Universidade de Lisboa

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Departamento de Biologia Animal

The role of microRNAs in X-Linked Myotubular Myopathy

Mariana Miranda Fontes

Mestrado em Biologia Humana e Ambiente

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Abstract

X-linked myotubular myopathy (XLMTM) is a congenital neuromuscular disorder characterized by profound hypotonia and severe skeletal muscle weakness in the affected newborn males. The pathology is associated with mutations in the *MTM1* gene leading to loss of function of the resulting encoded protein, myotubularin. Myotubularin is a phosphoinositol lipid phosphases known to be involved in endosome trafficking and membrane remodeling, however, the molecular mechanisms underlying myotubular myopathy are not yet clear.

MicroRNAs (miRNAs) are post transcriptional modulators of gene expression and play an important role in many developmental processes and diseases. To identify functional miRNA-protein networks that may be dysregulated in myotubular myopathy, we performed miRNA as well as mRNA expression profiling of skeletal muscle of *Mtm1* knockout mice. Bioinformatic analysis and real-time RT-PCR validation resulted in identification of 12 miRNAs that showed significantly differential expression in *Mtm1* mice. The functional targets of these miRNAs in myotubular myopathy were identified by a combinatorial approach in which computationally predicted targets genes of these 12 miRNAs were matched with statistically altered genes obtained by mRNA profiling of skeletal muscle tissues from *Mtm1* mice. Ontological classification of target genes revealed genes primarily belonging to skeletal muscle development and maintenance, regulation of cell cycle and differentiation of muscle fibers.

Expression analyses of miRNA-target genes identified from this study were also performed during earlier developmental time points (2 and 4 weeks) in *Mtm1* mice for a better comprehensive insight of miRNA-mRNAs in the progression of the disease. We observed that an increase in the severity of XLMTM is associated with an increase in the fold change of several miRNAs and their target genes, suggesting their crucial role in pathology of myotubular myopathy. We hope understanding the molecular pathways involving these miRNA-mRNA networks, which are disrupted in myotubular myopathy, will contribute to uncovering the mechanisms of muscle development and maintenance and the development of new therapies for myotubular myopathy.

Resumo

Miopatia miotubular associada ao cromossoma X (XLMTM) é a forma mais severa de um grupo de doenças musculares congénitas denominado miopatias centronucleares. XLMTM é caracterizada por uma marcada redução de tónus muscular e profunda debilitação do músculo esquelético em neonatais do sexo masculino. Como consequência, a maioria dos doentes falece nos primeiros oito meses de vida, devido a insuficiência respiratória. XLMTM tem uma estimada incidência de 1:50.000 neonatais, estando associada a mutações no gene *MTM1* que conduzem à perda de função da proteina por este codificado, miotubularina. A miotubularina é o membro prototipico de uma família evolucionariamente conservada de fosfatases de fosfatidilinositois (PtdIns), envolvida na regulação de tráfico endossomal e remodelação membranar. As vias bioquímicas reguladas por miotubularina estão fundadamente reconhecidas, no entanto o modo como a deficiência em miotubularina e consequentemente uma alteração nos níveis de fosfatidilinositol 3-P e fosfatidilinosital (3,5)P2 conduzem a um fenótipo musculo-esqueletico especifico é ainda indeterminado.

Estudos recentes atribuem à miotubularina um papel crucial no manutenção dos tubulos transversais (tubulos T). Os túbulos T são parte integral das triadas que constituem a região subcelular responsável pelo mecanismo de excitação e contração muscular. Os túbulos T são estruturas membranes que requerem constantes oscilações dos níveis de PtdIns, regulados por miotubularina. Segundo este modelo, uma deficiência em miotubularina conduz a uma descoordenação dos mecanismos de contração muscular, resultando na atrofia muscular observada nos doentes de miopatia miotubular.

Neste projecto pretendemos clarificar as vias afectadas pela ausência de miotubularina e compreender o envolvimento dos microRNAs na patofiosiologia da miopatia miotubular. Os microRNAs (miRNAs) são moléculas recentemente descobertas que modulam a expressão génica a nível pos-transcripcional. Após transcrição, transporte para o citoplasma e vários ciclos de processamento mediados pelas RNAses III Drosha e Dicer, os miRNAs funcionam como moléculas guia, que reconhecem por complementaridade as regiões 3'UTR dos mRNA alvo e promovem o seu silenciamento. Elevados graus de complementaridade miRNA:mRNA geram degradação do mRNA alvo enquanto que ligações com mismatch conduzem a repressão da tradução. A importância dos microRNAs no desenvolvimento muscular e em estados patológicos foi previamente demonstrada através da formação de Dicer knockouts condicionais e reportada em várias doenças neuromusculares.

Com o intuito de identificar potenciais vias reguladas por microRNAs alteradas na miopatia miotubular uma abordagem baseada em microarrays foi adoptada. O perfil de expressão de miRNAs e mRNAs foi primariamente efectuado e potenciais interações miRNA-target identificadas. miRNA profilling e subsequente validação por real time PCR revelaram expressão diferencial de miRNAs em ratinhos deficientes em miotubularina quando comparados com os controlos, demonstrando o seu envolvimento na miopatia miotubular. Na tentativa de decifrar redes miRNA/mRNA alvo relevantes para o desenvolvimento de XLMTM, o perfil de expressão de mRNAs foi comparado com a lista de potenciais alvos dos microRNAs obtidos como significantes nesta experiência por abordagens bioinformaticas. A combinação dos resultados de mRNA profiling (com 424 genes significantes) para a identificação de potenciais alvos dos 12 microRNAs obtidos como significantes nas análises de miRNA microarrays resultou em 142 genes alvos diretos de microRNAs. Este grupo de genes potencialmente regulados por miRNAs e diferencialmente expressos no contexto da patologia foram subsequentemente classificados em grupos funcionais sobre-representados usando o software GOstat. As categorias funcionais de contração muscular, regulação da progressão através do ciclo celular, morfogénese do músculo cardíaco, assim como desenvolvimento do músculo esquelético e componentes do sarcomero foram identificadas.

Dada a relevância biológica dos grupos funcionais obtidos, estudos posteriores basearam-se em genes presentes nestas categorias. Apenas genes que apresentaram um padrão de expressão diferencial contrário ao dos seus potenciais miRNAs foram considerados uma vez que os microRNAs regulam negativamente a expressão dos seus mRNAs alvo. Subsequente validação da expressão dos genes selecionados por qRT-PCR array permitiu a formulação de hipóteses quanto às vias moleculares envolvidas na XLMTM.

Estes estudos permitiram verificar que a atrofia muscular observada em pacientes de miopatia miotubular é dependente da expressão de FoxO1/Murf1/Mafbx. FoxO1 é um membro crucial da via de sinalização celular Akt/Pi3k que conduz a atrofia muscular como resposta a uma redução da actividade desta via. FoxO1 promove um aumento de expressão de componentes chave da via de ubiquitinacao-proteossoma. Em particular, Murf1 e Mafbx (moléculas sobreexpressas em XLMTM como foi detectado por análises de microarrays) foram identificados como marcadores necessários para a activação de atrofia muscular. Igualmente várias proteínas integrais do sarcomero previamente reportadas como envolvidas no processo de atrofia muscular foram encontradas sobre-expressas e potencialmente alvo de miRNAs sub-expressos na miopatia miotubular.

Estes resultados sugerem que estes miRNAs possam talvez não ter um papel específico nesta patologia, mas representar um mecanismo de regulação comum a outras doenças musculares. Desta forma, usando adenovirus para sobre-expressar estes miRNAs (sub-expressos em condições de atrofia muscular) poderá ser possível reduzir os níveis de proteínas implicadas no processo de atrofia e consequentemente atenuar o progresso da miopatia miotubular, entre outras doenças musculares. Adicionalmente foi também observada uma persistente sub-expressão de microRNAs alvo de genes inibidores de proliferação celular e respectiva sobre-expressão dos respectivos mRNA. Entre este os genes supressores de tumores p21, Gadd45a e Dusp4 representam alvos candidatos de microRNAs sub-expressos nesta doença. Igualmente, marcadores de diferenciação celular foram consistentemente detectados sobre-expressos.

Por outro lado, foi observado um aumento de expressão de genes (regulados por miRNAs) envolvidos no controlo da manutenção da população de células estaminais em ratinhos mutantes para miotubularina. Estas observações despoletaram o desenvolvimento de hipóteses explicativas da incapacidade do músculo esquelético de responder a estímulos regenerativos. É postulado que a incapacidade do músculo de ratinhos mutantes de promover hipertrofia muscular poderá estar correlacionada com um defeito no potencial proliferativo das células estaminais. De uma forma cooperativa vários miRNAs pertencentes a um mesmo cluster genomico (Dlk1-Dio3 cluster no cromossoma 12 em *Mus musculus*) poderão ser responsáveis pela sobre-expressão de genes inibidores do ciclo celular e igualmente genes indutores da diferenciação celular.

Estas evidências sugerem que uma redução na capacidade de proliferação celular acoplada a um aumento de proteinas de diferenciação nos ratinhos knockout deficientes em miotubularina, resultam na produção de apenas um pequeno pool de satellite cells activadas (células estaminais progenitoras de tecido muscular), insuficientes para uma eficiente reparação da injuria muscular. Esta incapacidade regenerativa poderá ser um dos mecanismos básicos fundamentais a contribuir para o desenvolvimento de atrofia muscular. Os genes responsáveis por estas duas vias (activação das células estaminais precursoras de mioblastos e indução de atrofia muscular) são regulados por vários microRNAs que formam uma rede altamente coordenada levando a que mesmo um pequeno defeito no circuito resulte no desenvolvimento de severa atrofia, incluindo associada às manifestações de miopatia miotubular.

Estudos de expressão génica realizados para diferentes tempos de desenvolvimento do ratinho usando a tecnologia de highthroughput qRT-PCR array permitiram demonstrar a relevancia do grupo de genes regulados por miRNAs selecionados. 26 genes envolvidos nas vias de diferenciação/proliferação

de células estaminais ou proteinas marcadores de atrofia muscular foram testados e demonstraram significativas alterações de expressao entre as classes wild-type e KO ratinhos ainda no estado pré-clinico da doenca. Desta forma, a hipotese de estas moléculas representarem apenas representarem um artefacto experimental ou apenas serem inespecificamente induzidas como consequência do estado geral de doença foi refutada. Os microRNAs encontrados diferencialmente expressos na XLMTM e identificados como responsáveis pelo estabelecimento de várias das vias funcionais desreguladas neste sistema constituem candidatos preferenciais para estudos futuros de terapia genética.

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1) Introduction

1.1) Linked Myotubular Myopathy

linked myotubular myopathy (XLMTM) is the most severe form of a group of congenital muscular disorders named centronuclear myopathies, and presents an estimated incidence of 1 in 50,000 newborn males. (D'Amico and Bertini, 2008) In contrast to the heterogeneous patterns found in the majority of congenital myopathies, XLMTM has a relatively homogeneous clinical presentation, where males are born with marked hypotonia and generalized muscle weakness, with respiratory difficulties often requiring ventilation. Most XLMTM patients die within 4-8 months as a consequence of respiratory failure. Even though some of them are able to survive several years (approximately 15%), and some achieving independent respiration, so far, it is not possible to accurately predict the severity of the phenotype at birth. (Jungbluth et al., 2008)

The morphological hallmark in muscle biopsies of XLMTM patients is the presence of numerous rounded hypotrophic fibers with a higher than expected occurrence of centrally placed nuclei in hematoxylin/eosin stained sections. This pattern resembles the structure of fetal myotubes, whereas in healthy individuals nuclei usually occupy a peripheral location. The percentage of these “myotubes” in a sample can vary widely among cases with a reported range between 2% to 60%. Based on this canonical characteristic this pathology previously was thought to be due to an arrest in muscle development at the myotube stage. (Sarnat, 1990) Subsequent studies, however, refuted this hypothesis and suggested instead that myotubular myopathy represents a defect in skeletal muscle maintenance. Typically found surrounding these nuclei in central locations is a clear perinuclear zone containing glycogen and mitochondria deposits, which can be observed in histochemical stains PAS and NADH-TR, respectively. (Tronchere et al., 2003)

Disease diagnostic results from an integrative approach compiling pathological findings, age of onset and, if available, genetic testing. The definitive diagnosis, however, is based on genetic screening for mutations in the *MTM1*, the only gene associated with the development of the pathology. (Laporte et al., 1996) So far, more than 200 different loss-of-function mutations have been identified throughout the entire coding sequence of *MTM1,* in more than 300 unrelated families. (Laporte et al., 2000) The *MTM1* gene spans approximately 100Kb at the genomic level, contains 15 exons and is located in Xq28 locus. MTM1 mRNA is expressed as of 3.9 Kb long transcript, ubiquitously expressed in all human tissues as idenfied by northern blot analysis. Interestingly, in skeletal muscle and testis, an additional smaller 2.5 Kb tissue-specific transcript is detected, resulting from the use of a different polyadenylation site. The biological significance of

this mechanism of gene regulation for *MTM1* remains unknown, pointing out the importance of further studies in *MTM1* expression control. (Pierson et al., 2005)

1.2) Myotubularin and the etiology of myotubular myopathy

The protein product of the ubiquitously expressed *MTM1* gene is myotubularin, a phosphatidylinositol (PtdIns) lipid phosphatase of 603 amino acids. Myotubularin contains an active phosphatase domain (PTP) and a prixomal PH-GRAM domain that binds phosphoinositides. Phosphoinositides (PI) are lipid second messengers that play key roles in signal transduction, trafficking and cellular homeostasis through the recruitment of effectors proteins to their subcellular target sites. The selection of the PI specie to be formed (in a set of seven) is dependent on the phosphorylation/ desphosphorylation balance in the position 3, 4 or 5 of their inositol sugar rings. These interconversions are controlled by their respective PI kinases and phosphatases. Myotubularins are 3'-phosphatases specific for PtdIns3P and PtdIns(3,5)P2, PIs involved in the endosomal–lysosomal pathway. (Robinson and Dixon, 2006)

Myotubularin is the archetypical member of a protein tyrosine phosphatase (PTP) superfamily of 14 closely related genes that share high levels of homology, present in a wide spectrum of eukaryotic organisms from yeast to mammals. (Clague and Lorenzo, 2005) In the myotubularin family only 8 members were defined has containing phosphatase activity. Other myotubularins that have an inactive phosphatase seems to be crucial for the stability and activation of the other catalytic active members. Most of the myotubularin family members are able to form homodimers or heterodimers with other members of the family by direct protein-protein interactions. The interaction between the inactive MTMR9 and active MTMR6 form reflects this essential function by leading to an increase in the 3-phosphatase activity of MTMR6 up to 6-fold. (Zou et al., 2009) Mutations in MTMR13 or its binding partner MTMR2 have been found in human patients of Charcot-Marie-Tooth disease further strengthing the importance of their interactions.

The biochemical functions of myotubularin have been intensively described, although, the biological pathways and regulatory mechanisms that when disrupted lead to myotubular myopathy, still remain uncertain. To address these questions several knockout/overexpression experiments were recently developed. Using siRNA-mediated technology it was demonstrated that knocking down the levels of myotubularin results in an increase of 60% to 120% of endogenous PI3K that accumulates on early endosomes. The sequential waves of PI(3)P synthesis

and degradation, regulated by the interplay of PI3 kinases and phosphatases and controlled by the activation of Rab GTPases, has been postulated to be the mechanism for signal transduction and receptor sorting necessary for acute recruitment, release or activation of the trafficking machinery components, during endocytosis. (Cao et al., 2008) A dysregulation in the orchestration of these antagonistic proteins seems to lead to impairment cellular metabolism, contributing to the development of several pathologies, including XLMTM.

Likewise MTM1, several MTM related genes (MTMR) have been established as involved in different types of neuromuscular disorders (e.g. MTMR2 and MTMR13 mutations are associated with Charcot-Marie-Tooth type 4B1 and 4B2 neuropathy, MTMR14 mutations result in autosomal centronuclear myopathy, MTMR1 splicing variants are associated with myotonic dystrophy and MIP/Mtmr14 mutations lead to muscular weakness and fatigue). (Azzedine et al., 2003); (Houlden et al., 2001); (Shen et al., 2009)

It is remarkable to note that ubiquitously expressed PI phosphatases acting in the same biological pathway and with similar catalytic active profiles are involved in pathologies affecting different specific tissues. As noted above, MTMR2 gene that shares 65% sequence identity with MTM1 affects primarily Schwann cells in peripheral nerves instead of skeletal muscle as MTM1 mutations. The reason for large amounts of apparently functional redundant PI proteins seems to be directly correlated with their specific spatiotemporal expression pattern and subcellular location. Both MTM1 and MTMR2 desphosphorylate pools of Ptdns(3)P and Ptdns(3,5)2P, however, MTM1 primary acts both on early and late endosomes, whereas MTMR2 is exclusively active on late endosomes. (Nicot and Laporte, 2008)

Although displaying specific subcellular functions, at least some MTM-related genes are able to compensate for the lack of expression of a particular family member, demonstrating a fine efficient mechanism of gene regulation, to restore cell homeostasis. MTM1 is ubiquitously expressed, although XLMTM is a muscle specific disorder. A possible justification for this phenomenon is the ability of MTMR1 and MTMR2 to compensate the loss of myotubularin in all but skeletal muscle tissue, where MTM1 is the main 3'-phosphatase. (Dowling et al., 2009)

The overexpression of myotubularin promotes formation of fillapodia-like structures and disrupts protein transport from late endosomes to lysosomes. (Tsujita et al., 2004) To deeper define the subcellular function of myotubularin in skeletal muscle, overexpression experiments were performed using adeno-associated virus (AAV) vectors expressing myotubularin. The formation of packed membrane assemblies and the presence of vacuoles positives for T-tubules

and plasma membrane markers were observed. Subcellular staining of myotubularin revealed the protein associates with sarcolemma and triads. Triads are highly specialized junctions where the control of calcium concentration, and therefore, regulation of excitation-contraction occurs. These structures are constituted by transverse T-tubules (plasma membrane invaginations) and terminal cisternae of sarcoplasmic reticulum. Myotubularin is suggested to be important for the maintenance of triads, remodeling of longitudinal T-tubules to become transverse T-tubules and plasma membrane homeostasis. The failure of these processes would lead to impaired excitation-contraction coupling and consequently generate muscular atrophy. (Buj-Bello et al., 2008); (Al-Qusairi et al., 2009)

In vivo studies using morpholino antisense technology to knockdown myotubularin, provides additional functional evidences supporting this hypothesis. Knockdown of myotubularin in zebrafish results in impaired motor function and histopathologic changes in skeletal muscle, resembling clinical XLMTM patient observations. Also, tubule-reticular structural abnormalities were found in MTM1-morpholino treated zebrafish and human patient biopsies, displaying disorganized and irregular patterns. T-tubule integrity is required for proper force generation, muscle contraction and more specifically for the process of excitation-contraction coupling. Defects in T-tubule structure and localization (T-tubule were found concentrated around the abnormal located nuclei in XLMTM patients) were also associated with T-tubule functional defects, as was demonstrated by abnormal mechanisms of excitation-contraction observed in skeletal muscle. (Dowling et al., 2009)

The physiological implications of T-tubules dysfunction in the etiology on myotubular myopathy can be hypothesized: T-tubules are membrane invaginations whose biogenesis and maintenance requires continuous recycling of membrane components, including phosphoinositides. If MTM1 function is disrupted, membrane recycling does not occur efficiently. Consequently, T-tubule function is impaired, leading to abnormal excitation-contraction mechanism, necessary to produce muscle force. Mutations of MTM1, causing loss of function, deregulate this process, and subsequently promote muscle wasting and atrophy, characteristics present in skeletal muscle of myotubular myopathy patients. (Dowling et al., 2009)

Recent findings, originated through investigation of MIP/MTMR14 mutant mice, remarkably elucidate the crucial function of MTMR14 in control of PtdInsP levels in establishing calcium homeostasis and muscle performance. Several PtdInsP were identified to directly bind and activate ryanodine receptors (RyR1) - the skeletal muscle calcium release channel in sarcoplasmic

reticulum, crucial for excitation-contraction coupling. These studies strongly help to understand how loss of myotubularin family proteins that are responsible for regulation of PtdInsP function lead to muscle contraction deficiencies. (Shen et al., 2009) (Treves et al., 2005)

Although considerable progress has been made in the understanding the pathogenesis of XLMTM, many critical aspects still remain mainly uncovered, in particular concerning the translation of the identified metabolic defects, into the skeletal muscle phenotype found in the disease.

In this project we will try to bring new insights to address these questions by studying gene expression control in the myotubularin-deficient mouse, model of XLMTM, using microarray analysis of mRNA as well as miRNA (endogenous modulators of gene expression) expression profiles. We aim to contribute to dissect primary molecular pathways disrupted in myotubular myopathy through the identification of miRNA-mRNA regulatory interactions and differential expression of relevant genes in the context of the disease, in knockout mice when compared to wild-type littermates.

1.3) Skeletal muscle development

The knowledge of the networks underlying muscle development and the regulatory mechanisms responsible for the homeostasis and maintenance of muscle integrity are a pivotal basis of muscle biology we hope to improve with our findings.

Skeletal muscle results from muscle progenitor cells derived from mesoderm. In humans, at approximately 3 weeks of gestation, these muscle stem cells generate myoblasts which fuse to generate myotubes at 7 weeks. The myotube stage is characterized by long multinucleated filaments with central located nuclei sharing the same basal lamina. Along the development, myotubes start to produce significant amounts of contractile components required for muscular function. Also, each nucleus acquires an individual basal lamina and become innervated. At this step, mature myotubes are called myofibers and possess peripheral located nuclei and contractile apparatus located in the central region. The determination of cell fate and coordination to myogenic lineage specific seems to be upstream regulated by *Pax3* and *Pax7*. (Maroto et al., 1997) (Seale et al., 2000) Pax3 acts mainly during primary myogenesis while Pax7 during the later stages of muscle growth.

After the initial phase of embryonic muscle development, muscle mass can be produced, according to functional needs, by mechanisms such as hypertrophy (the most common adult

muscle growth) and hyperplasia (primary occurs during embryonic development). During skeletal muscle growth through hypertrophy, myofibers increase their volume by increasing the amount of contractile components, requiring the incorporation of additional nuclei. Satellite cells are mononucleated cells able to fuse and increase the growing capacity of myofibers. (Sanger, 2004)

Satellite cells are the major muscle precursor cells and are involved in maintenance and repair of skeletal muscle. These stem cell-like structures derive from the dermomyotomal population (the dorsal compartment of the somites, where the embryonic myogenesis starts to take place), located in a specific niche between the basal lamina and the sarcolemma. Typically, there is a small pool of mitotically quiescent satellite cellswhich are activated in response to external stimuli that produce stress such as need for growth by hypertrophy or muscular injuries. Activated satellite cells proliferate and generate myogenic precursor cells (myoblasts), which undergo multiple rounds of cell division until they experience terminal differentiation. The resulting cells undergo fusion, leading to the formation of multinucleated myofibers.

The molecular mechanisms that underlie the regulation of the satellite cell pool and their self-renewal are still poorly understood. Myogenesis requires co-expression of myogenic regulatory factors (MRFs) such as Myf5, MyoD, Myogenin and MRF4 which promote cell cycle arrest and subsequent terminal differentiation into contractile muscle fibers. Concomitant with this process is the down-regulation of the levels of pax7. (Buckingham and Montarras, 2008) Several studies indicated pax7 as a key molecule in adult muscle cell remodeling pointing out its action as a negative regulator of MyoD and Myogenin and consequently delaying the successful adoption of the myotube phenotype. It has also been recently elucidated that myostatin controls self-renewal of satellite cells and their state of activation, through negative regulation of pax7 via the Erk1/2 pathway. (Bryson-Richardson and Currie, 2008; McFarlane et al., 2008)

Skeletal muscle development is organized by evolutionarily conserved networks of transcription factors that coordinate the expression of muscle-specific genes responsible for muscle growth, differentiation and contractibility. MADS-box transcription factors MEF2 (myocyte enhancer factor 2) and SRF (serum response factor), in combination with MyoD and Myogenin (basic helix-loop-helix factors) play a central role in the regulation of myogenesis through the activation of fine subsets of muscle specific genes according to developmental stages. (Chen et al., 2009)

1.4) microRNAs biogenesis and cellular function

Recently, an additional layer of complexity in skeletal muscle gene regulatory circuits started to be revealed. MicroRNAs (miRNAs) are pos-transcriptional gene expression modulators. Through their ability to coordinately regulate networks of genes, they enable a fast and precise cellular reaction to developmental, physiologic and pathologic signals in skeletal muscles. (Williams et al., 2009)

The recognition of fundamental catalytic activities carried out by RNA molecules on gene expression dates back to 1961 when Jacob and Monod proposed the role of RNAs in the inhibition of operons expression, through Watson-Crick base-pairing interactions with the operator sequence. (Jacob and Monod, 1961) However, the steeply growing knowledge and interest about these tiny molecules was triggered by the discovery of RNA interference mechanism in 1998 by Craig Mello's group. (Fire et al., 1998) By silencing the expression of specific genes in specific spatial-temporal limits this phenomenon of gene silencing generated by small non-coding RNAs (and effector proteins) is implicated in innumerous crucial cellular and biological processes and has been associated with human pathologies. MicroRNAs are defined as ssRNAs of ~22 nucleotides in length generated by the RNAse-III enzyme Dicer from an endogenous transcript containing a local hairpin structure. (Ambros et al., 2003) Bioinformatics methodologies have predicted that almost 30% of mammalian mRNAs of protein coding genes are regulated by these molecules, revealing their remarkable biological significance. (Bartel, 2004)

The first experiments highlighting the existence of miRNAs were conducted by the Ambros and Ruvkun labs. It was reported that short lin-4 RNA directly down-regulate lin-14 gene product by binding to specific repetitive sequences on the 3'UTR of the lin-14 messenger. This functional interaction promotes the progression from the first nematode larval stage to the second. (Lee et al., 1993) (Wightman et al., 1993)

Despite the fact that it is only seven years the next miRNA was discovered (let-7) (Reinhart et al., 2000) at the date of writing, the official miRNA database - miRBase (release 14.0, Sep 2009) - reports 10581 mature miRNAs in 115 species, including more than 700 human miRNAs. (http://www.mirbase.org) For a long period of time, miRNAs were thought exclusive to multicellular organisms, as a vehicle for the transition to a more complex organism design. Recent studies have identified microRNAs in *Chlamidomonas reinhardtii,* a unicellular alga, demonstrating the evolutionarily conserved nature of this mechanism of gene regulation. (Molnar et al., 2007)

With only one unique exception detected so far (Bao et al., 2004) microRNAs function at the post-transcriptional level. MicroRNAs regulate gene expression either by blocking mRNA translation or decreasing mRNA stability in the cytoplasm. New perspectives expect microRNAs to operate in almost every cellular process, including regulating pre-mRNA processing in the nucleus, acting as chaperones that modify mRNA structure or modulating mRNA-protein interactions. (Filipowicz et al., 2008)

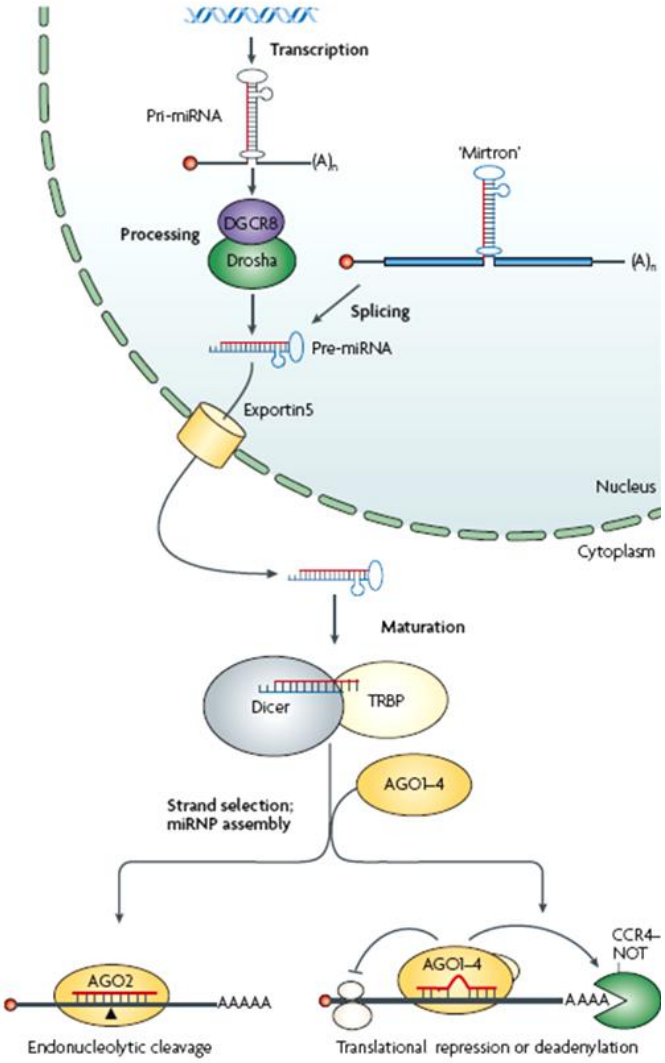

Figure 1: Schematic representation of miRNAs biogenesis and their mode of action. (Filipowicz, 2008)

miRNAs can either be transcribed as independent transcriptional units producing pri-miRNAs or as a result of splicing of intronic portions of protein-coding genes *(mirtrons)* leading to the direct formation of pre-mRNAs . Pri-miRNAs are processed by a complex that includes Drosha giving rise to pre-miRNAs.

These molecules are transported to the cytoplasm through exportin5 and cleaved by the RNAse III type Dicer to yield approximately 20 nucleotide miRNA duplexes. One of the strands is selected to form the mature miRNA being assembled into a miRNA-induced silencing complex (miRNP) while the other strand is subsequently degraded. High levels of complementarity between miRNA:mRNA target lead to endonucleolytic cleavage and sequences with several mismatches guide translational repression of mRNA targets.

The genomic localization and transcription process used for microRNAs is not deeply understood yet, however, different mechanisms seem to be involved. Initial studies have pointed out that the majority of microRNAs were encoded in intergenic regions (> 1kB away from the annotated/predicted gene) although the presence of microRNA codified in intronic regions in the

sense or antisense orientation were quickly reported: the notion of microRNAs as autonomous transcription units had emerged. Since more than 50% of the miRNAs are in close proximity with each other, and it was experimentally proven that they can be transcribed by their own promoters as a unique unit of clusters of pri-miRNAs, there is sufficient data to demonstrate that microRNAs might be transcribed as mono or polycistronic transcription units.

Their biogenesis is usually catalyzed in two major steps, processed by two RNAse III family proteins, namely Drosha and Dicer. (Figure 1) Once the precursors miRNAs (pri-miRNAs) are transcribed, typically by RNA Pol II (besides some exceptions reported (Cai et al., 2004)) they fold in a long hairpin-like structure containing an imperfectly base-pairing stem, often including several sequences for different miRNAs. Pri-miRNAs are processed by the endonuclease Drosha, complexed with a dsRNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8), leading to the release of ~70 nucleotide hairpins known as pre-miRNAs. As an RNAse III endonuclease, this enzyme cuts the RNA duplex at both strands of the stem near the base of the primary stem loop, resulting in a stem with 5' phosphate and 2 nucleotides 3' overhang. An alternative pathway that circumvents the requirement of Drosha-DGCR8 machinery was found in C. elegans, D. melanogaster and mammals and enable a subset of pre-miRNAs (*mirtrons*) to be generated from introns, by cooperative actions of spliceosome and lariat-debranching enzyme (LDBR). (Berezikov et al., 2007; Ruby et al., 2007)

In animals the pre-miRNAs are actively transported to the cytoplasm by the nuclear transport receptor exportin-5, in a cooperative complex with Ran-GTP, which after hydrolysis permits the release of the cargo. Once there, pre-miRNAs are subsequently subject to the second processing step by Dicer (associated with TAR RNA binding protein), generating the final ~22 nucleotides miRNA duplex product. This short duplex is incorporated into the functional miRNA-Ribonucleoprotein complex (miRNP), where the mature miRNA preferentially remains assembled in the complex.

The criterion for the selection of the strand to be matured seems to be the thermodynamic stability of the 5' end, with the less stable end to be generally elected and the other degraded. The key components of miRNP or miRISC (microRNA- induced silencing complex) are the Argonaute family proteins (AGO). This family comprises four AGO proteins with only AGO2 protein having the ability to participate in the RNAi-like mechanism developed by this complex. After assembly, the mature miRNA strand functions as the guide molecule to the complementary mRNA target to be silenced. The recognition of the exact mRNA to be targeted seems to be the

more controversial and critical issue in understanding the function of microRNAs in mammals. In plants the interactions between microRNA:mRNA are nearly perfect but when this relation is transposed to metazoans, miRNAs usually bind to mRNA by imperfect base-pairing, yielding variable degrees of miRNA-target mismatches. Consequently, this leads to large difficulties in the prediction of their target mRNAs. (Winter et al., 2009)

Although the mechanistic details of this interaction are poorly understood it is accepted that a stringent requirement must be present for a functional and productive binding: a contiguous and perfect base-pairing on the 2-8 nucleotides of the 5'end of the miRNA with the mRNA, denominated *seed region*. (Lewis et al., 2005) Further studies have also mentioned the importance of secondary structures on the 3' untranslated region (UTR) surrounding the target site (*seed*) in the target mRNA and the ability of complementarity at the 3' end of the cognate miRNA to compensate for imperfect *seed* matching. (Brennecke et al., 2005)

The molecular basis of this interaction is directly correlated with the selected pathway in miRNA-mediated gene regulation. If miRNA:mRNA-target interaction presents high affinity and nearly perfect complementary, the mRNP complex will trigger endonucleolytic mRNA cleavage by an RNAi-like process. On the other hand, when this match is imperfect, the most common event in mammals, miRNA lead to translation repression and possibly destabilization of the mRNA target. (Carthew and Sontheimer, 2009)

Despite the consensus about the role of microRNAs in promoting translation repression of mRNA targets, the mechanism through which miRNAs interfere with active translation, and, in particular, the step of translation inhibited, is a matter of controversy. Perhaps, the paramount open question is if miRNAs modulate gene expression by single or multiple mechanisms. Thus far, the model of miRNA gene regulation interfering with translation initiation stage has received increasing support by *in vivo* and *in vitro* studies. However, repression mechanisms starting after the initiation of translation mediated by miRNAs were also identified when miRNAs were found associated with actively translating polysomes. (Filipowicz et al., 2008)

miRNA regulatory pathways can not only affect the translation of cognate mRNAs but also might directly decrease their amounts, through the promotion of molecule destabilization. miRNAs control transcript levels by recruiting the machinery involved in mRNA decay. miRNPs recruit GW182 (a protein of the P-body structure), which subsequently recruits proteins involved in 3' poly-A cleaving. As a result, an unstable transcript is generated, susceptible to 3'-5' exonucleotidic activity, and vulnerable to decapping proteins with subsequent 5'-3' exonucleases

exposure. (Behm-Ansmant et al., 2006) P-bodies are highly dynamic cytoplasmatic structures, enriched in translation repressor molecules and mRNA-catabolizing enzymes. By conducting mRNA targets to P-bodies, miRNAs can promote the cleavage or storage of the repressed mRNAs, making miRNA regulation a versatile and wide ranging system. (Rana, 2007)

Furthermore, Vasudevan and colleagues have illustrated this versatility when they reported that miRNAs complexed with Ago2 and FXR1 (Fragile X mental retardation protein 1) can trigger upregulation of mRNAs target, in specific cellular conditions. MicroRNAs can switch from repression to activation according to cell cycle state: in proliferating mammalian cells they repress translation whereas in G1/G0 arrest, which usually precedes differentiation, they can potentially promote activation of gene expression. (Vasudevan et al., 2007)

As trans-acting molecules, miRNAs act in the maintenance of cellular homeostasis and development in cooperation with transcription factors. It is now clear that miRNAs have highly cell type-specific expression profiles and mutations in specific miRNAs that lead to the development of severe diseases.

1.5) miRNAs in skeletal muscle

The importance of the role of miRNAs in skeletal muscle development was identified through the generation of conditional Dicer null alleles under the control of MyoD regulatory elements (a muscle specific marker). The resulting Dicer-deficient mice show skeletal muscle hypoplasia associated with perinatal lethality. C2C12 experiments demonstrate increased apoptosis of cultured embryonic myoblasts. (O'Rourke et al., 2007)

Further studies revealed the involvement of particular muscle specific miRNAs (MyomiRs) in skeletal muscle proliferation and differentiation. (Chen et al., 2009; Williams et al., 2009) miR-1 and miR-206 were identified as enhancers of myogenesis and mir-133 was ascribed as a critical factor for myoblast proliferation and repression of myoblast differentiation. miR-1 promotes muscle cell differentiation by targeting histone deacetylase 4 (hdac4). Hdac4 is a main repressor of Mef2c, which in turn constitutes an essential muscle related transcription factor. Besides belonging to the same miRNA polycistron and being transcribed together, mir-133 triggers an opposite effect to mir-1. miR-133 stimulates myocyte proliferation, to a certain extent, by reducing the levels of serum response factor (SRF), a crucial regulator of muscle cell differentiation (Chen et al., 2006) Similarly to mir-1, mir-206 promotes myoblast differentiation. miR-206 has been shown to inhibit electrical coupling between myofibers via gap junctions through the

suppression of gap-junction protein connexin 43 (Cx43), a required step after fusion of skeletal myoblast during myogenesis (Anderson et al., 2006) Also, mir-206 is responsible for the suppression of cell proliferation by the repression of p180 subunit of DNA polymerase-alpha and inhibition of genes encoding follistatin-like 1 and utrophin in skeletal muscle. (Kim et al., 2006); (Rosenberg et al., 2006)

A surprising evidence of the connection of miRNAs to skeletal muscle disorders came from a direct genetic link, observed in a Texel sheep detected with exceptional muscularity. Fine mapping identified a mutation in the 3´-UTR of the myostatin gene (GDF8), a transforming growth factor family B (TGF-B) member, responsible for inhibition of muscle growth. This mutation creates an illegitimate microRNA target site for miR-1 and miR-206, muscle-specific miRNAs, that lead to specific translation repression of myostatin, and consequently promote muscular hypertrophy. (Clop et al., 2006)

As of late, miRNA overexpression or downregulation was associated with several primary muscular disorders, including muscular dystrophy and nemaline myopathy, contributing to progress in the understanding of these pathologies. (Eisenberg et al., 2007) Nevertheless, these studies have not been conducted for X-linked myotubular myopathy.

1.6) Mouse model of XLMTM

Given that XLMTM is a rare disorder, we decided to use a knockout mouse (KO) model, which reproduces the major features of the human disease, to facilitate sample collection and improve statistical relevance. The knockout mouse *Mtm1δ4* was generated for *Mtm1,* through homologous recombination followed by selection of mice with exon 4germline excision, by Professor Jean-Louis Mandel´s group. This excision causes absence of myotubularin protein production by creating a frameshift mutation that induces an early codon stop. Despite this deletion generating myotubularin-deficient viable mice, some degree of pre or neonatal lethality was observed with only 16.6% of the litter born, instead of the 25% expected. The resulting male mice recapitulate the histopathology of human XLMTM, showing morphological variation in muscle fiber size and accentuated hypotrophy (predominantly in type 1 fibers), with centrally located nuclei present in a higher proportion in the affected animals. A perinuclear halo containing ER glycogen and mitochondria accumulations was also detected, as well as myofibrillar disorganization with sarcomeric disarray and Z-line streaming. In accordance with the observed pathology in humans, *Mtm1δ4* mice manifest a muscle-specific disorder. These data corroborate

the tissue specific impact of myotubularin dysfunction in muscle integrity and the inability of other MTMRs to compensate for this defect in muscle. (Buj-Bello et al., 2002)

Although this model reproduces the human disease, some clinical differences are found and consequently should be considered when extrapolating conclusions. Mtm1 KO mice present with variable and progressive muscular weakness with associated severe reduced life expectancy following four characterized stages, based on distinctive clinical manifestations. M*tm1* $^{(-/-)}$ mice are born asymptomatic (phase I) manifesting a disease onset around 4-5 weeks of life with accentuated decrease in muscular hindlimb strength (phase II) that reaches forelimbs at 5~7 weeks and is associated with the manifestation of kyphosis (phase III). A reduction of 64% in muscle strength is observed in the later phase IV and leads to complete hindlimb paralysis in addition to severe respiratory difficulties that often result in cachexia and respiratory insufficiency, promoting death at 59 $\pm$ 19 days. The human disease is mainly nonprogressive, contrasting with the clinical evolution reported in the knockout. Different times of myogenesis may be the reason for these discrepancies since mouse muscle differentiation is completed much later during development (at birth) as compared with humans (which ends around 16 weeks of gestation). Analysis of *Mtm1*-deficient mice enabled identification of muscle weakness in XLMTM as a consequence of atrophy rather than hypoplasia, as well as demonstrated that the observed muscle fiber defects are not due to an arrest in myogenesis, but perhaps attributable instead to a defect in the maintenance of muscle structure. (Buj-Bello et al., 2002)

1.7) Study description and biological impact

To decipher the molecular mechanisms underlying XLMTM, it is crucial to improve the knowledge about *MTM1* gene regulation expression, and in particular, the process involved in its translation. In this project we will try to develop new insights about gene expression control in XLMTM by bioinformatic analysis of mRNA as well as miRNA expression profiles, using qRT-PCR for confirmation of obtained data.

Gene expression profiles of XLMTM patients were previously investigated by Nishino group. (Noguchi et al., 2005) Using custom cDNA microarrays, the molecular signature of eight XLMTM patients was characterized. An upregulation of extracellular/sarcolemmal proteins and cytoskeletal components was observed (in particular, actin-interacting proteins). On the other hand, proteins involved in muscle contraction and energy metabolism were found downregulated. These findings implicate myotubularin as a key regulator of membranous cytoskeletal actin

remodeling and suggest that a defect in myotubularin function contributes to disorganization of actin filament architecture, giving rise to the characteristic phenotype of XLMTM myofibers, with altered cell morphology and abnormal intracellular organelle distribution. (Noguchi et al., 2005)

Although the study was the first of its kind aimed at the understanding of the pathomechanisms of myotubular myopathy, the project was performed using a custom cDNA microarray with only 4,200 selected genes – muscle specific genes and others expected to be part of the dysregulated pathways in XLMTM. Furthermore, the patient group consisted primarily of infants was not well matched in age with the controls who were significantly older.

In the current investigation we developed a microarray-based approach, using oligonucleotide microarrays able to detect whole mice transcriptome, for an unbiased study of the molecular mechanisms involved in myotubular myopathy. Also, in a combinatorial methodology we analyzed simultaneous significant changes in mRNA and miRNA expression to recognize potential miRNA-based regulatory circuits in myotubular myopathy. mRNA and miRNA profiling by microarrays were evaluated in endstage mice (7 weeks), given that this is the mouse developmental disease time point that better recapitulated human pathology. However, for an accurate extrapolation of conclusions to human disease, final significant results should be experimentally repeated for confirmation in human skeletal muscle biopsies. Additionally, for a deeper understanding of the relevance of statistically significant identified miRNAs in the disease context, we analyzed the expression levels of these miRNAs and also several of their candidate cognate target genes, by qRT-PCR in two other developmental stages (14 and 27 days mice).

Through the study of differential regulated pathways in myotubular myopathy, as well as the identification of specific miRNAs potentially responsible for the development of the disease, we hope to dissect fundamental molecular features of disease and open new avenues for the generation of new therapies. The identification of miRNA signatures in diseases and their specific characteristics, such as small size and ability to affect the expression of several genes present in a given pathway, makes miRNA targeting a promising approach for pathology treatment. New approaches, such as locked nucleic acids (LNAs) and antagomiRs, which are synthetic complementary sequences able to inhibit the function of miRNAs, represent exciting new potential therapies to be used when a particular miRNA is upregulated and identified as promoter of a disease phenotype. Adeno-associated viruses (AAV) represent a very efficient delivery method to introduce miRNAs into cells of patients where these molecules are downregulated, potentially restoring health status. (Brown and Naldini, 2009); (Eisenberg et al., 2009); (Saunders and Lim,

2009) Thus, the findings achieved in this investigation constitute a comprehensive foundation for future research into microRNAs-mediated gene regulation in X-linked myotubular myopathy.

2) Methods

2.1) Samples preparation

Gastrocnemeus muscle from 14, 27 and 49 days mice were dissected under Rnase free conditions and immediately frozen using dry ice. Eight samples from 7 week-old wild-type (WT) mice and *Mtm1* KO mice (*Mtm1δ4)* were collected for microarray expression profiling. Four WT and four 4 week old mice, as well as 4 WT and 4 KO 2 week old mice gastrocnemeus samples were used for additional qRT-PCR experiments. All the protocols for mice experimentation were performed in compliance with IACUC (Institute of animal care and use committee) guidelines.

2.2) RNA extraction

Total RNA was isolated using mirVana isolation kit (Ambion, Austin, TX) accordingly to manufacturer´s instructions. The extraction was made according to the manufacturer's instructions. Yield and purity of RNA was examined using ND1000 Nanodrop (NanoDrop Technologies) and 2100 Bioanalyzer (Agilent Technologies).

2.3) miRNA Array analysis

miRNA profilling was performed by Asuragen Services (Austin, TX) applying a custom-manufactured Affymetrix® GeneChip from Ambion – DiscovArray™. RNA samples were processed according to the standard operating procedures of the company. (Shingara J, 2005) The miRNA-enriched fraction was obtained by total RNA fractionation through a flashPAGE Fractionator apparatus (Ambion) followed by clean-up and concentration using the flashPAGE Reaction Clean-Up Kit (Ambion). Subsequently, the resulted purified fraction was hybridized to the miRNA DiscovArray containing 623 human and 361 mouse probes plus additional species and >12,000 exploratory probes. GenePix 4200AL scanner (Molecular Devices) was used to scan processed arrays. Microarray signal processing included substraction of estimated background levels based on the median signal of a set of G-C-matched anti-genomic controls.

Raw data was also normalized through variance stabilization and normalization (VSN) method by Asuragen services. For each mature miRNA sequence a minimum of at least two

probes were available. After data processing, Asuragen designated the two probes as A and B, according to their hybridization performance and signal quality. Only probes A values were selected for subsequent statistical analyses.

2.4) mRNA microarray hybridization

mRNA expression profiles of *Mtm1* KO mice and controls were evaluated by hybridization of the isolated total RNA with GeneChip® Mouse Gene 1.0 ST Arrays (Affymetrix) containing approximately 27 probes for each of the 28,853 genes, covering the whole mouse transcriptome. The chip was processed by the Microarray Core Facility at Children's Hospital Boston, MA, according to the company standard protocol. Raw data was also further normalized using the VSN method using GeneSpring software.

2.5) Principal Component Analysis (PCA), Box-Whisker plot and Hierarchical Clustering

PCA was constructed using normalized scaling and mean centered values. Box whisker plot was created based on Pearson correlation. Hierarchical unsupervised 2D-clustering was processed for non-averaged significant entity lists on WT/MTM1 conditions via Euclidean distance metric and average linkage. GeneSpring GX 10.0.2 allowed these analyses.

2.6) Statistical Analysis of microarray data

For statistical analysis a combinatorial strategy was used. miRNAs were only considered statistical significant if both GeneSpring statistics and SAM identified the same miRNA as significant.

GeneSpring GX was used to apply the non-parametric unpaired Mann-Whitney U test with a cut-off p-value<0.05. The resulted mRNAs were subject to a filter of fold change> 1.5 and the genes still present in the final list considered significant by this statistic approach. In parallel the additional Statistical Analysis of Microarrays (SAM) method was also run. A False Discovery Rate (FDR) of 18% combined with a bootstrap of 5000 randomization of samples was selected to test T-statistic.

miRNAs matching both statistical criteria detected on GeneSpring and SAM analyses were selected for further research.

2.7) miRNA and mRNA Real Time PCR (qRT-PCR)

miRNA and mRNA expression were independently assed by real time PCR, using Taqman microRNA assays (containing specific primers to generate miRNA cDNA) and Taqman mRNA expression assays respectively (Applied Biosystems, Foster City, CA).

miRNA expression was evaluated using 5ng of initial RNA sample and 3 replicates per sample in a 7300 ABI Real Time PCR system. The relative amount of each miRNA was normalized to snRNA U6 endogenous control and the fold change calculated as described by the equation $\mathbf{2^{-\Delta\Delta Ct}}$, where $\Delta\Delta Ct = (Ct_{Wt\ miRNA} - Ct_{Wt\ U6}) - (Ct_{KO\ miRNA} - Ct_{KO\ U6})$.

mRNA expression was quantified by qRT-PCR using 48.48 Dynamic array (BioMark System, Foster City, CA). For each sample 3 technical replicates were examined with 100 ng of initial RNA sample used. Gapdh was select as the endogenous control and similarly fold change calculated accordingly to the $2^{-\Delta\Delta Ct}$ method.

2.8) miRNA target prediction and functional analysis

To predict miRNA target genes a multi-step sequential approach was performed.

The list of significant miRNAs obtained from the match of SAM and Genespring analysis was individually searched against the Argonaute database to identify already described and validated mRNA targets of the miRNAs in the study. (http://www.ma.uni-heidelberg.de/apps/zmf/argonaute/) The previously described miRNA targets found in this database were compared with the experimental mRNA expression profiles. Only mRNAs with differential expression were considered as potential miRNA target genes.

Statistically significantly changed miRNAs were subjected to *in silico* target prediction using the public TargetScan 4.2 algorithm incorporated in the GeneSpring software. (http://www.targetscan.org) Matching of subset gene lists obtained for statistically significant miRNAs predicted genes and mRNAs profiled by microarrays allowed the identification of most probable functional miRNA:mRNA interactions. Predicted genes, that didn´t show an inverse differential expression when compared with their associated miRNAs, were excluded from the group of genes of interest.

Genes targeted by several miRNAs differentially expressed in miRNA microarray analysis and overrepresented in significant functional categories were preferentially kept for further research.

To characterize gene ontology categories overrepresented in the knockout mouse model of XLMTM, GOstat software associated with the MGI (mouse genome informatics) GO database was used. The minimum of 3 genes per functional class and an associated p-value<0.02 were used as inclusion criteria. (http://gostat.wehi.edu.au) (Beissbarth and Speed, 2004)

The precise genomic location and nucleotide sequence of each miRNA was determined using miRBase (Wellcome Trust Sanger Institute; http://microrna.sanger.ac.uk). miRBase and BLAST allowed search for evolutionary conservation of microRNAs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). (Griffiths-Jones et al., 2006)

3) Results/Discussion

In this work we have used a genome wide microarray based approach to identify miRNA-mediated regulatory networks in XLMTM. miRNA expression profile was evaluated using a *Mtm1* mouse model to assess miRNAs potentially contributing to myotubular myopathy. In parallel, transcriptional profiles were generated to investigate the occurrence of miRNA/predicted target mRNA pairs anti-correlations. The resultant subset of target genes and associated enriched pathways might represent significant miRNA-regulated circuits in the pathophysiology of myotubular myopathy.

3.1) miRNAs are differentially expressed in XLMTM

In an attempt to identify miRNAs expression changes in myotubular myopathy, microarray experiments were performed for 8 *Mtm1* (49 days) mice as well as 8 age-matched WT control mice using Affymetrix DiscoVarry chips. These chips were based on miRBase 9.2 that contained >13000 miRNAs and representated by all miRBase organisms including *Mus musculus* (mmu) and *Homo sapiens*. In our studies, only results of hybridization of RNA samples with mmu probes were considered for analysis. Each miRNA was represented by two probes on the chip and depending on the efficiency of hybridization, they were classified in to two groups: A and B. Among these two groups, probes A (108 miRNAs) provided superior reliability and were further used for data analysis by Genespring Software.

Quality control analyses were done to identify potential outliers among 8 *Mtm1* and 8 WT control samples by performing Principal Component Analysis (PCA) and Pearson Correlation. All samples showed a good correlation by these approaches except two control samples: WT2 and WT5. Moreover, these two samples also showed showed a distinct pattern of expression when compared with the other samples of the same class by Hierarchical classification so were further excluded from the experiment. (Appendix Fig.1 and Fig.2)

After removing the outlier samples, the 3D-PCA aggregated samples in two different clusters which corresponding to two conditions in this study: *Mtm1* mice and WT control muscle groups. The clear separation of muscle samples in two different classes on PCA plot illustrated the unique miRNA expression profile in myotubular myopathy disease vs normal condition. (Fig.2)

These samples were then normalized using variance stabilization method. Normalized samples were analyzed by a box whisker plot to compare homogeneity among all samples. In each box plot, the box represents the main body of the data and the whiskers show the extreme values.

The variability is indicated by the size of the box and the length of the whiskers. A box whisker plot of all normalized samples showed a low variability among them suggesting high quality of hybridization data. (Fig. 2)

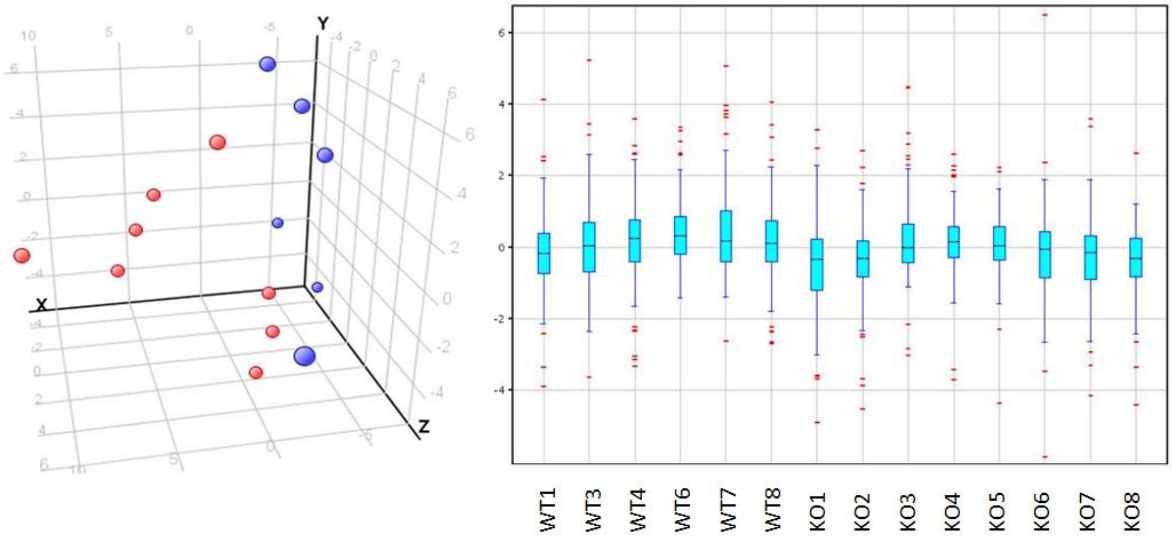

Figure 2: miRNA microarray data analysis for endstage mice. a) 3D-PCA analysis shows a robust distinction of the two classes (WT and KO) and reveals small variance intra-populations. b) Box Whisker Plot reflects a consistent homogeneity of the samples after normalization, as it is illustrated by the predominance of the values around 0.

Different kind of test statistics may be applied to microarray data. All of these tests are aimed at identifying p-values that help assess the likelihood that particular genes are regulated. Tests may be parametric or nonparametric. Parametric tests are applied to data sets that are assumed to follow a normal (Gaussian) distribution. Common parametric test for comparing two groups includes the *t*-test. Nonparametic tests do not make assumptions about the distribution of data. They rank the outcome variable (gene expression) from low to high and analyze the ranks. Mann-Whiteny and Wilcoxon tests are the most commonly used among nonparametric tests.

We analyzed our miRNA microarray data using both parametric as well as nonparametric statistical analysis methods to improve the confidence of obtained results. Only those miRNAs that were identified as significant in both methods were selected for further experimental validations. In nonparametric statistical analysis, GeneSpring GX software (version 10.0.2, Agilent Technologies) was employed to perform the Mann-Whitney test using a cut-off p-value<0.05. In parametric analysis SAM was used to perform *t*-statistics test with a FDR threshold of 18% to minimize the number of false positives. A high FDR value was selected to avoid exclusion of true

hits (Appendix Fig.3). A filter of fold change >1.5 was applied to the subset of list of significant results in both statistical methods.

27 miRNAs were obtained as significant in SAM and 16 miRNAs were identified using Genespring. 12 miRNAs matched both lists and therefore were chosen for subsequently studies (miR-127; miR-137; miR-337; miR-376a; miR-376c; miR-379; miR-434-3p; miR-434-5p; miR-410; miR-431; miR-489 and miR-541). (Table 1)

Table 1: Genespring and SAM results of miRNA microarrays data analysis for endstage mice. miRNAs highlighted in green were identified as significant using both statistical methods.

SAM				GeneSpring statistical Analysis			
Upregulated miRNAs	**q-value (%)**	**Downregulated miRNAs**	**q-value (%)**	**Upregulated miRNAs**	**p-value**	**Downregulated miRNAs**	**p-value**
mmu-miR-137	17,97	mmu-miR-434-5p	0	mmu-miR-137	0,0201	mmu-miR-127	0,002
mmu-miR-324-5p	17,97	mmu-miR-127	0			mmu-miR-329	0,010
mmu-miR-469	17,97	mmu-miR-376a	0			mmu-miR-337	0,020
mmu-miR-546	17,97	mmu-miR-541	0			mmu-miR-376a	0,020
mmu-miR-429	17,97	mmu-miR-434-3p	0			mmu-miR-376c	0,020
mmu-miR-344	17,97	mmu-miR-410	0			mmu-miR-379	0,002
mmu-miR-290	17,97	mmu-miR-431	8,66			mmu-miR-410	0,028
mmu-miR-367	17,97	mmu-miR-379	8,66			mmu-miR-411	0,010
mmu-miR-542-5p	17,97	mmu-miR-383	8,66			mmu-miR-431	0,039
mmu-miR-199b	17,97	mmu-miR-337	8,66			mmu-miR-434-3p	0,002
mmu-miR-465	17,97	mmu-miR-489	12,6			mmu-miR-434-5p	0,002
mmu-miR-464	17,97	mmu-miR-376c	12,6			mmu-miR-466	0,020
mmu-miR-295	17,97					mmu-miR-483	0,010
mmu-miR-224	17,97					mmu-miR-489	0,028
mmu-let-7d	17,97					mmu-miR-541	0,005

A hierarchical 2D-clustering constructed for these 12 statistically significant miRNAs using the Euclidean measure enabled to additionally investigate the clustering of the distinct patterns of expression of both groups. In agreement with the PCA comparison, the heatmap assorts miRNAs in mainly up or downregulated in knockout mice versus wild-type animals. Despite this observation, three mutant samples (MTM1-3, MTM1-5 and MTM1-8) showed an intermediate level of expression being separated from the remaining mutated samples on the clustering tree. (Fig.3)

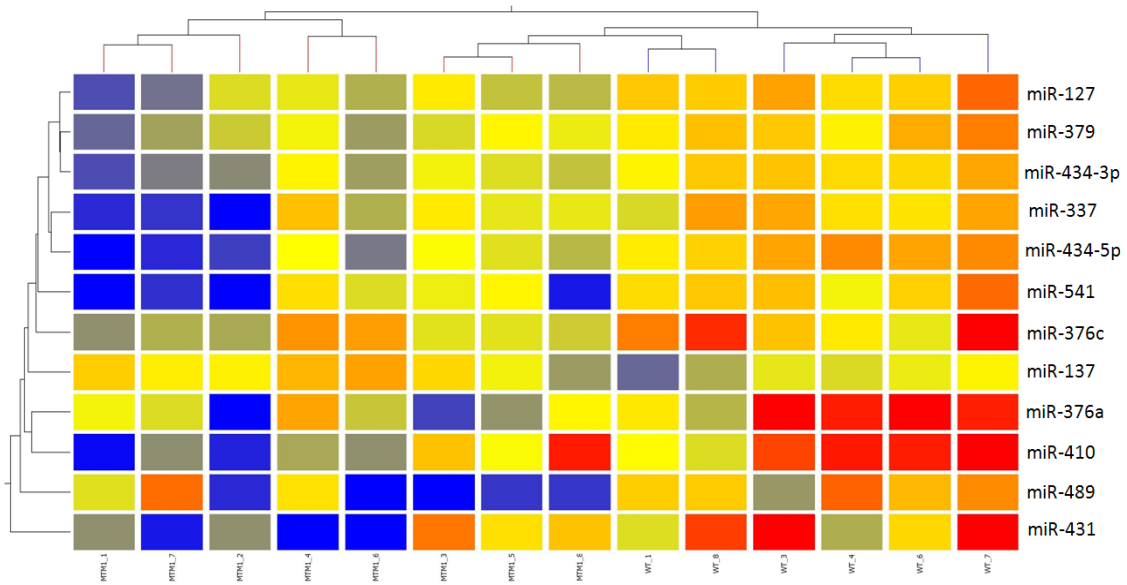

Figure 3: Hierarchical 2D-clustering produced for non-averaged significant miRNA lists via Euclidean distance metric and average linkage. The miRNA clustering tree is shown on the left and the sample clustering tree is shown on the top. The colored range represents the differential expression of miRNAs with red denoting <0 and blue >0.

3.2) Validation of differentially expressed miRNAs by qRT-PCR analysis

To validate the results obtained in microarray data analysis, qRT-PCR experiments were developed for 12 significantlly altered miRNAs identified from the match between SAM and statistical analysis using GeneSpring GX. (Table 2) All tested miRNAs, with the exception of miR-489, showed a good correlation between microarray and qRT-PCR results. (Figure 4) miR-24 and snRNA U6 were tested to use as internal control miRNAs. miR-24 showed significant up-regulation in mutants demonstrating not to be an appropriate reference miRNA for skeletal muscle expression experiments. snRNA U6 expression levels in mutant and control samples were indistinguishable. Thus, snRNA U6 was adopted for data normalization in qRT-PCR experiments.

To check the accuracy of our hypothesis that only those miRNAs that are common between two statistical analysis are significant, we also performed RT-PCR on few miRNAs that were excluded by parametric test, but considered significant by nonparametric and vice versa. No significant expression differences were observed for mir-411 (fold difference: significant by nonparametric) or miR-295 (fold difference: only identified as significant in parametric) between WT and *Mtm1* KO samples, corroborating the efficiency of the approach applied.

Table 2: Foldchange values obtained for microarray and qRT-PCR of 12 significant miRNAs p-value < 0.05 and FDR <18% was applied to Genespring and SAM analysis,respectively.

	Microarrays	qRT-PCR
mmu-miR-127	-3.09	-2.1
mmu-mir-137	1.75	2.36
mmu-miR-337	-3.27	-2.87
mmu-miR-376a	-6.89	-5.5
mmu-miR-376c	-3.16	-1.35
mmu-miR-379	-2.53	-1.4
mmu-miR-410	-5.62	-2
mmu-miR-431	-7.53	-3.4
mmu-miR-434-3p	-2.68	-2
mmu-miR-434-5p	-4.88	-2.01
mmu-miR-489	-4.39	-9.6
mmu-miR-541	-5.53	-4.4

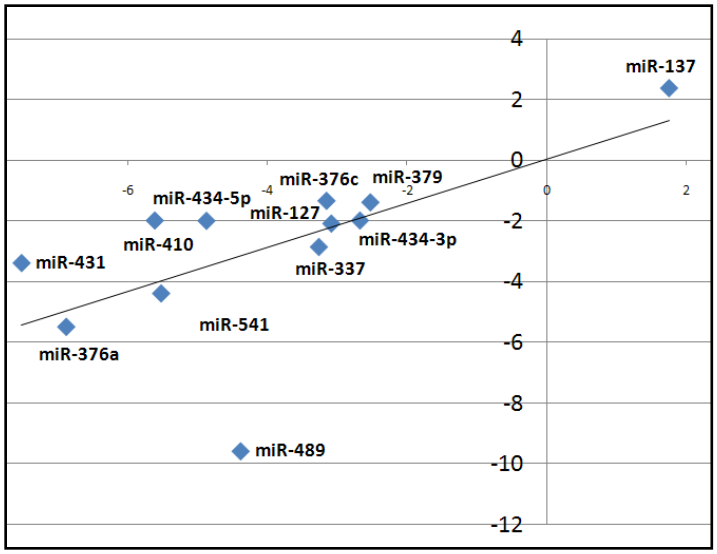

Figure 4: Linear correlation graph obtained with microarrays data in the X-axis and qRT-PCR values in the Y-axis for endstage mice.
Linear Trendline was applied. With the exception of miR-489, all miRNAs show a directly proportional variation of folchange between microarray and qRT-PCR values.

3.3) miRNAs genomic location

Several miRNAs identified in our study showed to be arranged in a tandem called Dlkq-Dio3 cluster array resulted from the processing of a single transcript designated *Mirg* (microRNAs-containing gene). *Mirg* is located in the Dlk1-Dio3 (Delta-like 1 homolog-type III iodothyronine deiodinase) imprinted domain in the distal portion of mouse chromosome 12 and respective human 14q32, and contains more than 40 miRNAs. (Table 3) (da Rocha et al., 2008)

miR-431, miR-127, miR-434, miR-379, miR-337, miR-541, miR-410, mir-376a, differentially expressed in our miRNA microarray, are located in this cluster. (Appendix Fig.4) miRNAs clustering is thought to represent a coordinated internal strategy to control more rapidly and efficiently common downstream pathways. Given that the same mRNA is often required to be target by several miRNAs, the expression of miRNAs regulated by the by same promoter sequence enables a powerful modulation of protein networks in response to a given stimulate. (Yuan et al., 2009) (Altuvia et al., 2005)

The biological importance of this domain, with ten genes detected so far, is illustrated by the resulted phenotypes obtained in Dlk1-Dio3 mutants. (Hagan et al., 2009) A single nucleotide mutation in Dlk1-Dio3 domain is responsible for the development of callipyge phenotype in sheep, characterized by extreme postnatal muscular hypertrophy, due to upregulation of Dlk1 and Rtl1 transcripts from this cluster. (Charlier et al., 2001) (Fleming-Waddell et al., 2009) Moreover, Dlk1 was reported to be involved in regulation of cell fate determination and skeletal muscle remodelling, showing high expression levels during development and on satellite cells in myopathic conditions. (Andersen et al., 2009)

Additionally, it was demonstrated the expression of Dlk1-Dio3 cluster requires Mef2 (myocyte enhancing factor 2) transcription factor activation in neurons. (Fiore et al., 2009) Mef2 is also a regulator of myogenesis, responsible for the coordination of muscle specific gene expression in developing embryos. These evidences suggest the potential induction and implication of this cluster in myogenenesis. *Mirg* expression (containing several microRNAs differentially regulated in this experiment) was never reported in skeletal muscle and mir-410 and miR-431 were identified as nervous system specific. (Wheeler et al., 2006) In this study we report for the first time the expression of miRNAs belonging to Dlk1-Dio3 domain in skeletal muscle of mice with 14, 27 and 49 days. The reason underling the non-detection of these miRNAs in previous experiments might correlate with the fact in situ analyses were developed for embryonic day 11.5. Primary mouse myogenesis starts between E12.5 and E14.5 and secondary fibers start to be established around E14-E16. (Buckingham et al., 2003) Further functional experiments, using in situ hybridization, should also be performed in adult mice sections to an accurate understanding of temporal expression patterns of identified differential expressed miRNAs.

miR-489 is an intronic miRNA located in the host gene calcineurin receptor precursor (CT-R)

miRNAs expression often correlates with expression of their respective host genes as well they involved in common biological pathways. In this study all miRNAs, with the exception of miR-489, are located either in the Dlk1-Dio3 cluster or in intergenic regions widespread in the genome. Interestingly, the host gene of miR-489 is the calcineurin-receptor precursor.

In adult animals, muscle growth is mainly mediated by hypertrophy, resulting in an increase in size, rather than in number, of pre-existing muscle fibers. Depending on external/internal stimulus, different molecular signaling pathways can be activated to increase intracellular Ca2+ levels, which subsequently promote not only muscle contractibility but also the induction of muscle specific genes required for hyperthrophy. Insulin-like growth factor (IGF-1) expression, mediated by the activation of phosphoinositide 3-kinase (Pi3K)/Akt/mTOR and PI(3)K/Akt/GSK3 pathways, is thought to be sufficient to induce hypertrophy. (Rommel C, 2001) However, Ca2+/calmodulin-dependent pathway, through the activation of calcineurin and subsequent interaction with GATA2 and NFATc1, was also reported to be required, although not sufficient, for hypertrophic mechanisms. (Musaro et al., 1999) (Semsarian et al., 1999)

Calcineurin is a Ca2+/calmodulin-activated phosphatase implicated in the phosphorilation state of NFAT (nuclear factor of activated T cell) transcription factor members. Calcineurin enables the activation and subsequent nuclei translocation of specific NFAT isoforms, which in cooperation with MEF2, are crucial in the modulation of muscle specific gene expression in different stages of skeletal muscle development. (Schulz and Yutzey, 2004) A direct role of calcineurin in myoblast differentiation was also observed in C2C12 myogenic cells. Calcineurin levels were demonstrated to be upregulated during terminal myogenesis, with the inhibition of calcineurin being responsible for a decrease myoblast ability to progress to myotube formation stage. (Delling et al., 2000)

Moreover, calcineurin signaling appears to be crucial in reprogramming of myofiber-specific gene expression, promoting the transition from resting IIb fiber types to slow contractile type I fibers (muscle fibers able to sustain high concentrations of intracellular calcium as a result of tonic motor nerve activity). (Naya et al., 2000) (Chin et al., 1998)

In this project it was not observed a significant change in the expression levels of calcineurin, however calmodulin levels were found to be downregulated. In several neuromuscular disorders, and mainly in congenital myopathies, higher incidence of atrophy in type I (slow) muscle fibers is mainly observed. (Imoto and Nonaka, 2001) In myotubular myopathy this phenomenon is not a constant observation, however type I predominance is often detected. (Pierson et al., 2005)

Table 3: Significant miRNAs identified by bioinformatic analysis and their genomic location in mouse. The data below was obtained from miRBase (miRNA database).

miRNA ID	Chromosome location	Genomic region	Gene description
mmu-miR-137	chr 3	Intergenic region	
mmu-miR-337	chr 12	Intergenic region	
mmu-miR-127	chr 12	Rtl1	Retrotransposon-like protein 1
mmu-miR-376a	chr 12	Intergenic region	
mmu-miR-376c	chr 12	Intergenic region	
mmu-miR-379	chr 12	Intergenic region	
mmu-miR-410	chr 12	Intergenic region	
mmu-miR-431	chr 12	Rtl1	Retrotransposon-like protein 1
mmu-miR-434-3p	chr 12	Rtl1	Retrotransposon-like protein 1
mmu-miR-434-5p	chr 12	Rtl1	Retrotransposon-like protein 1
mmu-miR-489	chr 6	Calcr	Calcitonin receptor Precursor
mmu-miR-541	chr 12	Intergenic region	

3.4) Biological relevance of identified miRNAs

Significant miRNAs involved in cell-cycle arrest and progression to differentiation state

It is noteworthy the observation that several miRNAs found differentially expressed in this study were consistently reported as cell cycle regulators in previous investigations. (Table 4)

miR-137, in cooperation with miR-124, was identified in glioblastoma multiforme tumors to inhibit cellular proliferation. Furthermore, it was revealed miR-137 induces differentiation of mouse neural stem cells. (Papagiannakopoulos and Kosik, 2008; Silber et al., 2008)

Conversely, miR-489 was found to be down-regulated upon differentiation and when inhibited promote differentiation of human mesenchymal stem cells - progenitor cells with multi-lineage capacity that can generate myocytes. (Schoolmeesters et al., 2009)

Despite different results were obtained for different tumors miR-127 was also associated with the modulation of cell cycle. In invasive squamous cell carcinomas patients, this microRNA was upregulated (suggesting the role in the promotion of proliferation). Also, it was identified as a potential biomarker for lymph nodes metastasis in this condition. (Lee et al., 2008) In contrast with these studies, in hepatocellular carcinoma miR-127 was shown to be downregulated in the early stages of the disease in a rat model. (Tryndyak et al., 2009)

In our data, miR-137 is overexpressed, while miR-127 and mi-489 are downregulated in the later, most severe stage of the disease. Overall, we might speculate the process of myogenesis

is being promoted while proliferation supressed, with miRNAs reported to trigger differentiation upregulated (miR-137) and miRNAs responsible to suppress this process downregulated (miR-489, miR-127) in endstage Mtm1-deficient mice.

Table 4: Previously described functions of 12 identified miRNAs

Genomic location	miRNA name	Cellular function
Cluster Dlk1-Dio3	miR-337, miR-376a, miR-376c, miR-379, miR-410, miR-431, miR-434-3p, miR-434-5p, miR-541	Cooperative role regulated by MEF2; Induction of myogenesis?
	miR-127	Cell cycle regulator
Intergenic and intronic region	miR-137	Inhibition of proliferation and induction of differentiation
	miR-489	Induction of stem cells differentiation

3.5) miRNAs expression differences during mouse development

Subsequently to the identification of statistical significant miRNAs by bioinformatic analysis of microarrays and their experimental validation by qRT-PCR we decided to assess their differential expression in two additional developmental time points. Through an integrative analysis of miRNA differential expression changes in 14, 27 and 49 days mutant animals, compared to respective controls, we aimed to deeper understand the role of selected miRNAs in the progression of myotubular myopathy severity.

Real-time PCR experiments detected the expression of all 12 tested miRNAs in skeletal muscle of 14, 27 and 49 days mice. (Figure 5) Comparing to endstage (49 days) mice which manifest significant levels of identified miRNAs downregulation, 14 and 27 days mice only present slight expression changes in KO/WT analysis. A pattern of increasing or decreasing foldchange sequentially from 14 days to 27 and finally 49 days, was not observed for miR-127, miR-376a, miR-376c, miR-379, miR-410, miR-431 and miR-434-3p. These results might indicate that the function of specified miRNAs is only triggered and biologically relevant in the most severe stage of the disorder. Conversely, miR-337, miR-434-5p, miR-489 and miR-541 show a significant progressive decrease in foldchange (KO/WT) values from the assymptomatic stage of the disorder (14 days

mice) to their terminal most severe stage (endstage 49 days mice). Based on these evidences we suggest a direct involvement of these microRNAs in the evolution and associated increase in phenotypic severity of myotubular myopathy. Finally, we highlight miR-137 and miR-489 for presenting a constant pattern of up and downregulation in mutants, respectively, throughout mouse development. miR-137 and miR-489 might represent main fundamental miRNAs essential for the etiology and manifestation of XLMTM.

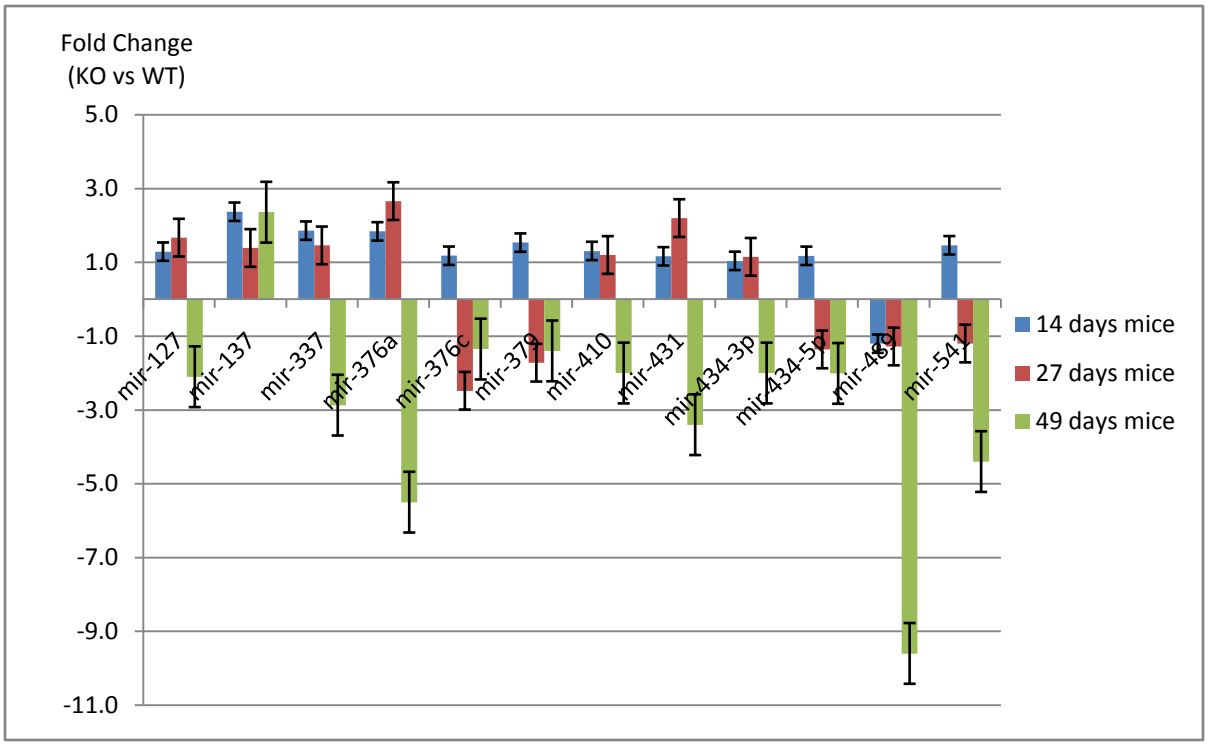

Figure 5: qRT-PCR foldchanges values for KO/WT 14 days, 27 days and 49 days mice. snRNA U6 was used as internal control with foldchange calculated using the ΔΔCt method.

3.6) mRNA expression profile of XLMTM

Following the identification of 12 differentially expressed miRNAs for subsequent *in silico* target prediction, we further restrict our analysis to mRNAs statistically differentially expressed in myotubular myopathy to increase the biological relevance of this study. Affymetrix Exon 1.0 ST array was used for mRNA hybridization that contains 27 probes for each of the approximately

29.000 genes. We applied the same criteria as for miRNA microarrays data analysis: VSN method for normalization, p-value<0.05 and fold difference of 1.5 as cut-off. 424 genes were found differentially expressed in XLMTM. In this experiment outliers identified in miRNA profiling were not included. An additional sample, KO7, was excluded by Spearman rank correlation quality controls performed by Affymetrix. (Appendix Fig.5)

In order to determine the functional categories potentially dysregulated in XLMTM, gene ontology enrichment using GOstat was estimated. GOstat assigns overrepresented functional categories according to associated significant p-values within a set of genes. Using as primary entity list the previously identified 424 differentially expressed mRNAs we obtained 8 significant functional groups (corrected p-value<0.02). In agreement with the expected, the resulted categories are directly associated with the pathophysiology of myotubular myopathy. Functional groups related to sarcomere structure, muscle development processes and protein network regulation were found overrepresented. (Appendix Table1) The muscle-specific phenotype characteristic of XLMTM rationalizes the data and corroborates the biological importance of further studies based on these genes.

3.7) Identification of miRNA-target interactions

miRNA profiling and subsequent qRT-PCR validation revealed differential expression of miRNAs in *Mtm1*-deficient mice compared to controls, demonstrating their involvement in myotubular myopathy. Each of the computational miRNA target prediction tool provides hundreds of target genes that may be regulated by a particular miRNA. Most of those predicted target genes may not be regulated in all tissue types or diseases sates. Further, a larger number of false positives also make the identification of real targets a very tedious process. To decipher functional miRNA/target gene networks in myotubular myopathy, we decided to identify significantly altered transcripts by mRNA profiling. All the genes that were thus identified were then matched with the list of 12 significantly altered miRNAs to identify their target genes in myotubular myopathy.

mRNA profiling was performed in the same Wt/*Mtm1* mice samples that were used for miRNA profiling earlier. This led us to identification of 424 transcripts that showed differential expression between *Mtm1* and WT mice. We next identified genes from these 424 genes that were a direct target of any of the 12 miRNAs that are significantly altered in Myotubular myopathy using Genespring.

There are several computational tools that are commonly used to identify miRNA target genes such as Targetscan, Miranda, PicTar and MicroCosm. Genespring has Targetscan integrated in its module and is widely used to identify targets with high accuracy. Combing the mRNA data (424 genes) to identify potential 12 miRNA targets resulted in 142 direct target genes of these miRNAs. (Figure 6)

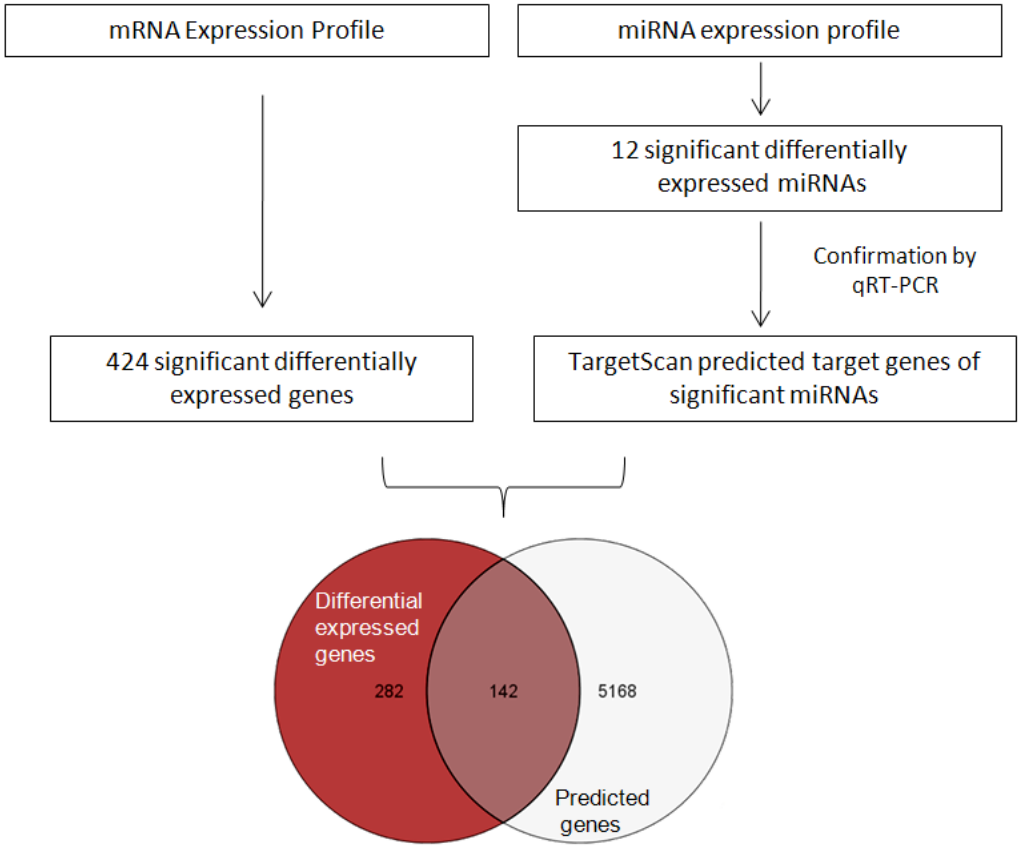

Fig 6: Skematic representation of combinatorial approach used to identify significant miRNAs and their mRNA target genes. miRNA as well as mRNA expression profile were performed using microarrays. Statistical analysis considered p-values<0.05 and foldchange>1.5 cut-offs. 424 significant mRNAs were overlapped with the predicted target genes for the 12 identified miRNAs, using TargetScan software. The resulted matching list contains 142 genes and is

3.8) Functional categories enriched in potential miRNA-regulated genes correlates with disease phenotype

The group of 142 candidate miRNA-regulated genes differentially expressed in XLMTM, was subsequently classified in overrepresented functional groups using GOstat. Interestingly, although the main categories involved in muscle development and sarcomeric composition overlapped the functional groups obtained for the 424 genes differentially expressed in mRNA expression profile, additional categories were highlighted for the same p-value<0.02.

Regulation of the progression through cell cycle, cardiac muscle morphogenesis and muscle contraction we also identified as enriched categories. (Table 5) These results are in conformity with previous reports suggesting the role of some of the identified miRNAs in the regulation of cell cycle. (Papagiannakopoulos et al., 2008) (Tryndyak et al., 2009) (Schoolmeester et al., 2009)

Furthermore, due to muscle injury in XLMTM patients and mice models, the observed overrepresentation of cardiac genes (skeletal muscle embrionary protein isoforms) might represent a biological compensatory mechanism to restaure muscle integrity and homeostasis. This strategy was previously described for several other congenital myopathies, including nemaline myopathy. (Laing NG, 2007) Patients with nemaline myopathy associated with null mutations in the alpha-skeletal muscle actin are only able to survive due to a re-expression of cardiac (embryonary) actin isoform that partially compensates for the absence of alpha-actin, required for muscle structure and function. (Nowak et al., 2007)

Finally, the identification of muscle contraction category is in agreement with recent studies that postulate myotubular myopathy results in defective excitation-contraction coupling due to transverse-tubules (T-tubules) structure destabilization and impaired ryanodine-mediated sarcomeric reticulum Ca^{2+} release. (Dowling et al., 2009) (Al-Qusairi et al., 2009) A deficiency in the production of myotubularin dysregulates the levels of phosphoinositides synthesis and degradation, required for proper regulation of endocytosis dynamics and membranous structures remodeling.

Muscle contraction requires the transfer of action potential from the sarcollema (in particular from T-tubules) to the sarcoplasmic reticulum to trigger Ca^{2+} release and subsequent Ca^{2+} dependent signaling pathways activation. T-tubules are sarcolemma invaginations constituent of the triads that transmit the signal to terminal cisternae promoting the induction of Ca^{2+} release from the internal Ca^{2+} storage compartment sarcoplasmic reticulum to the cytoplasm through

RyR1 channel. As membranous structures, T-tubules function, and consequently excitation-contraction coupling, is dependent on phosphoinositide activity regulated by PI3 kinases and phosphatases, such as myotubularin. (Flucher, 1992) (Frank and Oz, 1992)

Given the described evidences of biological relevance of the functional categories enriched in this experiment for myotubular myopathy development, we further decided to focus on miRNA-regulated mRNAs differentially expressed included in these groups.

Table 5: Gene ontology categories and respective genes integrated for p-value <0.02 of significant genes matching mRNA expression profile and predicted genes of significant miRNAs. Total of 142 genes analysed.

Most significant GO categories	**Genes**
GO:0005515 **(Protein binding)**	ube2g2 rb1 klhl31 abcd2 tcf12 lrrc2 trim63 app bhlhb3 phka1 adssl1 col8a1 btg2 mkl1 homer1 igfbp5 napb ablim1 csrp3 postn sorbs1 dmd gadd45a ranbp9 mafk flnc lrrn1 mstn jak2 tiam2 ncam1 esr1 erbb2ip frzb errfi1 lrrc58 mtap1b arntl atp1b2 tnni1 ctgf actc1 rasgrp3 ankrd1 enah cnksr1 atf3 cdkn1a atp2a2 gabarapl1 tlr4 dapk1 kcnn3 runx1 macf1 calm3 ar palld arl6ip5 calm2 fbxw7 hexa hdac4 aspn
GO:0043292 **(Contractile fiber)**	trim63 dmd actc1 ankrd1 myoz2 tnni1 tnnt2 csrp3
GO:0005856 **(Cytoskeleton)**	jak2 gabarapl1 ablim1 rb1 csrp3 sorbs1 dmd hspb7 app mtap1b macf1 myoz2 tnni1 tnnt2 flnc palld actc1 eml1 enah
GO:0005737 **(Cytoplasm)**	2610207i05rik ube2g2 oxct1 abcd2 msrb3 trim63 app adssl1 alas1 col8a1 mkl1 homer1 egln3 h6pd napb slc25a24 ablim1 csrp3 sorbs1 dmd ranbp9 flnc nploc4 jak2 tiam2 esr1 fmo2 cyb5r3 errfi1 hpgd mtap1b atp1b2 ces3 tnni1 tnnt2 actc1 rasgrp3 ankrd1 eml1 cnksr1 enah atp2a2 cdkn1a gabarapl1 bex2 chodl dapk1 glul nt5c2 psmd3 macf1 myoz2 ar calm3 tfrc ak3 palld arl6ip5 calm2 hexa snf1lk fbxw7 hdac4 atp6v1a
GO:0007517 **(Muscle development)**	app chrna1 rb1 tnni1 tnnt2 csrp3 dmd actc1 trp63
GO:0000074 **(Regulation of progression though cell cycle)**	app cdkn1a macf1 loh11cr2a rb1 esr1 trp63 gadd45a snf1lk
GO:0055008 **(Cardiac muscle morphogenesis)**	actc1 tnni1 tnnt2
GO:0006936 **(Muscle contraction)**	trim63 atp2a2 actc1 tnni1 tnnt2

Additional biological considerations were also taking into account to select genes for subsequent research. miRNAs negatively modulate gene expression by leading to mRNA target degradation, guiding of cognate mRNAs to P bodies for subsequent inactivation or complete degradation or by directly repressing their translation. (Bartel, 2004; Rana, 2007) In light of this mode of action if a miRNA is downregulated its target gene is expect to be upregulated, and the opposite respectively. Following this concept we next searched for mRNA target genes that showed an inverse differential expression comparing to their candidate differentially expressed miRNAs.

For further fine-mapping of potential important miRNA-mRNA interactions we prioritized genes overrepresented in common functional ontology categories and mRNA targeted by several miRNAs to reduce the number of false positives. (Ivanovska and Cleary, 2008; Krek et al., 2005)

This approach promoted the exclusion of miR-337, miR-376a and their single target genes from the list of miRNAs/mRNAs to deeper investigate.

The genes Ablim1, Actc1, Carp (Ankyrin 1), Cdkn1a (p21), Csrp3, Gadd45a, Jak2, Macf1, Mtap1b, Myoz2, Tnni1, Trim63 (Murf1), Trp63, Frzb, Hdac4, Homer 1, Igfbp5, Runx1 passed the described criteria and consequently were selected for subsequent analysis. In addition, a subset of genes differentially expressed in the mRNA expression profile that are not direct targets of miRNAs but are known to work in conjugation with the miRNA target genes were also included to identify regulatory networks operating in myotubular myopathy. (Table 6)

Among these Tnnt2, Rb1, Mstn, Foxo1, Fbxo32, Myog and Gas5 genes were found differentially expressed in XLMTM and selected based on their known role in muscle development and repair. (Table 7). To validate the differential expression of these genes qRT-PCR array was performed. qRT-PCR confirmed the results of microarray expression data in these mice, with the exception for plcd4 gene. (Fig. 9)

Table 6: Genes selected for further studies based on mRNA expression profile and predicted target genes of significant miRNAs list (142 genes). TargetScan was used to identify target miRNA target genes.

Gene Name	Description	miRNA target
Ablim1	actin-binding LIM protein 1	miR-434-3p, mir-376c
Actc1	actin, alpha, cardiac	miR-434-3p
Carp	ankyrin repeat domain 1 (cardiac muscle)	miR-431
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	miR-379
Csrp3	cysteine and glycine-rich protein 3 (muscle LIM protein)	miR-431
Gadd45a	growth arrest and DNA-damage-inducible 45 alpha	miR-489
Jak2	Janus kinase 2	miR-541
Macf1	microtubule-actin crosslinking factor 1	miR-431, miR-410, miR-379
Mtap1b	microtubule associated protein 1b	miR-376c, miR-379
Myoz2	Myozenin2	miR-410, miR-379
Tnni1	troponin I, skeletal, slow 1	mir-489
Trim63	tripartite motif-containing 63	miR-431, miR-410
Trp63	transformation related protein 63	miR-434-3p, miR-410
Hdac4	histone deacetylase 4	miR-127
Homer 1	Homer homolog 1 (Drosophila)	mir-410
Igfbp5	insulin-like growth factor binding protein 5	miR-137
Runx1	Runt related transcription factor 1	miR-541

Table 7: Genes selected from microarrays analysis for further studies. Potential genes involved in the pathways regulated by miRNAs were selected.

Gene Name	Description	miRNA target
Dusp4 (MKP-2)	dual specificity phosphatase 4	miR-434-5p
Plcd4	phospholipase C, delta 4	miR-410
Tnnt2	troponin T2, cardiac	
Rb1	retinoblastoma 1	
Mstn	Myostatin	
Foxo1	forkhead box O1	
Myog	Myogenin	
Fbx032	F-box protein 32	
Gas5	Growth arrest-specific 5	
Frzb	Frizzled related protein	

3.9) Biological pathways modulated by miRNAs relevant in the etiology of XLMTM

3.9.1) Muscle atrophy molecular pathways are upregulated in XLMTM

Our studies led us to identify several miRNA target genes that play a role in musclular atrophy. *Mtm1* knockout mice show remarkable atrophy in muscle fibers as the diseases progresses. Muscular atrophy represents a decrease in myofiber size due to a negative imbalanced ratio of protein synthesis versus protein degradation, hence shifting towards protein wasting. FoxO1 is a key regulator of muscular atrophy and found to be consistently upregulated in this study (1.63 folds upregulated in *Mtm1* mice). FoxO1 transgenic mice, overexpressing FoxO1 also show marked muscle loss and fiber atrophy. (Kamei et al., 2004) FoxO1 is an important member of Akt/Pi3K signaling pathway and leads to muscle atrophy in response to decreased Akt/PI3K signaling. It functions by upregulation of key components of ubiquitin- proteosome pathway that are involved in protein breakdown and are increased during atrophy (Sandri M, 2008) In particular, the muscle-specific E3 ubiquitin ligases Murf (also denominated Trim63) and Mafbx (also called Fbx032) are identified as required markers for atrophy that are activated by FoxO1 (Bodine et al., 2001). We found both Muscle RING finger protein 1 (Murf1) +2.68 as well as Muscle atrophy F-box protein (Mafbx or Fbxo32) +3.16 were overexpressed in mutants Therefore our results demonstrate the involvement of FoxO1 mediated activation of Murf1 and Mafbx and suggest the possible implication of supressed Pi3K/Akt pathway in myotubular myopathy.

We further identified two miRNAs, miR-431 and miR-410 that are downregulated in *Mtm1* KO mice and target Murf1 which we found to be upregulated and is a crucial regulator of atrophy. These results suggest multiple levels of activation of muscular atrophy pathways dependent/independent of miRNAs. Further functional studies will provide detailed insights in to contribution of these miRNAs in to muscular atrophy seen in myotubular myopathy. (Figure 7)

3.9.2) Structural sarcomeric proteins implicated in muscle integrity are overexpressed in XLMTM

In XLMTM, integrity of myofibrillar apparatus predominates, however, Z-streaming or complete sarcomeric loss is often reported. Coordinate expression changes in the levels of structural proteins harboring the link between the sarcomere and the nucleus might be crucial during disease status. Different specialized compartments in the sarcomere orchestrate distinct proteins relevant for intracellular signalling. In M-bands predominate protein-kinase regulated ubiquitin signaling and protein turnover mechanisms, while I-bands and Z-disks are mainly constituted by stretch sensitive pathways associated with transcription factors modifiers. (Laing NG, 2008)

Interestingly, we identified several structural sarcomeric proteins upregulated in *Mtm1* mice. Conversely, miRNA targeting these structural proteins were found to be downregulated in diseased state. Many of these sarcomeric genes have found to be upregulated in muscular atrophy in other myopathies as well. ankyrin repeat domain 1 (Ankrd1 or CARP) showed in microarrays analysis an upregulation in mutants of +7.30. Ankrd1 is a skeletal and cardiac muscle expressed transcription cofactor protein known to interact with titin/connectin and myopalladin. Mutations in Ankrd1 were described to be associated with the development of hypertrophic cardiomiopathies, revealing its requirement for muscle architecture. (Moulik et al., 2009) (Arimura et al., 2009) Ankrd2 was also upregulated in mRNA expression profile (+2.91). Ankrd2 shares high levels of homology with Ankrd1 and belongs to the same muscle ankyrin repeat proteins family (MARP). Ankrd2 is preferentially expressed in slow muscle fibers and cardiac muscle and was postulated to function in cooperation with Ankrd1 in the modulation of skeletal muscle gene expression programming in response to signaling pathways. Marp family function is still mainly unknown although increasing evidences support its role in sarcomeric structure integrity and function in the modulation of muscle-specific gene expression in muscular pathology context, such as atrophy. (Barash et al., 2007) (Miller et al., 2003) Ankrd1 and Ankrd2 were also suggested to

induce the expression of the cell cycle inhibitor gene p21 (+4.8 upregulated in our experiment and present in the 142 genes list), MLP (also +3.9 upregulated in 142 entity list) and regulate the expression of MyoD. (Kojic et al., 2004; Laure et al., 2009)

Other sarcomeric proteins such as Csrp3 (cysteine and glycine-rich protein 3 or muscle LIM protein) showed an upregulation of +3.9 and Myozenin2 (Myoz2) an upregulation of +2.4 in diseases muscles. Muscle LIM protein (encoded by the csrp3 gene upregulated in XLMTM) has a dual role by colocalizing with Z-disks and nucleus. In the first scenario, MLP function as a linker scaffolding protein fundamental to muscle integrity. (Barash et al., 2005) By presenting the ability of translocation to the nucleus MLP is involved in muscle sensory signaling pathways and promotion of myogenesis in differentiating muscle cells. (Arber et al., 1994; Flick and Konieczny, 2000) Interestingly, Csrp3 and Ankrd2 were also found upregulated in nemaline myopathy, a severe non-dystrophic congenital myopathy. (Sanoudou et al., 2006) In addition, Csrp3 was found to be upregulated in muscle injury conditions (Barash et al., 2004) while mutations associated with this gene promoted hypertrophic cardiomyopathy. (Geier et al., 2008; Knoll et al., 2002) Finally, Myozenin 2 (or Calsarcin-1) is an additional Z-disk interacting protein known to be mutated in cardiac hypertrophy conditions.

As noted earlier many miRNAs that target these sarcomeric proteins were found to be downregulated in myotubular myopathy. miR-431 that targets Ankrd1 and Crsp3 is significantly decreased (7.31 folds) in Mtm1 mice. miR-431 also targets Murf1 another gene that is upregulated in muscular atrophy as discussed earlier. Similarly, miRNA 379 that is found to be down regulated in diseases state also have several direct targets that are upregulated in Mtm1 mice as well in muscle atrophy. Therefore, our work identified few miRNAs that regulate a number of muscle atrophy causing genes. These studies suggests that these miRNA many not be specific to myotubular myopathy and many be operating in a number of muscle diseases involving muscle atrophy. Nonetheless, overexpression of these miRNAs may downregulate the genes involved in muscular atrophy and slow down the muscle damage and progression of myotubular myopathy. (Figure 7)

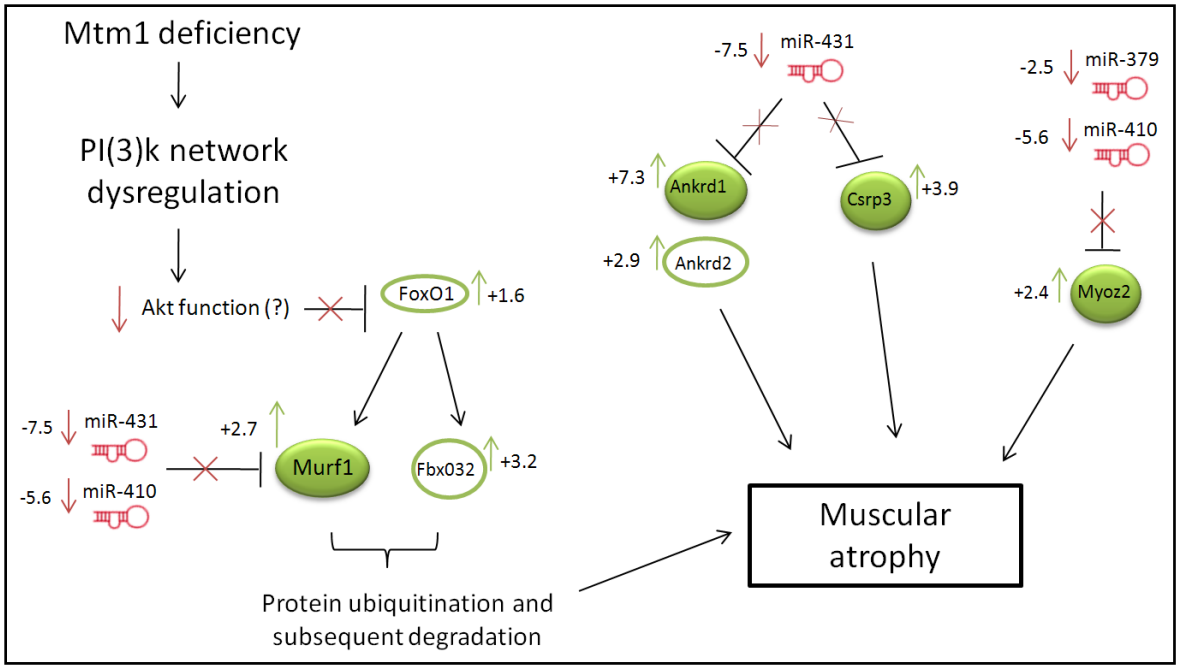

Figure 7: Putative molecular pathways involved in muscular atrophy are postulated to be mediated by miRNA gene expression regulation in XLMTM. The schematic diagram presented was constructed based on differential expression values in microarrays experiments. Positive values (and green molecules) represent gene or miRNA downregulated in mutants comparing to controls, while negative values and associated red molecules illustrated genes or miRNAs upregulated. Green filled schematic genes represent identified potentially miRNA-regulated genes in the 142 gene list. White filled molecules surrounded by green line represent genes only significantly differentially expressed in the mRNA expression profile, present in the 424 gene list.

3.9.3) Inhibition of proliferation and induction of differentiation in satellite cells

Myogenesis requires the coordinate activation of genes responsible for cell cycle withdrawal and muscle lineage commitment or differentiation. We identified a robust upregulation of cell cycle arrest genes as well as muscle differentiation markers in diseased muscles. (Figure 8) p21, a predicted target of miR-379 upregulated in XLMTM, is a cyclin-dependent kinase inhibitor of G1 cell cycle progression that directly suppresses the activity of cyclin-CDK2 and cyclin-CDK4 complexes that are required for cell cycle progression. p21 expression is required for irreversible cell cycle exit upon myocyte differentiation. In agreement with these data and gene expression profile in XLMTM, retinoblastoma 1 (Rb1) and myogenin (Myog) were also found upregulated in this condition. Rb1 is a tumor suppressor gene that negatively regulates the progression from G0 through to G1 and into S phase. (De Falco et al., 2006; Mal et al., 2000) Rb together with myogenin (a marker of differentiation) also plays a key positive role in the activation of muscle specific genes during myogenesis. Also described to promote cell cycle arrest, Gadd45a (growth-arrest and DNA-damage inducible 45 alpha) was shown upregulated in this experiment, possibly as a result of the decrease expression of its candidate negative regulator miR-489.

Additionally, the actin-binding protein 1 (ablim1) previously described as a tumor suppressor gene, was also detected upregulated in this experiment and is a target of miR434-3p and miR-376c. It will be interesting to identify if each of these miRNA acts independently or miR-434-3p and miR-376c cooperatively regulate ablim1 expression. Downregulation of these miRNAs and subsequent upregulation of ablim1 in XLMTM might contribute to cell cycle exit. (Kim et al., 1997)

Another upregulated protein with tumor suppressor gene function associated with XLMTM was dusp4, a predicted target of miR-434-5p. Dusp4 (also named MKP-2) is a dual-specificity phosphatase of the activated MAP kinases ERK1 and ERK2, which plays an essential role in mitogen-regulated growth factor signal transduction. (Armes et al., 2004) Erk1/2 functions by phosphorylation of MAPKK in response to growth factors and/or ligand molecules. An increase in Dusp4 can result in a subsequent decrease in Erk1/2 activation affecting MAPK and or Akt signaling pathways. These are the first studies that hint towards an involvement of MAPK signaling pathway in myotubular myopathy. Our hypothesis is further strengthened by a recent study in which inhibition of Erk1/2 kinases led to reduction of myotube size and protein content. This

effect of MAPK pathway on muscle growth is specific to Erk1/2 as inhibition of other MAPK pathways i.e. JNK or p38 did not show any affect on muscle growth (Shi et al., 2009) Further over-expression of one of the MAPK-phosphatases, MAPK1 in soleus as well as gastrocnemeus muscles decreased the fiber size. These studies clearly suggest the role of MAPK signaling in skeletal muscle maintenance as inhibition of this signaling result in muscular atrophy.

In addition to an increase of cell cycle inhibitors in *Mtm1* KO mice we also found an upregulation of several genes involved in the control of stem cell growth, particularly satellite cells. Muscle satellite cells account for 2-5% of the total cell population in adult skeletal muscle where they play a major role in muscle regeneration. Under normal conditions satellite cells are mitotically quiescent in the adult skeletal muscle. When activated by muscle damage, they proliferate, differentiate and fuse with each other or injured fibers and eventually regenerate mature myofibers. Importantly, a small fraction of activated satellite cells exit the cell cycle and return to the quiescent satellite state during muscle regeneration to maintain their number and regenerative capacity of muscle. Thus, normal functioning of satellite cells are indispensable for the integrity of skeletal muscle, and the cells themselves are an important source of cells for cell therapy of muscle diseases, making it valuable to clarify the molecular regulation of maintenance, activation/proliferation, and differentiation in satellite cells.

Syndecan-4, a marker of satellite cells, was seen upregulated in *Mtm1* mice. Syndecan-4 expression in adult mice is restricted to satellite cells and in particular into the quiescent population, suggesting its role in the maintenance of quiescent satellite cell population.. (Cornelison et al., 2001) Gas5 (growth arrest-specific gene 5) another marker of quiescent satellite cells was also found upregulated in *Mtm1* mice. (Coccia et al., 1992) Trp63 (transformation related protein 63) that is necessary for adult stem cells maintenance was also seen to be upregulated in diseased muscles. (Su et al., 2009) Trp63 is a target gene of miR-410 and miR-434-3p and may be co-regulating by these miRNAs. We also observed a number of other satellite cell activation markers such as Pax3, Myf5, Eya1 downregulated in *Mtm1* mice by other expression analysis. Furthermore, recent studies demonstrated that a reduction in frizzled levels (also downregulated in *Mtm1* KO mice) leads to an increase in Wnt signaling pathway which consequently is associated with a reduction in the ability of maintenance of stem cells potential in mice. (Brack et al., 2007)

These observations make us hypothesize that the inability of atrophic skeletal muscle to respond to regenerative stimuli and promote hypertrophy might result from defective proliferation as well as activation of satellite cells (myogenic stem cells progenitors). In a

cooperative mode miR-379, miR-410, miR-434-3p, miR-434-5p, miR-376c and miR-489 might control the overexpression of genes that promote cell cycle arrest, as well as induce muscle differentiation. This suggests that a lack of cell proliferation coupled with an increase in differentiation proteins in Mtm1 KO mice result in only a small number of activated satellite cells, insufficient for proper muscle repair and constitutes one of the crucial mechanisms that ultimately result in muscular atrophy in *Mtm1*-deficient mice. The genes responsible for these pathways are regulated by a number of miRNAs thus forming a coordinated network where any defect in the circuit can elicit a response eventually leading to muscle atrophy.

Some of these miRNAs such as miR-379 and miR-410 show a significant contribution in multiple processes: cell cycle defects as well as muscular atrophy. This suggests that muscle atrophy is not a final manifestation of diseased muscle state but a process in which genes are tightly regulated from very early stages of disease. This is clearly seen from expression of several muscle atrophy genes that show an increase in *Mtm1* mutant mice in preclinical stage (this difference increases further as the disease progresses). Therefore, over expression of miR-379 and miR-410 in *Mtm1* KO mice may lead to correct satellite cell activation as well as muscular atrophy defects and provides a promising potential therapeutic approach for treatment of myotubular myopathy.

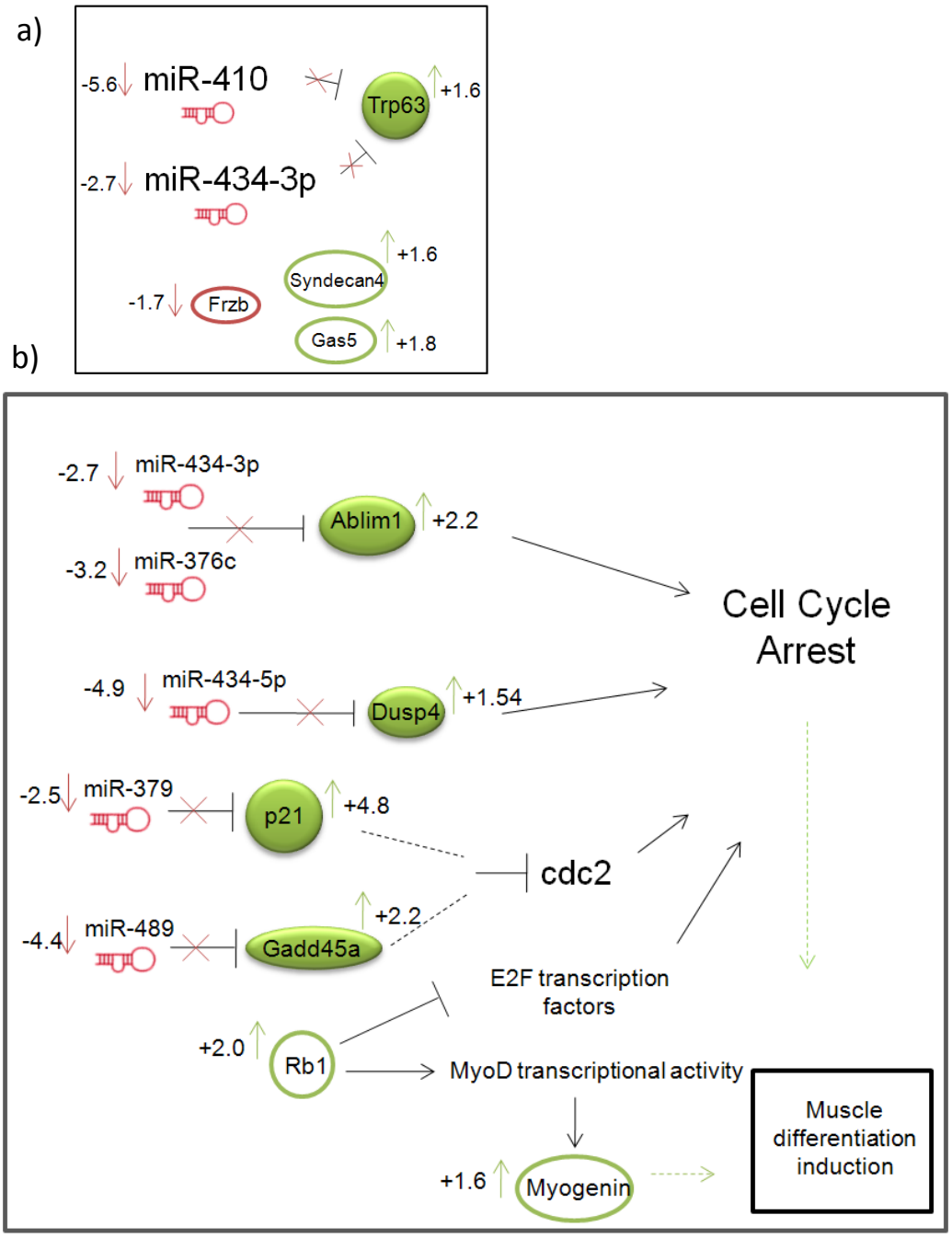

Figure 8: Diagrams illustrating the hypothesized miRNA-mediated pathways implicated in the regulation of satellite cells function. a) Microarray expression of genes and potential regulator miRNAs reported to be crucial for stem cells maintenance. B) Microarray expression of miRNAs and cognate predicted target genes responsible for regulation of cell cycle. Of note, genes involved in the promotion of cell cycle arrest and promotion of cell differentiation are upregulated in mutants. Positive values (and green molecules) represent gene or miRNA downregulated in mutants comparing to controls, while negative values and associated red molecules illustrated genes or miRNAs upregulated. Green filled schematic genes represent identified potentially miRNA-regulated genes in the 142 gene list. White filled molecules surrounded by green or red line represent genes only significantly differentially expressed in the mRNA expression profile, present in the 424 gene list.

3.9.4) Overexpression of Type I myofiber genes

An additional pathway seems to be also upregulated in XLMTM and correlate with the phenotype observed in individuals with myotubular myopathy. A significant overepresentation and upregulation of genes involved in the switch from fast to slow muscle fibers was observed.
Troponin I, skeletal, slow 1, cysteine and glycine-rich protein 3 (muscle LIM protein), Myozenin2 and ATPase, Ca++ transporting, cardiac muscle, slow twitch 2, Troponin T2, cardiac, were found significantly upregulated. (Table 8) Their inverse correlation with miRNA predicted targets suggest the involvement of miRNAs in regulation of this biological mechanism associated with several neuromuscular disorders. Further analyses in this topic are presently being developed.

Table 8: Biological pathways hypothesized to be regulated by miRNAs and disrupted in XLMTM pathology. Genes highlighted in bold are predicted targets of significant identified miRNAs.

Biological Process	Associated Genes
Muscle atrophy	**Murf1**, Mafbx, FoxO1,
Sarcomeric integrity	**Ankrd1**, **Csrp3**, **Myoz2,** Ankrd2
Differentiation/ Proliferation	**Ablim1, Dusp4, Gadd45a, Igfbp5, Frzb, p21, Runx1, Trp63,** Rb1, Myog, Syndecan-4, Gas5
Type I myofibers	**Tnni1, Myoz2, Csrp3,** Tnnt2, Atp2a2

3.10) Differential expression of candidate miRNA-regulated genes during development

After the identification of potential significant miRNA-regulated pathways involved in the etiology of myotubular myopathy we further decided to evaluate their association with the progression of the pathology observed in knockout mice. Graphic X represents the obtained folchanges in qRT-PCR array for KO/WT gene expression of 26 selected genes in three developmental disease stages (14, 27 and 49 days). The expression of all genes for all stages was successfully achieved, with the exception of plcd4 to which signal was never observed (indicating the probe used was unsuitable to hybridize with our samples). (Figure 9)

As the results illustrate, there is a significant and consistent increase in the changes of expression of the identified genes during development, refecting their fundamental role in the development of XLMTM progression. It is striking to note that 21 out of 26 analysed genes showed the same pattern of differential expression in the three developmental time points verified (Ablim1, Actc1, Ankyrin 1, Cdkn1a, Csrp3, Gadd45a, Macf1, Mtap1b, Myoz2, Tnni1, Trim63, Trp63, Hdac4, Runx1, Tnnt2, Rb1, Foxo1, Fbxo32, Myog, Gas5 and Dusp4). These data suggest that identified overepresented pathways regulated by miRNAs do not represent experimental artifacts but are involved in the basis of myotubular myopathy since the assymptomatic stage (14 days).

Interestingly, all genes that present an opposed pattern of foldchange showed a slight upregulation in the assymptomatic and mild disease condition stages and a significant higher downregulation in endstage mice. Therefore, we suggest the expression of Frzb, Igfbp5, Homer1 and Mstn might represent a general consequence of muscle injury.

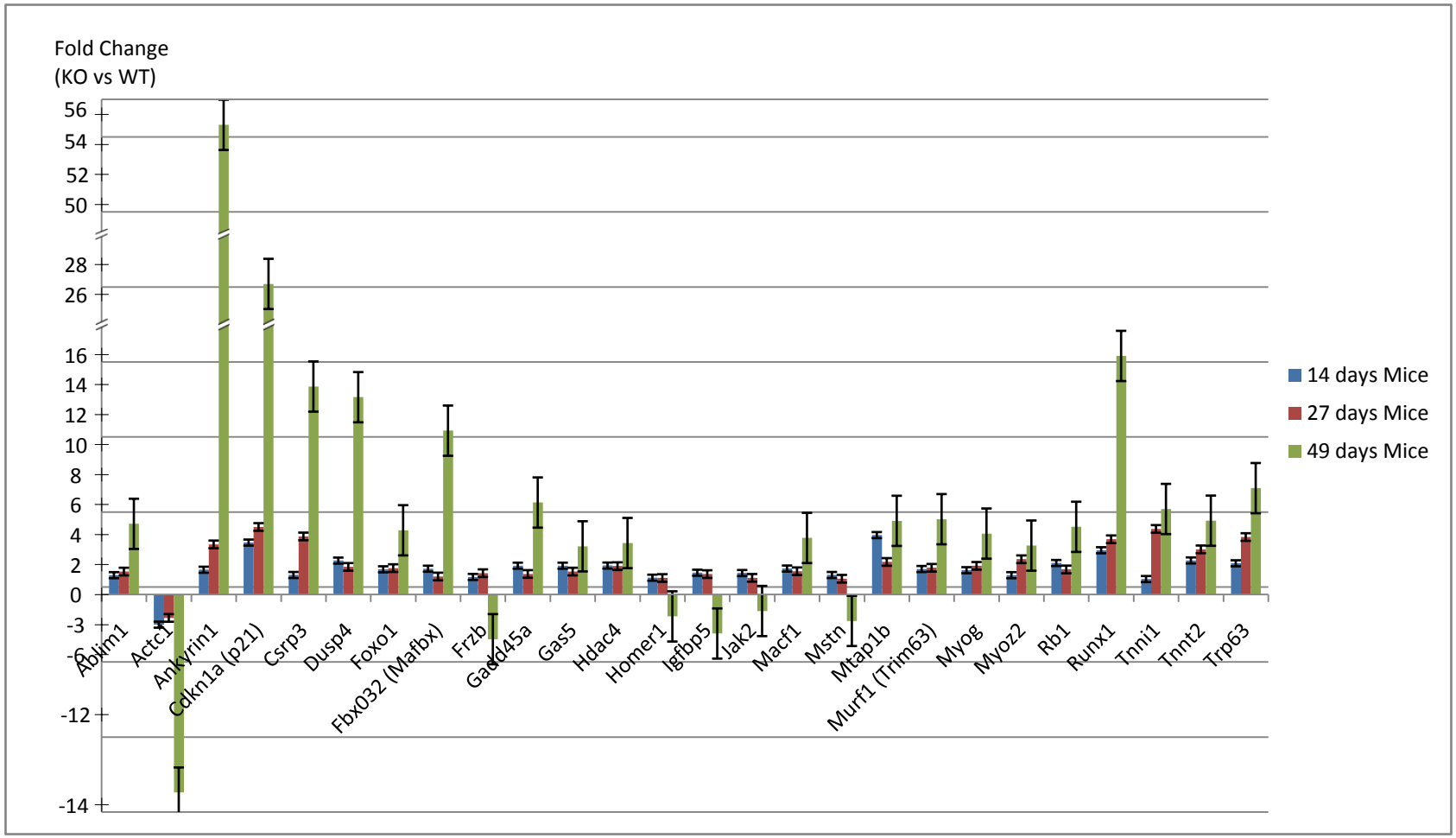

Figure 9: Graphic illustrating foldchange differences obtained in qRT-PCR array for 26 selected genes. For each gene, three developmental time points were evaluated: 14 days, 27 days and 49 days. For each stage 4 WT and 4 KO samples were used. For each sample 3 technical replicates were used and the data was normalized to GAPDH expression values. FoldChange was calculated using ΔΔCt method. Error bars were design on excel using pattern error. 49 days mice show a significant higher difference of folchange KO/WT, correlating with the severity of the disease and consequently corroborating the hypothesis of the involvement of these genes in the pathology of XLMTM.

4) Concluding remarks

In the current investigation we have explored the potential role of microRNAs in X-linked myotubular myopathy. The relationship between miRNAs and their target genes is complex and available miRNA target prediction methods are still mainly imprecise. To dissect miRNA-regulated pathways with functional significance in the disease context, we used a microarray-based combinatorial approach that integrates differential expressed genes in XLMTM and *in silico* predicted targets of differentially expressed miRNAs.

We identified miR-127, miR-137, miR-337, miR-376a, miR-376c, miR-379, miR-410, miR-431, miR-434-3p, miR-434-5p, miR-489 and miR-541 as strong candidate molecules to be implicated in these interactions. It is interesting to remark that the expression of these miRNAs was never previously described in skeletal muscle. miR-337 and miR-376a target genes were, however, not recognized as significant in the pathophysiology of XLMTM. miRNAs are pos-transcriptional modulators of gene expression thought to influence cell function through translation repression of their specific mRNA targets or promotion of mRNAs degradation. (Sontheimer and Carthew, 2005) (Eulalio et al., 2008)

Accordingly, direct effects of miRNAs in their targets genes might not be reflected at the mRNA level in conditions of translation repression. miR-337 and miR-376a may be involved in XLMTM but not detected their target genes by the methodology applied, highlighting the importance of further studies in this field. Nonetheless this possibility, miRNAs may indirectly trigger a down-stream cellular feedback mechanism to promote mRNA destabilization of translational repressed molecules. Moreover, miRNAs can directly promote mRNA degradation as well as mRNA target sequestration to P bodies for subsequent inactivation and degradation. (Wu and Belasco, 2008) These effects correlate with decreasing messenger RNA levels, detected in microarray experiments. Thus, we preferred to follow this approach to reduce false discovery values.

Ultimately, the goal of this study was to decipher how miRNA-mediated regulation of gene expression is associated with the disrupted molecular pathways of XLMTM. For this purpose functional categories enrichment for differentially expressed genes overlapping significant miRNA predicted genes was assessed. The functional groups muscle development, cardiac morphogenesis, regulation of cell cycle and muscle contraction were recognized. Further bioinformatic analysis with parallel experimental validation by qRT-PCR arrays of miRNAs and their

potential target genes enabled a comprehensive analysis of the role of identified miRNAs during muscle development and disease.

An overall analysis integrating miRNA/mRNA data allowed us to suggest potential significant biological pathways regulated by miRNAs that may underlie myotubular myopathy. Our preliminary data strongly supports the involvement of miRNAs in the regulation of muscular atrophy process, severely observed in this pathology, through the activation of a dependent FoxO1/Murf1/Fbxo32 molecular network. Furthermore, we speculate the inability of skeletal muscle to respond to muscle injury signaling pathways and promote regeneration might be associated with miRNA-regulated defects in the activation and proliferation of satellite cells, required for adult muscle growth and repair. (Buckingham and Montarras, 2008; Shi and Garry, 2006)(Gangaraju and Lin, 2009) Additionally, an overexpression of type I myofiber genes was observed to be targeted by differentially expressed miRNAs in this study, corroborating known associations in the literature that report predominance of slow fibers in XLMTM. (Pierson et al., 2005)

These findings propose an intricate control of gene expression in X-linked myotubular myopathy both at the transcriptional and miRNA-mediated pos-transcriptional level, providing a foundation for future functional studies. We warrant the requirement for additional validation studies in human biopsies and larger sample sets as well as the possibility of other miRNAs discovered since this project was initiated also contributing to disease development.

Knockout and over-expression experiments are in progress and might give further insights into the regulatory interactions between miRNAs and their cognate genes and potentially contribute to the identification of novel therapeutic targets.

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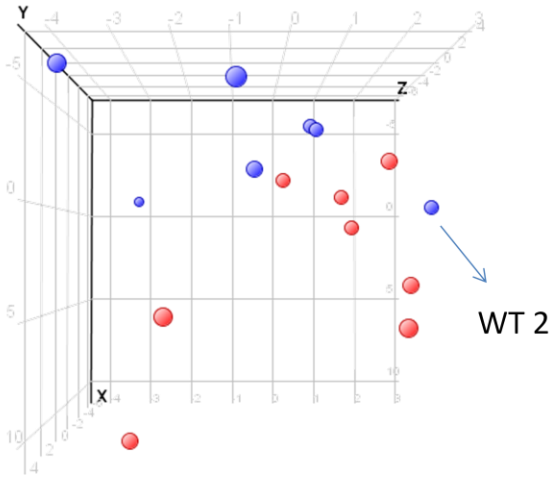
6. Appendix

a)



b)

Identifier	WT vs KO	Predicted(WT vs KO)	Confidence Measure(WT vs KO)
MTM1_4	KO	[KO]	1.000
MTM1_3	KO	[KO]	1.000
MTM1_7	KO	[KO]	1.000
MTM1_5	KO	[KO]	1.000
MTM1_2	KO	[KO]	1.000
MTM1_1	KO	[KO]	1.000
MTM1_8	KO	[KO]	1.000
MTM1_6	KO	[KO]	1.000
WT_8	WT	[WT]	0.857
WT_7	WT	[WT]	0.857
WT_4	WT	[WT]	0.857
WT_1	WT	[WT]	0.857
WT_6	WT	[WT]	0.857
WT_3	WT	[WT]	0.857
WT_2	WT	[WT]	0.143

Removal of WT-2

Identifier	WT vs KO	Predicted(WT vs KO)	Confidence Measur...
WT_1	WT	[WT]	1.000
WT_4	WT	[WT]	1.000
WT_3	WT	[WT]	1.000
WT_8	WT	[WT]	1.000
WT_7	WT	[WT]	1.000
WT_6	WT	[WT]	1.000
MTM1_5	KO	[KO]	1.000
MTM1_1	KO	[KO]	1.000
MTM1_3	KO	[KO]	1.000
MTM1_7	KO	[KO]	1.000
MTM1_8	KO	[KO]	1.000
MTM1_4	KO	[KO]	1.000
MTM1_2	KO	[KO]	1.000
MTM1_6	KO	[KO]	1.000

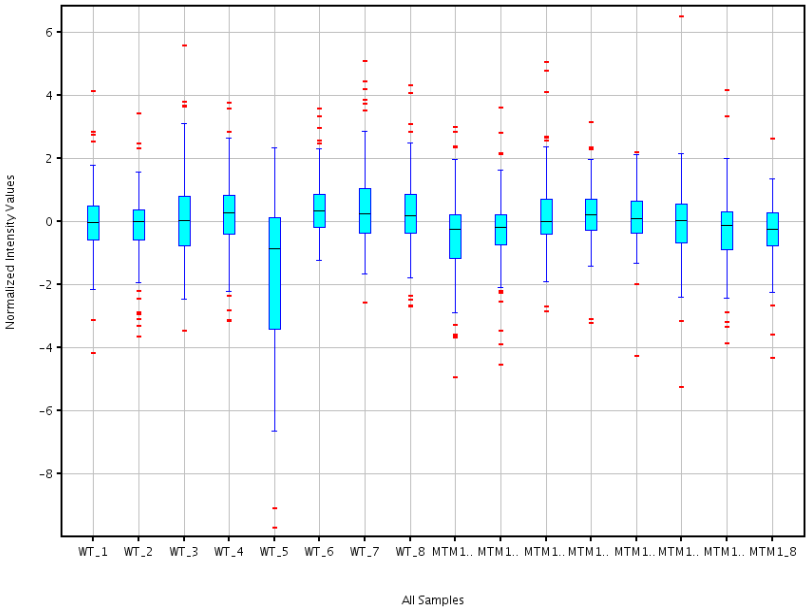
Supplementary Fig. 1: miRNA microarrays data analysis using 7 WT samples and 8 KO samples.

a) 3D-PCA graphic exhibiting a clear preferential association of WT2 sample with the knockout samples classe. b) Genespring software class prediction application through randomization of the samples and posterior inclusion in specified groups includes WT2 in the class of mutants.

a)

WT_1	WT_2	WT_3	WT_4	WT_5	WT_6	WT_7	WT_8	MTM1_1	MTM1_2	MTM1_3	MTM1_4	MTM1_5	MTM1_6	MTM1_7	MTM1_8
1.0	0.86575276	0.8325796	0.8962242	0.5318947	0.8888058	0.8534661	0.8800489	0.845195	0.8535238	0.8451372	0.89443517	0.90087175	0.8752945	0.8507628	0.88647234
0.86575276	1.0	0.8310005	0.8698011	0.5978084	0.8735335	0.8245473	0.86621076	0.8149265	0.8470202	0.82891035	0.9122708	0.926245	0.88356614	0.8669351	0.9034942
0.8325796	0.8310005	1.0	0.8552943	0.5081137	0.92600393	0.90393794	0.87243766	0.8248959	0.82683194	0.894574	0.8508309	0.87896204	0.80637705	0.8187586	0.87268025
0.89622426	0.86980116	0.85529435	1.0	0.5723753	0.8959646	0.8472899	0.9160544	0.8071167	0.87390363	0.8496493	0.9035996	0.89855397	0.8528436	0.8490596	0.8802357
0.5318947	0.5978084	0.5081137	0.5723753	1.0	0.5677065	0.49771044	0.5201235	0.605332	0.615566	0.5171365	0.56606346	0.5543353	0.5923349	0.6235258	0.58416754
0.8888058	0.8735335	0.92600393	0.8959646	0.5677065	1.0	0.90323555	0.8769877	0.8584795	0.8681317	0.90779513	0.91593724	0.9137065	0.8792629	0.86094564	0.90114105
0.8534661	0.8245473	0.90393794	0.8472899	0.49771044	0.90323555	1.0	0.866933	0.8100464	0.809901	0.8665735	0.8667013	0.8596487	0.8365698	0.8143047	0.87359095
0.8800489	0.86621076	0.87243766	0.9160544	0.5201235	0.8769877	0.866933	1.0	0.82670414	0.86449087	0.8787402	0.8762013	0.9011083	0.8418968	0.8511369	0.8888361
0.845195	0.8149265	0.8248959	0.8071167	0.605332	0.8584795	0.8100464	0.82670414	1.0	0.8694821	0.833126	0.86558837	0.8589646	0.8378295	0.8675695	0.85815734
0.8535238	0.8470202	0.82683194	0.8739036	0.615566	0.8681317	0.809901	0.86449087	0.8694821	1.0	0.8681052	0.8764211	0.8891829	0.86388844	0.88100857	0.8634721
0.8451372	0.82891035	0.894574	0.8496493	0.5171365	0.90779513	0.8665735	0.8787402	0.833126	0.8681052	1.0	0.8836645	0.8920652	0.860987	0.81825775	0.8772695
0.89443517	0.9122708	0.8508309	0.9035996	0.56606346	0.91593724	0.8667013	0.8762013	0.86558837	0.8764211	0.8836645	1.0	0.9221398	0.91987497	0.89091325	0.89670163
0.90087175	0.926245	0.87896204	0.8985539	0.5543353	0.9137065	0.8596487	0.9011083	0.8589646	0.8891829	0.8920652	0.9221398	1.0	0.9004189	0.8809813	0.9336411
0.8752945	0.88356614	0.80637705	0.8528436	0.5923349	0.8792629	0.8365698	0.8418968	0.8378295	0.86388844	0.860987	0.91987497	0.9004189	1.0	0.8639675	0.85758024
0.8507628	0.8669351	0.8187586	0.8490596	0.6235258	0.86094564	0.8143047	0.8511369	0.8675695	0.88100857	0.81825775	0.89091325	0.8809813	0.8639675	1.0	0.9028208
0.88647234	0.9034942	0.87268025	0.8802357	0.58416754	0.90114105	0.87359095	0.8888361	0.85815734	0.8634721	0.8772695	0.89670163	0.9336411	0.85758024	0.9028208	1.0

b)

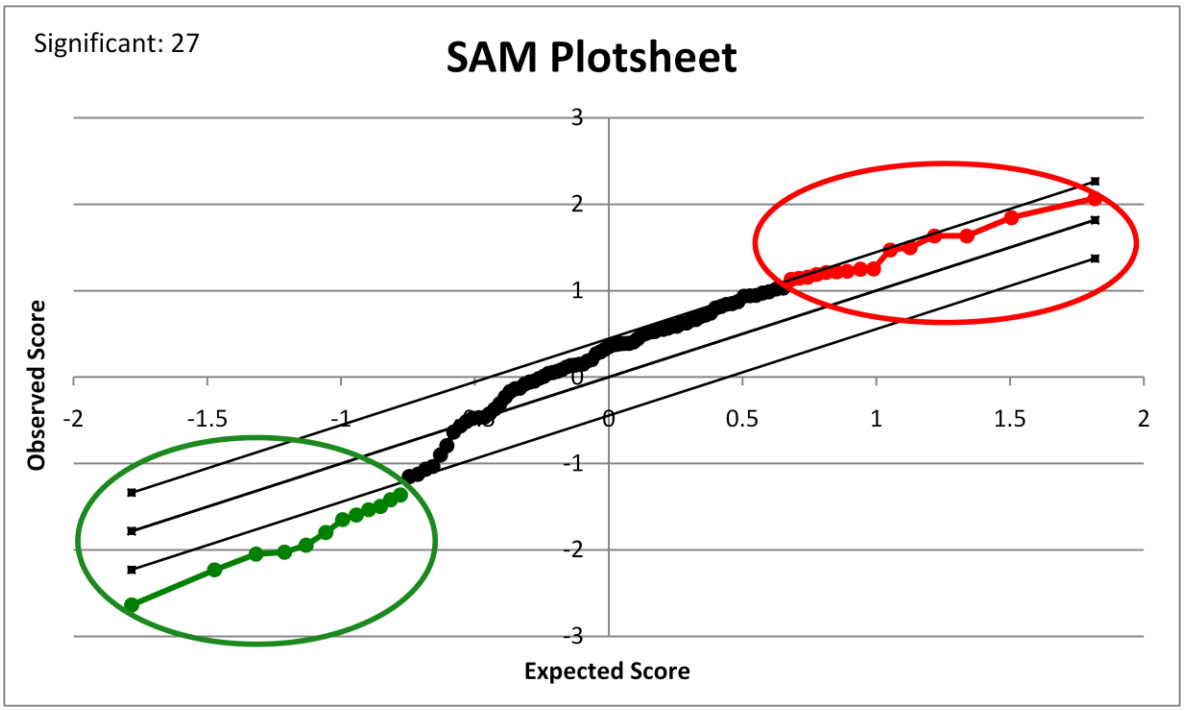


Supplementary Fig. 2: miRNA microarray of endstage mice (49 days)

a) Pearson correlation table shows a dramatic reduction in the values of linear association for wild-type 5 sample. High values of correlation approximate 1, while 0 represents no correlation between normalized samples. b) Box Whisker Plot translates the distinct pattern of expression of normalized wild-type 5 sample.

Bone contamination in the collection of gastrocnemeous muscle might explain this discrepancy in miRNA expression profile.

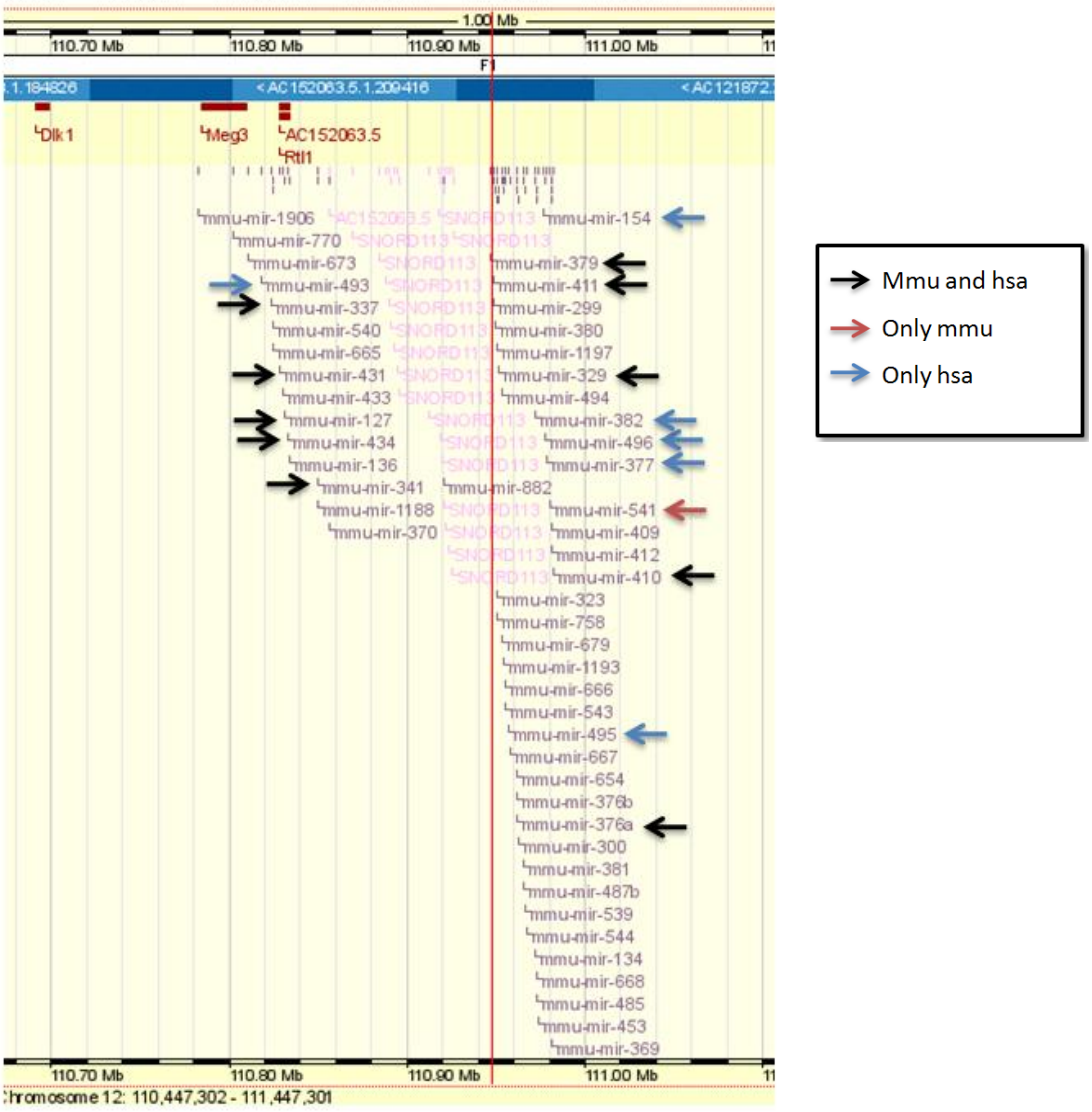
a)



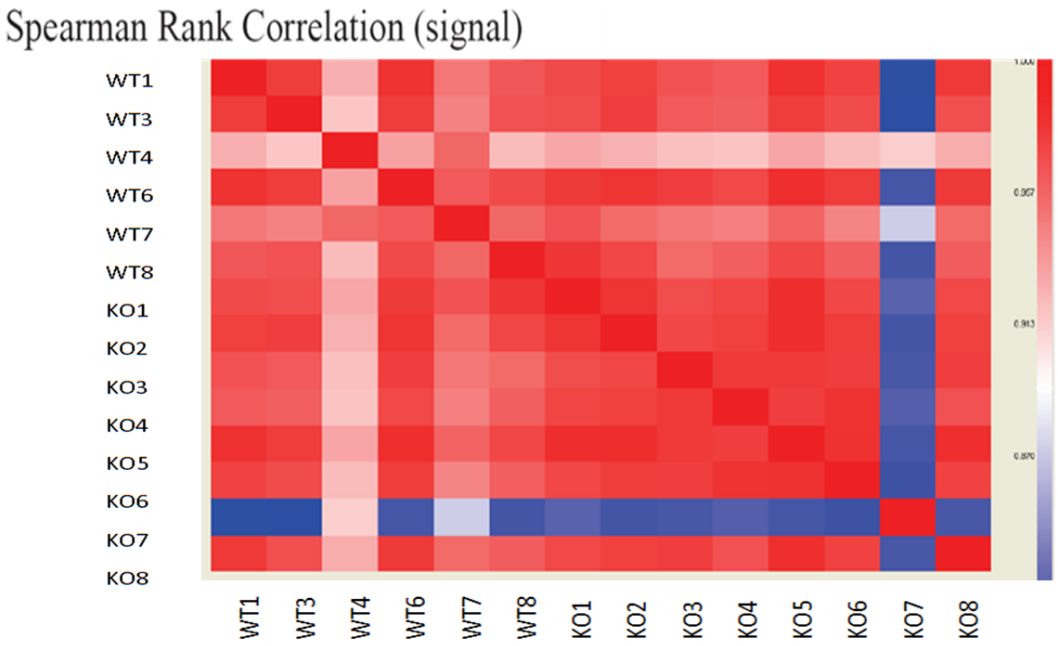
b)

Delta Value	0.407	0.447	0.55	0.6	0.604	0.8	0.86
FDR (%)	25.7	17.96	11.5	6.93	6.93	0	0
Upregulated genes	21	15	0	0	0	0	0
Downregulated genes	14	12	12	11	10	5	0

Supplementary Fig. 3: Statistical Analysis of Microarrays (SAM) used to examine miRNA expression profile in endstage mice. Different tried FDR values with the respective significant genes obtained are shown.



Supplementary Fig 4: Dlk1-Dio3 representation from Ensembl highlighting with arrows the genomic location of miRNAs identified in human (has) and mouse (mmu) probes available in the miRNA microarray as significant (black arrow), only significant changes in mmu probes (red arrow) and only in hsa probes (blue arrow). All the miRNAs found only differentially expressed in hsa probes didn't have the homologous probe for mmu in the chip. Moreover, BLAST search enabled to identify homology between significant hsa identified miRNAs and mmu miRNAs as shown in the diagram. Their possible involvement in the disease should consequently be addressed in further studies.



Supplementary Fig. 5: Spearman Rank Correlation quality control performed by Affymetrix representative company. This coefficient is a non-parametric measure of the correlation between two variables. Samples WT4 and KO7 were considered potential outlier, however only KO7 was significantly considered as outlier and

Supplementary table 1: Gene ontology enriched categories for 424 statistically significant genes, identified on mRNA expression profile

GO Term	Corrected p-value
Muscle development	0.017
Myofibril	2.97E-07
Sarcomere	8.79E-08
Z disc	0.007760731
I band	7.72E-06
Contractile fiber	8.79E-08
Protein binding	0.0030
Cytoplasm	4.37E-04