DNA strand (Shirane, Toh et al. 2013).

DNA is mostly de-methylated and re-methylated at an early embryonic stage and partially during cell division and differentiation. Alterations of the DNA methylation profile also occur with aging (Issa 2014), in cancer and several other diseases (Robertson 2005). It has recently been appreciated that DNA methylation is a dynamic phenomenon (Kangaspeska, Stride et al. 2008) influenced by environmental factors (Herceg 2016) and that these changes may be transmitted intergenerationally (Barres and Zierath 2016).

Most recent studies have focused on CpG methylation which is the most abundant DNA methylation mark. However, a role for non-CpG methylation is emerging. Non-CpG methylation is more frequent in embryonic and induced pluripotent stem cells (iPSCs) than in differentiated cells (Ziller, Muller et al. 2011). Non-CpG methylation is unlikely to be transmitted during cell division and would thus need to be re-established after the division (Patil, Ward et al. 2014). Nevertheless a few studies have provided evidence that non-CpG methylation might play a role, especially in rarely dividing cells such as neurons or skeletal muscle (Patil, Ward et al. 2014).

All epigenetic mechanisms are linked and the sum total of the different effects determines the final cellular program. How much each epigenetic mechanism contributes and how different mechanisms interact together to alter the structure of chromosomal regions and activity states is still to be clarified.

Understanding epigenetics is of clinical relevance. Epigenetic marks, such as methylation of the *SEPT9* gene, are already used in the field of cancer as biomarkers to stage disease risk and progression (Mikeska and Craig 2014; Tahara and Arisawa 2015; Rasmussen, Krarup et al. 2016). Epigenetic changes linked to genes encoding for transporters and metabolizing enzymes can significantly alter pharmacokinetics of a drug and could be predictive factors of drug response (Ivanov, Kacevska et al. 2012). Drugs targeting epigenetic proteins (epidrugs) are already used in the clinic. The DNMT1 inhibitor 5-aza-2′deoxycytidine (Azacitidine) has been approved for the treatment of myelodysplastic syndrome (Muller and Florek 2010). Altogether, progresses in epigenetic research should lead to improved prevention, personalization of treatment and may even provide new treatments.

2 AIMS

Physical activity and diet affects skeletal muscle function which in turn has effects on other organs such as the brain. The overall aim of this thesis is to identify regulators of gene expression relevant for insulin sensitivity and glucose metabolism in healthy, obese and type 2 diabetic skeletal muscle and to determine how exercise and diet may impact gene expression and function. Progress in these areas could reveal new regulatory mechanisms involved in the protective effect of exercise or the pathophysiology of type 2 diabetes and obesity, providing avenues for potential improvements in the management and treatment of metabolic diseases.

Specifically, the aims are to:

1. Determine whether the transcription factors TWIST1 and TWIST2 are involved in the regulation of glycogen storage and inflammatory genes in skeletal muscle.
2. Analyze effects of exercise on the kynurenine pathway in people with normal glucose tolerance or type 2 diabetes.
3. Determine whether endurance training in lean and obese rats alters DNA methylation changes in skeletal muscle.
4. Determine whether insulin directly alters skeletal muscle DNA methylation.

3 METHODOLOGICAL CONSIDERATIONS

3.1 HUMAN COHORTS

Five human cohorts are included in the present work. Biopsies of the *vastus lateralis* skeletal muscle were obtained to analyze gene expression and DNA methylation. From these biopsies we also retrieved human primary myocytes that were used in **paper 4**. We used two different techniques to collect the biopsies as detailed below.

3.1.1 Open Biopsy

In **paper 3**, for the *in vitro* studies, we collected skeletal muscle strips using the "open biopsy" technique (Koistinen, Galuska et al. 2003; Pfenninger and Fowler 2011). For this the patient lies down in a relaxed position, the skin overlying the skeletal muscle sample to be taken is washed with antiseptic and local anesthesia is administered. An incision (4 cm) in the *vastus lateralis* is made 15 cm proximal of the patella. A muscle biopsy (around 2 g) is excised and placed in oxygenated Krebs-Henseleit buffer. Hemostasis and closing of the wound requires electrocautery and stiches of the skeletal muscle fascia and skin. This technique allows for the retrieval of a long portion of the same group of fibers that can then be cut in smaller strips as described in **paper 4**. We subsequently treated each muscle strip with different compounds and determined response.

3.1.2 Needle Biopsy

Skeletal muscle biopsies used in **papers 1, 2** and for the *in vivo* study in **paper 3** were retrieved using a "needle biopsy" technique. Washing and local anesthesia are prepared as described for the "open biopsy". A small incision (~ 5 mm) is made in the *vastus lateralis* and a Weil-Blakesley conchotome is introduced to retrieve a skeletal muscle biopsy (~ 100 mg). Several samples can be collected. Wound closing does not require stiches. The needle technique is less invasive and technically easier to perform, but the amount of tissue collected is smaller.

3.1.3 Study Participants

Clinical characteristics of the five human cohorts presented in this thesis are in **Tables 3 and 4**. We used three different cohorts where we compared people with normal glucose tolerance (NGT) to type 2 diabetic subjects and 2 other cohorts where only healthy sedentary subjects were enrolled.

Table 3: **Anthropometric and Metabolic Characteristics of Type 2 Diabetic and Normal Glucose Tolerant Volunteers**

	Paper 1		Paper 2		Paper 4	
	NGT	**T2D**	**NGT**	**T2D**	**NGT**	**T2D**
n	10	10	12	12	12	12
Sex (F/M)	5/5	4/6	0/12	0/12	0/12	0/12
Age (y)	58 ± 2	63 ± 1*	59 ± 2	58 ± 2	60 ± 3	63 ± 1
Height (cm)	171 ± 3	170 ± 4	179 ± 2	177 ± 2	178 ± 2	180 ± 1
Weight (kg)	84.8 ± 3.6	90.8 ± 5.8	84.8 ± 3.4	86.4 ± 4.1	80.8 ± 2.3	91.5 ± 2.0
BMI (kg/m^2)	29.1 ± 0.6	31.3 ± 1.2	26.4 ± 1.0	27.6 ± 1.0	25.4 ± 0.5	28.3 ± 0.6*
Waist to hip ratio					0.92 ± 0.01	0.98 ± 0.01*
Waist. circumference (cm)	93.2 ± 2	106.0 ± 4*				
Body fat %			27 ± 2	30 ± 2		
SBP (mmHg)	133 ± 5	140 ± 3			133 ± 5	135 ± 3
DBP (mmHg)	79 ± 3	78 ± 2			81 ± 3	85 ± 2
FBG (mmol/L)	5.5 ± 0.0	7.9 ± 0.2*	5.0 ± 0.2	7.7 ± 0.5*	5.3 ± 0.1	8.1 ± 0.4*
2-h PG (mmol/L)	6.6 ± 0.4	14.5 ± 1.4*	5.6 ± 0.5	14.3 ± 1.1*	5.9 ± 0.3	15.8 ± 1.0*
Insulin (pmol/L)	51.4 ± 12.5	92.5 ± 16.6*	42.4±6.3	68.8±7.6*	51.2 ± 6.4	100.6 ± 13.9*
HbA1c (%)	4.6 ± 0.1	5.3 ± 0.2*	5.3±0.1	6.6±0.3*	5.5 ± 0.1	6.9 ± 0.2*
HbA_{1c},mmol/mol					35 ± 1	49 ± 3*
HDL (mmol/L)	1.58 ± 0.14	1.24 ± 0.05*	1.39 ± 0.12	1.22 ± 0.08	1.3 ± 0.06	1.3 ± 0.08
LDL (mmol/L)	3.24 ± 0.18	2.74 ± 0.28	3.24 ± 0.18	2.15 ± 0.19*	3.4 ± 0.14	2.8 ± 0.26*
TG (mmol/L)	1.45 ± 0.33	1.24 ± 0.11	0.96 ± 0.10	1.26 ± 0.16	0.98 ± 0.16	1.22 ± 0.15
Total cholesterol, (mmol/L)			5.07 ± 0.21	3.94 ± 0.20*	5.1 ± 0.2	4.6 ± 0.2
HOMA-IR	1.97 ± 0.10	3.48 ± 0.30*	1.38±0.24	3.30±0.53*	1.59 ± 0.2	5.42 ± 0.9*

NGT, normal glucose tolerant subjects; T2D, type 2 diabetes subjects; BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic blood pressure; FPG, Fasting Plasma Glucose; 2-h PG, Plasma Glucose two hours after oral glucose ingestion; HbA1c, glycated hemoglobin; HDL, high density lipoproteins; LDL, low density lipoproteins; TG, Triglycerides; HOMA-IR, homeostatic model assessment-insulin resistance; Data mean ± SEM. * $p < 0.05$, calculated using Student t-test.

Table 4: **Anthropometric and Metabolic Characteristics of Healthy Volunteers**

	Paper 1		**Paper 4**
	Pre-training	**Post-training**	***Muscle strip donors***
n	13		8
Sex (F/M)	7/6		0/8
Age (y)	25 ± 3		53 ± 2
Height (cm)	174 ± 10		181 ± 2
Weight (kg)	70.0 ± 11.3	69.6 ± 10.5	82.4 ± 2.7
BMI (kg/m^2)	22.8 ± 2.2	22.7 ± 1.9	25.24 ± 0.6
Waist to hip ratio		0.89 ± 0.02	0.89 ± 0.02
Body fat %	24 ± 8	23 ± 8	
SBP (mmHg)			125 ± 5
DBP (mmHg)			81 ± 3
FBG (mmol/L)			5.3 ± 0.1
Insulin (pmol/L)			36.9 ± 8.0
HbA1c (%)	4.3 ± 0.3	4.2 ± 0.3*	5.4 ± 0.1
HbA_{1c},mmol/mol			35.9 ± 0.6
HDL (mmol/L)	1.2 ± 0.29	1.2 ± 0.23	1.4 ± 0.08
LDL (mmol/L)	2.24 ± 0.54	2.17 ± 0.54	3.7 ± 0.2
TG (mmol/L)	0.82 ± 0.34	0.67 ± 0.24	0.85 ± 0.14
Total cholesterol, (mmol/L)	3.84 ± 0.65	3.67 ± 0.62	5.3 ± 0.3

NGT, normal glucose tolerant subjects; T2D, type 2 diabetes subjects; BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic blood pressure; FPG, Fasting Plasma Glucose; 2-h PG, Plasma Glucose two hours after oral glucose ingestion; HbA1c, glycated hemoglobin; HDL, high density lipoproteins; LDL, low density lipoproteins; TG, Triglycerides; HOMA-IR, homeostatic model assessment-insulin resistance; Data mean ± SEM. * $p < 0.05$.

Type 2 diabetic patients included in the studies had good control over their blood glucose based on the fact that their mean HbA1c was below 7%. LDL cholesterol was also lower in the diabetic subjects as compared to respective controls. Study participants were matched for many parameters. Nevertheless, a few parameters were not matching in all cohorts. In **paper 1** the groups are not matched for waist circumference, while in **paper 4,** NGTs and T2Ds were not matched for waist to hip ratio, suggesting that these type 2 diabetic patients have more abdominal fat than the controls. Abdominal fat has been associated with type 2 diabetes and cardiovascular disease (Smith 2015). Type 2 diabetic patients matched for BMI with normal glucose tolerant subjects (**paper 1**) or with a higher BMI (**paper 4**) than normal

glucose tolerant subjects are thus expected to have more abdominal fat. Fasting glucose and insulin concentration in plasma were higher in type 2 diabetic compared to control subjects as expected.

3.2 MOUSE COHORT

In **paper 1** we used C57BL/6J and C57BL/6J-*ob/ob* mice. Although strain differences are widely recognized and have been studied (Kulkarni, Almind et al. 2003), C57BL/6J remain the most commonly used mouse strain. Mouse assembly and gene annotation are based on C57BL/6J genome. Their popularity makes it also suitable to compare and reproduce studies.

3.3 RAT COHORT

In **paper 3** we used Wistar rats, a commonly used outbred albino rat, in two distinct experiments. In the first experiment we had two variables: diet (chow or high-fat) and exercise (sedentary rats or trained rats) thus finally resulting in 4 groups: Rats in the first group ate a chow diet and remained sedentary. In the second group, rats received also a chow diet but underwent training. In the third and fourth group, rats were fed a high-fat diet while rats in the third group remained sedentary and rats in the fourth group underwent training. Training consisted of swimming sessions of three hours separated by 45 minutes of rest each day for 5 days as described somewhere else (Galuska, Kotova et al. 2009).

In the second experiment, no *in vivo* treatment was performed but *tibialis anterior* muscles from Wistar rats were collected for *in vitro* contraction performed as described in **paper 3**. In brief, rats were anesthetized and both *tibialis anterior* muscles were collected. One *tibialis anterior* muscle was then subjected to electrically-stimulated *in vitro* contraction for one hour while the other TA remained untouched. Muscles were then transferred to oxygenated buffer for four hours before snap freezing in liquid nitrogen.

3.4 CELL CULTURES

We used two major types of cells in culture: primary cells and immortalized cell lines. Immortalized cell lines are primary cells selected or engineered to evade senescence and can theoretically be grown for indefinite passages *in vitro*. Transformed cells usually proliferate rapidly and are quite resistant to external stressors. Primary cells are directly extracted from tissue. As tissues contain multiple sorts of cells, purification is a challenge. Once in culture *in vitro*, primary cells have a limited lifespan. After a certain number of cell divisions they stop proliferating or lose their ability to differentiate.

In **paper 1 and 3** we used well established mouse C2C12 and rat L6 cell lines purchased from ATCC (Manassas, VA, USA). In **paper 4** we used primary human skeletal muscle cells

isolated in our laboratory from the "muscle strip donors" cohort described in **Table 4**. Not all isolations procedures were successful and some cultures had to be abandoned either because the cells would not grow, because macroscopic inspection revealed contamination with other cell types or because molecular markers specific for muscle cells were not expressed by the primary cells in culture.

3.5 mRNA EXPRESSION ANALYSIS

mRNA analysis in this thesis involved 4 steps: sample collection, mRNA extraction, reverse transcription of the RNA into cDNA and real-time quantitative PCR (qPCR). Clear guidelines for all these steps have been described (Bustin, Benes et al. 2009). Sample collection requires that the tissue or cells are immediately frozen or kept at 4°C and put into a protective lysis buffer to prevent RNA degradation before extraction. Trizol is the most widely used method for RNA extraction, but several commercial suppliers offer columns system for purification of RNA of all sizes or co-purification of RNA and DNA. We used TRIzol reagents (Thermo Fisher Scientific) to extract RNA from mouse muscle in **paper1** and column-based (from Qiagen) methods for all other extractions. Details are described in the respective papers.

RNA concentration and purity has been assessed by measurement of absorbance properties of the sample using a spectrophotometer (Nanodrop 1000 from Thermo Fisher Scientific). Other methods have been described and compared (Bustin 2005).In **paper 3**, RNA integrity has been measured using the Experion Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, CA).

cDNA synthesis is easily performed using ready-made kits from commercial suppliers including random primers and protocols ensuring complete conversion of RNA into cDNA. cDNA is more stable than RNA and can be stored over long periods of time at -20°C. In all papers in this thesis the High-capacity cDNA RT Kit (Thermo Fisher Scientific) was used.

Abundance of the transcript in the original sample is measured by increased fluorescence emission throughout a PCR reaction including most commonly either Taq-Man reagents or SYBR Green dye. Taq-Man polymerase and Taq-Man probes (Thermo Fisher Scientific) were used in **paper 1**. Taq-Man Probes purchased from Thermo Fisher Scientific are guaranteed to detect exclusively the gene of interest and to have 100% efficiency, meaning that every PCR cycle doubles the amount of cDNA of the target gene. Custom Taq-Man probes can also be designed. In **papers 2, 3 and 4** we designed our own primers and performed the qPCR using SYBR Green dye.

When designing our own primers, we evaluated specificity *in sillico* (through blasting using NCBI/ Primer-BLAST https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and subsequently performed a melting curve to ensure that qPCR amplification was limited to one specific product. Self-designing primers is inexpensive and permits targeting specific splice variants.

As mRNA input for cDNA synthesis and qPCR is imprecise, a normalization step using housekeeper genes is necessary. A suitable housekeeper gene is not affected by the differences between groups or by the treatment. We usually assessed three or more potential housekeeper genes and using NormFinder (Pfaffl, Tichopad et al. 2004), selected the most suitable gene or combination of genes for normalization. The house keeping genes for each study are specified in the experimental details related to each paper.

3.6 IMMUNOBLOT ANALYSIS AND ANTIBODIES

Immunoblots were performed as described in the papers. Most antibodies used have been tested, validated and used in previous publications from our laboratory. New antibodies from Abcam (ab, Cambridge, UK), Santa Cruz Biotechnology (sc-, Dallas, TX, USA) and Sigma-Aldrich (sig, Saint-Louis, MO, USA) were tested for TWIST1(sig6451,sc-15393 and sc-6269), TWIST2 (ab66031), SPRY1 (ab75492 and sc-30048), Nuclear Respiratory Factor 1(NRF1) (sc-721) and Early Growth Response 1 (EGR1) (sc-189).

In **paper 1**, overexpression of Twist1 and Twist2 was examined using qPCR and metabolic assays. Immunoblotting confirmed overexpression of TWIST2 protein. Despite using 3 different antibodies, we could not identify and measure TWIST1 protein as no band corresponded to the expected molecular weight or presented an overexpression pattern between samples.

In **paper 3**, we tested 2 different antibodies to measure SPRY1 protein expression. In our setup, Abcam's SPRY1 antibody (ab75492) bound to many unspecific sites and was discarded. Santa Cruz's antibody (sc-30048) bound to 3 proteins in the vicinity of SPRY1 expected molecular weight. HepG2 cells highly express SPRY1 and allowed us to identify the band corresponding to SPRY1. No difference in SPRY1 protein expression was noted between groups (see **Figure 10** on page 32).

Antibodies against NRF1 and EGR1 were used in a supershift assay in **paper 3**. These antibodies have been successfully used in supershift and chromatin immunoprecipitation (ChIP) assays for several years and by multiple research groups, however, the results we obtained were inconclusive and will require further optimization.

3.7 ELECTROPHORETIC MOBILITY SHIFT ASSAY AND SUPERSHIFT ASSAY

We used electrophoretic mobility shift assay (EMSA) to study protein-DNA interaction at the Spry1 promoter and assess if methylation of the promoter would enhance or prevent binding of skeletal muscle nuclear proteins. We adapted a previous protocol (Hellman and Fried 2007) as detailed in the method section of **paper 3**. To methylate the probe we used CpG-only Methyltransferase (M.SssI) (New England Biolabs, Ipswich, MA). To our surprise,

complete methylation of the CpGs in the probe resulted in increased binding of nuclear factors.

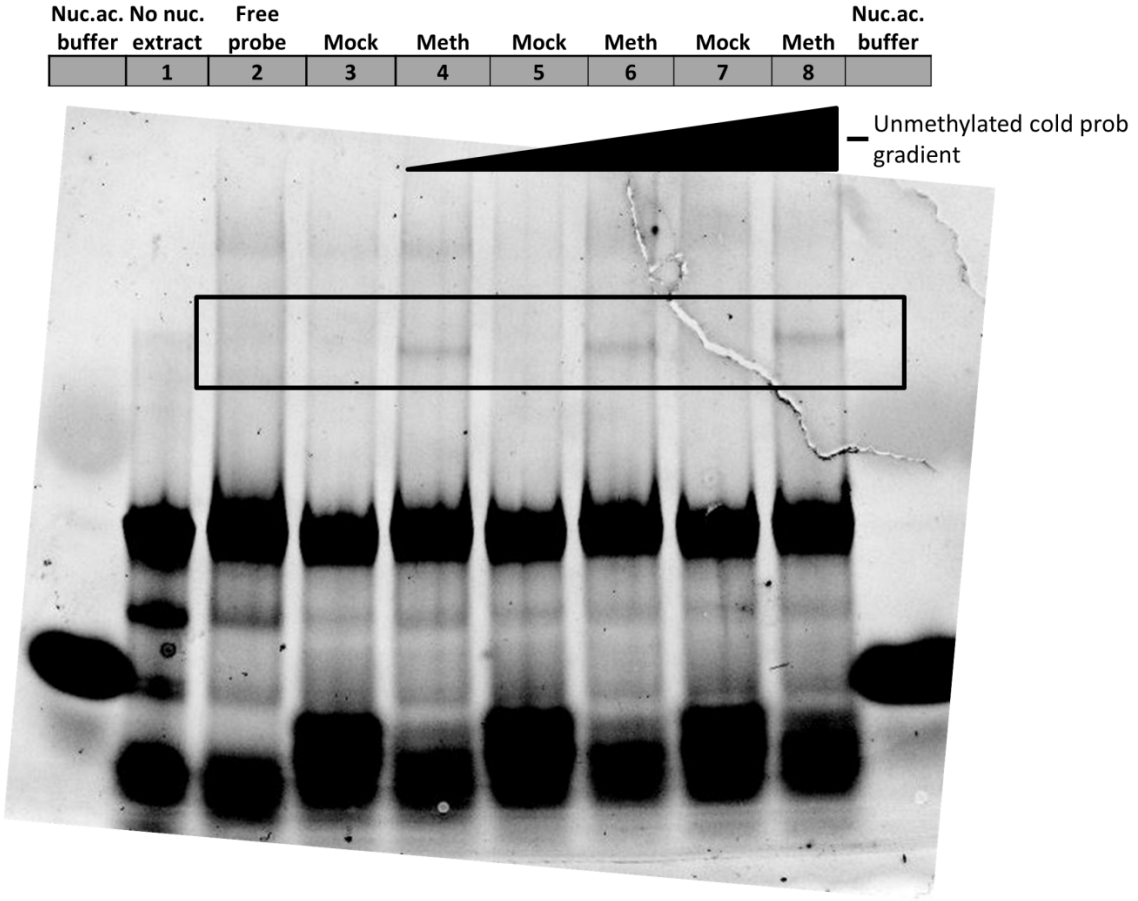

Figure 5: EMSA: unmethylated unlabeled competitor (cold probe) in excess did not affect nuclear extract binding to the methylated labeled probe.

In the search to identify which transcription factor/s bind to the methylated *Spry1* promoter, we used FIMO version 4.11.0 (Grant, Bailey et al. 2011) to scan for individual motifs recognized by transcription factors. We used two different motif databases: JASPAR_CORE_2016_vertebrates and Uniprobe_mouse. There was unfortunately no available database specific for rats. From this analysis, we selected NRF1 as an interesting potential target as it has been linked to metabolism and growth (Cam, Balciunaite et al. 2004; van Tienen, Lindsey et al. 2010). When extending the sequence 24 bases away from the TSS of the gene, a motif recognize by EGF1 transcription factor was found.

We performed a super-shift assay as described (Dhar and Wong-Riley 2009) with modifications stated in the methods of **paper 3 ,** where we added either one of the antibodies or IgG as a control together with nuclear extract and labeled methylated probe. This should allow us to test if incubation with the specific antibody will bind with the transcription factor and result in a shift of the protein-DNA complex. However no such shift in binding was noticed as compared to the IgG control lane.

3.8 BISULFITE CONVERSION

Sequencing techniques do not discriminate between a cytosine and a methylated cytosine. Bisulfite treatment of DNA samples transforms unmethylated cytosines into uracil while methylated cytosines remain unaltered (**Figure 6**). Subsequent PCR amplifies the target region while tyrosine is incorporated in lieu of uracils which represent also the unmethylated cytosine of the original sequence (**Figure 7**). Complete bisulfite conversion without DNA breakdown is a challenge. Commercially available kits have been developed and optimized for this purpose. We tested both EpiTect Fast Bisulfite Conversion Kit (Qiagen) and EZ DNA Methylation-Gold Kit (Zymo research, Ivrine, CA) and both performed equally well. Pyrosequencing controls for complete bisulfite conversion by testing a random CpH (meaning a non-CpG site), supposed unmethylated in the original DNA sequence, for complete conversion into thymidine during the sequencing step.

Figure 6: Bisulfite-mediated conversion of cytosine to uracil.
(Image under CC0 1.0 Universal Public Domain Dedication).

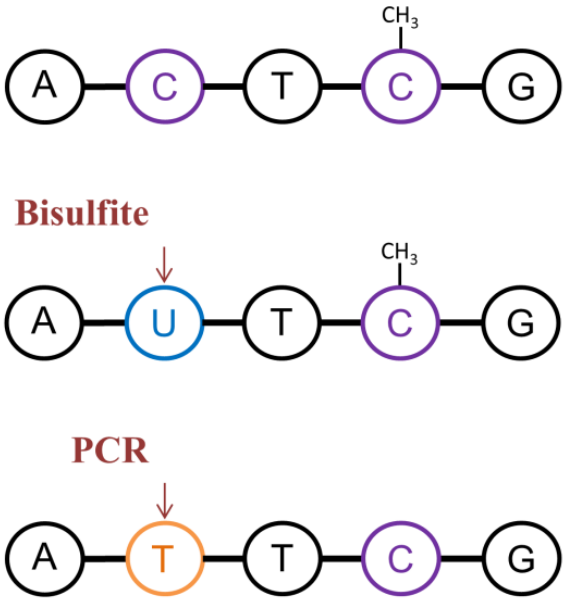

Figure 7: Base substitution during bisulfite conversion and PCR amplification before pyrosequencing. A: Adenosine; C: Cytosine; T: Thymidine; G: Guanine; CH_3: methyl-group.

3.9 PYROSEQUENCING

Pyrosequencing is a method used for *de novo* sequencing and, when combined with bisulfite treatment, to determine methylation status of short regions of DNA. Pyrosequencing was performed as described in papers 3 and 4. The challenge we encountered with pyrosequencing was to design primers that would successfully amplify and sequence our target region. Caveats in the design were CpG density and repetitive motifs. If the density of the CpG is high, there will be expected changes in the sequence due to the bisulfite conversion step. Because of the density, primers will overlap with CpGs and binding might not be effective or biased. In case of a repetitive sequence, primers might bind at several locations resulting in amplification of several products. These multiple products will be detected during the electrophoresis step. About 25% of our primer's designs resulted in successful sequencing. Once the primers are established, Pyrsequencing is a straightforward technique as described in handbooks.

3.10 STATISTICAL ANALYSIS

Data in the studies are presented as mean ± SEM. Student's t-test and paired- Student's t-test were used to compare two groups. Repeated measures one-way ANOVA followed by Bonferroni's multiple comparisons test was used to compare two groups across multiple time points. Two-way ANOVA followed by Bonferroni's multiple comparisons test was used in experiments with more than two groups. Pearson's correlation coefficient was used to analyze the relationship between two variables.

4 RESULTS AND DISCUSSION

4.1 TWIST PROTEIN IN SKELETAL MUSCLE

Type 2 diabetes and obesity are characterized by low-grade inflammation. Several pro-inflammatory cytokines are up-regulated in the insulin resistant state including Tumor necrosis factor (TNFα) and Interleukin 6 (IL6) (Donath and Shoelson 2011). On the contrary, exercise training is known to have long-term anti-inflammatory effects (Woods, Vieira et al. 2006). IL6, a cytokine upregulated by exercise and in type 2 diabetes, appears to have differential effects whether acting on the liver, adipose tissue or in skeletal muscle (Castanon, Von Stetina et al. 2001; Glund and Krook 2008). Transcription factors regulate the gene expression of multiple targets, thus affecting cell function. TWIST proteins play a role in metabolism and inflammation as Twist1 silencing reduces fatty acid oxidation and modulates pro-inflammatory cytokine expression and secretion in adipose tissue (Pettersson, Laurencikiene et al. 2010; Pettersson, Mejhert et al. 2011). We hypothesized that TWIST1 or TWIST2 would play a role in metabolic disease and cellular process within skeletal muscle.

In **paper 1** we studied *Twist1* and *Twist2* overexpression in myoblasts *in vitro* and in the *tibialis anterior* skeletal muscle of mice *in vivo*. We found that overexpressing *Twist* 1 or *Twist* 2 in skeletal muscle had profound effects on mRNA expression and protein abundance of genes related to inflammation and energy metabolism in skeletal muscle. We found signs of glycolysis enhancement: increased Akt signaling was accompanied by decreased *Pdk4* mRNA expression, reduced Acetyl-CoA carboxylase (ACC) and glycogen synthase (GS) protein expression and decreased glycogen stores (**Figure 8**). Moreover, mRNA expression of the inflammatory markers *Il1β*, *Il6*, and *TNFα* was upregulated.

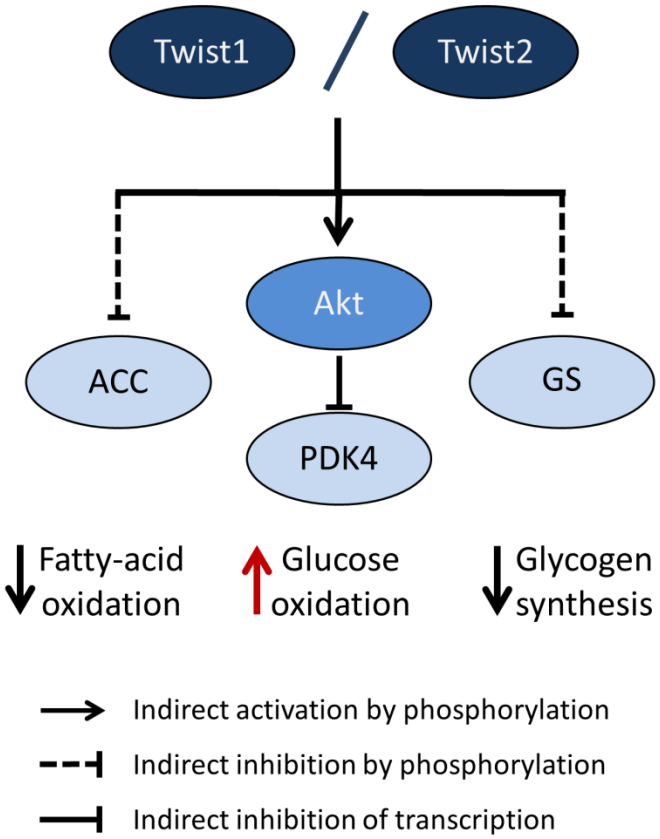

Figure 8: *Twist1* or *Twist2* overexpression increases protein kinase B (Akt) signaling while inhibiting acetyl-CoA carboxylase (ACC) and glycogen synthase (GS), thus promoting energy expenditure and cell growth

While we focused on the role of the TWIST proteins in glucose utilization and glycogen stores, the important role of TWIST1 in skeletal muscle development should not be overlooked. As TWIST1 is a known inhibitor of MyoD and could reverse myogenesis (Hjiantoniou, Anayasa et al. 2008), we used C2C12 myoblasts and not myotubes. Furthermore, the plasmid we used could not be efficiently transfected into dividing skeletal muscle cells, while it was well incorporated into myoblast cultures. To verify the potential effects of TWIST1 and 2 on differentiation, we measured mRNA expression of *MyoD*, *Pax7*, and myogenin in cells and *tibialis anterior* muscle. Only myoblasts with *Twist1* overexpression presented an increase in *Pax7* mRNA expression, but at the same time *MyoD* and *myogenin* trended to be up-regulated, highlighting the role of TWIST1, but not TWIST2 in skeletal muscle differentiation. These changes were not visible in *tibialis anterior* muscle overexpressing *Twist1*, suggesting that differentiated cells *in vivo* might have counter-mechanisms against TWIST1-induced de-differentiation.

A limitation in the interpretation of our results is that we overexpress Twist1 or Twist2 mRNA by more than 3 fold. As TWIST proteins are basic helix-loop-helix proteins, a dose-response experiment could potentially reveal contrasting effects depending on the magnitude

of the overexpression. TWIST protein concentration probably impacts dimers formation, partner protein availability for dimerization and consequently, downstream effects.

We further measured *Twist* 1 and 2 mRNA expression in different metabolic states: obese and diabetic *ob/ob* mice, healthy and diabetic humans and in skeletal muscle biopsies obtained before and after exercise training. We found no difference in *Twist 1* and *2* mRNA expression in these various metabolic states. mRNA expression does not always reflect protein abundance and activity. Nevertheless, our results suggest that despite being expressed in skeletal muscle, *Twist1* and *2* might not be altered by these metabolic conditions. This conclusion does not preclude TWIST proteins to be of potential benefit in metabolic diseases.

We focused on overexpression of the *Twist* genes as previous reports suggested that *TWIST1* was not expressed in mammalian skeletal muscle (Pettersson, Laurencikiene et al. 2010). However, using different sets of Taq-Man primers, we showed that *TWIST1* and *TWIST*2 mRNA is present in mouse and human skeletal muscle. As overexpression of the *Twist* genes increases inflammatory and glycolysis markers, silencing their expression could be beneficial to reduce inflammation and increase fatty acid oxidation.

4.2 THE KYNURENINE PATHWAY AND ACUTE EXERCISE

KATs are enzymes expressed in skeletal muscle and several other tissues where they convert, among other substrates, kynurenine into kynurenic acid (Perkins and Stone 1982; Han, Cai et al. 2010). This conversion prevents kynurenine from reaching the brain as kynurenic acid cannot cross the brain blood barrier, thus reducing the risk of depression (Myint, Kim et al. 2007; Oxenkrug 2010; Agudelo, Femenia et al. 2014; Campbell, Charych et al. 2014). Exercise training and PGC1α-induced increases in skeletal muscle KAT expression have been linked to decreased circulating kynurenine levels and resistance to stress-induced depression (Agudelo, Femenia et al. 2014). We tested the hypothesis that acute exercise in humans is sufficient to impact circulating kynurenine levels, and asked whether type 2 diabetic patients would respond to the same extent as healthy volunteers.

In **paper 2** we observed that acute exercise alters kynurenine levels in plasma. Plasma tryptophan and kynurenine concentrations were reduced, while kynurenic acid levels increased. Our results are consistent with earlier evidence that exercise or increased PGC1α expression are associated with enhanced conversion of kynurenine into kynurenic acid (Agudelo, Femenia et al. 2014). Changes in the kynurenine pathway noted in response to exercise were similar between type 2 diabetic and healthy volunteers, suggesting that this mechanism is preserved in people with type 2 diabetes.

We also correlated metabolite concentrations at baseline and three hours after the exercise bout with clinical parameters. Kynurenine levels correlated with BMI regardless of diabetes. As obesity has been linked to depression (Mannan, Mamun et al. 2016; Muhlig, Antel et al. 2016), elevated kynurenine levels may play a role in obesity-related depression. However, we

did not have mood or depression rating scales to measure possible acute changes of mood in this cohort.

In our study we noticed that plasma tryptophan was 10 times more concentrated than kynurenine, which is 1,000 times more concentrated than kynurenic acid. After acute exercise, both tryptophan and kynurenine plasma concentrations were reduced by 20-30%, while kynurenic acid was increased by 15%. Thus the increase in kynurenic acid does not fully explain the decrease in tryptophan and kynurenine. These decreases could be explained by a shunt of tryptophan conversion from kynurenine to serotonin, thus reversing a major etiological process in depression (Lapin and Oxenkrug 1969). However, we did not have assays to measure serotonin and the other arm of the kynurenine pathway, quinolinic acid and intermediary products. Assessment of these species would be informative and should be performed in future studies.

The low plasma concentration of kynurenic acid could be partly explained by the fact that kynurenic acid is an acid and therefore does not easily diffuse through a phospholipid bilayer such as the plasma membrane (Cooper 2000). Although kynurenic acid transporters have been described (Uwai, Hara et al. 2013), they do not appear to be expressed in skeletal muscle (according to the human protein atlas accessed on August 18th 2016 (Uhlen, Fagerberg et al. 2015).

In **paper 2**, *KATs* expression measurements revealed that three hours after an acute bout of exercise *KATs 1,2* and *3* mRNA was unaffected, while *KAT4* mRNA decreased. Absence of an increase in *KATs* mRNA expression is in contrast to previous results in mice where *Kats* 1,3 and 4 mRNA expression was increased after 8 weeks of voluntary wheel running (Agudelo, Femenia et al. 2014). This discrepancy could be due to several factors. *KATs* mRNA induction may require more than three hours or may require several bouts of exercise as observed for the expression of other genes (Yang, Creer et al. 2005). As plasma concentration of the metabolites is already altered after an acute bout of exercise, it is probable that the activity of the KATs enzymes in skeletal muscle or other peripheral tissues is already increased by acute exercise. Type 2 diabetic volunteers exhibited reduced levels of *KAT1* and *KAT2* mRNA compared to their healthy counterparts without any measurable consequence on plasma concentration of kynurenine or kynurenic acid suggesting that mRNA might not reflect protein level or enzymatic activity.

The clinical setup has some limitations. Resting blood chemistry and biopsy were taken after an overnight fast on a different day than the samples taken immediately after the acute bout of exercise and after a three hours recovery period. Furthermore, volunteers had a light breakfast on the day of the exercise bout. These variables add confounding factors for the time course study, but are likely not sufficient to explain the differences seen between rest, exercise or recovery. Especially, kynurenic acid concentration was increased by exercise, but was similar between rest and recovery.

To monitor exercise intensity, we used maximum heart. .Heart rate is a simple measure, but with some drawbacks. Intensity measured by VO_2 or energy output in watts is stable throughout the exercise bout and reproducible to a certain extent between bouts. Heart rate on the contrary is influenced by multiple, rapidly changing, factors such as fever, fatigue or hydration. During the exercise, heart rate is not stable. If intensity is maintained, heart rate will increases over time. This phenomenon is known as "cardiac drift" and is especially prominent if, as in our design, hydration is not provided (Coyle and Gonzalez-Alonso 2001). Thus, using heart rate as a measure of exercise intensity may have resulted in reduced exercise intensity. Nevertheless, the exercise bout was sufficient to induce changes in tryptophan, kynurenine and kynurenic acid plasma concentration.

Despite the limitations mentioned, our study sheds light on the effects of acute exercise on the kynurenine pathway and provides a potential mechanism for the anti-depressive effects of exercise. Interestingly, exercise-mediated changes in kynurenine metabolism are preserved in type 2 diabetic patients, highlighting the therapeutic potential of exercise in this population.

4.3 DNA METHYLATION IN SKELETAL MUSCLE

In skeletal muscle, DNA methylation has been shown to respond to and be modulated by multiple stimuli including exercise, diabetes and diet (Barres, Osler et al. 2009; Barres, Yan et al. 2012; Nitert, Dayeh et al. 2012; Lindholm, Marabita et al. 2014). For selected genes, these studies have suggested a possible role of DNA methylation in cell metabolism by drawing positive or negative correlations with mRNA expression or protein abundance.

4.3.1 Exercise, Diet and DNA Methylation

The multiple health benefits of physical activity and exercise training are the result of repeated skeletal muscle contraction, but also involve and impact most organs and tissues in the body. The health benefits of regular physical activity include but are not limited to, a reduction of relative risk of dementia, diabetes, depression, hip fracture and several cancers including breast and colon cancer (Rethorst, Wipfli et al. 2009; Khan, Thompson et al. 2012). Understanding which exercise-mediated changes are driving health benefits is an area of active research. In this thesis, the effects of exercise are studied in three out of four studies.

As the lack of exercise and an unhealthy diet are important factors in the development of obesity and type 2 diabetes, we used four different cohorts in **paper 3**: The first cohort was composed of sedentary rats on a chow diet as controls, the second of rats trained by swimming for 5 days, the third of sedentary rats fed a high-fat diet, the fourth of both trained and fed rats (**Figure 9**). Molecular markers of exercise response and high-fat feeding had already been assessed in another study (Galuska, Kotova et al. 2009). We assessed the effects

of exercise training on DNA methylation. We hypothesized that exercise-mediated changes in skeletal muscle mRNA would in part be dependent on altered DNA methylation.

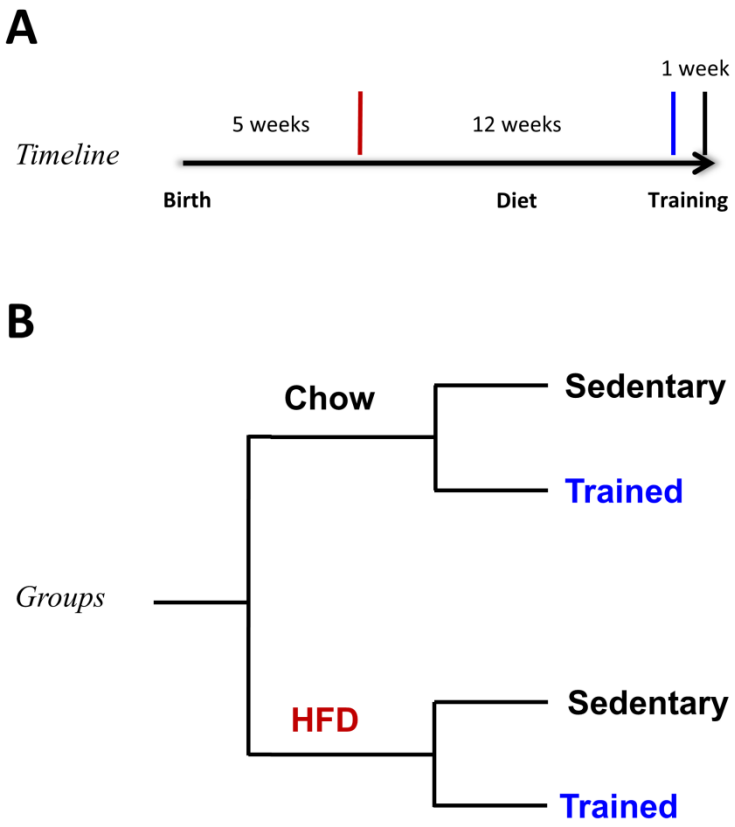

Figure 9: Design of the Rat Cohorts study in paper 3. A: Design of the timeline. B: Design of the groups and treatments.

Using methylated DNA immunoprecipitation sequencing (MeDIP-sequencing) we revealed genome-wide differences in the DNA methylation profile induced by exercise, before validating some specific targets at base-pair resolution in all four groups. DNA methylation changes were not accompanied by changes in mRNA or protein levels of nearby genes (**Figure 10**), raising the question discussed later in this thesis of the physiological consequences of the methylation changes in these specific targets.

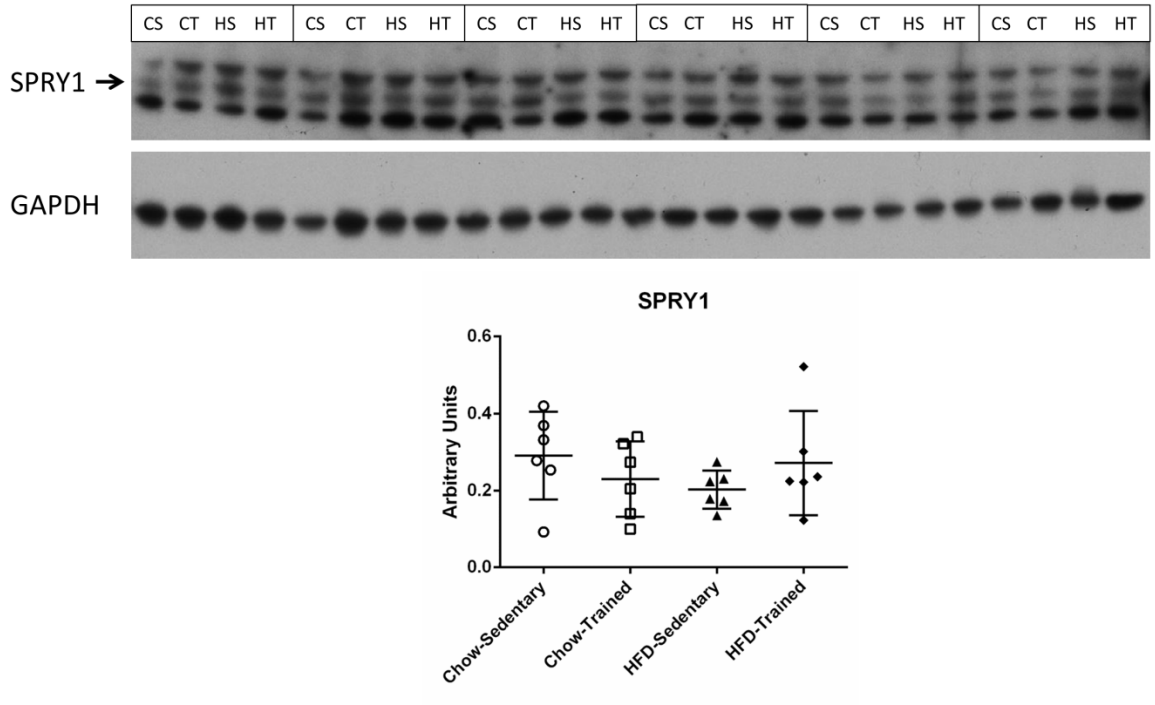

Figure 10: SPRY1 immunoblot.
CS: chow fed & sedentary rats; CT: Chow fed & trained rats; HS: high-fat fed & sedentary rats; HT: high-fat fed & trained rats. Results are presented as individual values and mean ± SEM arbitrary units.

In studies investigating human skeletal muscle DNA methylation in a metabolic context, DNA methylation changes affected only a limited percentage of all the cells in the samples, raising the question of the significance and of the origin of the cells affected (Barres, Osler et al. 2009; Barres, Yan et al. 2012; Nitert, Dayeh et al. 2012; Lindholm, Marabita et al. 2014).For example, is only one cell type affected or a sub-group of multiple cell types? Is it a fiber type specific matter? DNA methylation changes could be due to changes in the composition of the muscle as satellite cells start proliferating and new nuclei are added in the case of muscle growth. With training, vascularization of the muscle changes (Gustafsson 2011), possibly altering the DNA methylation profile of a whole-muscle biopsy. Cellular composition of the biopsy subjected to DNA methylation profiling may also reflect changes in cell type due to neutrophil infiltration following exercise (Schneider and Tiidus 2007). Contamination of the biopsy by vascular or subcutaneous fat tissue during the surgical procedure is another possible confounding factor.

In **paper 3** we used several methods in attempt to unravel the origin of the DNA methylation changes. In recent studies on skeletal muscle DNA methylation and exercise performed in our laboratory and by others, the differences in methylation between groups were subtle (Barres, Yan et al. 2012; Nitert, Dayeh et al. 2012; Lindholm, Marabita et al. 2014). Satellite cells represent approximately 8% of the nuclei of a skeletal muscle, while the rest is half myonuclei and half stromal nuclei (Schmalbruch and Hellhammer 1977). We hypothesized that DNA methylation changes observed after exercise could be due to satellite cells being reprogrammed into myoblasts and later on into differentiated myotubes.

DNA sequences in the promoter region of *Spry1*, a regulator of satellite cell quiescence, presented DNA methylation changes resulting from both training and diet interventions. EMSA analysis revealed that nuclear proteins bind to this *Spry1* promoter region when CpGs are fully methylated. We attempted to perform a supershift assay using antibodies against two transcription factors possibly binding around that region, but no changes in binding were noted. To further test the hypothesis that the DNA methylation could be related to changes in satellite cell proliferation and differentiation, we examined the DNA methylation profile of the *Spry1* promoter between proliferating and differentiated L6 myotubes. No changes in DNA methylation were noted, suggesting that changes in DNA methylation of the *Spry1* promoter are not linked to differentiation of myoblasts *in vitro*. However, differences in the DNA methylation landscape between cells *in vivo* and *in vitro* have been noted (Nestor, Ottaviano et al. 2015). Further experiments isolating satellite cells and skeletal muscle fibers *in vivo* would be needed to address this more fully. Moreover, DNA methylation of *Spry1* could be measured at several time points during differentiation, as changes are possibly transient during the process of differentiation.

Subtle differences in DNA methylation could be explained by factors extrinsic to skeletal muscle or changes in the cellular composition of the skeletal muscle rather than changes in the epigenome of the myonuclei. To explore this hypothesis, we isolated *tibialis anterior* muscles from rats and subjected them to *in vitro* contraction followed by four hours of recovery. There was no difference in DNA methylation between contracted and control muscles suggesting that an external factor or "crosstalk" between skeletal muscle and other organs might be necessary to induce the DNA methylation changes observed in *Spry1*. However, such extrinsic factors appear not always necessary, as a previous study from our research group highlighted cases where similar DNA methylation changes were induced by exercise in a human cohort, as by *in vitro* contraction in mouse skeletal muscle but only 45 minutes after the end of the contraction protocol and not at other time points (Barres, Yan et al. 2012). Nevertheless, a biopsy after training and *in vitro* contraction are two different models and many variables can account for differences between experimental results. For example, we did not mimic the five days training protocol: the *in vitro* contraction protocol was only sustained for one hour and the muscle specimen had four hours recovery following the exercise.

4.3.2 Insulin and DNA Methylation in Skeletal Muscle

In **paper 4** we assessed if insulin triggers epigenetic modifications in skeletal muscle. Using the 450k DNA methylation array, a genome-wide technique, we revealed that a one hour insulin treatment of human skeletal muscle *in vitro* induces broad changes in the DNA methylation profile. A genome wide approach allowed us to subsequently investigate expected targets (*HK1* and *HDAC5*), as well as potentially novel targets of insulin (*DAPK3* and *ATP2A3*). Base pair resolution pyrosequencing confirmed insulin-induced increase in DNA methylation in the gene body of *DAPK3* at one specific CpG and the changes in the 3'UTR region of *ATP2A3* at two different CpGs. To further validate these results, we

exposed primary human myotubes to insulin *in vitro* and observed a trend in increased *DAPK3* methylation mirroring the result observed in the biopsies.

To translate our results to an *in vivo* setting, skeletal muscle DNA methylation was also assessed in biopsies obtained from normal glucose tolerant and type 2 diabetes subjects (**Table 3**) before and after an oral glucose challenge. *DAPK3* methylation was reduced in skeletal muscle from type 2 diabetic patients as compared to controls at baseline, and a glucose challenge further decreased *DAPK3* DNA methylation in both groups. Hyperglycemia and loss of insulin sensitivity have additive effects on skeletal muscle DNA methylation in human skeletal muscles, but the difference in *DAPK3* DNA methylation between the insulin- and glucose-stimulated conditions is difficult to reconcile as both of these factors induce insulin resistance. Additional systemic factors induced by hyperglycemia probably affect *DAPK3* DNA methylation. Furthermore, *DAPK3* DNA methylation was inversely correlated with plasma glucose concentration two hours after the glucose challenge, thus potentially involving *DAPK3* in nutrient signaling pathways.

Further experiments in myocyte cultures *in vitro* could help separate the direct roles of glucose and insulin on skeletal muscle DNA methylation. Furthermore, measurement of the skeletal muscle mRNA expression of *DAPK3* between type 2 diabetic and normal glucose tolerant subjects is lacking.

Basal *DAPK3* methylation was lower in the muscle strips than in biopsies from control subjects before the glucose challenge. Differences in methylation could be due to the different techniques used to collect the biopsies, differences in clinical parameters between the cohorts such as age, or to the fact that muscle strips are not flash frozen in liquid nitrogen but transported to the laboratory before incubation. During transport and incubation, a possible hypoxia could induce changes in the DNA methylation profile. This possibility could be assessed by measuring DNA methylation in strips frozen immediately after collection. Furthermore, the type 2 diabetic and normal glucose tolerant volunteers included in this study presented differences in BMI that may also explain the differences observed in the DNA methylation. However, blood glucose levels were more tightly correlated with DNA methylation than BMI, thus glucose levels are more likely to explain changes in DNA methylation of *DAPK3*.

In vitro insulin stimulation of skeletal muscle strips did not correlate with mRNA changes of *DAPK3* or *ATP2A3* after one hour of insulin stimulation. As most mRNA expression changes following insulin stimulation in skeletal muscle appear after three hours of stimulation (Pandini, Medico et al. 2003; Rome, Clement et al. 2003), we stimulated myotubes *in vitro* and collected DNA and mRNA four hours after the insulin stimulation. We found that *DAPK3* and *ATP2A3* mRNA was unaltered four hours after insulin treatment, while DNA methylation only trended to be altered by the insulin stimulation. A larger number of subjects would be necessary to be able to conclude these observations more firmly.

4.3.3 DNA Methylation and Gene Expression

The addition of a methyl group to a cytosine is commonly considered as a modification that will repel transcription factor binding, thus making DNA methylation a repressive mark. However, DNA methylation can also lead to increased transcription factor binding (Rishi, Bhattacharya et al. 2010). In **paper 3**, methylation of the *Spry1* promoter increases skeletal muscle nuclear factor binding to the promoter.

Promoter hypermethylation correlating with gene silencing or promoter demethylation leading to de-repression of mitogenic genes is a well-known phenomenon in cancerous cells (Jones and Baylin 2002). *In vitro* studies using luciferase assays comparing gene expression relative to promoter methylation have often demonstrated that complete methylation of all CpG in the promoter leads to reduced expression of the gene (Yang, Dayeh et al. 2012; Ronn, Volkov et al. 2013). Nevertheless, some hypermethylated promoters are transcriptionally active, while unmethylated promoters can be inactive (Boyes and Bird 1992; Weber, Hellmann et al. 2007). Recent genome-scale studies found negative associations, positive associations or no association between DNA methylation and gene expression (Eckhardt, Lewin et al. 2006; Ball, Li et al. 2009; Lister, Pelizzola et al. 2009). Overall, epigenetic studies in the field of metabolism found that a minor proportion of the regions with altered DNA methylation also exhibited differential mRNA expression (Jacobsen, Brons et al. 2012; Nitert, Dayeh et al. 2012; Ronn, Volkov et al. 2013; Lindholm, Marabita et al. 2014). In **paper 3 and 4** we did not find correlation between DNA methylation changes and gene expression, suggesting that other factors such as histone modifications and CpG density (Weber, Hellmann et al. 2007) influence the expression of the genes we investigated in these studies. DNA methylation in the regions we studied could potentially alter expression of distant genes in a similar fashion as the *FTO* variant influences expression of the distant IRX3 gene (Smemo, Tena et al. 2014). Studying other epigenetic mechanisms in insulin- or exercise-treated samples as described in **paper 3 and 4** could help understanding the interplay between the multiple layers of epigenetic regulation and gene expression.

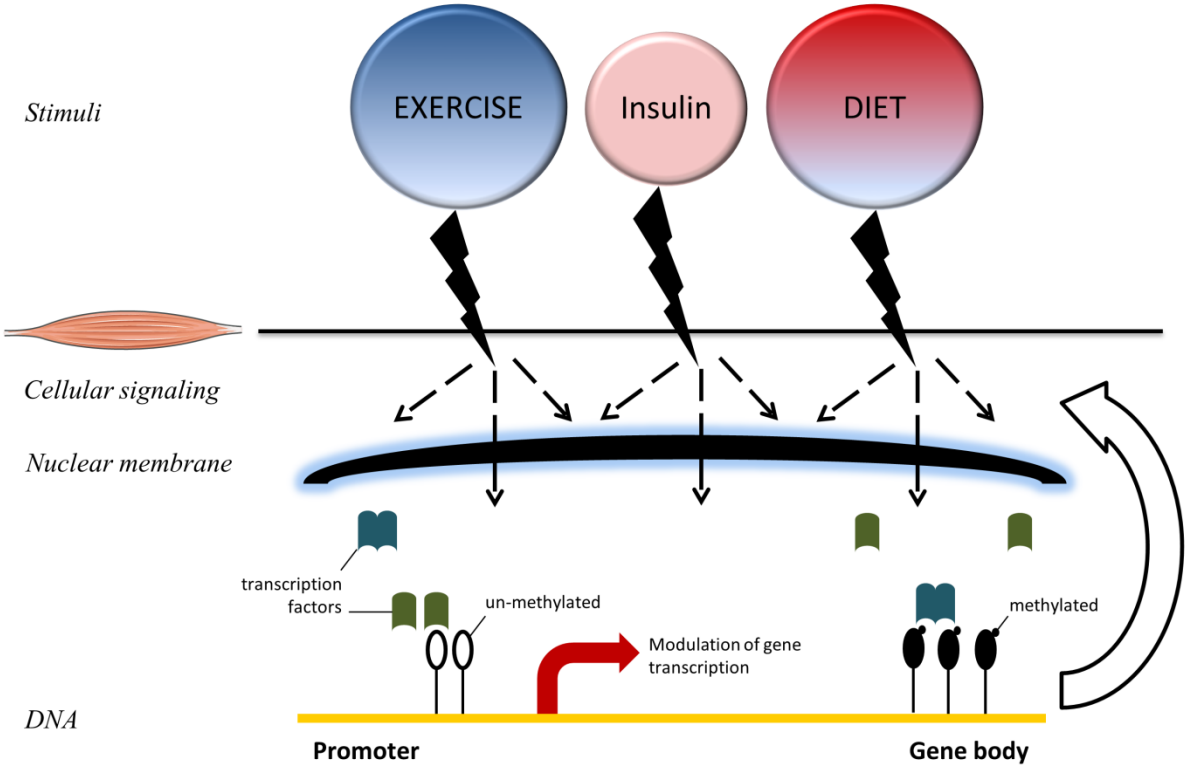

Figure 11: Schematic Summary of the Main Resuls from Papers 3 and 4: Stimuli affect skeletal muscle signaling and epigenetic marks, which in turn affect the cellular program. (Muscle schematic adapted from Servier Medical Art)

4.3.4 Techniques in DNA Methylation Studies

In this thesis, **paper 3 and 4** aim to understand how DNA methylation participates in the adaption of skeletal muscle to exercise training and diet and how hormonal signaling by insulin may influence these responses. We used whole genome MeDIP-sequencing and genome wide human 450k methylation array followed by pyrosequencing to measure changes in DNA methylation of skeletal muscle.

Several genome-wide methods have been recently developed to study DNA methylation on a large scale. Several reviews have described and compared methods (Bock, Tomazou et al. 2010; Laird 2010; Nair, Coolen et al. 2011; Walker, Bhagwate et al. 2015). The cutting edge techniques in 2012-2013 were: methylated DNA immunoprecipitation sequencing (MeDIP-sequencing), DNA capture by affinity purification (MethylCap-sequencing), reduced representation bisulfite sequencing (RRBS), and Human Methylation 450 Bead Chip (450k array). Whole genome bisulfite sequencing was, at the time, very costly and bioinformatics tools were not available.

The 450k array is an affordable and reliable way to assess genome-wide DNA methylation at over 485’000 selected human CpG sites covering 99% of the human RefSeq genes with multiple sites per gene. With a cost of approximately 1500 SEK (200 US dollars) per sample the 450k array is now considered an excellent choice to study DNA methylation in large

groups. The 450k array was our method of choice in **paper 4.** In **paper 3**, as we studied rats, we had to find a different assay.

MethylCap-sequencing more accurately identifies differentially methylate regions (DMRs) than MeDIP-sequencing (Bock, Tomazou et al. 2010), but has a lower sensitivity than MeDIP-sequencing in lowly methylated regions (Nair, Coolen et al. 2011). MethylCap-sequencing was not the appropriate method for our project because promoter and gene bodies of expressed genes are usually lowly methylated.

MeDIP-sequencing provides whole-genome coverage but relies on antibodies and thus depends on the affinity of the antibodies. MeDIP-sequencing is considered a qualitative rather than quantitative method as it is not totally accurate and reproducible (Bock, Tomazou et al. 2010). RRBS uses the combination of restriction enzymes and bisulfite sequencing to enrich for areas with high CpG density and provide reliable data at a base-pair resolution (Bock, Tomazou et al. 2010). RRBS coverage is however limited. We selected MeDIP-sequencing for a broader coverage. Given that DNA methylation alterations between metabolic conditions are subtle, RRBS would probably have detected more DMRs.

Genome-wide analyses provide a large amount of data requiring further bioinformatic analysis. Multiple tools are available: commercial all-in-one solutions like Ingenuity IPA, free packages for "R" or online tools such as DAVID (Database for Annotation, Visualization and Integrated Discovery) or Webgestalt. We mainly used Webgestalt as it is a published and freely available tool. Pathway analysis in Webgestalt is based on the Kyoto encyclopedia of genes and genomes (KEGG). Based on analysis from the sequencing companies and the one we performed in-house, we selected DNA regions to be technically validated on a base-pair resolution using pyrosequencing.

Pyrosequencing is a method where bisulfite-converted DNA is amplified by polymerase chain reaction (PCR) and then sequenced to determine if the bases of interest were methylated or not (Tost and Gut 2007). It requires the design of a primer pair for PCR and a sequencing primer. Out of all the regions we aimed to investigate, many could not be sequenced as either the PCR or the sequencing failed due to problem in primer binding. When amplification and sequencing are successful, pyrosequencing is a precise tool to measure DNA methylation at base-pair resolution.

We used pyrosequencing in **papers 3 and 4**. Interestingly, DNA methylation of *Spry1* and *Myh7b* in gastrocnemius and *tibialis anterior* muscles from Wistar rats presented a very similar profile, illustrating that DNA methylation is stable between animal species and across muscle biopsies (**Figures 12 and 13**). On the contrary, in **paper 4**, DNA methylation in *DAPK3* of *in vitro* skeletal muscle at basal is more variable between donors than the magnitude of the changes induced by the insulin treatment (**Figure 14**). More pronounced inter-individual differences *in vitro* may reflect DNA methylation profile changes induced by cell culture *in vitro (Nestor, Ottaviano et al. 2015)*. Further experimental parameters such as

the purity of the cell culture or the multiplicity of stages of satellite cells differentiation could also account for inter-individual differences.

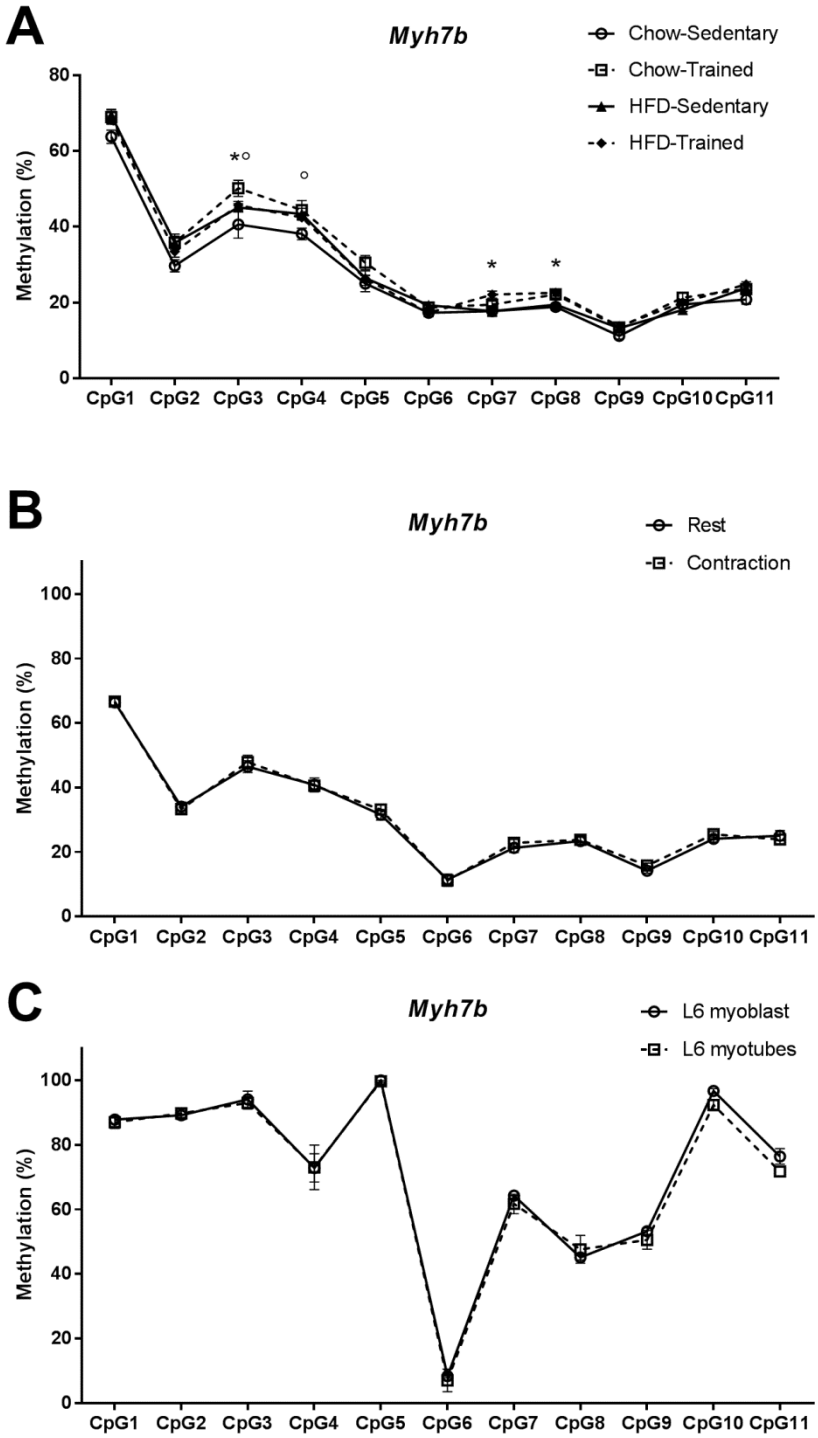

Figure 12: Comparison of *Myh7b* DNA methylation from 3 different origins.
A: From Rat gastrocnemius muscle; B: From rat *tibialis anterior* muscle C: from L6 rat muscle cell line. Data are presented as the mean ± SEM. * $p<0.05$, for exercise effect within one single CpG, ° $p<0.05$, for an interaction between exercise and diet within one single CpG.

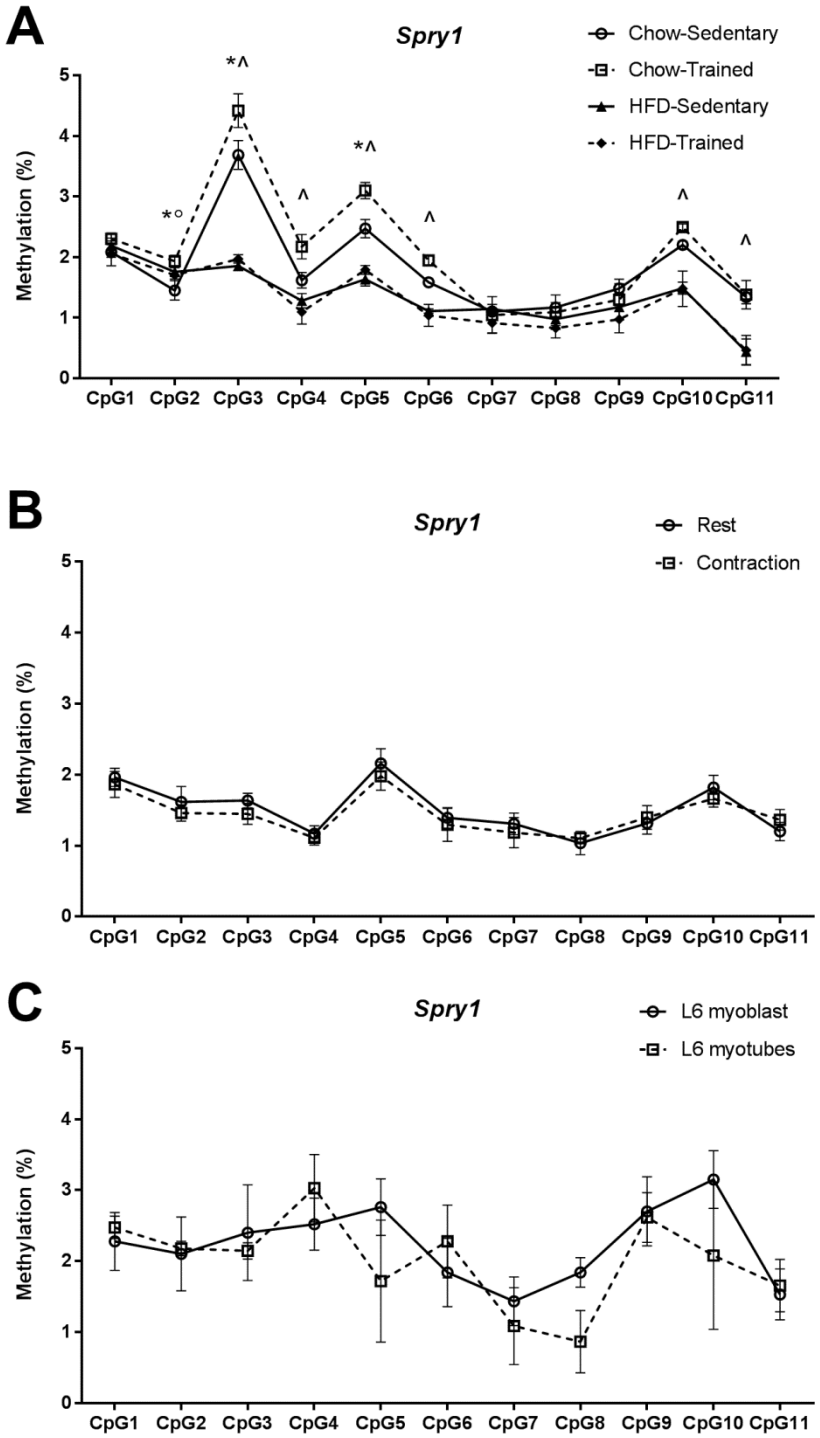

Figure 13: Comparison of *Spry1* DNA methylation from 3 different origins.
A: From Rat gastrocnemius muscle; B: From rat *tibialis anterior* muscle C: from L6 rat muscle cell line. Data are presented as the mean ± SEM. * $p<0.05$, for exercise effect within one single CpG, ^ $p<0.05$, for diet effect within one single CpG. ° $p<0.05$, for an interaction between exercise and diet within one single CpG.

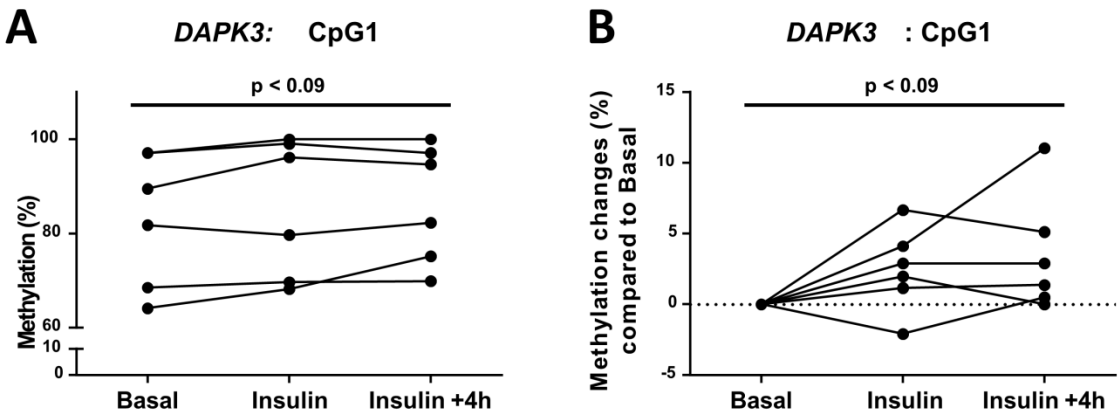

Figure 14: Individual changes in DNA methylation of *DAPK3* at CpG1 at Basal, after insulin treatment and four hours after insulin treatment. Data are presented as percentage of DNA copies methylated (A) or relatively to the basal level (B). Each dot represents a subject at a specific time point. DNA methylation of *DAPK3* and *ATP2A3* varied between donors, while absolute changes in response to insulin treatment were more consistent.

4.3.5 Approaches to Study Skeletal Muscle Methylation in Different Metabolic States

In **papers 3 and 4** we used a similar approach to compare DNA methylation in various metabolic contexts. In both cases we started with a genome-wide method comparing two conditions (sedentary and trained in **paper 3**, insulin stimulated or not in **paper 4**). We then used this data to target more specific regions. When target regions had been confirmed, we used other samples to assess the reproducibility of the findings in similar but different models (**Figure 15**).

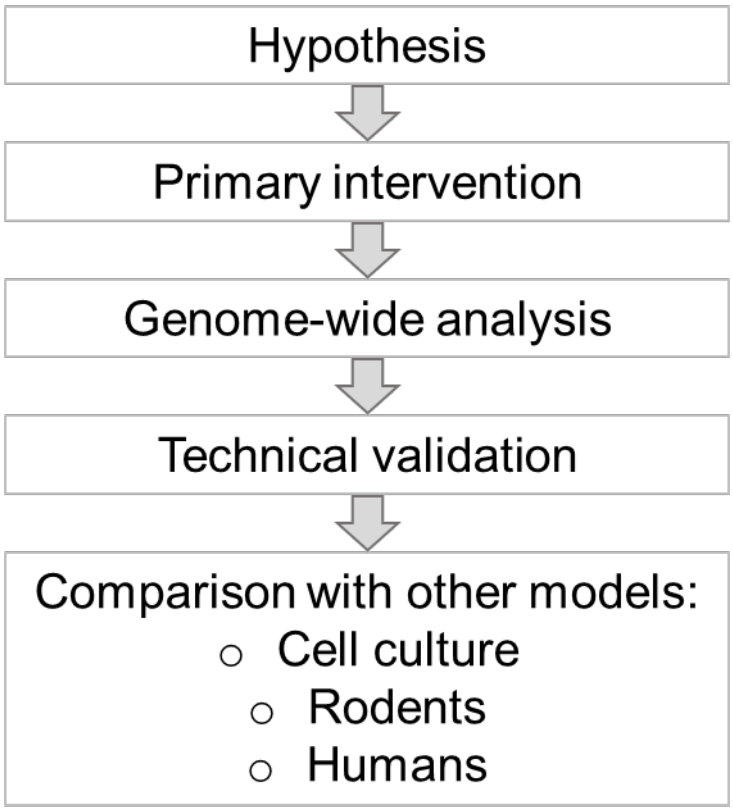

Figure 155: Schematic of the systematic approach used in papers 3 and 4

For example, the majority of mRNA transcripts regulated by insulin are significantly altered no earlier than three hours after insulin stimulation (Pandini, Medico et al. 2003; Rome, Clement et al. 2003). As we did not find mRNA changes in the genes of interest in the skeletal muscle strips after a one hour of *in vitro* stimulation, we used primary cell culture to assess if *DAPK3* and *ATP2A3* mRNA changes would appear four hours after the stimulation, as in the case of *PDK4*. The insulin treatment was effective as exemplified by the insulin-responsive gene *PDK4* (**Figure 16**), but neither *DAPK3* nor *ATP2A3* gene expression was affected.

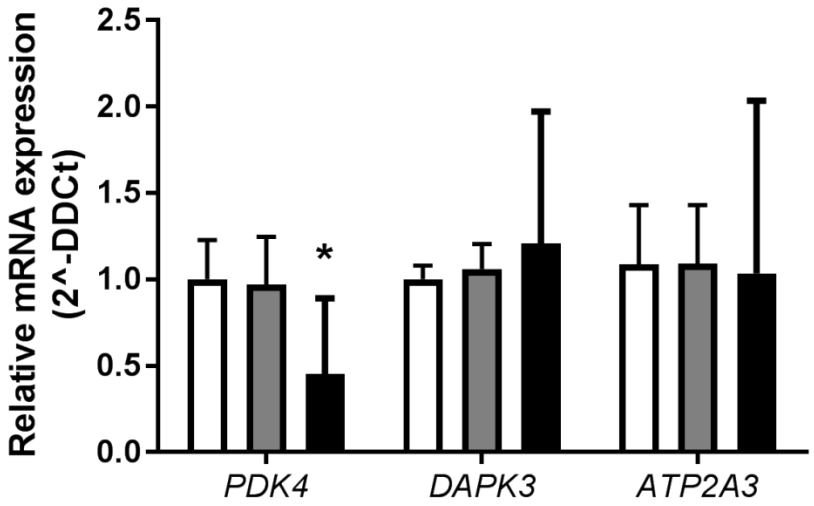

Figure 166: mRNa expression of *PDK4, DAPK3* and *ATP2A3* in primary skeletal myotubes. Samples were collected at basal (white bar), immediately after one hour of insulin stimulation (grey bar) or incubated with insulin and collected four hours after stimulation (black bars). Data are mean ± SEM. *p<0.05, Basal vs four hours post-stimulation.

In **papers 3 and 4** we did not find any correlation between the changes in DNA methylation and mRNA expression. Depending on the gene, mRNA changes can occur up to 4 days after the last bout of exercise (Neubauer, Sabapathy et al. 2014). It is possible that we missed the correct window to capture changes in mRNA. Nevertheless, we tested whether methylation of the *Spry1* promoter would alter binding of transcription factors to its sequence. Surprisingly, while methylation is usually associated to transcription factor binding inhibition, EMSA showed that methylation of *Spry1* promoter increased its binding of nuclear proteins.

5 CONCLUSION

The main findings in this thesis are:

1. TWIST1 and TWIST2 proteins alter skeletal muscle glucose metabolism and mRNA transcription of cytokines. Skeletal muscle *TWIST1* and *TWIST2* mRNA expression is not altered in a range of metabolic conditions.

2. Acute exercise alters plasma concentration of tryptophan, kynurenine and kynurenic acid in normal glucose tolerant healthy volunteers, as well as in type 2 diabetic subjects. Kynurenine concentration in plasma correlates with BMI in both healthy volunteers and type 2 diabetic subjects.

3. Exercise training and diet alter the methylome in skeletal muscle. DNA methylation does not necessarily shown an inverse correlation with mRNA. DNA methylation increases binding of nuclear proteins to *Spry1* promoter.

4. Insulin stimulation acutely alters DNA methylation in skeletal muscle. Skeletal muscle *DAPK3* DNA methylation is altered in type 2 diabetic patients. A glucose challenge reduces skeletal muscle *DAPK3* DNA methylation in both normal glucose tolerant healthy volunteers and type 2 diabetic patients .

Type 2 diabetes, obesity and exercise, all have profound effects on skeletal muscle gene expression and metabolism. The work included in this thesis reveals the metabolic effects of TWIST1 and TWIST2 overexpression in skeletal muscle; provides evidence for the acute effect of exercise on the kynurenine pathway and maps changes in the methylome following exercise training and insulin stimulation supporting the notion that DNA methylation is a rapidly adaptive epigenetic mark in somatic cells. Our studies further suggest the kynurenine pathway as a potential mechanism for some of the anti-depressive effects of exercise, and highlights glucose as a potential modulator of DNA methylation.

Our work on DNA methylation in particular regions of the genome underscores recent results from genome-wide studies (Jacobsen, Brons et al. 2012; Nitert, Dayeh et al. 2012; Ronn, Volkov et al. 2013; Lindholm, Marabita et al. 2014) that methylation often does not correlate with gene expression of the neighboring gene. However, DNA methylation increases nuclear protein binding as in the *Spry1* promoter,

Altogether, the work in this thesis reveals new mechanisms involved in the protective effect of exercise and the pathophysiology of type 2 diabetes and obesity, offering new opportunities for improvements in the management and treatment of metabolic diseases.

6 FUTURE PERSPECTIVES

6.1 THE TWIST PROTEINS

Work in this thesis confirms that TWIST1 and TWIST2 are expressed and play a role in skeletal muscle metabolism, growth and differentiation. Although we did not find alterations in *TWIST* mRNA in trained or type 2 diabetic people and obese mice, protein abundance and functional activity of TWIST proteins was not measured. Additionally, a map of DNA binding regions for TWIST by chromatin immunoprecipitation (ChIP) with DNA sequencing and a better understanding of TWIST dimerization and protein-protein interaction using Cross-linking/mass spectrometry would help refine understanding of the role of TWIST in skeletal muscle. Furthermore, *TWIST1* and *TWIST2* gene silencing studies could reveal beneficial effects in reducing inflammation and favoring fatty acid oxidation.

6.2 THE KYNURENINE PATHWAY

The kynurenine pathway has been linked to psychiatric disorders. Exercise reduces the risks of depression, possibly through the kynurenine pathway in mice, healthy humans and as described here, in type 2 diabetic subjects. Studying depressed subjects undergoing exercise training would allow direct correlation of depression score with tryptophan, kynurenine and other metabolites plasma concentration. Furthermore, the measurement of other metabolites such as quinolinic acid would broaden our understanding of the pathway equilibrium.

Understanding the regulation of the balance between the transformation of tryptophan into kynurenine or serotonin and further, the transformation of kynurenine into kynurenic acid and quinolinic acid could increase our understanding of the role of tryptophan and kynurenine metabolism in depression. So far, only the role of skeletal muscle on plasma kynurenine has been studied. Further studies on other tissues such as fat and liver could increase our understanding of plasma kynurenine regulation.

6.3 DNA METHYLATION IN SKELETAL MUSCLES

DNA methylation was long thought to remain constant in differentiated cells. Recently, we and others have observed rapid DNA methylation profile changes in response to diverse stimuli including exercise, diet, lipids, and now insulin (Barres, Osler et al. 2009; Barres, Yan et al. 2012; Nitert, Dayeh et al. 2012; Ronn, Volkov et al. 2013; Lindholm, Marabita et al. 2014; Dekkers, van Iterson et al. 2016). As noted in this thesis work, in skeletal muscle, methylome remodeling appears to be limited to a few of the DNA copies present in the samples. The exact origin of the changes remains unclear and warrants further investigations to determine which cells are affected, and what are the functional consequences of the DNA

methylation changes. Sorting of the cells and single cell sequencing could be helpful methods to achieve this goal.

Furthermore, the interplay between DNA methylation marks and chromatin configuration, transcription factors binding, and mRNA transcription remains incompletely understood. Studying several of these aspects in a simpler model such as an immortalized cell line *in vitro* could help bridge the gap between the different levels of epigenetic regulation. For example in the case of *Spry1* promoter, ChIP with DNA sequencing could help speed up the identification of regulatory transcription factors that bind in a methylation-dependent manner.

Insulin and glucose have broad effects on growth and metabolism. Here we expand their potential roles to include direct effects on skeletal muscle DNA methylation, thereby providing an additional level of regulation. The epigenetic response to systemic factors associated with type 2 diabetes might be useful as biomarkers in the development of skeletal muscle insulin resistance or as targets for future treatments. The opposite effect of insulin stimulation *in vitro* compared to glucose stimulation *in vivo* on *DAPK3* methylation may potentially be explained by changes in yet undescribed systemic factors. The identification of the factors overriding insulin signaling to control DAPK3 methylation would provide a new insight into DNA methylation regulation.

Further work in these areas of research could provide a deeper insight into the mechanisms behind metabolic diseases and open new avenues to improve care management and create novel treatments.

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