Deep RNA profiling identified Clock and molecular clock genes as pathophysiological signatures in collagen VI myopathy

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Abstract

Collagen VI myopathies are genetic disorders due to mutations in collagen 6 A1, 2, and 3 genes, ranging from the severe Ullrich congenital muscular dystrophy to the milder Bethlem Myopathy, which is recapitulated by collagen VI null ($Col6a1^{-/-}$) mice. Abnormalities in mitochondria and autophagic pathway have been proposed as pathogenic causes of collagen VI myopathies, but the link between collagen VI defects and these metabolic circuits remains unknown. To unravel the expression profiling perturbation in muscles with collagen VI myopathies we performed a deep RNA profiling in both $Col6a1^{-/-}$ mice and ColVI patients. Interactome map identified common pathways suggesting a previously undetected connection between circadian genes and collagen VI pathology. Intriguingly, $Bmal1^{-/-}$ mice, a well-characterized model displaying arrhythmic circadian rhythms, showed profound deregulation of the collagen VI pathway and autophagy-related genes.

The involvement of circadian rhythms in collagen VI myopathies is new and links autophagy and mitochondrial abnormalities. It also opens new avenues for therapies of hereditary myopathies to modulate the molecular clock or potential gene-environment interactions that may modify muscle damage pathogenesis.

Introduction

Collagen VI (ColVI) is a major component of the extracellular matrix (ECM) crucial for muscle cell adhesion, stability and regeneration. COLVI forms a microfibrillar network involved in maintaining tissue integrity through a structural link between different components of connective-tissue basement membranes and cells (Allamand et al., 2011). COLVI is a heterotrimeric molecule composed of three α-chains encoded by distinct genes (*COL6A1*, *COL6A2*, *COL6A3*). Equimolar association of the three alpha–chains into a triple helical monomer is followed by formation of dimers and tetramers stabilized by disulfide bonds. After secretion, the tetramers form a network of microfilaments that bridge the surface of muscle cells with the connective tissue (Bonaldo et al., 1990). Mutations occurring in the three ColVI genes cause two major disease phenotypes: a mild form, Bethlem myopathy (BM), and a severe form, Ullrich congenital muscular dystrophy (UCMD) (Gualandi et al., 2012). Three additional ColVI genes have recently been described, COL6A4, COL6A5 and COL6A6, and code for the α4(VI), α5(VI) and α6(VI) protein chains, respectively. However, only α6(VI) is highly expressed in skeletal muscle, although not associated with basement membranes (Tagliavini et al., 2014a) and none of these additional ColVI genes are known as disease genes in humans.

COLVI myopathies are well defined conditions, characterized by a broad spectrum of clinical features, ranging from prenatal onset with decreased fetal movements; hypotonia or torticollis, early-childhood onset - by delayed motor milestones, muscle weakness and contractures; and adult onset (4th-6th decade) - by proximal weakness and Achilles tendon or long finger flexor contractures (De Visser et al., 2004). As a result of slow but ongoing progression of the condition, more than two-thirds of affected individuals over age 50 years need supportive means for outdoor mobility (Jöbsis et al., 1999).

The severe phenotype with early onset, UCMD, is characterized by severe muscle weakness, striking hyperlaxity of distal joints, and proximal joint contractures. In contrast to BM, UCMD is marked by an early loss of ambulation and a rapid disease progression leading to early death caused by respiratory failure (Maraldi et al., 2009). In addition, two other conditions have been associated with COLVI myopathy: autosomal dominant limb-girdle muscular dystrophy (LGMD), and autosomal recessive myosclerosis myopathy (Merlini et al., 2008; Scacheri et al., 2002). Recently, the COL12A1 gene was shown to be mutated in patients with a BM-like phenotype (Hicks et al., 2014).

Despite this broad clinical spectrum, both muscle function and respiratory performance evaluation can address the diagnosis. Nevertheless, UCMD and BM can be inherited accordingly to both dominant and recessive models, and generally, neither the type of mutation (missense, nonsense, splicing insertion or deletion), nor the effect of the mutation on the protein struc-

ture/function allows precise discrimination between two phenotypes (Allamand et al., 2011; Maraldi et al., 2009; Bönnemann, 2011). This suggests that the genetic background of patients may influence the mutated genes expression, therefore impacting on the resulting phenotype.

The $Col6a1^{-/-}$ mouse is a well characterized animal model for COLVI myopathies. These mice carry a targeted inactivation of the α1(VI) chain and exhibit an early onset myopathic phenotype that strongly resembles BM (Bonaldo et al., 1998). Muscle fibres of $Col6a1^{-/-}$ mice show loss of contractile strength and a number of structural defects in the hind limb muscle and especially in the diaphragm. Although the pathology associated with collagen VI myopathy is nonspecific and variable, it has been widely documented (Grumati et al., 2010; Urciolo et al., 2013). Defective mitochondrial function with ultrastructural alterations, increased spontaneous apoptosis and abnormal propensity of the permeability transition pore (PTP) to open, have been characterized in both animal models and patients (Irwin et al. 2003; Bernardi and Bonaldo, 2008; Tagliavini et al., 2014b).

A further link between COLVI myopathies and mitochondrial dysfunction has been established by the discovery that autophagy is defective in $Col6a1^{-/-}$ mice and UCMD/BM patients (Grumati et al., 2011). This supports a mechanism of disease pathogenesis that might be in part due to defective regulation of autophagy, which leads to accumulation of abnormal mitochondria and sarcoplasmic reticulum. Moreover, in $Col6a1^{-/-}$ mice, changes in metabolic proteins resulting in decreased glycolysis and affecting the tricarboxylic acid (TCA) cycle fluxes lead to a different fate of a-ketoglutarate triggering lipotoxicity. The metabolic changes are associated with changes of proteins involved in mechanotransduction at the myotendineous junction/costameric/sarcomeric level and in energy metabolism (De Palma et al., 2013). Recently, a strong depletion of the α6(VI) chain in skeletal muscle has been reported as potential biomarker of COLVI myopathies (Tagliavini et al., 2014a). In spite of these recent achievements, a complete understanding of COLVI disease pathogenesis remains elusive.

Gene expression profiling and RNA sequencing are powerful tools for exploring the alterations of transcriptional behavior in disease tissues (Sánchez-Pla et al., 2012). It brought great advances in cancer research and disclosed deregulated transcripts that are now being considered as prognostic/therapeutic biomarkers of neoplastic diseases (Sevov et al., 2012; Campo, 2013). Considering the availability of novel therapeutic possibilities for rare muscle disorders (Aartsma-Rus and Muntoni, 2013), finding prognostic biomarkers for disease severity or drug response will be of great benefit for patient care and treatment (Ferlini et al., 2013; Scotton et al., 2014). In this work, we explored RNA profiling of $Col6a1^{-/-}$ mice and BM/UCMD patients to identify transcriptional patterns correlating with disease state. Such patterns can identify specific pathway alterations and pathophysiological biomarkers. This unbiased approach revealed marked changes

in the expression of genes involved in the circadian rhythm pathway. We further supported these findings by protein studies performed both in mice and in patients. In addition, RNA and protein studies in the *Bmal1*^{-/-} mice, a known animal model of circadian rhythm disruption, showed profound deregulation of *Col6a6* as well as of the autophagy-related genes such as p62/SQSTM1 and AMPK. Although it has been previously reported that disruption of circadian rhythms are linked to skeletal muscle remodeling, function, performance and aging, the involvement of the molecular clock genes in hereditary myopathy as COLVI disease is a novel finding. These findings open new perspectives on potential target genes involved in muscle damage pathogenesis and offers new therapeutic approaches.

Results

Overall gene expression profile in *Col6a1*^{-/-} muscles reveals changes in genes associated with muscle function

Gene expression array profiles of wild-type (WT) and *Col6a1*^{-/-} mice were compared in three muscle types: diaphragm, tibialis anterior (TA) and gastrocnemius. To determine genes with statistically significant differential expression (p-value < 0.05 in limma package) we analysed each muscle type both separately and in pools (averaged expression change over all muscle types). The degree of transcriptional deregulation was different among *Col6a1*^{-/-} muscles with 11,170 differentially expressed genes in TA, 6,790 in diaphragm and 3,759 genes in gastrocnemius. To further support the finding of different tissue susceptibility to COLVI deficiency, we performed sample clustering of expression profiles from each muscle type in both *Col6a1*^{-/-} and WT mice. The resulting dendrogram is shown in supplementary figure 1A. We found that expression profiles of *Col6a1*^{-/-} and WT gastrocnemius were clustered together and therefore similar. Indeed this supports the existing knowledge about muscle involvement in the *Col6a1*^{-/-} mouse model, where gastrocnemius is very mildly affected, whereas tibialis and even more diaphragm are severely affected (De Palma et al., 2013). In contrast, diaphragm and TA *Col6a1*^{-/-} muscles clustered together and clearly separate from their WT counterparts, suggesting that COLVI deficiency severely and similarly affects these muscle types (Fig. S1).

Expression of genes associated with muscle development and function was altered in *Col6a1*^{-/-} mice (supplementary table 1). The changes affected multiple aspects of muscle biology including structure, metabolism, transcription and regulation. There was variability between tested muscle types. 60% (26 out of 44) of muscle-specific genes changed their expression in an opposite direction in TA versus diaphragm, 23% in TA versus gastrocnemius (7 out of 30) and 67% in gastrocnemius versus diaphragm (12 of 18). TA displayed differential expression of myosin, troponin and calsequestrin isoforms (predominantly cardiac and slow), and increased expression of

Pax3. Genes differentially expressed in diaphragm and TA included almost all members of the dystroglycan complex (dystrophin, dystrobrevin, dystroglycan 1, syntrophin, α-, β-, γ-sarcoglycans) and several transcription factors important for muscle-related processes, such as MyoD1, MyoG, MYF6, NFATc2 and Pax7. In the diaphragm, embryonic isoforms of myosin were upregulated as well as Pax7, a marker of satellite cell number, which together can be regarded as a sign of muscle regeneration. These muscle type variability in the transcription profile is not surprising since these three muscles have different metabolic properties and different fiber type composition (Bonaldo et al., 1998).

Gene ontology groups and key expression regulators analyses show deregulation of the circadian rhythmic process in *Col6a1*^{-/-} mice

Among differentially expressed genes between *Col6a1*^{-/-} and WT mice, 479 changed expression in the same direction in all tissues with p-value < 0.05. We used Gene Set Enrichment analysis (GSEA) to identify gene ontology (GO) groups enriched with such genes. The top 10 GO groups are listed in Supplementary figure 2. Identified GO groups were broad categories not specific enough to yield an insight regarding the biological effects occurring in *Col6a1*^{-/-} mice. We also performed GO analysis of 334 genes differentially expressed in the "pooled" data from all three muscle groups compared with "pooled" WT muscles. This GSEA identified the GO groups shown in Figure 1. One of the top 10 GO groups was "rhythmic process" containing genes regulating circadian rhythms as well as known genes that are regulated downstream of the molecular clock, such as DBP, and all were down-regulated in *Col6a1*^{-/-} muscles.

To identify key regulators that change their activity in *Col6a1*^{-/-} muscles, we used sub-network enrichment analysis (SNEA) in Pathway Studio. Our first SNEA was done using the list of genes differentially expressed in one direction in all muscle types as an input. Regulators identified by this approach are shown in Figure 2. Genes changing their expression in one direction in all muscle types are primarily regulated by inflammatory cytokines and their receptors. In good agreement with the GO analysis, SNEA of the "pooled" dataset found ARNTL (gene encoding the molecular clock factor, BMAL1), CLOCK, PER1, and PER3 regulators that belong to cellular circadian rhythm pathway (Fig. 2 and table S2). We then explored the intersection between the two SNEA analyses and found consistent results (Fig. 2 and table S2). Indeed, we found CLOCK, ARNTL, GFI1B, IL2, CD28, PIM3 and inflammatory cytokines key regulators upstream of genes differentially expressed in all comparisons. In total 46 key regulators were identified by the three comparisons.

To validate the 46 key regulators selected by SNEA, we explored the differential expression of these transcripts using TaqMan low-density arrays (TLDA). Comparison of the data obtained

from both the microarray experiments and those with the TLDA cards resulted in the same trend of fold change in 37 out of 46 genes (80%) of the diaphragm and TA, whereas in the gastrocnemius we found 32 out of 46 genes (70%) with these characteristics (Figs. S3A, S3B).

The TLDA and microarray data for regulators of the rhythmic process (ARNTL, ATF5, CLOCK, DBP, EGR1, FKBP5, PER1, PER2 and PER3) and transcription factors important for muscle-related processes (MYOD, MYOG, MYF6, PAX7) were in good agreement in both diaphragm and TA and to a lesser extent in gastrocnemius, confirming the deregulation of these two pathways in the $Col6a1^{-/-}$ mice (Fig. S3B).

Western blot revealed that CLOCK levels were significantly (Student's t-test, n = 3, p-value < 0.05) increased in diaphragm and TA muscles of $Col6a1^{-/-}$ mice compared with their respective WT controls, whereas they remained unchanged in gastrocnemius. MAT2A protein levels did not change significantly in the diaphragm, whereas in TA and gastrocnemius muscles they were, respectively, increased and decreased (Fig. 3A).

Abnormal collagen VI expression in $Bmal1^{-/-}$ mice, a model for circadian rhythm deregulation

There are multiple genetic mouse models in which molecular clock factors have been knocked out, but of these models only the $Bmal1^{-/-}$ mice exhibit complete loss of behavioral rhythm under normal housing conditions (Bunger et al., 2000). We adopted this last model in order to have a homogeneous phenotype, with complete failure of circadian control. Western blot analysis of gastrocnemius muscles of $Bmal1^{-/-}$ mice revealed significant changes in the levels of COLVI chains, with decreased α6(VI) and increased α3(VI) levels. Interestingly, down-regulation of *COL6A6* was also recently observed by us in UCMD and BM muscles (4). In addition to COLVI, components of the autophagy pathway were also affected in the muscle of $Bmal1^{-/-}$ mice with increased LC3 and decreased p62/SQSTM1 levels, whereas Beclin 1 levels were unchanged. We also found increase in the activation status of AMPK, as defined by higher phospho-AMPK/total AMPK, however this was driven in part by a significant decrease in the levels of total AMPK (Fig. 3B).

Circadian genes show deregulation in muscle from UCMD and BM patients.

Gene expression profiles in the 3 patients (1 UCMD and 2 BM) compared to healthy controls by RNAseq identified several altered transcripts grouped into GO categories (Fig.4A). Consistently deregulated genes in muscles from UCMD/BM patients are reported in Table 1. In all three patients, we identified 7 consistently upregulated genes belonging to transcription factor, metabolism process and cellular stress response categories. The 24 consistently downregulated genes

are involved in signaling, metabolism, immunity and cell adhesion, axon guidance and circadian rhythm including DNA repair GO categories (Fig. 4B). Consistent with a link to circadian rhythms we found that 8 of the genes listed in Table 1 are known to be circadian in mouse skeletal muscle and/or changed in expression in the *Bmal1*$^{-/-}$ mouse.

In order to find GO groups deregulated in different samples, we performed GSEA analysis (p-value 0.05 cut-off) for each patient separately. The bi-clustering was performed using Jaccard-based distance between GO groups (based on the number of common genes in groups). This method allowed finding common deregulated "patterns" in different patients, even though the GSEA method shows different GO groups. We found genes coding for postsynaptic membrane/cell junction/synapse, regulation of ion transmembrane transport, protein homo-oligomerization/protein specific binding, cell surface receptor linked signalling pathway and DNA repair/response to DNA damage, including circadian rhythm genes (Figs. 4A, 4B).

Considering the evidence from both mice and human studies for the deregulation of circadian genes in muscles with COLVI defects, we performed expression analysis by real time PCR of CLOCK and MAT2A genes in other 11 UCMD and 12 BM patients. We found that CLOCK gene was significantly downregulated (p value < 0.0001) in all UCMD muscles. The MAT2A transcript showed a milder downregulation, albeit not statistically significant (p value: 0.0753), only in UCMD patients (Fig. 3C). BM patients do not show any significant deregulation in the analysed circadian genes. Western blot in 4 UCMD and 3 BM patient muscles showed significantly increased CLOCK protein levels (ANOVA + Tukey, n = 3, p-value < 0.05) in UCMD patients only, whereas MAT2A protein levels were increased in BM patients only (Fig. 3D).

Discussion

ColVI myopathies have been both clinically and genetically defined, and alterations in the apoptosis and autophagy circuits have been reported. Nevertheless, the link between mutations of *COL6A1-COL6A3* genes and the muscle dysfunction seen in ColVI myopathy still remains elusive. We therefore adopted RNA profiling to study muscle from both *Col6a1*$^{-/-}$ mice and BM/UCMD patient biopsies, searching for unique transcriptional patterns. The idea was to detect specific signatures that may enhance our understanding of the pathogenesis of ColVI myopathies. In order to strengthen our findings, we also evaluated protein expression of the transcripts that we found to be consistently deregulated in both mouse and patient muscle tissue.

***Col6a1*$^{-/-}$ and *Bmal1*$^{-/-}$ mice shows connection between ColVI myopathy and circadian genes**
To our knowledge this is the first attempt to study *Col6a1*$^{-/-}$ mice by RNA profiling. Proteomic studies were performed by Irwin et al. (Irwin et al., 2003) in *Col6a1*$^{-/-}$ mice, revealing the dia-

phragm as the most severely affected muscle, having a deeply subverted ultrastructure and evident loss of contractile strength, accompanied by changes in proteins involved in mechanotransduction. Our pooled data identified 334 differentially expressed genes in *Col6a1*^{-/-} versus WT, showing consistent deregulation of the genes involved in metabolic processes and regulation of transcription, known to be affected in myopathies, as well as of the circadian CLOCK gene. This is an entirely new observation.

We show major transcriptional changes in the diaphragm and TA, accordingly to the proteomic findings (De Palma et al., 2013). All muscles shared similar alterations in the genes involved in the inflammatory response, cell proliferation, transcriptional regulation, and metabolism, but differed substantially in the sign of expression of a number of genes associated with muscle functions, including structure, metabolism, transcription, and regulation – a not entirely unexpected finding considering that these three muscles have different morphogenetic origins and functional requirements (Eberhard and Jockusch, 2004; Merrell and Kardon, 2013).

The CLOCK gene deregulation, we also confirmed at the protein level in *Col6a1*^{-/-} mice, suggests a previously unreported relationship between circadian rhythms, the molecular clock and myopathy. *Col6a1*^{-/-} model shows both muscle and osteotendinous junction damage (Izu et al., 2011; Izu et al., 2012), reflecting defective costamerogenesis and altered mechanotransduction (De Palma et al., 2013). Specific activation of either MRTFs (myocardin-related transcription factors) and TCFs (ternary complex factors), whose targets are regulators of cytoskeleton dynamics and mechanosensing, have potential to reset the molecular clock via regulation of PER1, PER2, ARNTL and other CLOCK genes (Esnault et al., 2014). This first link between the ECM and the profound alterations we detected in the circadian rhythm/molecular clock pathway (ARNTL, ATF5, CLOCK, DBP, EGR1, FKBP5, PER1, PER2 and PER3 genes) supports the known data demonstrating that physical activity/exercise can regulate circadian gene expression in skeletal muscle (Wolff and Esser, 2012). In addition, providing a further bridge between protein and RNA data, intersection between the individual muscle groups and the pooled transcription data, to identify key regulators, showed that inflammatory cytokines and their receptors, known to be regulated by PER1-3 genes, are significantly under-expressed. The connection between ColVI and circadian genes is also strongly supported by our protein findings in *Bmal1*^{-/-} mice, which exhibit significant changes in the levels of COLVI chains as well as alterations of the autophagy pathway (Fig.3B). Since our expression analyses were only performed at one time point, our results can only allow us to conclude that molecular clock pathway is disrupted. Further studies are needed to determine whether the clock factors are phase shifted, damped or arrhythmic.

RNA profiling of COLVI patients shows profound deregulation in ECM-mediated structural muscle remodeling and circadian genes

In the 3 ColVI patients (BM, BMs and UCMD) studied, the highly efficient RNAseq analysis revealed many changes in transcription profile, as expected for this progressive, chronic and severe myopathy. The major changes observed, shared by all patients, were under- or overexpression in membrane/cell junction/synapse, ion transmembrane transport, protein homo-oligomerization/protein-specific binding, cell-surface-receptor-linked signaling pathway and circadian gene pathways (Fig. 4B). The overexpressed genes we detected (ZNF384, ZNF526, SCP2, SUOX, KIF1B, PCSK6 and SESN1) are all involved in the control of gene expression and metabolic pathways. As reported in UCMD muscles (Paco et al., 2013), increased expression of these transcripts is associated with muscle regeneration defects and inflammatory signatures. Our RNAseq data confirm that this myopathy is associated with structural alterations coupled with either signaling or regeneration failure (Bönnemann, 2011). The 24 down-regulated genes we identified mainly involved transcripts associated with cell-cell communication/adhesion and signaling. Deregulation of transcription, homeostasis, and cell response was also evident. This RNAseq data combines with that obtained by Paco et al. (Paco et al., 2013) using array technology to indicate that COLVI deficiency drives both structural remodeling of muscle tissue, mediated by ECM, adhesion, cell-signaling and metabolism genes, and a massive immune response. The central role of metabolism in muscle remodeling has also been confirmed by De Palma et al. (De Palma et al., 2014), who described changes in the unfolded protein response coupled with metabolic dysregulation, autophagic impairment, and mechanotransduction signaling alterations.

Most intriguingly, and in line with the mouse data, our RNAseq analysis also revealed that circadian-related CRY2 and MAT2A genes were profoundly deregulated. CRY2 is a well-established negative regulator of the molecular clock mechanism that underlies circadian rhythms, and MAT2A has a link with circadian rhythms through regulation of the enzyme required for melatonin synthesis, and mediates photoneural-circadian regulation of the pineal gland (Kim et al., 2005). Using array technology, Paco et al. (Paco et al., 2013) also identified significant deregulation of the CLOCK pathway gene EGR1, which also supports circadian rhythm involvement in the pathogenesis of COLVI in both mice and patients. This finding prompted us to profile both MAT2A and CLOCK in a larger patient cohorts, and the CLOCK gene was significantly downregulated (p value < 0.0001) in all UCMD, the more severe form of ColVI myopathy. All these data support that circadian genes and CLOCK in particular, have a role in ColVI myopathy pathogenesis.

Circadian genes and hereditary myopathies: a new perspective

Circadian rhythms are approximate 24hr oscillations in physiology and behavior driven by the molecular clock, a transcription-translation feedback mechanism. A link between circadian genes and skeletal muscle has been identified in both animal models and in humans (Schroder and Esser, 2013). In particular, expression profiling has identified genes expressed in a circadian manner in the gastrocnemius, soleus and TA muscles of mice (McCarthy et al., 2007; Dyar et al., 2013; Hodge et al., 2015). Analysis of human muscle samples taken 12 hours apart have also shown changes in clock-gene expression, consistent with the findings in mice (Zambon et al., 2003). Indeed, Zhang et al. (Zhang et al., 2012) found that the molecular clock factors BMAL1:CLOCK transcriptionally regulate expression of MyoD1 in adult muscle, thereby suggesting a link between the daily clock cycle and periods of muscle repair and maintenance. We add to this growing body of evidence by demonstrating, by RNA and protein analysis, a novel link between COLVI myopathy and deregulation of CLOCK circadian gene expression in both mouse and human tissues.

In mice, the protein data fully support the transcription results. Altered expression of CLOCK seems to be a marker of COLVI disease severity, being upregulated in the most profoundly affected muscles (TA and diaphragm). In this way, CLOCK involvement appears to provide the missing link between autophagic pathway alterations and the physiopathogenesis of COLVI myopathies (Fig. 5). Supporting this link, many mitochondrial genes show a circadian oscillation in expression and are significantly downregulated in the muscle of Clock mutant mice (McCarthy et al., 2007); in addition, the circadian animal models (both $Clock^{\Delta19}$ and $Bmal1^{-/-}$) have a dramatic reduction (40% less) in the mitochondrial volume. In these mutant mice the remaining mitochondria display a pathological morphology characterized by swelling and disruption of cristae, associated with an increased uncoupling of respiration (Andrews et al., 2010). These findings suggest that circadian genes can modulate the respiratory defects. Unsurprisingly, therefore, protein analysis in the $Bmal1^{-/-}$ mice identified a reduced expression of the α6(VI) chain. Interestingly, a severe depletion of the α6(VI) chain has recently been identified both in skeletal muscle and muscle cell cultures of COLVI patients, and this protein change has been proposed as a diagnostic marker of COLVI diseases (Tagliavini et al., 2014a). Indeed, timing plays a crucial role in muscle remodeling. For instance, Dadgar et al., (Dadgar et al., 2014) demonstrated that asynchrony drives fibrosis and regeneration failure in the mdx model, finding a link between profound deregulation of global muscle transcription and the muscle damage seen in various dystrophies, including dystrophinopathies and several LGMD types.

Recently, BMAL1 was found to be involved in myogenic response to muscle damage, orchestrating the regeneration processes in adult skeletal muscle (Chatterjee et al., 2015)

Although these studies suggest that circadian circuit plays a critical role in muscle function and regeneration, the mechanism mediating these effects in skeletal muscle-wasting diseases remains to be elucidated.

Our results demonstrate, for the first time, a deregulation of circadian genes in skeletal muscle of COLVI myopathy that may lead to characterizing the involvement of circadian clock in many other muscle-wasting diseases, opening new therapeutical perspectives.

Putative models linking collagen VI myopathies with circadian rhythm deregulation

The new data suggest a pathogenic connection between circadian-rhythm-gene deregulation and collagen-VI-related myopathies. It appears that CLOCK gene deregulation could provide a link between autophagy, mitochondrial abnormalities, and COLVI diseases. In light of the asynchrony recently described as the mechanism driving regeneration failure (Dadgar et al., 2014), this relationship could be expressed as one of the two following scenarios:

1) *Circadian rhythm deregulation is a downstream effect of COLVI deficiency and contributes to muscle damage pathogenesis* **(Fig. 6A).** In this hypothesis, the critical player interconnecting COLVI deficiency with circadian rhythm could be the inappropriate persistence of AKT1 activation. Due to the reduced COLVI expression, this would lead not only to the down-regulation of autophagy (Grumati and Bonaldo, 2012), but also to the activation of HIF1A transcription factor and the consequent downregulation of GSK3-beta kinase. Indeed, muscle regeneration is a finely regulated process which drives the transcription of myogenic factors through the orchestration of cell contact, growth factors and various hormones. Murine models lacking or constitutively expressing AKT genes show severe muscle deficiency or extensive hypertrophy, respectively (Wilson and Rotwein, 2007; Peng et al., 2003, Lai et al., 2004), implicating AKT genes (1 and 2) in both the development and regeneration of muscle. Serra et al. (Serra et al., 2007) hypothesize that this may arise through convergence of the AKT pathway and the p38 circuit at the chromatin level controlling the assembly of myogenic transcriptome and driving the regeneration process.

2) *Circadian rhythm deregulation in COLVI myopathies is an independent event acting as a secondary modifier of disease severity* **(Fig. 6B).** Our observation that the collagen VI α6 chain is downregulated in a mouse circadian model could suggest that circadian genes are genetic modifiers able to influence the primary COLVI defect. It is well recognized that the molecular clock in skeletal muscle can be modified by environmental factors including time of feeding or time of physical activity (Wolff and Esser, 2012; Hatori and Panda, 2015). Merging this concept with the known direct control that circadian genes have on MyoD (muscle development), PGC1 (mitochondria) and ATG14 (autophagy circuit) proteins provides the model through which circadian rhythm disruption could function independently to modify disease severity. It may therefore be enlightening to explore circadian genes in the many COLVI patients whose causative

mutations are still unknown (recently mutations in *COL12A1* gene have been linked to the UCMD/BM phenotype) (Hicks et al., 2014).

In conclusion, our demonstration of circadian gene involvement in the pathogenesis of COLVI myopathy in both an animal model and human patients opens novel avenues for both pathogenesis and treatment of hereditary muscle diseases. Timing and synchronization may have different effects on gene expression, depending on the specific gene mutation (COL6 or DMD). Different key regulators, such as AKT1 for COL6 myopathies and TGFB1 for dystrophin mutations (Dadgar et al., 2014), may be implicated, as these two muscle diseases have different pathogenic signatures, represented by defective autophagy and massive muscle necrosis, respectively. Finally, since CLOCK is downregulated only in the severe UCMD phenotype, it should be further explored as a severity biomarker in patients with myopathies characterized by a severe muscle remodeling, with a view to applications in both disease stratification in patients and monitoring in clinical trials.

Material and Methods
Analysis of *Col6a1*^{-/-} mice
RNA extraction

For the focus of this work, we used standardized timing of muscle tissue collection from the *Col6a1*^{-/-} mice and the *Bmal1*^{-/-} mice. Mice in all colonies were maintained in normal 12L/12D schedules with lights on at 7am (Zeitgeber time - ZT0). Muscle tissues were collected between 10am to 12pm (ZT3-5) and frozen immediately in liquid N_2. By following these strict time of collection procedures this allows comparisons across tissues and between animals. Both WT and *Col6a1*^{-/-} mice belong to C57BL/6 strain regarding the fiber type composition of different muscles as previously described (Augusto et al., 2004)

Total RNA was isolated from three muscles (diaphragm, gastrocnemius and tibialis) of four male mice for each condition (wild type and knock out, aging 6 months) using the TRIzol extraction (Invitrogen) as specified by the manufacturer. RNA from each muscle was quantified with Nanodrop (Thermo scientific) spectrophotometer. Its integrity was evaluated with a 2100 Bioanalyser (Agilent) prior to pooling equal amounts of RNA from muscles of the same kind.

Whole genome expression microarray analysis

We used the Whole Mouse Genome gene expression kit composed by four arrays of 44,000 probes (Agilent Technologies, G4122F) to measure transcriptional profile of muscle tissue samples of four mice. Sample labelling and hybridization were performed in triplicates according to

protocols provided by Agilent (One-Colour Microarray-Based Gene Expression Analysis version 5.0.1). The hybridized array was processed by Agilent scanner and Feature Extraction software (v9.5). Uniformness of array hybridization was verified via Agilent Quality Controls (Spike-in), consisting of a mixture of 10 in vitro-synthesized, polyadenylated transcripts derived from the Adenovirus E1A gene, premixed at concentrations spanning six logs and differing by one-log or half-log increments.

Microarray data analysis

Agilent's Feature Extraction Software (v9.5) was used for array image processing. Intensity data with local background adjustment by spatial detrending were log-transformed and quantile-normalized. Synonymous probes from the same gene were averaged. Software selected 28,052 probes for further analysis. To perform multi-way comparisons we utilized limma package (Smyth et al., 2005). This package fits a separate linear model for each gene and uses moderated t-statistics to estimate differences between arrays. Empirical Bayes analysis is used to improve statistical power in small sample sizes. P-values were corrected for multiple testing using the Benjamini & Hochberg correction (Benjamini and Hochberg, 1995).

To ascribe biological changes in KO vs. WT muscles we used Gene Ontology term enrichment analysis. GO terms enriched with differentially expressed genes were identified using Fisher exact test. We also performed Gene Set Enrichment analysis (GSEA), (Subramanian et al., 2005) using Pathway Studio 9 from Elsevier.

Potential transcription regulators of the observed expression changes were identified using sub-network enrichment analysis (Sivachenko et al., 2007) with default parameters implemented in Pathway Studio. This algorithm identifies sub-networks consisting of a single expression regulator (for example, transcription factor) and its downstream genes showing concordant changes in expression between two conditions. Information about gene-gene regulations is extracted from the literature using natural language processing and stored in Pathway Studio database. At the time of analysis, the database contained over 250,000 Expression relations between proteins. Relations were extracted by natural language processing of more than 22 million PubMed abstracts and 880,000 full-text articles.

Microarray data analysis validation by fluidic cards (TLDA)

46 genes were selected for fluidic card analysis based on both differential expression between wild type and KO mice and literature analysis in Pathway Studio to find genes associated with circadian rhythm pathway, muscle regeneration, autophagy, and apoptosis. Selected genes were used to set up a custom TaqMan low density array (TLDA or Fluidic card) according to manu-

facturer specifications in order to validate results obtained using the microarray platform. MIP gene (major intrinsic protein of lens fiber) was selected as negative control for its absence of expression in the microarray data. Single fluidic card can analyse 48 genes (47 test and the 18S control housekeeping gene) simultaneously in eight samples and was run five times for each sample, it also included major autophagic effector protein beclin-1 (Grumati et al., 2010).

150 ng for each RNA pool was retrotranscribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to manufacturer's instructions and loaded in a single port of the TLDA run on ABI 7900HT System (Applied biosystem) for 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles for 15 s at 97 °C and 1 min at 60 °C. Data analysis was performed following the ΔΔCT Method (Applied Biosystems User Bulletin #2) and using the 18S gene as calibrator and the WT samples as controls with respect to the KO samples. Calculated mean 2-ΔΔCT has been Log transformed and compared with normalized fold change microarray data.

Western blotting and RNA analysis in *Bmal1$^{-/-}$* mice

Immunoblotting was used to evaluate protein levels of LC3/MAP1LC3A (Rabbit polyclonal, Thermo scientific, PA1-16930, 1:1000), BECLIN1 (D40C5 Rabbit mAb, Cell SignalingTech, 3495, 1:1000), P62/SQSTM1 (Guinea pig polyclonal, Progen GP62-C, 1:1000), AMPK (Rabbit polyclonal, Cell SignalingTech, 2532, 1:1000), p-AMPK (Rabbit polyclonal, Cell SignallingTech, 2531, 1:1000), COL6A1 (COL6A1 H-200, Santa Cruz Biotech, sc-20649, 1:1000), COL6A3 and COL6A6 (rabbit polyclonal antisera against mouse a3(VI) and a6(VI) were kindly

supplied by R. Wagener, Colonie, Germany). Gastrocnemius muscles from 30-35 week old male wild type (n=4) and Bmal1$^{-/-}$ (Andrews et al., 2010; Bunger et al., 2000) mice (n-4) were homogenized using extraction buffer (2% SDS, 10% glycerol, 2% 2-mercaptoethanol, 50mM Tris-HCl, pH8.8). The homogenized samples were immediately incubated at 80°C for 5 min and protein concentration was measured using BioRad DC protein assay kit. The protein samples (50μg/lane) were then subjected to 7.5% (for ColVI) or 10% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membrane (Millipore). The membrane was blocked in Odyssey blocking buffer (LI-COR) for 1 hour and then incubated with primary Ab in 50% Odyssey blocking buffer (LI-COR) diluted with 1 X PBS containing 0.1% Tween for 1 hour. The membrane was then washed and incubated with HRP-conjugated secondary Ab (for all Collagen VI proteins and p62) or Alexa Fluor 680 secondary Ab diluted in in 50% Odyssey blocking buffer (LI-COR). The protein signal was detected either by incubation with ECL substrate and signals was developed by exposing the membrane to CL-Xposure film (for HRP-conjugated secondary Ab), or by using the Odyssey infrared imager (LI-COR, for Alexa Fluor 680 Ab). The protein

bands were analyzed by using Image J. The plots in the figures are average of four samples for both wild type and *Bmal1*$^{-/-}$. * indicates p<0.05.

All animal procedures were conducted in accordance with institutional guidelines for the care and use of laboratory animals as approved by the AAALAC accredited, University of Kentucky Institutional Animal Care and Use Committees with the protocol number: 00728M2004.

Analysis of BM/UCMD patients

RNA extraction

The three ColVI patients have the following mutations

- UCMD patient: heterozygous c.819_833del p.Pro274_Gly278del in COL6A1 gene

- severe BM patient: homozygous c.1393C>T p.Arg465* in COL6A3 gene

- BM patient: heterozygous c.428+1G>A p. Tyr122_Gly143del in COL6A1 gene

We obtained muscle biopsies from ColVI patients after informed consent and approval of the Ethics Committee of the University of Ferrara (N. 02/2009, 26th of February 2009).

Muscle biopsy procedures were taken at a time according to the local clinics rules, ranging from 8-10 am (central Europe time) or 10-12 am (Greenwich time), depending on the sites. Fresh biopsies were frozen following routine procedures. Because of the similarity in time of collection, comparison of molecular clock components across patient samples can be made. The muscle fiber composition in these three muscles shows a predominance of fiber type I (Fig. S4).

Generally, hereditary myopathies, including the collagen VI myopathies, are characterized by the fiber type disproportion with type I fiber hypotrophy and predominance. (Schessl et al. 2008).

Total RNA was isolated from skeletal muscle biopsies of three patients with different phenotypes: UCMD, dominant and recessive BM (BMs) and from one healthy control biopsy using the RNeasy-kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. We verified high-quality of isolated RNA and RNA integrity using an Agilent Technologies 2100 Bioanalyzer. The isolated RNA had RNA Integrity Number (RIN) value greater than 8.

RNAseq

1 µg of total RNA sample was used for polyA+ mRNA selection using streptavidin-coated magnetic beads, followed by thermal mRNA fragmentation. Fragmented mRNA was retro-transcribed using reverse transcriptase (Super-Script II) and random primers. The cDNA was converted into double stranded cDNA, its ends were repaired using Klenow fragment, T4 polynucleotide kinase and T4 polymerase, and then cDNA was ligated to Illumina paired end (PE) adaptors. Size selection was performed using a 2% agarose gel, generating cDNA libraries ranging in size from 200 to 250 bp. Finally, the libraries were enriched using 15 cycles of PCR (30 seconds at 98° C; 15 cycles of 10 s at 98°C, 30 s at 65°C, and 30 s at 72°C; then 5 minutes at

72°C) and purified by the QIAquick PCR purification kit (Qiagen). The enriched libraries were diluted with Elution Buffer to a final concentration of 10 nM. Each library was run at a concentration of 7 pM on one Genome Analyzer (GAIIe) lane using paired-end 100 bp sequencing.

We performed mapping of reads to human genome using TopHat software. TopHat, the splice junction mapper for RNA-Seq reads, was run with default parameters. Supplied annotations from hg19 Refseq refGene were downloaded from the UCSC Genome Browser. The mapping results from TopHat were then passed to Cuffdiff, a tool which finds significant changes in transcript expression. In Cuffdiff quartile normalization was turned on, Bias Correction was set on, Min Alignment Count was set to 10, and all other parameters were set to default. Both TopHat and Cuffdiff were run on the free pubic Galaxy server (http://galaxyproject.org/). To identify consistently deregulated genes, differential probes were identified by OK test in Cufflinks (Trapnell et al., 2010). Log-ratios were calculated as difference between patient and control.

Quantification of CLOCK and MAT2A transcripts by real-time PCR

To validate the deregulated expression of CLOCK and MAT2A genes, we enlarged our patient cohort. We analysed twelve UCMD, nine BM, three patients with intermediate phenotypes and a pool made of muscle controls. All ColVI patients have been clinically characterized with regard to "circadian involvement" - date and time of biopsy, if the patients were fasting or taking drugs, if the patients were ambulatory and the presence of ventilation at the time of biopsy (Table S3). Total RNA was isolated from muscle sections using RNeasy Kit (QIAGEN, Chatsworth, CA) and reverse transcribed by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). In order to quantify the steady state level of CLOCK and MAT2A transcripts, commercially available TaqMan expression assays (Applied Biosystems) were used (CLOCK: Hs00231857_m1 NM_001267843 exon boundary 19-20; MAT2A: Hs01553622_g1 NM_005911 exon boundary 5-6) as for β-actin as housekeeping reference gene (ACTB Endogenous Control). Real-time PCR was performed in triplicate on the Applied Biosystems Prism 7900HT system, using 10 ng of cDNA and default parameters. Evaluation of transcripts levels was performed by the comparative CT method (ΔΔCT Method). Statistical analysis were performed with Student's *t*-test.

Western blotting analysis in *Col6a1^{-/-}* mice and ColVI patients

For immunoblotting analysis, frozen muscle tissues from both patients and mice were ground in a dry ice-cooled mortar and stored at -80°C. An aliquot of each frozen muscle was suspended in lysis buffer (urea 7M, thiourea 2M, CHAPS 4%, Tris 30mM, and PMSF 1mM) and solubilized by sonication on ice. Samples were pooled and protein concentrations were determined using

PlusOne 2D-Quant kit (GE Healthcare, Milan, Italy). Total protein extracts (50μg) from pooled *Col6a1*$^{-/-}$ (5 mice) and wt (5 mice) animal models, and from pooled UCMD (4 patients), BM (8 patients), 30 unrelated patients with Duchenne (DMD) or Becker (BMD) muscular dystrophies, and healthy control (5 subjects) muscles, were loaded in triplicate and resolved on 14-8% gradient polyacrylamide gels. Blots were incubated with rabbit polyclonal primary antibodies as follows: anti-MAT2A (Novus Biologicals, NB110-94158, 1:1000), anti-CLOCK (Santa Cruz Biotechnology, sc-25361, 1:500). After washing, the membranes were incubated with anti-rabbit (GE Healthcare) secondary antibody conjugated with horseradish peroxidase (1:5000). The signals were visualized by chemiluminescence using the ECL Prime (GE Healthcare) detection kit and the Image Quant LAS 4000 (GE Healthcare) analysis system. Statistical analysis was performed applying the ANOVA and Tukey tests (n=3, $p<0.05$).

Manipulations of animals, required for the experiments described, are conform to the rules of the Animal Experimental Commission of the "Complesso Interdipartimentale A. Vallisneri, University of Padua (Italy)", where the work has been carried out. These regulations represent the application of the D.L. 27/1/92 n. 116 of the Italian Government, which meets Directive 86/609/EEC of the EU Council concerning the protection of animals used for experiments and scientific purposes. The presented procedures have been approved by the Animal Experimental Commission with the certificate n. 22675.

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Figure legend

Fig. 1. Differentially GO groups expressed in sample from pooled tissues from three muscle tissues, diaphragm, gastrocnemius and tibialis anterior, of four Col6a1^{-/-} and WT mice. Representation of the 334 genes differentially expressed in the "pooled" data from all three muscle groups (diaphragm, gastrocnemius and TA) of *Col6a1*^{-/-} mice compared with "pooled" wild-type muscles.

Fig. 2. Regulators identified by SNEA upstream genes differentially expressed between *Col6a1*^{-/-} and wild-type mice muscle tissues (p-value <0.05). Blue highlighting represents regulators whose targets are enriched by genes differentially expressed in samples "pooled" from three muscle types, diaphragm, gastrocnemius and tibialis anterior, of four Col6a1^{-/-} in respect to WT mice yellow highlighting indicates regulators whose targets are enriched with differentially expressed genes that have the same direction of change in all muscle types; green highlighting represents regulators whose targets are enriched with differentially expressed genes with the same direction of change using both "pooled" and individual muscle approaches.

Fig. 3. Circadian genes' transcription and expression analysis on mice and human muscles. (A) Western blotting of CLOCK and MAT2A in three different muscle tissues (diaphragm, gastrocnemius and TA) of five, male 6 months aging, *Col6a1*^{-/-} and wild-type mice revealed a significant upregulation (t-test, P value<0,05) of Clock protein levels in the diaphragm and TA, whereas the MAT2a protein level was significant altered in TA and gastrocnemius, which showed an increase and decrease, respectively. (B) Immunoblotting to evaluate the protein levels of p62, α3(VI), α6(VI), α1(VI), Beclin 1, LC3, AMPK-P, AMPK and α-tubulin in the gastrocnemius muscle of four, male 30–35 week old, wild-type and *Bmal1*^{-/-} mice. The analysis identified a downregulation of α6(VI), and upregulation of α3(VI). Components of autophagic pathway were also affected, expressed as an increase in LC3 and decrease in p62. (C) Real-time analysis of of CLOCK gene (black bars) and MAT2A gene (white bars) transcription in the cohort of 12 BM and 12 UCMD patients. The analysis identified a significant deregulation of the CLOCK gene in the UCMD patients only (t-test, P value<0,0001). (D) Western blotting of CLOCK and MAT2A in four BM, eight UCMD patients, and 30 patients affected by other diseases (BMD and DMD), as well as five healthy controls, showed a significant increase (ANOVA, P value <0.05) in Clock protein levels in UCMD patients only; MAT2A was slightly but significantly increased in BM patients. All the above described experiments were performed in triplicate.

Fig. 4. Gene ontology groups in ColVI patients RNAseq output. (A) Representation of the differentially expressed GO genes in the ColVI patients (UCMD, BMs, BM) compared to control. (B) Venn diagrams showing the number of genes, identified by RNAseq analysis, constantly de-

regulated in the ColVI patients. The patients shared an upregulation of genes involved in postsynaptic membrane, cell junction and synapse pathways, and a downregulation of genes belonging to response to DNA damage stimuli and DNA repair pathways.

Fig. 5. Interactome pathway linking ColVI and circadian rhythms. This model is based on the literature data analyzed by Pathway Studio, and shows the connections between autophagy and circadian pathways. In this model the proposed pathophysiological mechanism of UCMD is that mTOR kinase activation due to ColVI deficiency yields not only downregulation of autophagy but also activation of HIF1A transcription factor, which in turn represses the expression of autophagy components via FoxO3 inhibition. In addition, HIF1A physically interacts with several major components of the circadian rhythm pathway, bringing about a reduction of MYOD levels. AKT1 directly inhibits GSK3-beta by phosphorylation. GSK3-beta is a central negative regulator of circadian rhythm that phosphorylates its components. GSK3-beta repression by AKT1 further de-represses HIF1A, which is under GSK3-beta negative control.

Fig. 6. Proposed models (A and B) that potentially link collagen VI myopathy and circadian genes:

Mechanism A: Circadian rhythm deregulation is a downstream effect of collagen VI deficiency and contribute to muscle damage pathogenesis. The inappropriate persistence of AKT1 activation could follow reduced ColVI expression, leading not only to the downregulation of autophagy (also related to mTOR overexpression) but also to the activation of HIF1A transcription factor and the downregulation of GSK3-beta kinase. HIF1A physically interacts with several central components of the circadian rhythm pathway: BMAL1/ARNTL/MOP3 protein (Hogenesch et al., 1998), PER1 (Chilov et al., 2001), PER2 (Koyanagi et al., 2003) and ROR-alpha. HIF1A activation has been shown to increase levels of PER1 and CLOCK proteins (Chilov et al., 2001). The increased levels of PER1, the negative regulator of circadian rhythm, may lead to decreased transcriptional activity of BMAL1-CLOCK heterodimer, thereby slowing down circadian oscillations. GSK3-beta also plays a role in the post-translational regulation of molecular clock components, targeting BMAL1 for degradation at the end of the circadian cycle and stabilizing Rev-ErbA, a negative regulator of BMAL1 transcription (Pritchett et al., 2012).

Mechanism B: Circadian rhythm deregulation in collagen VI myopathies is an independent event acting as a secondary modifier of disease severity. The collagen VI α6 chain is downregulated in the mouse circadian model, suggesting that circadian genes can influence the primary COLVI defect, as supported by the direct control circadian genes have on MyoD (muscle development) and ATG14 (autophagy circuit) proteins. Downregulation of CLOCK induces ATG14 persistence and autophagy activation, whereas MyoD overexpression alters regeneration and induces muscle damage).

Table 1: List of the seven upregulated and 24 downregulated genes, identified by RNAseq, consistently represented in all three patients.

Table 1

| GENE	NAME	UP/DOWN REGULATED	ACCESSION NUM-BER	OMIM	FUCTION	CORRELATION WITH NEUROMUS-CULAR DISEASE	FOLD-CHANGE		
							BM	BMs	UCMD
ZNF384	Zinc Finger Protein 384	Up	NM_001039919	*609951	Transcription factor that regulate the promoters of the extracellular matrix genes MMP1, MMP3, MMP7 and COL1A1		39,3095	37,233	39,7226
ZNF526	Zinc Finger Protein 526	Up	NM_133444	*614387	Transcription factor		2,7601	1,6734	1,6813
SCP2	Sterol Carrier Protein 2	Up	NM_001007250	*184755	SCP2 protein is involved in the lipid me-tabolism	leukoencephalopathy with dystonia and motor neuropathy	2,0683	2,2456	1,4437
SUOX	Sulfite Oxidase	Up	NM_000456	*606887	The SUOX enzyme catalyzes the oxidation of sulfite to sulfate	Sulfite oxidase deficien-cy	37,8363	38,1009	39,0129
KIF1B	Kinesin Family Member 1B	Up	NM_015074	*605995	This gene encodes a motor protein that transports mitochondria and synaptic ves-icle precursors.	Charcot-Marie-Tooth disease, type 2A1	3,1132	2,5426	1,9332
PCSK6	Proprotein Convertase Subtili-sin/Kexin Type 6	Up	NM_138321	*167405	PCSK6 encoded protein is a calcium-dependent serine endoprotease that can cleave precursor protein at their paired basic amino acid processing sites. Some of its substrates are - transforming growth factor beta related proteins, proalbumin, and von Willebrand factor.		1,7039	2,2606	1,9061
SESN1	Sestrin 1	Up	NM_014454	*606103	SESN1 play a role in the cellular response to DNA damage and oxidative stress		1,8542	1,5004	1,4777

CRY2	**Cryptochrome Circadian Clock 2**	**Down**	**NM_021117**	***603732**	**Circadian gene necessary for the generation of circadian rhythms**		**-40,4144**	**-1,1962**	**-40,3619**
MAT2A	**Methionine Adenosyltransferase II, Alpha**	**Down**	**NM_005911**	***601468**	**MAT2A catalyzes the production of S-adenosylmethionine (AdoMet) from methionine and ATP**		**-2,7992**	**-2,2145**	**-3,7638**
ARTN	**Artemin**	**Down**	**NM_001136215**	***603886**	**ARTN is involved in signaling of dopaminergic CNS neurons**		**-1,9588**	**-3,0809**	**-4,4802**
WNT3A	**Wingless-Type MMTV Integration Site Family, Member 3A**	**Down**	**NM_033131**	***606359**	**Ligand for members of the frizzled family of seven transmembrane receptors. Wnt3a play roles in cell-cell signaling**		**-1,7748**	**-1,5573**	**-1,6244**
PTPN11	**Protein Tyrosine Phosphatase, Non-Receptor Type 11**	**Down**	**NM_002834**	***176876**	**Protein tyrosine phosphatases (PTPs) are a group of enzymes that catalyze the removal of phosphate groups from tyrosine residues by the hydrolysis of phosphoric acid monoesters. PTPN11 participates in the signal transduction from the cell surface to the nucleus**		**-1,8989**	**-3,1835**	**-1,9862**

CD3G	CD3g Molecule, Gamma (CD3-TCR Complex)	Down	NM_000073	*186740	The protein encoded by this gene is the CD3-gamma polypeptide, which together with CD3-epsilon, -delta and -zeta, and the T-cell receptor alpha/beta and gamma/delta heterodimers, forms the T-cell receptor-CD3 complex. This complex plays an important role in coupling antigen recognition to several intracellular signal-transduction pathways		-38,1546	-37,7322	-3,2284
CDH1	Cadherin 1, Type 1, E-Cadherin (Epithelial)	Down	NM_004360	*192090	CDH1 is a calcium dependent cell-cell adhesion glycoprotein		-1,6719	-2,049	-1,3894
DMAP1	DNA Methyltransferase 1 Associated Protein 1	Down	NM_001034024	*605077	DMAP1 is involved in transcription repression and activation.		-37,8224	-37,4289	-37,7671
ZNF181	Zinc finger 181	Down	NM_001145665	*606741	May be involved in transcriptional regulation		-1,4527	-1,0334	-1,6816
ZNF233	Zinc finger 233	Down	NM_181756	-	May be involved in transcriptional regulation		-1,7889	-0,9786	-1,4194
ZNF619	Zinc finger 619	Down	NM_001145083	-	May be involved in transcriptional regulation		-3,487	-1,1514	-1,6887

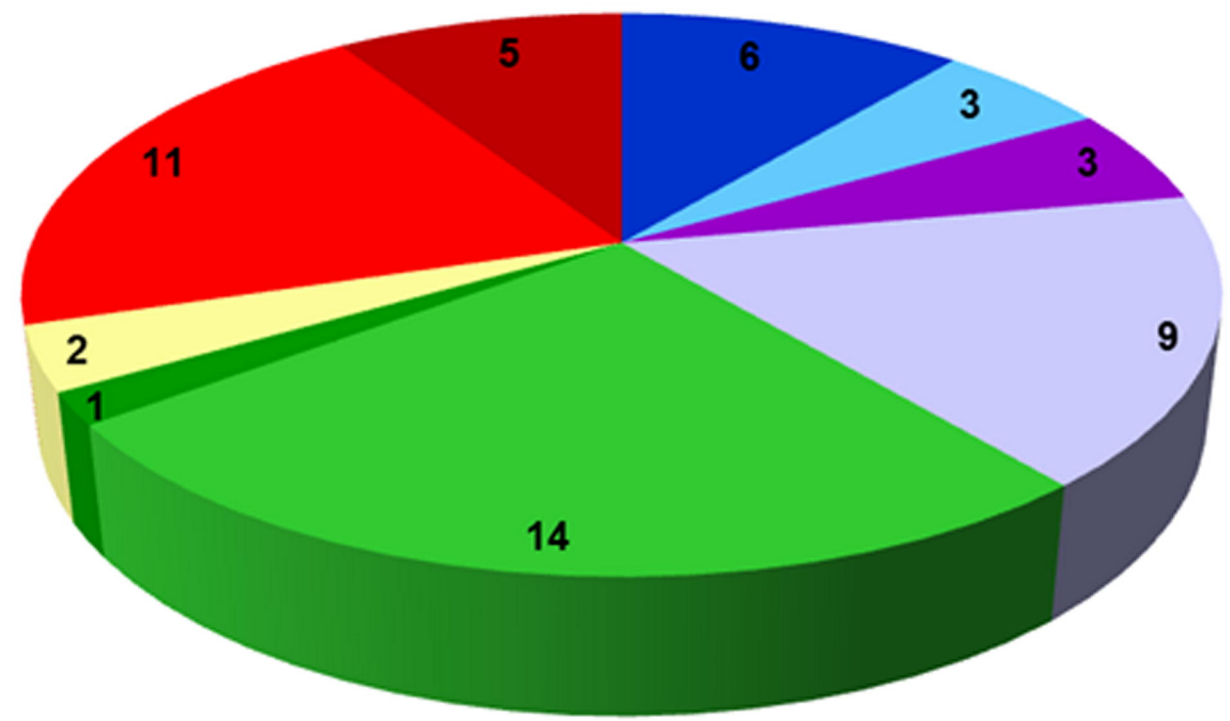| Gene	Name		NM	OMIM	Description		Value 1	Value 2	Value 3
GCKR	Glucokinase (Hexokinase 4) Regulator	Down	NM_001486	*600842	GCKR is a regulatory protein that inhibits glucokinase in liver and pancreatic islet cells by binding non-covalently to form an inactive complex with the enzyme.		-2,2272	-1,2255	-2,0378
PGLS	6-phosphogluconolactonase	Down	NM_012088	*604951	Hydrolysis of 6-phosphogluconolactone to 6-phosphogluconate		-2,4972	-2,65	-2,341
SCO1	SCO1 Cytochrome C Oxidase Assembly Protein	Down	NM_004589	*603644	Thought to play a role in cellular copper homeostasis, mitochondrial redox signaling or insertion of copper into the active site of COX		-1,7047	-1,5227	-2,4417
GPR15	G Protein-Coupled Receptor 15	Down	NM_005290	*601166	This gene encodes a G protein-coupled receptor that acts as a chemokine receptor for human immunodeficiency virus type 1 and 2.		-2,1248	-1,7917	-1,7865
ITPRIP	Inositol 1,4,5-Trisphosphate Receptor Interacting Protein	Down	NM_033397	-	ITPRIP encodes a membrane-associated protein that binds the inositol 1,4,5-trisphosphate receptor (ITPR). The encoded protein enhances the sensitivity of ITPR to intracellular calcium signaling.		-4,7234	-5,8684	-4,4783
ADCY4	Adenylate Cyclase 4	Down	NM_001198568	*600292	ADCY4 is membrane-associated enzymes that catalyze the formation of the secondary messenger cyclic adenosine monophosphate (cAMP)		-3,4624	-2,0732	-2,4598

Gene	Name	Direction	Accession	OMIM	Description	Disease	Value 1	Value 2	Value 3
NEDD1	Neural Precursor Cell Expressed Developmentally Down-Regulated Protein 1	Down	NM_001135177	*600372	NEDD1 is required for mitosis progression. Promotes the nucleation of microtubules from the spindle		-40,2291	-39,7942	-40,1467
HECTD3	HECT Domain Containing E3 Ubiquitin Protein Ligase 3	Down	NM_024602	-	HECTD3 mediates ubiquitination of TRIOBP and its subsequent proteasomal degradation, thus faciliting cell cycle progression by regulating the turn-over of TRIOBP. Mediates also ubiquitination of STX8		-1,7798	-1,0458	-1,6116
ERP27	Endoplasmic Reticulum Protein 27	Down	NM_152321	*610642	ERP27 is a noncatalytic member of the protein disulfide isomerase family of endoplasmic reticulum (ER) proteins	ataxia	-5,3233	-2,0534	-2,0794
MID1IP1	MID1 Interacting Protein 1	Down	NM_021242	-	MID1IP1 plays a role in the regulation of lipogenesis in liver.		-2,015	-1,4034	-1,8172
C1orf64	Chromosome 1 Open Reading Frame 64	Down	NM_178840	-	Involved breast and colorectal cancer		-38,3313	-3,1196	-38,2086
TMEM110	Transmembrane Protein 110	Down	NM_198563	-	Structural protein		-1,6315	-1,1639	-1,1536
PRSS42	Protease, Serine, 42	Down	NM_182702	-	serine-type endopeptidase activity		-2,0183	-1,6536	-1,726

**A** Genes are significantly different for pooled muscles in KO vs WT: <u>upregulated genes</u>
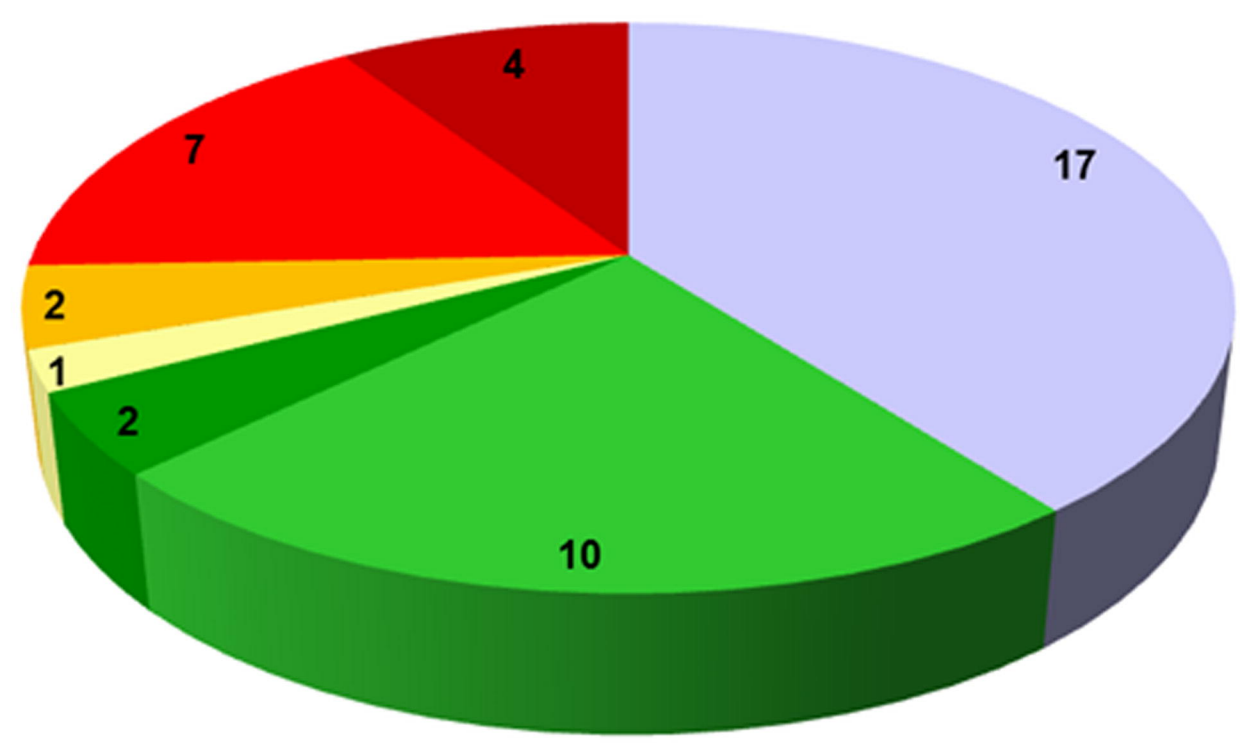
17 — regulation of transcription
10 — ATP binding
2 — Wnt-protein binding
1 — response to cadmium ion
2 — thyroid hormone generation
7 — transport
4 — apoptosis

Legend:
- regulation of transcription
- ATP binding
- Wnt-protein binding
- response to cadmium ion
- thyroid hormone generation
- transport
- apoptosis

B Genes are significantly different for pooled muscles in KO vs WT: <u>downregulated genes</u>

6 — rhythmic process
3 — nuclear localization sequence binding
3 — retinol metabolic process
9 — regulation of transcription
14 — ATP binding
1 — Wnt-protein binding
2 — response to cadmium ion
11 — transport
5 — apoptosis

Legend:
- rhythmic process
- nuclear localization sequence binding
- retinol metabolic process
- regulation of transcription
- ATP binding
- Wnt-protein binding
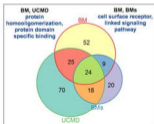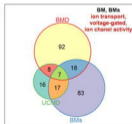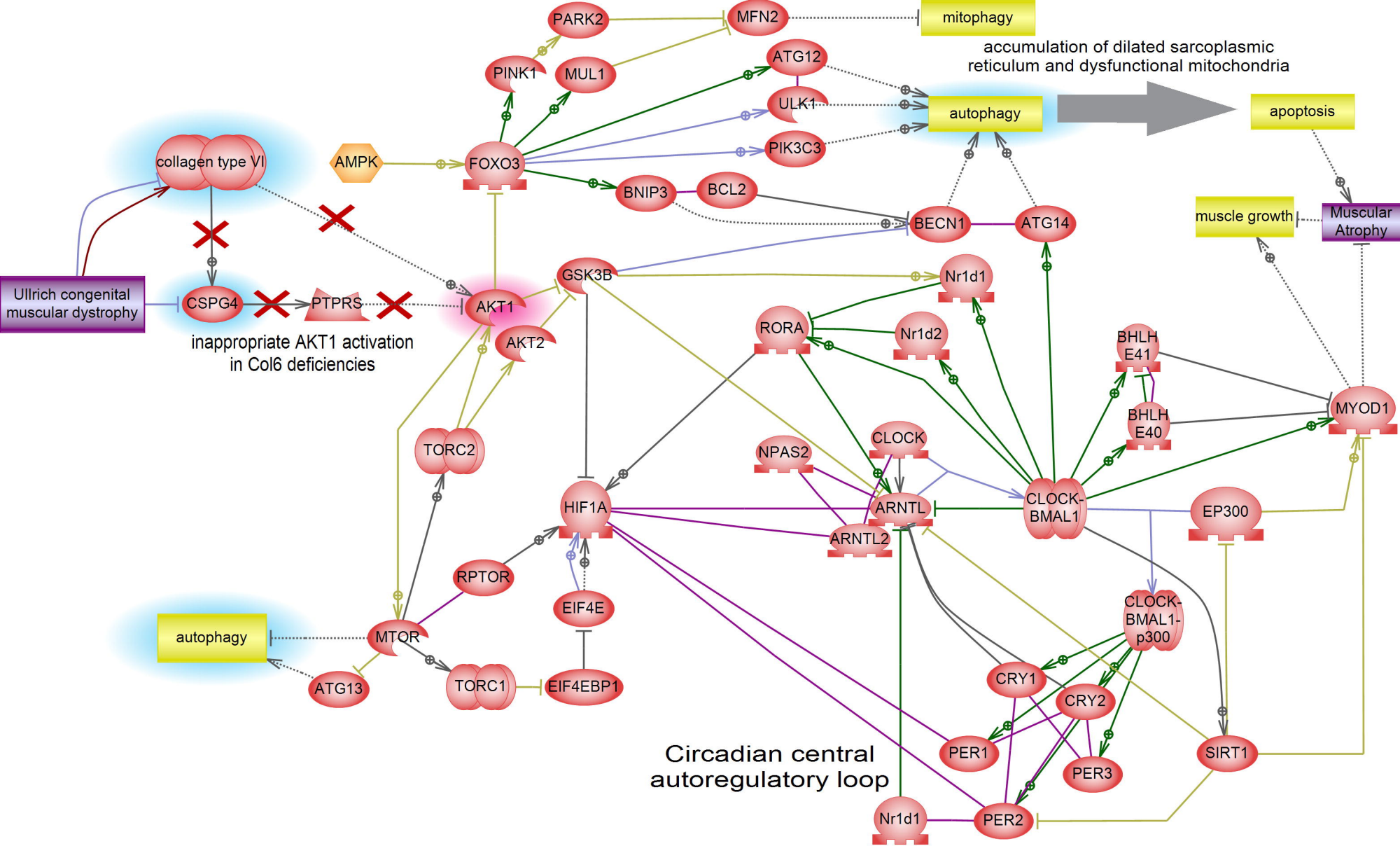- response to cadmium ion
- transport
- apoptosis

A
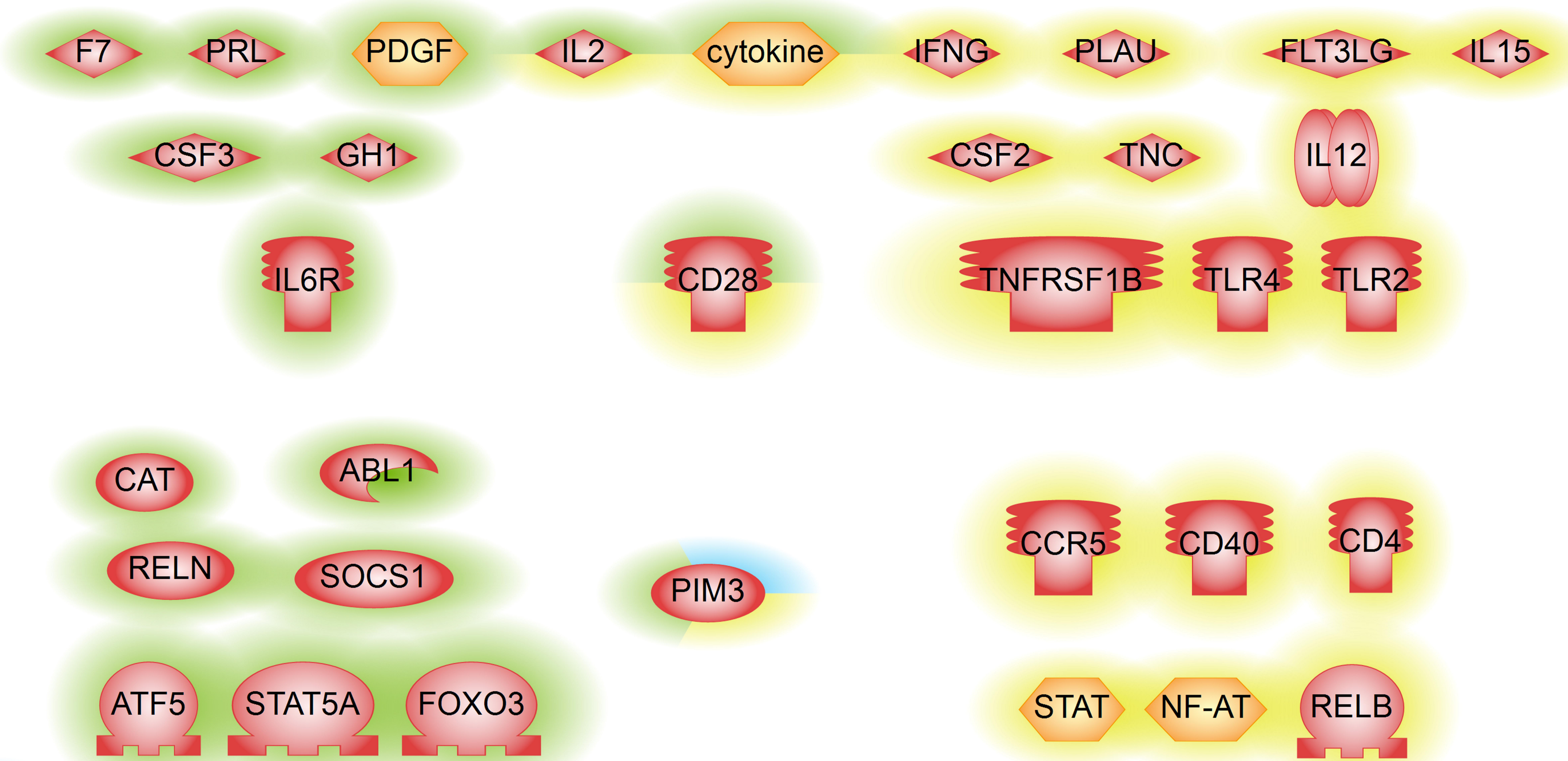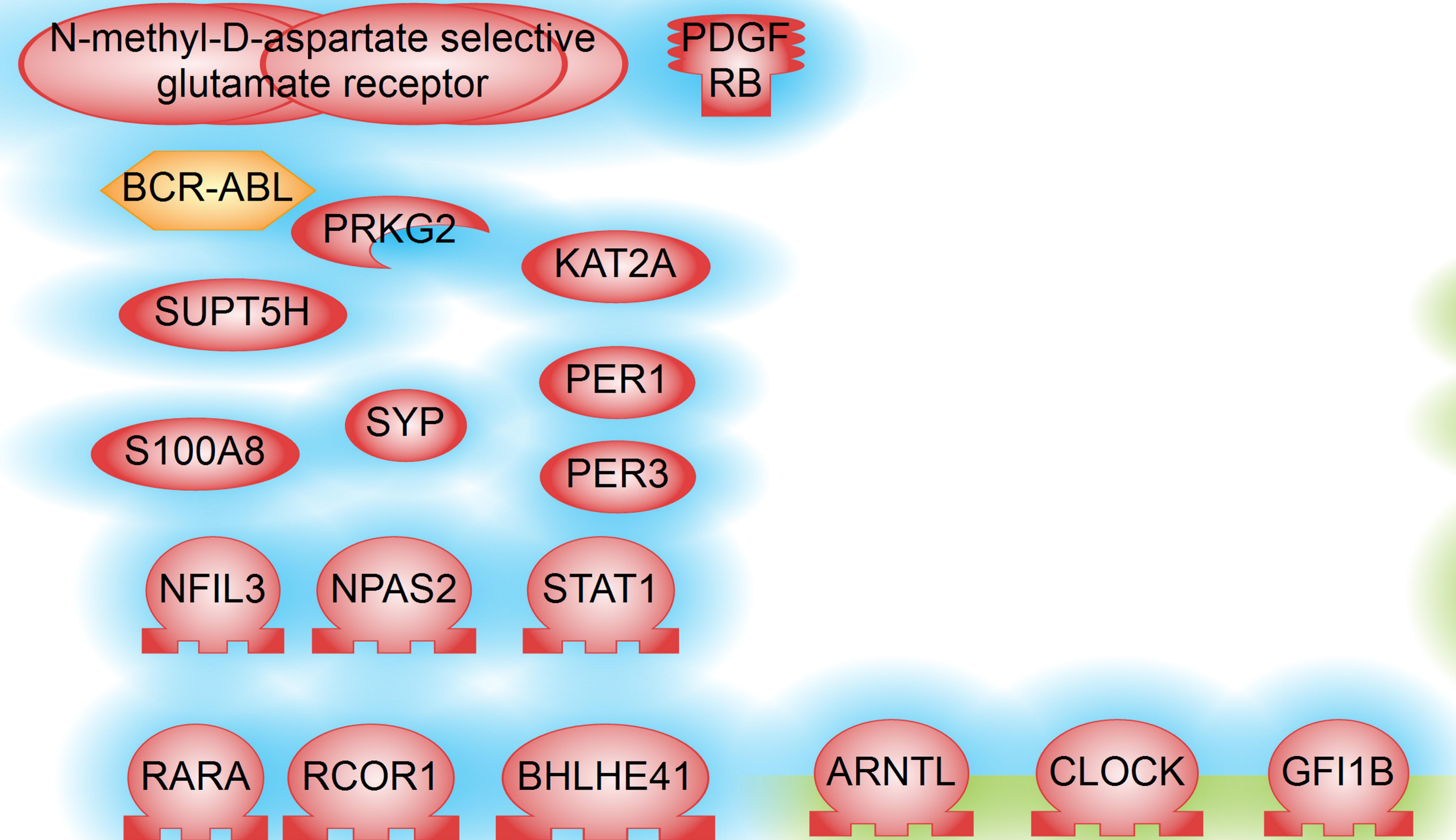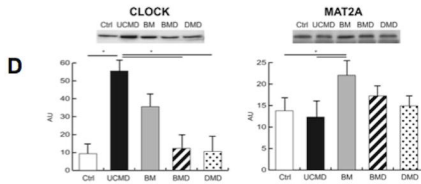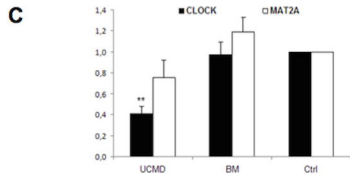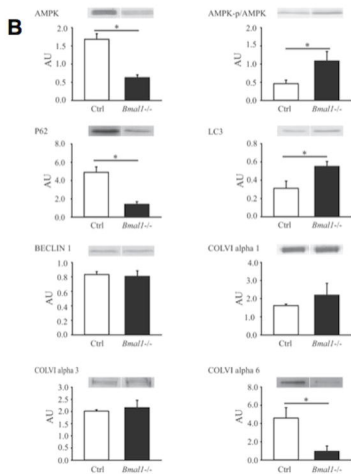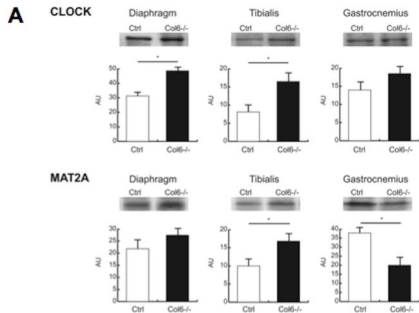
GO groups in COL VI patients vs control

- postsynaptic membrane
- synapse
- response to DNA damage stimuli
- voltage-gated ion channel activity
- ion transport
- protein complex
- transmembrane receptor activity
- cell junction
- DNA repair
- regulation of ion transmembrane transport
- calcium ion transport
- protein homeostatization
- protein complex binding
- cell surface receptor linked signaling pathway

B

ion transport,
voltage-gated,
ion channel activity

protein
homeostatization,
protein domain
specific binding

cell surface receptor,
linked signaling
pathway

All samples unregulated
post-synaptic membrane, cell junction, synapse

All samples downregulated
DNA repair, response to DNA damage stimulus

accumulation of dilated sarcoplasmic reticulum and dysfunctional mitochondria

inappropriate AKT1 activation in Col6 deficiencies

Circadian central autoregulatory loop

A

COLLAGEN

FOXO3
mTOR

AKT1

CALPAIN

CASPASE

GSK3

ATG14

HIF1A

PER
CLOCK
REV-ERB
BMAL

AUTOPHAGY

CIRCADIAN DEREGULATION

APOPTOSIS

MYOD   PGC1

MUSCLE DAMAGE

B

COLLAGEN

CALPAIN

CASPASE

AKT1

FOXO3   mTOR

CIRCADIAN DEREGULATION

ATG14

MYOD   PGC1

APOPTOSIS

AUTOPHAGY

MUSCLE DAMAGE

◆ inhibition   biological process
→ regulation   protein