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Genetic and functional studies of
Hereditary Myopathy with Lactic Acidosis

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TO Oscar

I may not have gone where I intended to go, but I think I have ended up where I intended to be.

-Douglas Adams

Abstract

Hereditary myopathy with lactic acidosis (HML, OMIM#255125) is an autosomal recessive disorder which originates from Västerbotten and Ångermanland in the Northern part of Sweden. HML is characterized by severe exercise intolerance which manifests with tachycardia, dyspnea, muscle pain, cramps, elevated lactate and pyruvate levels, weakness and myoglobinuria. The symptoms arise from malfunction of the energy metabolism in skeletal muscles with defects in several important enzymes involved in the TCA cycle and the electron transport chain. All affected proteins contain iron-sulfur (Fe-S) clusters, which led to the suggestion that the disease was caused by malfunctions in either the transportation, assembly or processing of Fe-S clusters.

The aim of my thesis was to identify the disease causing gene of HML and to investigate the underlying disease-mechanisms. In **paper I** we identified a disease-critical region on chromosome 12; a region containing 16 genes. One of the genes coded for the Fe-S cluster assembly protein ISCU and an intronic base pair substitution (g.7044G>C) was identified in the last intron of this gene. The mutation gave rise to the insertion of intron sequence into the mRNA, leading to a protein containing 15 aberrant amino acids and a premature stop. In **paper II** we investigated why a mutation in an evolutionary well conserved protein with a very important cellular role, which in addition is expressed in almost all tissues, gives rise to a muscle-restricted phenotype. Semi-quantitative RT-PCR analysis showed that the mutant transcript constituted almost 80% of total ISCU mRNA in muscle, while in both heart and liver the normal splice form was dominant. We could also show that, in mice, complete absence of Iscu protein was coupled with early embryonic death, further emphasizing the importance of the protein in all tissues. These data strongly suggested that tissue-specific splicing was the main mechanism responsible for the muscle-specific phenotype of HML. In **paper III** the splicing mechanisms that give rise to the mutant ISCU transcript was further investigated. We identified three proteins; PTBP1, IGF2BP1 and RBM39, that could bind to the region containing the mutation and could affect the splicing pattern of ISCU in an *in vitro* system. PTBP1 repressed the inclusion of the intronic sequence, while IGF2BP1 and RBM39 repressed the total ISCU mRNA level though the effect was more pronounced for the normal transcript. Moreover, IGF2BP1 and RBM39 were also able to reverse the effect of PTBP1. IGF2BP1, though not a splicing factor, had higher affinity for the mutant sequence. This suggested that the mutation enables IGF2BP1 binding, thereby preventing the PTBP1 induced repression seen in the normal case.

In conclusion, we have determined the genetic cause of HML, identifying a base pair substitution in the last intron of the *ISCU* gene that gives rise to abnormally spliced transcript. The muscle-specific phenotype was also analyzed and tissue-specific splicing was identified as the main disease-mechanism. Furthermore, nuclear factors with ability to affect the splicing pattern of the mutant *ISCU* gene were identified. This work has thoroughly investigated the fundamental disease mechanisms, thus providing deeper understanding for this hereditary myopathy.

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Abbreviations

A	Adenine
Aa	Amino acid
AS	Alternative splicing
ATP	Adenosine triphosphate
bp	Base pair
C	Cytosine
CFTR	Cystic fibrosis transmembrane conductance regulator
cM	Centimorgan
CNS	Central nervous system
CPT II	Carnitine palmitoyltransferase II
DNA	Deoxyribonucleic acid
EMSA	Electrophoretic mobility shift assay
Fe-S	Iron-sulfur
FRDA	Friedreich ataxia
FXN	Frataxin
G	Guanine
GLRX5	Glutaredoxin 5
GOF	Gain-of-function
HML	Hereditary myopathy with lactic acidosis
IGF2BP1	Insulin-like growth factor 2-binding protein 1
ISCU	Iron-sulfur cluster assembly protein

LOD score	Logarithm of the odds score
LOF	Loss-of-function
Mb	Mega bases
MIBG	Meta-iodobenzylguanidine
mRNA	Messenger RNA
PCR	Polymerase chain reaction
PTBP1	Polypyrimidine tract binding protein 1
PYGM	Glycogen phosphorylase, muscle
qRT-PCR	Quantitative real-time reverse-transcription PCR
RBM39	RNA-binding motif 39
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription PCR
SDH	Succinate dehydrogenase
SFRS14	Splicing factor, arginine/serine-rich 14
SNPs	Single nucleotide polymorphisms
ss	Splice site
T	Thymin
TCA-cycle	Tricarboxylic acid cycle
wt	Wild-type (normal variant)
XLASA/A	X-linked sideroblastic anemia with cerebellar ataxia

Original Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

Paper I

Olsson A, Lind L, Thornell LE, Holmberg M. Myopathy with lactic acidosis is linked to chromosome 12q23.3-24.11 and caused by an intron mutation in the ISCU gene resulting in a splicing defect. *Hum Mol Genet* 2008;17:1666-72.

Paper II

Nordin A, Larsson E, Thornell LE, Holmberg M. Tissue-specific splicing of ISCU results in a skeletal muscle phenotype in myopathy with lactic acidosis, while complete loss of ISCU results in early embryonic death in mice. *Hum Genet* 2011;129:371-8.

Paper III

Nordin A, Larsson E, Holmberg M. The defective splicing caused by the ISCU intron mutation in patients with myopathy with lactic acidosis is repressed by PTBP1 but can be de-repressed by IGF2BP1. In press, *Human Mutation*.

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Introduction

The topic of this thesis is a rare monogenic disorder called hereditary myopathy with lactic acidosis (HML) originating from the counties of Västerbotten and Ångermanland in the Northern part of Sweden. The disease affects the mitochondrial metabolism of muscle cells, which results in severe exercise intolerance and the development of lactic acid even at a low workload. In this thesis the initial chromosome localization of the disease-causing gene, the identification of the mutation and functional studies which explain the muscle-specific phenotype of the disease are described.

Genetic Diseases

Genetic diseases are defined as diseases which are caused by different forms of abnormalities in the DNA. There are different types of genetic diseases, including monogenic, complex and chromosomal. Chromosomal disorders are caused by large chromosomal rearrangements such as duplications, deletion or translocations as well as numeric alterations, for example trisomi 21 which causes Down's Syndrome. Many of these defects arise during meiosis, the cell division process during which the haploid germ cells are formed. Complex diseases are caused by several susceptibility genes in combination with environmental factors and include many common diseases such as diabetes, heart disease and cancer. There are also diseases which are caused by mutations in the non-chromosomal DNA that is found in the mitochondria. These diseases are very rare and are inherited in a maternal fashion. I will however focus on the group of monogenic diseases.

Monogenic Diseases

Monogenic diseases are caused by mutation(s) in one single gene and are inherited in a Mendelian fashion. Since the diseases are caused by changes in only one gene there is a more obvious correlation between the gene and the disease phenotype compared to more complex disorders. This is a great advantage because it simplifies the analysis of the pathways leading to disease, including genetic mechanisms, interaction partners and cellular mechanisms. The existence of monogenic forms of common diseases, such as diabetes, Alzheimer's disease and different forms of cancer can therefore help to increase the understanding of the pathways involved in the complex forms thus benefitting the population at large¹⁻².

Monogenic diseases have simple modes of inheritance. They are recessive or dominant and are caused by changes in genes situated either on the non-sex (autosomal) chromosomes or the sex chromosomes (most often X-linked but in extremely rare cases Y-linked). For recessive diseases two identical copies of the disease-causing gene are needed in order to give a phenotype, while for dominant diseases only one copy is needed. Disorders coupled to the sex-chromosomes have a more complex inheritance because females carry two copies of the X-chromosome (XX) while men carry one (XY). The effect of a mutation located on the X-chromosome will therefore differ depending on the sex of the carrier, while Y-linked disorders without exception affect males. By studying pedigrees of an affected family, coupled with a thorough

family anamnesis, the inheritance pattern of a monogenic disease can be determined. This information is very important when trying to identify disease-causing mutations.

Studying Genetic Diseases

The identification of disease-causing genes by linkage analysis takes advantage of the recombination events which take place during meiosis I. Recombination is a process in which genetic material is exchanged between the maternal and the paternal chromosomes to ensure correct segregation and genetic diversification with each new offspring. The likelihood of recombination between two loci, for example a genetic marker (usually microsatellites or single nucleotide polymorphisms, SNPs) and a disease-causing gene, increases with the distance between them. Centimorgan (cM) is a unit which is used to define the relative distance between loci based on the rate of recombination, where 1% of recombination equals 1 cM. If a genetic marker and a disease locus are located close to each other they are more likely to be inherited together and are therefore said to be linked. In a linkage analysis you search for genetic markers with known chromosomal locations which are inherited together with the trait of interest. The logarithm of the odds (LOD) score is a statistical method used to calculate the likelihood of a genetic marker and a disease being linked to each other³.

When linkage to a certain chromosomal region has been found, haplotype analysis is used to limit the disease-critical region. For this purpose genetic markers closely spaced and covering the chromosomal region are analyzed and the alleles for each individual are genotyped. Haplotypes are then constructed and the haplotype inherited together with the disease is determined, thus further defining the disease-critical region. Once the chromosomal region harboring the disease-causing gene is determined, the genes located in the region can be identified using different genomic databases. The most likely candidate genes, based on function and expression pattern, are then subjected to mutational analysis in order to determine if there are any sequence variations which show the same inheritance pattern as the disease. The mutation analysis has traditionally focused on the coding parts of a gene, the exons, since changes in these sequences can cause direct changes in the protein. However, the non-coding, intronic, sequences may contain regions important for gene regulation and splicing and changes in these regions may therefore also be pathogenic. Once a variation common to all affected individuals is found, one must prove that it is the disease-causing mutation. The first step in this process is to

determine the frequency of the mutation in the general population. Since monogenic diseases are quite rare one would not expect to find a disease-causing mutation outside of the affected family. For recessive diseases heterozygous carriers can be found in the population, but at a low frequency. The frequencies of the most common sequence variations are available today in different databases, but a screen of the actual population is still needed in order to confirm association with the disease.

Functional Studies

DNA mutations can be of several types; substitutions, deletions, insertions, translocations or expansions. The severity of a mutation is dependent, not only on mutation type, but also on localization and the extent of the change. Changes in a coding region may induce amino acid (aa) substitutions, insertions or deletions, but can also lead to a premature stop or a change of the reading frame, thereby introducing severe changes into the protein. Coding mutations can also be silent, meaning that no aa change is induced. However, silent mutations may cause changes in regulatory domains, usually affecting splicing, hidden within the coding parts of the gene and can thereby cause disease⁴. Mutations found in non-coding parts can be situated in regions important for gene regulation and may affect either the transcription or the splicing of the gene. The result of the mutations can be either loss-of-function (LOF), where the gene product is either missing (in part or completely) or dysfunctional, or gain-of-function (GOF), where the protein has acquired new properties, for example changed expression pattern or function.

Functional studies are initially used in order to prove that the identified mutation is indeed the cause of disease in an affected family. Once this has been established functional studies can be used to increase the understanding of both basal and disease associated mechanisms of the gene and protein in question. The approach, which is used to study the effect of the mutation, is adapted to the type of mutation, inheritance pattern of the disease and function of the gene (if known). If the function of the gene is unknown, the phenotype of the disease may lead to new discoveries in that area. LOF mutations often lead to recessive diseases such as cystic fibrosis, which is caused by mutations in the gene coding for the chloride ion channel protein CFTR⁵. In contrast, GOF mutations often result in dominant disorders such as Huntington's disease, a neurodegenerative disorder where selective cell loss in the brain is caused by an expanded CAG-repeat in the gene coding for the protein huntingtin⁶. The reason for the recessive

inheritance of LOF mutations is that the level of expression from one single normal allele is often sufficient to uphold a normal phenotype. In some cases there is also an up-regulation of the normal allele to compensate for the decreased expression. The GOF mutations on the other hand often lead to acquisition of toxic properties which cannot be overcome by the functional allele, therefore resulting in a dominant phenotype.

Alternative Splicing

Genes are made up of exons spaced by the non-coding introns. After transcription, in which a single-stranded RNA molecule is formed, the introns of the gene are removed and the exons are fused in a process known as splicing. The splicing is performed by a spliceosome complex and is crucial for the formation of a mature messenger RNA (mRNA) molecule, which will later be used as a template to make proteins in a process called translation. With the sequencing of the human genome, the number of genes proved to be only 20,000-25,000⁷, which was much lower than the approximate 100,000 genes expected when the Human Genome Project was launched in the early 1990s⁸. One reason for this is that a phenomenon referred to as alternative splicing (AS), where two or more transcripts can be formed from the same gene (isoforms), is much more common than was earlier believed. Today it is estimated that approximately 94% of all human genes produces two or more isoforms of their mRNA⁹, thus providing a way of creating different forms of proteins without increasing the amount of genetic material. The higher rate of AS events in more complex organisms has led to the belief that AS is also an important evolutionary mechanism¹⁰.

Splicing and Disease

AS is an important part of the regulation of gene expression. The regulated inclusion or exclusion of specific parts of a gene (see Figure 1 for different forms of alternative splicing) can affect, among other things, mRNA levels, intracellular localization, protein stability, post translational modifications and protein function¹¹. AS is also involved in regulating the expression of stage specific or tissue-specific isoforms of proteins, such as for troponin t¹² and the α -tropomyosin protein¹³. Since the splicing machinery is such an important part of the protein regulation it is essential that it runs smoothly. To ensure this there are certain sequences which direct the spliceosome complex and help to define the exons and introns. The most important of these sequences are the 5' splice site (ss), the 3'ss, the polypyrimidine tract and the branch point¹⁴. There are also other elements with enhancing or suppressing roles located in introns and exons which further direct the splicing machinery to the correct sites and ensure that cryptic splice sites are not used¹⁵. However, in higher eukaryotic organisms these splice site sequences are not highly conserved, which leads to high variability but also higher risk of malfunctions¹⁶.

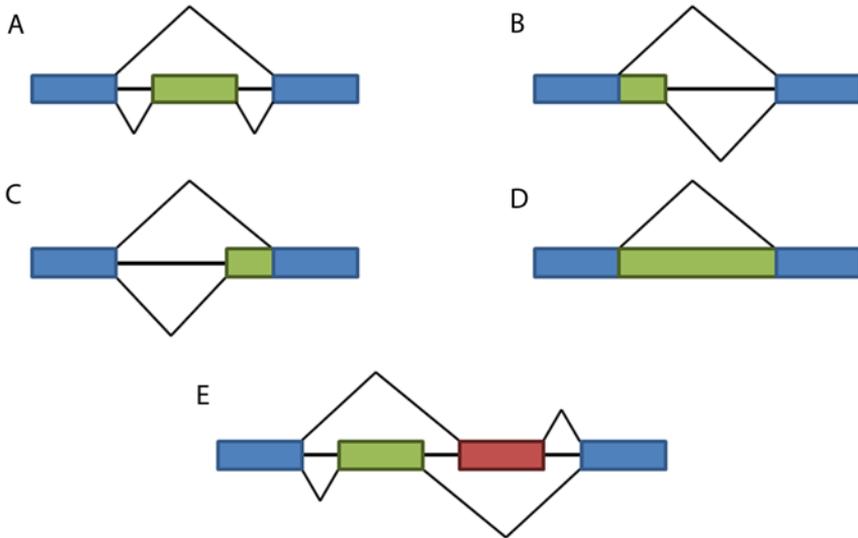


Figure 1: Basic modes of alternative splicing. **A** Cassette exon (exon inclusion/exclusion). **B** Alternative 5'ss. **C** Alternative 3'ss. **D** Intron retention. **E** Mutually exclusive exons.

AS has often been associated with human disease, either as a modifier of disease phenotype or as a direct cause. These diseases can arise from mutations in *cis*-acting elements, which is a common name for the splicing regulatory elements within a gene, or from mutations in splice site-like regions¹⁵. They can also be caused by mutations in *trans*-acting factors, which are the factors needed for normal function and regulation of the splicing machinery¹⁵. This means that if a mutation has emerged in a *cis*-acting element, only the splicing of the gene harboring the mutation is affected. Conversely, mutations in a *trans*-acting factor can affect the splicing in general. The aberrant AS causing disease follows the same basic modes as normal AS (Figure 1), the difference being that it will unerringly lead to misregulation resulting in for example a change in expression pattern, cellular localization or function.

Myopathy – Disease of the Muscles

“Neuromuscular disorders” is a collective name for a very heterogeneous group of diseases which affect the muscles, the innervating neurons or the neuromuscular junctions. These types of diseases often show symptoms such as muscle weakness, cramps, stiffness and wasting. However, depending on the primary site of disease the severity can vary significantly. If the primary site of disease lies within the muscles the disorder is called a myopathy. Myopathies can be caused by infections or toxins, but can also have autoimmune characteristics or be of hereditary nature¹⁷⁻¹⁹. Hereditary myopathies are often subdivided into four groups depending on symptoms and cause; dystrophies, ion channel diseases, congenital muscle diseases and metabolic muscle diseases¹⁹. In this thesis the focus will be on the group of metabolic myopathies.

Metabolic Myopathies

Metabolic myopathies are caused by defects in the various metabolic pathways of the cells, mostly by defects in the energy producing glycogen, lipid or mitochondrial metabolisms. The common characteristics of these diseases are that they all result in exercise intolerance and/or progressive weakness^{17, 20}. Exercise intolerance in itself may not sound as a very serious symptom, but it can in fact be very disabling and have both medical and social implications. Patients with exercise intolerance often present with muscle cramps and episodes of rhabdomyolysis, which is an acute breakdown of muscle fibers, often resulting in brownish pigmented urine called myoglobinuria²⁰. Presence of myoglobin in the urine can appear as a consequence of any type of muscle damage. The danger with this condition is that the myoglobin can occlude the filtration system of the kidneys thereby causing severe damage which in the worst case scenario can be lethal²¹.

Muscles utilize different energy sources depending on the state of the muscle; for example, if it is resting or if it performs work of different types. This means that the symptoms of a metabolic myopathy will vary depending on which metabolic pathway is affected. A brief overview of the different energy-producing processes in muscle cells is presented in Figure 2.

Disorders of the glycogen metabolism are caused by defects which either directly or indirectly affect the anaerobic glycolysis. Usually patients suffering from myopathic forms of defects in the glycogen metabolism

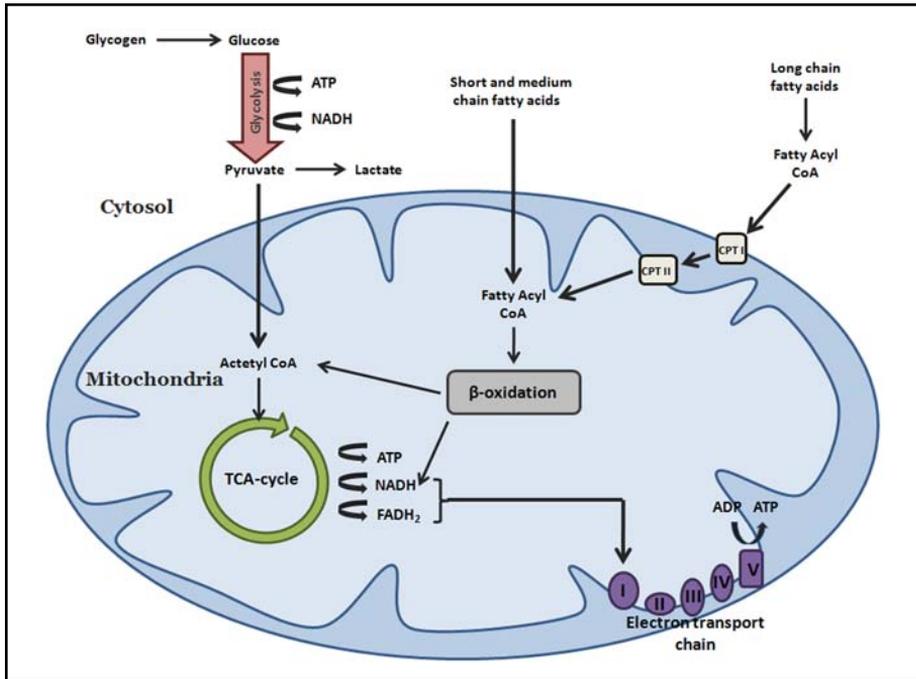


Figure 2: Energy metabolism in muscle.

experience muscle cramps and extreme fatigue during exercise, especially during the first minutes of exercise and during exercise of high-intensity²². This is due to increased rate of anaerobic metabolism during the initial stage of exercise and during high-intensity training. However, after the initial stage the blood supply to the muscles increases and there is a switch to aerobic metabolism which enables the muscles to rely more on the TCA-cycle and electron transport chain for energy. This can also be observed in the patients, especially those suffering from McArdle's disease, where after initial exercise muscle cramps and pain arise, but after a few minutes rest the patient can keep going without these problems (second wind phenomenon)²³.

Disorders of the lipid metabolism are caused by defects in the β -oxidation or in enzymes involved in the transport system of fatty acids into the mitochondria, for example CPT II²⁴. Patients suffering from myopathic forms of lipid disorders can be more or less asymptomatic unless put under metabolic stress, such as infections, fasting or extensive exercise²⁵. The

patients can usually perform short-term exercise with high-intensity, while endurance training causes cramps, muscle pain and rhabdomyolysis²².

Disorders affecting the mitochondrial metabolism arise due to defects mainly in the electron transport chain, which can be caused by both mitochondrial and nuclear DNA mutations. Many of the mitochondrial myopathies are in fact systemic and affect several organs, especially those with high energy demand, but there are also pure myopathic forms²⁴. Symptoms vary depending on the organs involved, but exercise intolerance and fatigue are common. This thesis deals with a form of mitochondrial myopathy originating from Northern Sweden; hereditary myopathy with lactic acidosis.

Hereditary Myopathy with Lactic Acidosis

Hereditary myopathy with lactic acidosis (HML, OMIM#255125) is an autosomal recessive disorder which originates from the coastal areas of the counties of Västerbotten and Ångermanland in the Northern part of Sweden (Figure 3)²⁶. A total of 30 patients from 18 families have been identified thus far (see Table 1), out of whom one patient is of Norwegian nationality. Nine of the families have been shown to be connected through genealogical analysis, suggesting a common ancestor²⁶.

HML was first described in the early 1960s when Larsson and co-workers presented the finding of a new form of myopathy. The study was based on 14 patients from five families who suffered from severe exercise intolerance with myoglobinuria²⁷. Exercise intolerance with myoglobinuria is, as described in the previous section, a fairly common symptom for a myopathy and often presents in cases with metabolic myopathies. However, the HML patients also presented elevated levels of both lactate and pyruvate in the blood during exercise which suggested an abnormally high rate of glycolysis²⁷, indicating defects in the mitochondrial metabolism.



Figure 3: Map of Sweden, black dot indicates location of Stockholm, dark grey indicates the counties where HML has its origin.

Table 1: Families suffering from HML

Family	Total no of sibs	Affected sibs	Reference
A	8	4	27-30 (Family patient P3 in ref 30)
B	7	2	27, 29
C	3	3	27, 29, 31 (Family D in ref 29)
D	8	4	27, 29, 32 (Family C in ref 29 and F in ref 32)
E	7	1	27, 29
F	2	1	29, 33
G	6	2	29, 31, 33-35
H	2	1	29-30, 36-37 (Family patient P1 in ref 30)
I	2	1	32
J	5	1	Family I in ref 29
K	2	1	Family patient P2 in ref 30
L	2	1	Family A in ref 32
M	2	1	Family B in ref 32
N	2	1	Family C in ref 32
O	1	1	Family D in ref 32
P	3	2	Family E in ref 32
Q	1	1	38
R	3	2	unpublished

Symptoms and Clinical Findings

HML debuts in early childhood, where complaints of shortness of breath and muscle aches are common³¹. The disease is chronic with recurrent acute episodes²⁷⁻²⁸, and the HML patients have been thoroughly examined in both stages^{27-28, 33-36, 39}. In the chronic stage the patients experience fatigue, shortness of breath and palpitations even during exercise with low work load²⁷. However, the extent of exercise tolerated by the patients seems to differ, both between different muscle groups within a patient²⁷ and between different patients. In a patient survey performed during the fall of 2010, four patients described being heavily challenged when trying to walk only a few hundred meters, while the remaining patients (n=9) experienced it as only a

mild challenge or no challenge at all (Nordin et al., unpublished data). If the work continues for an extended period of time the muscles become hard and tender and cause pain²⁷. Acute episodes of the disease, which can be provoked by prolonged strenuous exercise or fasting, are characterized by severe muscle pain, resting dyspnea, tachycardia, pareses in proximal muscle groups, myoglobinuria due to breakdown of muscle fibers, nausea and vomiting^{27, 31}. The acute stage of the disease can be life-threatening due to pareses in breathing muscles, severe acidosis and renal failure. Indeed, there have also been a few cases where patients have died from an acute episode^{26-27, 34}.

Patients have been subjected to exercise tests in order to discern the properties of the exercise intolerance. It was observed that the patients had hyperkinetic circulation during exercise and an abnormally high blood flow compared to oxygen uptake. It was also noted that during exercise the lactate and pyruvate concentrations in the blood rose more than what was expected from the work load. This suggested an abnormal usage of glycolysis in muscle consistent with defects in the mitochondrial metabolism²⁷⁻²⁸.

Histological and Molecular Findings

Histological analysis has shown that, at rest, the patients' muscles contained a large amount of lipid droplets and glycogen, while after exercise the muscles were depleted of glycogen but still contained lipid droplets³⁴. Analysis also showed reduced activity of succinate dehydrogenase (SDH) in the muscle fibers³⁴. SDH consists of four subunits, A-D, and is found in the inner membrane of the mitochondria. It is part of both the tricarboxylic acid (TCA) cycle and the electron transport chain (complex II) and lowered levels of this protein complex would have a negative impact on the energy production of the mitochondria. There are hereditary forms of pure SDH deficiency, even though they are not very common. However, the result of these forms of deficiency is often early onset encephalomyopathy (*SDHA* mutations)⁴⁰⁻⁴² or susceptibility to paraganglioma and pheochromocytoma (*SDHB-D* mutations)⁴³⁻⁴⁴, which is quite far from what is seen in HML.

Biochemical analysis confirmed deficiency of complex II in the HML patients, and more specifically of subunit B, the subunit containing iron-sulfur (Fe-S) clusters³⁶. The patients were furthermore found to have reduced levels of mitochondrial aconitase³⁶. The mitochondrial aconitase is part of the TCA-cycle, where it is involved in the conversion of citrate to isocitrate. Aconitase is, like SDHB, a Fe-S containing protein which, together

with the finding that the mitochondria contained electron-dense iron-rich inclusions, suggested a possible malfunction of the iron metabolism^{36, 39}.

It was later determined that complexes I and III of the electron transport chain also had defects³⁷. Closer examination showed that all proteins affected in HML contained Fe-S centers. This led to the suggestion that these patients suffered from dysfunction in synthesis, import, processing or assembly of Fe-S clusters³⁷.

Diagnosis and Treatment

Traditional methods for determining a correct diagnosis have included a full anamnesis, physical inspection, muscle biopsies and test of work capacity on a bicycle ergometer together with analysis of blood lactate and pyruvate levels³¹. The risk of exposing the patients to too much strain when measuring work capacity this way should however advise against using this method for diagnosis. Muscle biopsies have been an important tool for making the correct diagnosis. Histological analysis of pathological changes in muscle structure, such as iron deposits in mitochondria, together with determination of SDH and aconitase activity would be important signs indicating HML. However, it has been hard to establish a correct diagnosis in the past and several patients have not received a correct diagnosis until adult age. This is both due to many differential diagnoses and because it is a very rare disease which few practicing physicians have come into contact with.

There is a lack of good treatment for patients with HML. Some patients are or have been treated with vitamins, cofactors or other nutritional supplements, such as coenzyme Q₁₀, carnitine, nicotinamide, riboflavin and arginine hydrochloride. It is quite common to treat mitochondrial myopathies with supplements such as those described above. The thought behind this is that it might help to “boost” the remaining activity for example in the electron transport chain, thus providing the patients with a little more ATP production. This type of treatment is however subjected to some debate, since clinical benefits have been reported in just a few individuals suffering from mitochondrial disease⁴⁵. The effect observed in the HML patients has accordingly been found to be only moderate (personal communication). The best way to control HML is still to avoid physical exertion and to eat regularly, preferentially a diet rich in carbohydrates since glycolysis is the only energy producing pathway intact in the patients’ muscles³¹.

Fe-S Clusters

Fe-S clusters are inorganic compounds essential for the function of numerous proteins. The Fe-S proteins can be found in all life forms, from bacteria to higher eukaryotes. Most proteins involved in the Fe-S assembly process are evolutionary well conserved, which suggests that the system is very sensitive to changes. Many of the proteins involved in the Fe-S biogenesis have been selectively deleted in different animal models, such as yeast, *C. elegans* (roundworm), zebrafish and mouse⁴⁶⁻⁴⁹, several with lethal outcomes. This confirms the importance of a well functioning Fe-S assembly machinery.

The Fe-S cluster assembly process involves several steps which depend on proteins that function as sulfur donors, iron donors, scaffold proteins, chaperones and transporters. The complex machinery is not completely understood, but studies on model organisms, such as yeast, have provided us with much of the knowledge available today. In addition various forms of human disease caused by problems in the Fe-S system have emerged over the years and have contributed to progress in the field.

Fe-S Cluster Assembly and Disease

In mammalian cells the Fe-S proteins are important for various processes such as the electron transport chain, the TCA cycle, iron metabolism, DNA repair and protein translation initiation (subject reviewed in Sheftel et al., 2010)⁵⁰. In humans there are diseases which are known to be caused by problems in the Fe-S system, the most common being Friedreich ataxia (FRDA).

FRDA is an autosomal recessive neurodegenerative disorder, often associated with cardiomyopathy. In most cases the disease is caused by an intronic GAA-triplet repeat expansion in the gene *FXN*, coding for the protein frataxin⁵¹. The role of frataxin in Fe-S assembly has been the subject of much debate, but the fact that frataxin can bind iron and that it associates with the scaffold protein has led to speculation that it might function as an iron-donor⁵². In both FRDA and HML one can find iron-rich inclusions in mitochondria and deficiency of the Fe-S containing complexes I, II and III of the electron transport chain and aconitase. However, in FRDA patients these changes are not found in muscle tissue, but in the brain and heart⁵³⁻⁵⁴. The

distinct similarities between HML and FRDA can be seen as further evidence regarding the importance of the Fe-S system in the pathogenesis of HML.

Other defects in the Fe-S system have been shown to lead to multiple mitochondrial dysfunction syndromes caused by mutations in the alternative scaffold protein NFU1 and the proposed chaperone BOLA3⁵⁵⁻⁵⁶. Various forms of anemia and iron overload have also been found, such as deficiency of GLRX⁵⁷, which have a proposed role downstream of the scaffold protein, and XLASA/A which is caused by mutations in the ABCB7 transporter protein located in the mitochondrial inner membrane⁵⁸.

Aim of this thesis

The overall aim of this thesis was to identify the cause of hereditary myopathy with lactic acidosis (HML) and to investigate the underlying disease mechanisms. The specific goals were:

- ∅ To determine the chromosomal localization of the disease-causing gene and to find and verify the disease-causing mutation (paper I)

- ∅ To determine why HML results in a muscle-specific phenotype (paper II)

- ∅ To investigate the complete absence of the *Iscu* gene in a mouse model (paper II)

- ∅ To identify factors that affect the splicing pattern of the *ISCU* gene (paper III)

Results and Discussion

Genetic Mapping and Mutation Identification

Patients with HML have been thoroughly investigated using both clinical and molecular approaches, as described previously in this thesis. The genetic background was however unknown for a long time, although it was initially stated that the mode inheritance appeared to be autosomal recessive²⁷. Based on the assumption of an autosomal recessive inheritance pattern a genome-wide screen was performed on 50 individuals, including 15 patients, from 9 families with HML (Figure 4) in order to identify regions of shared homozygosity among the affected. Using this approach we could map the disease-causing gene to a region on the long arm of chromosome 12 (12q23.3-24.11). Significant LOD-scores were obtained for a number of markers in the region and a disease-critical region of 1.6Mb was established (**paper I**, Table 1). The disease-critical region was found to contain 16 genes (**paper I**, Figure 2A), including the gene coding for the Fe-S cluster

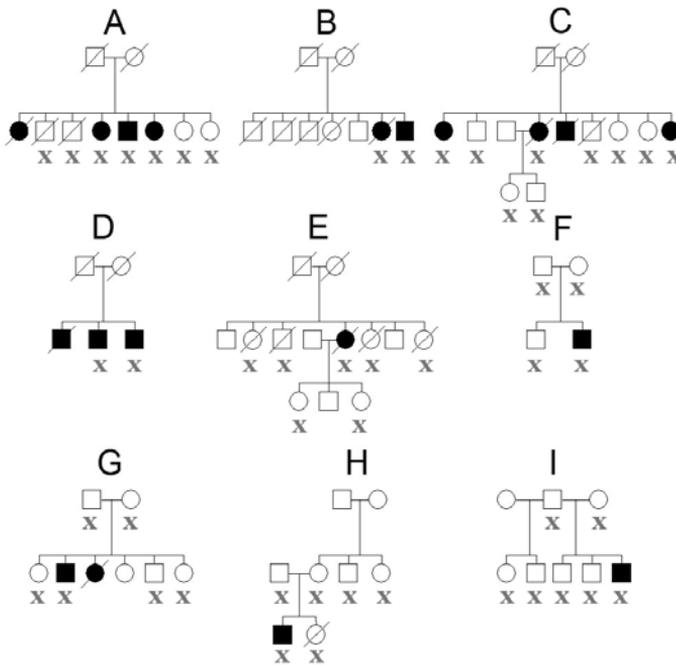


Figure 4: Pedigrees of the nine families included in the mapping study.

assembly protein ISCU. ISCU is an evolutionary well conserved protein with an important role in the assembly of Fe-S clusters. It works as a scaffold on which Fe-S clusters are formed before they can be delivered to the target proteins, for example in the TCA-cycle and the respiratory chain⁵⁹⁻⁶⁰. Considering the defects of Fe-S cluster-containing proteins found in the HML patients^{34, 36-37}, this protein was considered an excellent candidate gene. All exons, exon-intron junctions and the promoter region were analyzed by sequencing, but no disease-specific changes could be found. However, a disease-specific single bp substitution was identified, G→C, 382 bp into the last intron (g.7044G>C, **paper I**, Figure 2B). Analysis of population controls showed that only one out of 177 individuals was heterozygous for this mutation.

Functional Analysis of the ISCU Mutation

Deep intronic mutations do not affect the functionality of the protein in an obvious fashion and the way in which it causes disease is therefore harder to prove. Many intronic regions have been shown to have regulatory roles in processes such as transcription and splicing. Analysis of the intronic region harboring the HML mutation using DNA-EMSA revealed that nuclear factors were able to bind to both the normal and the mutant sequences. However, the binding pattern differed between the two (**paper I**, Figure 3A and B). This suggested that the intronic mutation could in fact have functional implications. The possible role in transcriptional regulation was analyzed using a luciferase gene expression system where the ISCU intron sequence, with or without mutation, was inserted in front of the promoter. The region was found to have enhancer activity, but the activity was the same for both sequences (**paper I**, Figure 4), suggesting that the mutation does not interfere with transcriptional regulation. We then analyzed whether the mutation may have an effect on the splicing of the gene. For this, RNA from muscle biopsies from two patients and one control were analyzed using RT-PCR. Because ISCU has been shown to have two alternatively spliced transcripts - a cytosolic form and a mitochondrial form⁶¹ - the primers were designed to amplify and separate all possible transcripts. A distinct difference was observed between the transcripts seen in patient and control samples. In the control sample the mitochondrial form of ISCU was found to be dominant, while the patient transcript was 100 bp larger, consistent with the size of the cytosolic form (**paper I**, Figure 5A). However, sequencing of the RT-PCR products revealed that the transcript found in patient muscle was of the mitochondrial form, but with 100 bp of intron sequence inserted

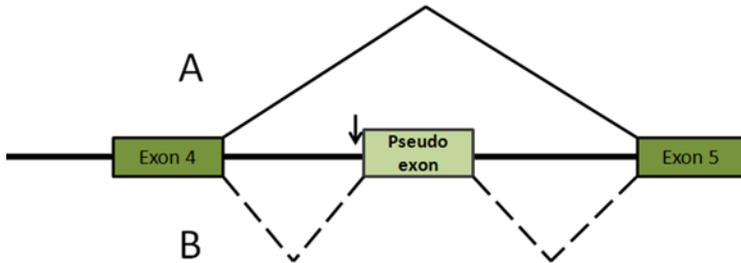


Figure 5: Schematic drawing of the splicing of 3'-end of the *ISCU* gene. **A** denotes the normal splicing where exon 4 and 5 are fused. **B** denotes the abnormal splicing where the pseudoexon is inserted into the mRNA as a consequence of the intronic mutation (arrow).

into the mRNA between exon 4 and 5 (Figure 5 and **paper I**, Figure 5B). The region was found to contain cryptic splice sites, which becomes activated as a consequence of the mutation. The inserted sequence, or pseudoexon, was found to cause a change of the highly conserved c-terminal of the protein, giving rise to 15 aberrant aa and a premature stop, most likely giving rise to a dysfunctional protein. A simultaneously published article, which reported the same results using three patients, also showed that there was a decrease in the total *ISCU* mRNA level and that the *ISCU* protein was almost completely absent from patient muscle³⁰, indicative of nonsense-mediated decay. A more recent publication reported that the intron insertion can actually be either 86 bp or 100 bp³². The two different pseudoexons share the same 3' acceptor splice site, but have different 5' donor splice sites. The protein resulting from the shorter transcript will however contain the same aberrant aa:s and premature stop as the one containing the 100 bp insertion.

Tissue-Specific Phenotype

The *ISCU* protein is expressed in various tissues in the human body, with the highest levels in skeletal muscle and the heart⁶¹. However, the HML patients only show skeletal muscle involvement, with a drastic decrease of the *ISCU* protein in muscle³⁰. Given the drastic reduction of proteins involved in the energy metabolism as a consequence of the HML mutation observed in patient muscle, one would expect other energy-demanding tissues, such as the heart and central nervous system (CNS), to also be affected. Energy-demanding tissues are generally sensitive to defects in the Fe-S assembly system. This is shown by the involvement of the CNS and the heart in

FRDA⁵³⁻⁵⁴, caused by defects in the suggested iron-donor frataxin, and by the involvement of CNS in multiple mitochondrial dysfunction syndromes caused by defects in the alternative scaffold protein NFU1 or the proposed chaperone BOLA3⁵⁵⁻⁵⁶. This further emphasizes the question of why there is no obvious involvement of tissues other than skeletal muscle in HML. To investigate whether there are differences in ISCU protein content between different patient tissues, we analyzed protein samples from muscle, kidney, heart and liver from one patient. As reported previously, ISCU was almost completely absent from patient muscle, but was present in the other tissues examined (**paper II**, Figure 2A). Something which is noteworthy is the fact that the lowest levels of ISCU were observed in patient muscle and heart. These are the two tissues which have been reported to have the highest levels⁶¹, which suggested that the level of ISCU is also reduced in patient heart. A comparison of patient and control samples from muscle and the heart confirmed a drastic decrease of ISCU in both tissues (**paper II**, Figure 2B). However, the reduction of ISCU in the heart did not affect aconitase or SDH (**paper II**, Figure 4), suggesting that even though there is a drastic reduction of the level of ISCU, there is still enough protein to sustain the Fe-S assembly needed for these processes. To rule out other dysfunctions, such as problems in the iron metabolism, patient muscle, heart, pons and liver were stained with Perls' iron staining. Patient muscle had an accumulation of iron in the cells, but the other patient tissues did not differ from the healthy control (**paper II**, Figure 3), giving further evidence regarding the tissue-specificity of HML.

There are two hypotheses which could explain this tissue-specific phenotype:

- 1) There are other proteins with roles overlapping that of ISCU in non-skeletal muscle tissue. These proteins either have the main responsibility for the Fe-S cluster assembly in non-muscle tissue, or work as a back-up system, stepping in when the ISCU levels go down.
- 2) There are tissue-specific differences in the splicing of the mutant ISCU transcript which results in more defect protein in muscle than in other tissues.

However, the fact that patients who are compound heterozygous for the HML mutation and a missense mutation in exon 3 (c.149G>A) suffer from cardiomyopathy as well as severe exercise intolerance³² argues against hypothesis 1. The condition of the compound heterozygous patients also seems to be slowly progressive, something which is not seen in HML patients. Another difference is that the ISCU protein is present at almost normal levels in muscle tissue from these patients³². However, the missense

mutation disrupts a completely conserved residue, suggesting that the protein formed in the compound heterozygous patients is inactive. These data clearly show that the ISCU protein is important for the Fe-S assembly in other energy demanding tissues, such as the heart, thus favoring hypothesis 2.

Knock-Down of ISCU

To confirm the essential role of ISCU we wanted to analyze the effect of complete absence of the ISCU protein in mice. If the muscle-specific phenotype of HML patients is caused by tissue-specific splicing of mutant ISCU transcript, and no other protein can rescue ISCU malfunction in other tissues, the complete absence of the protein would lead to a more severe and universal phenotype. *ISCU* homologues have been knocked-out in other species, such as *Trypanosoma brucei*, *Saccharomyces cerevisiae* and various bacterial strains, with results varying from decreased viability to complete lethality^{46, 62-64}. However, ISCU had never before been depleted in a higher eukaryotic organism. *FXN*, the gene coding for frataxin, which has a proposed role as the iron-donor in the Fe-S assembly, has previously been inactivated in mice, with early embryonic death as a result⁴⁹. This is an indication that the Fe-S system is also conserved in higher organisms. However, one cannot rule out the possibility that back-up systems have developed in higher organisms for certain parts of the Fe-S machinery.

In **paper II** we used ES-cell lines obtained from the knock-out mouse project (KOMP) repository to generate *Iscu* knock-out mice. The heterozygous mice (*Iscu*^{+/-}) were identical to their wt littermates both in physical and behavioral phenotype. These phenotypes were also evaluated in a pilot experiment exposing the mice to a high fat diet, but no apparent difference was observed between the wt and *Iscu*^{+/-} mice (Nordin et al., unpublished observations). Moreover, no difference was observed in the levels of SDHB and aconitase between wt and *Iscu*^{+/-} mice, indicating an efficiently functioning Fe-S assembly (**paper II**, Figure 5B). However, cross-breeding of *Iscu*^{+/-} mice did not result in any homozygous *Iscu*^{-/-} offspring. Furthermore no *Iscu*^{-/-} embryos were detected at E7.5-10.5, but at pre-implantation stage E3.5 (blastocyst stage) *Iscu*^{-/-} embryos were identified (**paper II**, Figure 5A). This suggested that *Iscu* is vital for the implantation and/or early embryonic development in mice and is further support of the hypothesis that tissue-specific splicing of the mutant ISCU transcript is responsible for the muscle-specific phenotype of HML.

Tissue-Specific Splicing of ISCU

Several pieces of evidence pointed towards tissue specific splicing being the cause of the muscle specific phenotype seen in HML. To determine whether this was indeed the case, the proportions of normal and mutant transcripts were analyzed in muscle, heart and liver samples from patients and controls using semi-qRT-PCR (Figure 6 and **paper II**, Figure 1B). The mutant transcripts in patient muscle were found to constitute 80% of the total ISCU mRNA, which was in sharp contrast to the liver and heart where the normal splice variant dominated. In liver the incorrectly spliced form constituted nearly 50% of total ISCU mRNA, whilst in heart the corresponding number was only 30%. In patient heart a large reduction of ISCU was observed on the protein level (**paper II**, Figure 2B) and we therefore expected quite a large portion of total ISCU mRNA to be incorrectly spliced. The fact that less than a third of all ISCU transcripts in heart were incorrectly spliced was thus quite surprising. However, analysis of the mRNA levels of ISCU in heart using qRT-PCR, showed that there was a reduction in the patient sample compared to controls. This, together with the aberrant splicing, could explain the dramatic loss of ISCU protein in patient heart. The reason for the reduction of ISCU mRNA observed in patient heart remains unknown. Low amounts of incorrectly spliced ISCU could also be found in all control tissues, with the levels ranging from 7% in muscle to 3% in liver. The fact that we also see differences between the control tissues, with constantly higher levels of mutant transcripts in muscle, supports the theory that tissue-specific differences may affect the splicing of the pseudoexon. Work by Sanaker et al. supported the finding that the major part of ISCU is incorrectly spliced in patient muscle³⁸. Furthermore, they observed a more

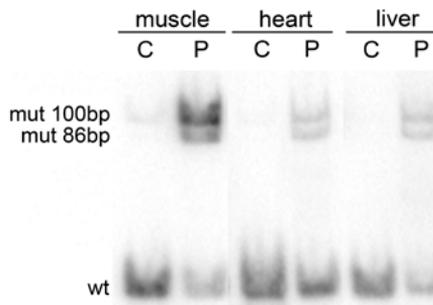


Figure 6: Semi-qRT-PCR of muscle, heart and liver samples from patients and controls. Gel run as to separate the wt and mutant splice variants as well as the two mutant splice forms.

equal distribution of the wt and mutant transcripts in myoblasts, fibroblasts and blood from a patient³⁸. Together these data strongly suggest that tissue-specific splicing is what causes the muscle-specific phenotype found in HML patients.

RNA-Binding Proteins Interacting with the ISCU Intron Region

Tissue-specific splicing was found to be the main mechanism leading to the muscle-only phenotype in HML patients. However, the factors which regulate the inclusion/exclusion of the pseudoexon and how they are affected by the HML mutation had not previously been investigated. In **paper III** we wanted to investigate these splicing events further and find out if there is any difference between the factors that bind to the intronic region in the absence or in the presence of the HML mutation and how this affects the splicing pattern of ISCU. The region where the HML mutation is situated strongly resembles a polypyrimidine tract, which is an important splicing element. The mutation strengthens this element, but the question is which factors are involved in the process.

As a first step we analyzed whether there was any difference in the binding of nuclear factors to the sequence containing the mutation when compared to the normal intron sequence. Using RNA-EMSA, interaction of nuclear proteins with the intronic sequence could be confirmed. The binding pattern observed with the wt and mutant probes appeared more or less the same, but the interaction was much stronger with the mutant sequence (**paper III**, Figure 1A). This suggested that there are one or more nuclear proteins with increased affinity for the intron region as a result of the mutation. The mutant and wt RNA-probes were then used for purification of RNA-protein complexes, which were subsequently separated using SDS-PAGE (**paper III**, Figure 1B). Protein bands were then cut out and analyzed using mass-spectrometry. Using this approach five nuclear proteins with affinity to the ISCU intron sequence were identified; Matrin 3, SFRS14, IGF2BP1, RBM39 and PTBP1, including one (IGF2BP1) with higher affinity for the mutant sequence (**paper III**, Figure 1B and C and supp. Table S1). The proteins all had mRNA binding capacity, and three were known splicing factors (SFRS14, RBM39 and PTBP1). The ability to affect the splicing pattern in a cell-based system was then investigated using an ISCU minigene with either normal or mutant sequence (**paper III**, supp. Figure S1A and B).

Regulation of Splicing in HML

Three factors were found to affect the splicing of the ISCU minigene; PTBP1, RBM39 and IGF2BP1 (Figure 7 and **paper III**, Figure 2A, B and C). PTBP1, which is a known regulator of alternative splicing and often occupies a splicing inhibiting role⁶⁵⁻⁶⁷, was found to have a repressive effect on the inclusion of the psuedoexon. In contrast, the addition of RBM39 or IGF2BP1 resulted in lower levels of total ISCU transcripts, but the repression of the normal transcript was more pronounced. The decrease of the total level of ISCU transcripts is somewhat hard to explain. However, as mentioned above, there have been reports showing reduced levels of ISCU mRNA^{30, 38} in patient muscle. This suggested that what we see in the *in vitro* system might reflect a mechanism present in the HML patients. RBM39 and IGF2BP1 were also found to be able to de-repress the PTBP1 induced repression of the incorrect splicing (**paper III**, Figure 2D and E). RBM39, as mentioned above, is a protein which is known to be involved in splicing. It belongs to a family of splicing factors called U2AF-like proteins and several of these splice factors interact with polypyrimidin tracts, thus facilitating the splicing process⁶⁸. However, as there was no difference in the affinity for the intronic region of *ISCU* with or without the HML mutation, we believe that it is not involved in the specific regulation of the splicing of the mutant *ISCU* gene, but might have a more general role in the process. Conversely, IGF2BP1, was found to have a stronger affinity to the mutation-containing sequence. IGF2BP1 has not been previously implicated in splicing, but is associated with the regulation of IGF-2 translation⁶⁹. However, IGF2BP1 has been shown to be able to compete with PTBP1 for binding site, often in a manner dependent on Mg²⁺ concentration⁶⁹. We suggested that normally PTBP1 binds to the polypyrimidine tract upstream of the ISCU pseudoexon, thus preventing inclusion into the mRNA. The HML mutation favors IGF2BP1 binding over PTBP1 binding, thus leading to increased inclusion of the psuedoexon, but lower total levels of ISCU mRNA.

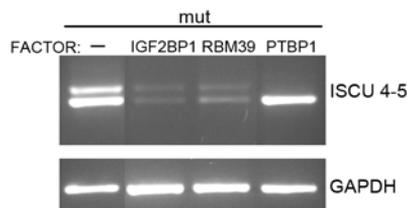


Figure 7: Splicing pattern of the mutant minigene without (lane 1) or with addition of nuclear factors (lane 2-4). Upper band corresponds to the mutant transcripts, whereas the lower band belongs to the wt splice form.

Splicing and Hypoxia/ATP Depletion, Unpublished Data

ISCU expression has been shown to be negatively regulated by microRNA-210 during hypoxic conditions with subsequent repression of mitochondrial respiration⁷⁰. Because of the malfunctions in the electron transport chain and the TCA-cycle in the muscles of HML patients, a state of “pseudo hypoxia” could develop, at least during exercise. ATP-depletion is also a problem in patient muscle and could well have an impact on the splicing pattern. To investigate whether hypoxia or ATP depletion have an effect on the splicing pattern of ISCU, RD4 cells (generous gift from Dr Tracey Rouault) were transfected with either the normal or mutant ISCU minigene as described in **paper III** (**paper III**, supp. methods and supp. Figure S1A and B), and then subjected to CoCl₂-induced hypoxia (500μM CoCl₂) or MIBG-induced ATP-depletion (20-200μM MIBG) for 21-48h. However, neither state was able to provoke any significant changes to the splicing pattern (Figure 8).

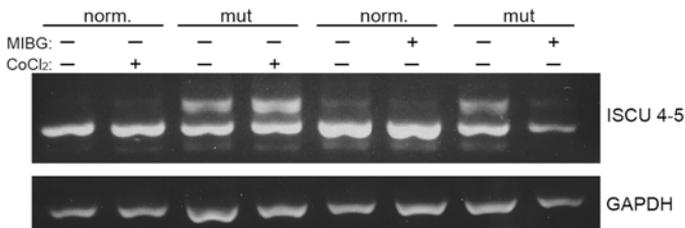


Figure 8: Effects of hypoxia and ATP depletion on the splicing of the ISCU pseudoexon. RT-PCR performed on RD4 cells transfected with either the normal or mutant minigene with or without the addition of CoCl₂ (induced hypoxia) or the ATP inhibitor MIBG.

ATP also has the ability to affect the activity of certain parts of the splicing machinery, including PTB^{66, 71}. We therefore analyzed whether the PTBP1-induced splicing pattern changed when the cells were deprived of ATP. Cells were transfected co-transfected with the ISCU minigene and the different factors (**paper III**, supp. methods) and were then exposed to MIBG-induced ATP-depletion. However, no difference could be seen between the ATP-depleted samples and the samples with normal levels, neither in the presence of PTBP1 nor any other factor studied (Figure 9A and B).

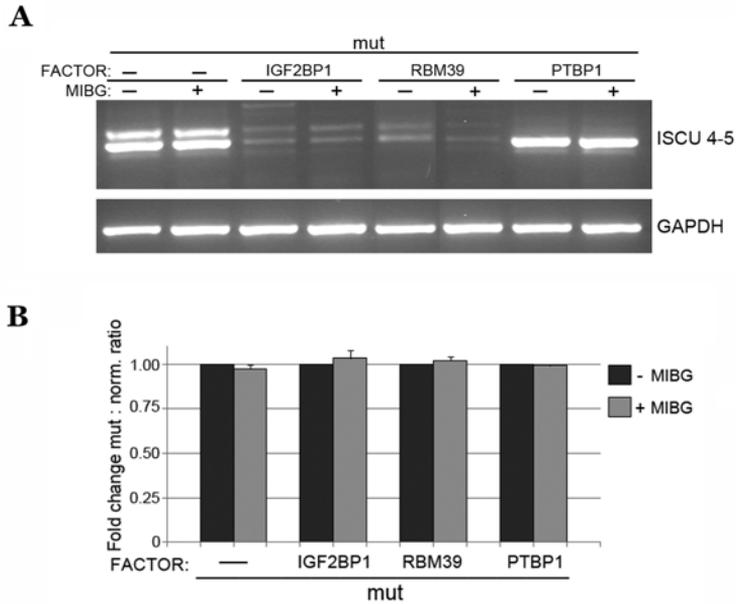


Figure 9: Effects of ATP-depletion on the splicing pattern induced by IGF2BP1, RBM39 and PTBP1. A) RT-PCR analysis of RD4 cells co-transfected with the ISCU mutant minigene and expression vectors for IGF2BP1, RBM39 or PTBP1 with or without the addition of the ATP inhibitor MIBG. B) Semi-qRT-PCR. The graph presents the mean fold change of the mutant:normal transcript ratio \pm SD from at least three independent experiments with the minigene alone or in the presence of IGF2BP1, RBM39 or PTBP1 with or without the addition of the ATP inhibitor MIBG.

Conclusions

- ∅ HML is caused by a single bp substitution in the last intron of the gene coding for the iron-sulfur cluster scaffold protein, ISCU, localized on chromosome 12.

- ∅ The intronic mutation (g.7044G>C) leads to aberrant splicing of the *ISCU* gene, inserting intronic sequence into the mRNA.

- ∅ Complete absence of the Iscu protein in mice results in early embryonic death, while mice heterozygous for the Iscu deletion are phenotypically normal.

- ∅ The muscle-specific phenotype observed in HML patients is caused by tissue-specific splicing, generating more incorrectly spliced ISCU in muscle compared to other tissues.

- ∅ The aberrant splicing can be repressed by PTBP1 and de-repressed by IGF2BP1 or RBM39 in a cell based system.

- ∅ The absolute levels of ISCU transcripts seem to be decreased in the presence of IGF2BP1 or RBM39.

Populärvetenskaplig Sammanfattning

Hereditär myopati med laktacidosis (HML), även kallad Linderholms sjukdom, är en autosomt recessiv sjukdom (två anlag krävs för att bli sjuk) som har sitt ursprung i Västerbotten och Ångermanland. Totalt sett har 30 individer från 18 familjer diagnostiserats med sjukdomen, varav en är av norsk härkomst. Patienter som lider av denna sjukdom har en mycket låg fysisk prestationsförmåga och drabbas redan vid lättare ansträngning av mjölksyra, muskeltkramp, hjärtklappning och andnöd. Svåra episoder av sjukdomen, kan till exempel utlösas av för hård ansträngning eller fasta. I dessa kan patienten drabbas av svår störning av syrabalansen i kroppen, förlamning av andningsmusklerna och njursvikt – tillstånd som kan vara direkt livshotande.

HML beskrevs för första gången i början av 1960-talet, då 14 patienter från fem familjer undersöktes på grund av nedsatt fysisk förmåga, med symptom som myoglobinuri (mörkfärgad urin till följd av nedbrytning av muskelfiber) och förhöjda laktat- och pyruvatnivåer i blodet vid ansträngning. I början av 1990-talet upptäckte man att dessa patienter hade mycket låga nivåer av succinatdehydrogenas (SDH) i sina muskler. SDH är ett protein som är involverat i både citronsyracykeln och elektrontransportkedjan, två mitokondriella processer som svarar för en stor del av cellens energiproduktion. Senare fann man även brist av andra proteiner involverade i dessa processer. Den gemensamma nämnaren för de påverkade proteinerna visade sig vara att de kräver järn-svavelkluster (Fe-S kluster) för att fungera som de ska. Detta ledde till hypotesen att HML orsakas av ett fel i antingen sammansättningen, processningen eller transporten av Fe-S kluster. Fe-S kluster är icke-organiska föreningar som är mycket viktiga för funktionen hos ett stort antal olika proteiner i kroppens celler. Många av dessa proteiner är viktiga för de metabola processerna, men Fe-S proteiner har även andra viktiga funktioner i cellen som att reparera skadat DNA. Sammansättningen av Fe-S kluster sker med hjälp av en rad olika proteiner. Detta system är evolutionärt välbevarat och återfinns i organismer så varierande som bakterier, jäst, växter och däggdjur. Studier har också visat att förändringar i de proteiner som är involverade i syntesen av Fe-S kluster oftast leder till drastiska konsekvenser för organismen.

Syftet med dessa studier har varit att ta reda på den genetiska orsaken till HML, samt att undersöka de bakomliggande sjukdomsmekanismerna, med fokus på sjukdomens vävnadsspecificitet.

I det första arbetet var målet att hitta den genetiska orsaken till HML. Vi fann koppling mellan HML och en region på kromosom 12 som innehöll 16 gener. En av generna kodade för ett protein, ISCU, som är involverat i sammansättningen av Fe-S kluster och utmärkte sig därför som en perfekt kandidatgen. Sekvensering av genen visade att varken promotorn eller exonerna innehöll några sjukdomsspecifika förändringar, däremot upptäcktes ett basparsutbyte i den sista intronen (g.7044G>C). Mutationen visade sig leda till att intronsekvens felaktigt infogas i mRNA:t, vilket resulterar i ett protein med nedsatt aktivitet.

ISCU är ett evolutionärt välbevarat protein som fyller en mycket viktig funktion i cellen och som uttrycks i nästan alla kroppens vävnader. Trots detta ger den drastiska HML mutationen upphov till en sjukdom som enbart verkar påverka skelettmuskulaturen. Anledningen till detta undersöktes i det andra arbetet. Genom att använda en semi-kvantitativ analysmetod kunde vi jämföra fördelningen mellan normalt och mutant transkript (splicingmönstret) i olika vävnader. I muskelvävnad från patient visade sig 80% av totala mängden ISCU mRNA innehålla intronsekvensen, medan det normala transkriptet visade sig vara dominerande i både lever och hjärta. Vi kunde också visa att total avsaknad av ISCU proteinet i möss är oförenligt med liv, en upptäckt som ytterligare visar på vikten av ISCU för alla kroppens vävnader. Tillsammans tyder dessa fynd på att intronsekvensen infogas i mRNA:t på ett vävnadsspecifikt sätt (vävnadsspecifik splicing) och att detta är den främsta anledningen till den muskelspecificitet som ses i HML.

I det tredje arbetet undersökte vi vilka mekanismer som styr splicingen och ger upphov till det mutanta ISCU transkriptet. Vi identifierade 5 proteiner som binder in till den region där mutationen är lokaliserad. Tre av dessa; PTBP1, RBM39 och IGF2BP1, hade dessutom förmåga att påverka splicingmönstret av ISCU genen. PTBP1 förhindrade den felaktiga splicingen av intronsekvensen. IGF2BP1 och RBM39, däremot, gav upphov till en nedreglering av den totala nivån av ISCU mRNA, men det normala transkriptet påverkades i högre utsträckning än det mutanta. Vidare hade IGF2BP1 och RBM39 förmåga att motverka effekten av PTBP1. IGF2BP1 visade sig dessutom binda in starkare till den muterade intronregionen. Detta kan innebära att mutationen möjliggör inbindning av IGF2BP1, vilket i sin tur förhindrar inbindning av PTBP1, och därmed möjliggör att intronsekvensen felaktigt introduceras i ISCU mRNA:t. Samspelet mellan dessa faktorer skulle därför kunna vara en viktig del i processen som leder till den vävnadsspecifika splicingen i HML.

Sammanfattningsvis så har vi identifierat den genetiska orsaken till HML, som visade sig vara ett basparsutbyte i den sista intronen av *ISCU* genen och som ger upphov till ett felaktigt spliceat transkript. Vi kunde vidare visa att den muskelspecifika fenotypen hos patienterna orsakas av vävnadsspecifik splicing av det muterade *ISCU* mRNA:t. Vi har även identifierat nukleära faktorer som har kapacitet att påverka splicingen av *ISCU* transkriptet. Detta arbete har ökat vår förståelse för de grundläggande sjukdomsmekanismerna som leder till HML och kan förhoppningsvis i förlängningen leda till bättre behandling av sjukdomen.

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~*~

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