Ca^{2+} handling abnormalities in early-onset muscle diseases: novel concepts and perspectives

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ABSTRACT

The physiological process by which Ca^{2+} is released from the sarcoplasmic reticulum is called excitation-contraction coupling; it is initiated by an action potential which travels deep into the muscle fiber where it is sensed by the dihydropyridine receptor, a voltage sensing L-type Ca^{2+}channel localized on the transverse tubules. Voltage-induced conformational changes in the dihydropyridine receptor activate the ryanodine receptor Ca^{2+} release channel of the sarcoplasmic reticulum. The released Ca^{2+} binds to troponin C, enabling contractile thick-thin filament interactions. The Ca^{2+} is subsequently transported back into the sarcoplasmic reticulum by specialized Ca^{2+} pumps (SERCA), preparing the muscle for a new cycle of contraction. Although other proteins are involved in excitation-contraction coupling, the mechanism described above emphasizes the unique role played by the two Ca^{2+} channels (the dihydropyridine receptor and the ryanodine receptor), the SERCA Ca^{2+} pumps and the exquisite spatial organization of the membrane compartments endowed with the proteins responsible for this mechanism to function rapidly and efficiently. Research over the past two decades has uncovered the fine details of excitation-contraction coupling under normal conditions while advances in genomics have helped to identify mutations in novel genes in patients with neuromuscular disorders. While it is now clear that many patients with congenital muscle diseases carry mutations in genes encoding proteins directly involved in Ca^{2+} homeostasis, it has become apparent that mutations are also present in genes encoding for proteins not thought to be directly involved in Ca^{2+} regulation. Ongoing research in the field now focuses on understanding the functional effect of individual mutations, as well as understanding the role of proteins not specifically located in the sarcoplasmic reticulum which nevertheless are involved in Ca^{2+} regulation or excitation-contraction coupling. The principal challenge for the future is the identification of drug targets that can be pharmacologically manipulated by small molecules, with the ultimate aim to improve muscle function and quality of life of patients with congenital muscle disorders. The aim of this review is to give an overview of the most recent findings concerning Ca^{2+} dysregulation and its impact on muscle function in patients with congenital muscle disorders due to mutations in proteins involved in excitation-contraction coupling and more broadly on Ca^{2+} homeostasis.

KEY WORDS: Ca^{2+} homeostasis, sarcoplasmic reticulum, excitation-contraction coupling, congenital myopathies, ryanodine receptor, dihydropyridine receptor, SERCA, mutations.

1 INTRODUCTION

1.1 The sarcoplasmic reticulum and Ca^{2+} regulation.

The sarcoplasmic reticulum (SR) is a specialized intracellular membrane compartment present in striated muscles and almost completely dedicated to Ca^{2+} regulation. The SR can be subdivided into different functional domains that are each enriched in specific Ca^{2+} handling and structural proteins. The transverse tubules (T-tubules) are invaginations of the plasma membrane and contain the voltage sensing dihydropyridine receptor (DHPR). The portion of the SR terminal cisternae membrane facing the T- tubules is called junctional face membrane and contains the ryanodine receptor Ca^{2+} release channel (RyR1), regulatory and structural proteins such as JP-45, triadin and junctin as well as a number of other proteins that are part of the RyR1 Ca^{2+} release channel macromolecular structure [1-4]. In skeletal muscle, the triad, i.e. the structure formed by the T-tubule and two sarcoplasmic reticulum terminal cisternae is the membrane domain directly involved in excitation-contraction coupling. Figure 1 illustrates the main protein components of the excitation-contraction coupling machinery in mammalian skeletal muscle.

The sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) is the main protein of the longitudinal sarcoplasmic reticulum, accounting for >70% of its total protein content. SERCAs belong to class P-type ATPases and are responsible for replenishing the SR with Ca^{2+} after its release following excitation- contraction coupling [2,4,5]. In striated muscle, SERCA activity can be modulated by two small regulatory proteins, sarcolipin and phospholamaban [5,6]. Sarcolipin decreases the affinity of SERCA1a and SERCA2a for Ca^{2+} thereby inhibiting its re-uptake in the SR and diminishing the SR Ca^{2+} stores. In humans it is expressed in both fast and slow twitch muscles as well as in cardiac muscle [7,8]. Phospholamban, which is expressed in cardiac and slow twitch muscles, acts as an inhibitor of SERCA2a when present in the unphosphorylated form, but upon phosphorylation by PKA its inhibitory effects

are abolished [9,10]. Interestingly sarcolipin and phospholamban share ~30% identity and have been proposed to bind to the same domain on SERCA2a [5,6,11]. Myoregulin is a recently identified skeletal muscle-specific micropeptide sharing structural similarities with sarcolipin and phospholamban. Myoregulin interacts with SERCA1 and by so doing decreases the pump's activity [12]. Its deletion in mice enhances Ca^{2+} handling and improves exercise performance, suggesting that myoregulin plays an important physiological role in regulating skeletal muscle Ca^{2+} homeostasis.

1.2 Excitation-contraction coupling

Excitation-contraction coupling (ECC) is the process whereby an electrical signal generated by an action potential is converted into a chemical gradient, that is, an increase in the myoplasmic [Ca^{2+}], leading to muscle contraction [13,14]. Efficient ECC relies on a highly sophisticated subcellular architecture, where voltage sensing L-type Ca^{2+} channels (DHPRs) lie opposite RyR1 in a highly organized conformation resembling a checkerboard, with every other RyR1 tetramer facing four DHPRs arranged in a characteristic square shape called a tetrad [15, 16]. The DHPR is a macromolecular complex made up of at least five subunits present in a 1:1:1:1:1 ratio, including $Ca_v1.1$ (or $\alpha1.1$), the channel pore that interacts through its II-III loop with the RyR1, as well as the $\alpha2$, ß, γ and ∂ subunits [17]. Several diseases have been linked to mutations in *CACNA1S,* the gene encoding $Ca_v1.1$, including some forms of hypokalemic periodic paralysis and malignant hyperthermia [18-20].

The RyR1 is a huge macromolecular complex of >2.5 MDa made up of 4 homotetramers of ≈565 kDa each that assemble into a functional Ca^{2+} release channel. The first 4000 amino acids make up the large cytoplasmic domain that contains binding sites for various modulators, while the last 1000 amino acids constitute the carboxy terminal pore-forming domain [21-22]. Dominant and recessive mutations in *RYR1,* the gene encoding the RyR1, are associated with a range of early-onset neuromuscular disorders including the core myopathies central core disease (CCD) and multi-minicore disease (MmD), congenital fiber type disproportion, (CFTD) centronuclear myopathy (CNM), exertional rhabdomyolysis/myalgia as well as the pharmacogenetic disorder malignant hyperthermia (MH) [23-26].

Aside the RyR1, the junctional sarcoplasmic reticulum is enriched in several other proteins including the structural proteins triadin and junctin, as well as minor constituents such as JP-45, junctate and humbug, mitsugumin-29, SRP-27/TRIC-A and junctophilin-1 [4, 27-29]. These minor constituents play a role in the fine regulation of Ca^{2+} release from the SR or are involved in maintaining the structural integrity of the Ca^{2+} release machinery. Another major constituent of the junctional SR is the high capacity, low affinity Ca^{2+} binding protein calsequestrin. In the presence of millimolar Ca^{2+} (that is, at a concentration similar to that present at rest in the lumen of the SR), calsequestrin polymerizes and forms a mesh-like structure in an area adjacent to the RyR1 where it acts as the principal SR Ca(2+) storage protein. [29-31]. The luminal domain of junctin interacts directly with calsequestrin and together with triadin forms a complex that is thought to have a dual role: (i) maintaining calsequestin adjacent to the RyR1 and (ii) sensing the amount of Ca^{2+} in the lumen of the SR [31, 32]. Two isoforms of calsequestrin have been identified: calsequestrin1 that is predominantly expressed in fast twitch skeletal muscles and calsequestrin 2 that is expressed in cardiac and slow twitch muscle. Mutations in *CSQ2* (the gene encoding calsequestrin 2) have been linked to catecholaminergic polymorphic ventricular tachycardia (CPVT), sudden cardiac arrest and heart failure [33,34] and will not be discussed in this review, whereas mutations in *CSQ1* (the gene encoding calsequestrin 1) have been implicated in rare cases of malignant hyperthermia and vacuolar aggregate myopathy [35,36].

2.1 Pathological consequences of mutations in genes encoding proteins directly involved in ECC

2.1.1 Disorders associated with *RYR1* mutations

The first mutation identified in *RYR1,* the gene encoding the skeletal muscle RyR1, was linked to Malignant Hyperthermia Susceptibility (MHS), a pharmacogenetic disorder triggered by halogenated anesthetics and by the muscle relaxant succinylcholine [37]. Since this initial discovery in the early 90s, more than 200 mutations have been identified in patients with a variety of inherited myopathies including MHS, exertional rhabdomyolysis/myalgia and a range of congenital

myopathies including CCD, as well as subgroups of MmD, CNM and CFTD [23-26]. Because of the sheer size of the *RYR1* gene which spans 106 exons and >15 kb DNA, initial mutation searches were confined to hotspot domains originally implicated in autosomal dominant MHS and CCD. However, thanks to technical advances in DNA sequencing and genome analysis, a more systematic examination of the *RYR1* gene became possible and revealed that mutations, especially recessive ones, can affect the entire coding region of the *RYR1* gene. The calculated frequency of *RYR1* mutations is approximately 1:3000 making it one of the most commonly mutated genes associated with neuromuscular disorders [38-40]. A number of reviews concerning the frequency, disease associations and functional impact of *RYR1* mutations have been published in recent years and the reader is referred to these for a more in depth description (for example see [12-24,41-45]. The disease phenotype resulting from *RYR1* mutations largely depends on their location within the *RYR1* coding sequence and whether the mutations are dominant or recessive. Dominant mutations are more commonly associated with the MHS or exertional rhabdomyolysis and CCD phenotypes, while most cases of MmD, CNM and CFTD are associated with recessive mutations. From a general point of view, dominant *RYR1* mutations affect the biophysical properties of the Ca^{2+} channel while the mode of action of recessive mutations has been more difficult to decipher.

For gain-of-function dominant MHS-linked mutations that render the Ca^{2+} channel hypersensitive and thus more prone to be in the open conformation, the amino acid substitutions induce local protein misfolding or affect the intersubunit domain-domain interfaces; the resulting tetrameric channels are deregulated leading to a leak of Ca^{2+} into the cytoplasm or to an excessive amount of Ca^{2+} release from the SR [22, 24, 46-49]. This results in a number of direct effects in the muscle fibers including a higher resting cytosolic [Ca^{2+}], a shift in the sensitivity to activating signals (depolarization, pharmacological stimulation) or greater depolarization-induced Ca^{2+} influx, all of which can lead to a hypermetabolic state which is the underlying feature of MHS.

Dominant mutations associated with CCD induce weak muscles either because they render the RyR1 channels less efficient at transporting Ca^{2+}, or because they lead to intraluminal SR Ca^{2+} store depletion [50,51]. Although understanding precisely the functional impact of the mutation is important in order to develop an appropriate

pharmacological approach, both types of mutations lead to changes in the biophysical properties of the RyR1 and cause weak muscles because Ca^{2+} is not available to bind to the contractile proteins and initiate contraction.

Two important aspects concerning the effects of dominant *RYR1* mutations still require clarification. One relates to the protomer composition of the Ca^{2+} channel in individuals carrying dominant *RYR1* mutations, while the other is related to secondary downstream effects. It is very difficult, if not impossible, to establish the exact protomer composition of the RyR1 protein complex in individuals carrying dominant *RYR1* mutations. A variation in the proportion of protomers encoded by wild type and mutated alleles within the tetrameric RyR1 Ca^{2+} channel complex will undoubtedly affect the biophysical properties of the Ca^{2+} channel [52], possibly contributing to the phenotypic variation seen in patients carrying the same *RYR1* mutations. The primary defects in Ca^{2+} regulation caused by dominant *RYR1* mutations will also lead to secondary downstream effects in the muscle fibers, such as increased release of inflammatory cytokines, mitochondrial abnormalities, modifications due to the excessive generation of reactive oxygen and nitrogen species, and changes in transcriptional regulation, including those that are dependent on activation of the Ca^{2+}–sensitive NFAT transcription factors [50,53,54]. An example of such an effect is illustrated in figure 2, showing that in myotubes from patients with CCD carrying dominant *RYR1* mutations, the nuclear to cytoplasmic ratio of NFATc1 is higher than in myotubes from control individuals, under resting non-stimulated conditions. Furthermore, the nuclear to cytoplasmic ratio of NFATc1 increases dramatically after KCl-induced depolarization (Fig. 2 A and B) [53]. Moreover, even in the absence of inflammatory conditions (as suggested by the lack of upregulation of the C-reactive protein, a marker of inflammation) the serum content of pro-inflammatory cytokines such as IL-6 is chronically elevated in individuals carrying dominant *RYR1* mutations (panel C), but the functional consequences of such changes are largely unknown.

From figure 2 it is also apparent that the control and MmD (expressing recessive *RYR1* mutations) groups are more similar in their serum IL-6 levels and this is probably linked to the functional effect of the mutations. Indeed, in myotubes from patients with recessive *RYR1* mutations no dysregulation of Ca^{2+} homeostasis was detected [55]. This result is puzzling since muscle biopsies from the same patients

show significant depletion of RyR1 protein, which should lead to a pronounced reduction of the amount of Ca^{2+} released following RyR1 activation [55-57]. In a recent study we addressed the pathophysiology of recessive *RYR1* mutations and in particular the epigenetic mechanism(s) responsible for bringing about such a drastic decrease of RyR1 protein in the muscles of patients [55]. Interestingly, most patients harboring recessive *RYR1* mutations exhibit a 6-16 fold increase in class II histone de-acetylase protein content (HDAC-4 and HDAC-5) and hypermethylation of (at least) a CpG island within the *RYR1* gene. Class II HDACs can bind to and sequester the muscle transcription factor mef-2 [58], thus one would expect that an increase in HDAC-4/5 expression would prevent the transcription of mef-2 dependent genes. The transcription of miR-1 and miR-133 has been shown to be mef-2 dependent [59] and the 5' region of the *RYR1* contains a mef-2 binding domain [60]. Furthermore, since miR-22 and miR-124 have binding sites within the 3'UTR of the *RYR1* their transcription may be linked to that of their binding partner *RYR1*. These results are compatible with and explain our findings in muscle biopsies of patients with recessive *RYR1* mutations who not only showed reduced RyR1 protein levels, but also reduced myomesin content (a mef-2 dependent protein) and a significant down-regulation of muscle specific micro RNAs including miR-22, miR-124, miR-1 and miR-133 [55]. Our working hypothesis is illustrated in figure 3 where it is shown that the down-regulation of RyR1 is a consequence of the activation of a pathological epigenetic loop in skeletal muscle. These results are novel and will require further investigations in animal models, but if confirmed they will open new pathways for the treatment of muscle disorders which would then not target directly the primary genetic defect, but the downstream effects (in this case methylation and HDAC activation) which are hyperactivated in patients with neuromuscular diseases due to recessive *RYR1* mutations and possibly also in patients with neuromuscular disorders due to mutations in other genes, increasing the significance of these findings.

2.1.2 Disorders associated with *CACNA1S* mutations

In skeletal muscle, the RyR1 and DHPR constitute a signaling complex whereby orthograde (or forward) coupling between the two complexes activates Ca^{2+} release from luminal SR stores via the opening of RyR1 channels, while retrograde signaling from the RyR1 back to the DHPR regulates the magnitude of the L-type inward Ca^{2+} current from the extracellular space into muscle cells carried by the

DHPR [13,14,21]. In primary myotubes, depolarizing stimuli that do not substantially deplete SR Ca^{2+} stores can rapidly activate extracellular Ca2+ entry by a mechanism called excitation coupled Ca^{2+} entry (ECCE) that occurs through a complex made up of RyR1 and DHPR, the latter having dual functions not only as the voltage sensor for EC coupling but also as an L-type Ca2+ channel [61,62]. Experimental evidence supporting a pathogenic role of enhanced ECCE in MHS was provided by Cherednichenko et al. [63] in mice knocked in for the *RYR1* p.R163C mutation. Studies on skeletal muscles from these mice demonstrated: (i) that they exhibited enhanced depolarization-induced Ca^{2+} entry, (ii) that this influx of Ca^{2+} did not occur via store activated Ca^{2+} entry, but via ECCE and (iii) that it is sensitive to dantrolene, the drug that is clinically used to revert MH reactions. Increased ECCE in myotubes from patients with dominant *RYR1* mutations was also observed by Treves et al. [53] providing further experimental evidence that pathological dysregulation of Ca^{2+} homeostasis is not only caused by effects on the RyR1 Ca^{2+} release, but also by alteration of extracellular Ca^{2+} influx into skeletal muscles.

Although more than 50% of MHS cases are caused by *RYR1* mutations that render the Ca^{2+} channel hypersensitive, rare mutations either at the heterozygous or homozygous state have been identified in *CACNA1S,* the gene encoding the α1.1 subunit of the DHPR [18-20,64]. The presence of the *CACNA1S* p.R174W mutation affects Ca^{2+} homeostasis by altering the regulation of the RyR1 by $Ca_v1.1$. The RyR1 becomes leaky, resulting in an increased resting cytosolic [Ca^{2+}] and partial depletion of SR Ca^{2+} stores. Though no studies on Ca^{2+} influx or altered ECCE were performed on cells carrying this mutation, the increased resting [Ca^{2+}] was insensitive to nifedipine suggesting that, at least in this case, the pathological effect is due to the lack of inhibition of RyR1-mediated Ca(2+) leak by the DHPR at rest [65]. Similar conclusions were reached when studying the effects of the *CACNA1S* p.R1086H mutation in myotubes, although in the latter case the *CACNA1S* mutation results in the enhanced sensitivity of the RyR1 to activation by both endogenous and exogenous activators [66].

2.1.3 *STAC3* mutations and Native American myopathy

The first indication that patients with Native American myopathy, a peculiar myopathy affecting the Native American Lumbee population, may have a defect in

Ca^{2+} regulation was the finding that they have an increased susceptibility to Malignant Hyperthermia, in the absence of *RYR1* mutations [67]. Many individuals with Native American myopathy however, present a phenotype that is reminiscent of that of patients with *RYR1* mutations, including progressive scoliosis, short stature and dysmorphic features resembling *RYR1*-related King Denborough syndrome (KDS [67, 68]. The first report regarding the underlying molecular mechanism of this disorder was the identification of the recessive *STAC3* p.W284S mutation in patients with Native American myopathy. Subsequently genetic characterization of a spontaneous zebrafish mutant showing defective swimming revealed that it carried a point mutation in the fish homologue of *STAC3*. The mutation disrupted a splice donor site, leading to the inclusion of intron 4 and a premature stop codon and to the absence of the stac3 protein from skeletal muscle [69 and Muntoni et al, personal observation]. The ECC mechanism of these mutant fish was severely compromised as shown by the reduced Ca^{2+} transients in both fast and slow twitch muscles, but there were no changes in the architecture of the T-tubules or SR membranes, even though Stac3 forms a complex with the DHPR and RyR1.

Stac3 KO mice die soon after birth because of breathing impairment [70,71]; muscles from newborn mice exhibit a decrease in muscle mass, centrally located nuclei and importantly, no contraction and no Ca^{2+} release in response to depolarization. Skeletal muscles from Stac3 KO mice respond normally to the RyR1 pharmacological agonist 4-chloro-m-cresol, indicating that RyR1 function is normal [71]. These results indicate that the absence of Stac3 affects the voltage sensing and L-type Ca2+ channel DHPR but not the SR Ca^{2+} channels, a result that was confirmed recently when it was shown that in skeletal muscle Stac3 is required for surface expression of $Ca_v1.1$ [72]. Clearly much remains to be investigated on how defects in *STAC3* cause such a strong muscle phenotype and are linked to MHS.

2.1.4 *CASQ1* mutations and vacuolar aggregate myopathy

Calsequestrin 1, the Ca^{2+} storing protein of skeletal muscle, is a low affinity high capacity Ca^{2+} binding protein present in the lumen of the SR. At luminal resting [Ca^{2+}] calsequestrin forms polymers that are thought to make available large amounts of Ca^{2+} in close proximity to the RyR1. In the past two years, two reports have appeared linking one mutation in *CASQ1* to vacuolar aggregate myopathy [35,73].

Patients carrying the heterozygous *CASQ1* p.D244G mutation, affecting a residue that lies within the Ca^{2+} binding domain of calsequestrin 1, have a mild myopathy, suffer from muscle cramps, elevated CK levels, reduced muscle strength and fatigue. From a functional point of view, muscle fibers isolated from these patients show (i) decreased Ca^{2+} release following caffeine administration, (ii) increased glycogen content and mis-oriented SR junctions, (iii) an increase by approximately 25% of calsequestrin 1 content and (iv) a lower content of polymerized calsequestin [35,73]. Interestingly a report on the physicochemical properties of wild type and p.D244G mutated *CASQ1* showed that the mutation reduces the Ca^{2+} binding properties of calsequestrin 1 and causes it to form large aggregates *in vitro* [36]. It should be pointed out that all the patients identified so far were heterozygous for the causative *CASQ1* mutation, while the functional studies were performed on homozygous mutated calsequestrin 1 molecules. Thus it is likely that the mild phenotype of the patients may be due to the presence of wild type calsequestrin 1 molecules within the SR. Of interest, CASQ1 KO mice exhibit heat intolerance leading to an MH-like phenotype, enhanced oxidative stress, mitochondrial damage and reduced levels of releasable Ca^{2+} [74,75].

2.2 Congenital myopathies due to mutations in genes involved in Ca^{2+} entry pathways and SR Ca^{2+} uptake

Skeletal muscle fibers express two distinct mechanisms of extracellular calcium entry: (i) calcium entry activated by depletion of sarcoplasmic reticulum stores (Store Operated Calcium Entry, SOCE) [76-78] and (ii) Ca^{2+} entry via the voltage sensing DHPR α1.1 subunit, also referred to as ECCE (see section 2.1.2 where disorders associated with *CACNA1S* mutations are described), which is triggered either by a train of action potentials or by prolonged depolarization [61,62,79].

2.2.1 Disorders of SOCE: tubular aggregate myopathy

Dominant mutations in *STIM1* and *ORAI1* have been linked to muscle disorders classified as tubular aggregate myopathy. This group of myopathies comprises rare, slowly progressive disorders causing either weakness or stiffness of proximal muscles [80-84]. *STIM1* and *ORAI1* encode respectively stromal interacting protein 1 (Stim1) and Orai1 or Calcium Release Activated Ca^{2+} Influx channel

(CRAC), proteins that are ubiquitously expressed and play a major role in SOCE [85]. Remarkably, recessive loss-of-function mutations are associated with immune deficiency syndromes characterized by T-cell dysfunction [80,81]. Two different models have been proposed for the activation of SOCE, one is slow and occurs in most cells, while the other is rapid and occurs in skeletal muscle. In resting conditions, Stim1 binds Ca^{2+} and is distributed throughout the ER membrane, while Orai1 channels are localized on the plasma membrane. Upon Ca^{2+} store depletion, Ca^{2+} unbinds from Stim1 causing its re-localization to ER punctae adjacent to the plasma membrane. The latter event leads to the recruitment of Orai1 into the plasma membrane domain associated with ER punctae, and the conformational coupling of Stim1 to Orai1 results in the activation of SOCE. This process is slow, requiring tens of seconds to develop in non-muscle cells [85]. In patients with loss of function mutations in *STIM1* and *ORAI1* Ca^{2+} influx in T cells is severely affected, resulting in impaired T-cell proliferation [80,81,86].

Evidence that depletion of Ca^{2+} from the SR activates Ca^{2+} entry in skeletal muscles was unambiguously provided by Kurebayashi and Ogawa [76], who demonstrated that store depletion activated Ca^{2+} influx is sensitive to Ni^{2+} and resistant to nifedipine and that it is inhibited by membrane depolarization. These results suggest that the properties of this Ca^{2+} influx pathway are similar to CRAC channels and some transient receptor potential canonical (TRPC) channels. TRP channels are a family of relatively non-selective cation channels predominantly expressed on the plasma membrane that are often part of signaling complexes. There are 7 TRP subfamilies encoded by 28-30 genes; members of the TRPC subgroup play a role in Ca^{2+} influx. Using an elegant experimental approach Launikonis and Rios [87] provided experimental results supporting a role for SOCE in mammalian skeletal muscles. They showed that in isolated rat EDL, SOCE: (i) is activated by release of Ca^{2+} from the SR but does not require complete depletion of Ca^{2+} stores; (ii) its rate of activation is independent of the magnitude of the Ca^{2+} transient; and (iii) occurs rapidly, requiring less than 1 second following Ca^{2+} release from the SR. This rapid SOCE activation in skeletal muscle is most likely due to the lack of Stim1 re-localization from the SR/ER membrane to the plasma membrane because of its pre-clustering at the junctional SR membrane close to Orai1 prior to store depletion [87]. Thus Stim1 and Orai1 are likely (but probably not the only) candidates to mediating

SOCE in mature mammalian skeletal muscles. A link between mutations in proteins mediating SOCE and myopathies was unequivocally provided (i) by the creation of a STIM1 KO mouse model, (ii) the identification of dominant mutations in *STIM1* and *ORAI1* in patients with tubular aggregate myopathy and (iii) the finding that the absence of Orai1 not only leads to immunological defects but also to a subtle myopathic phenotype. Homozygous *STIM1* KO is almost always neonatally lethal, the few surviving pups being substantially smaller, with reduced body weight and having weak fatigable muscles [88]. Histological examination of muscles from KO mice revealed an increase in the number of centrally located nuclei, swollen mitochondria and small atrophic fibers; such changes were accompanied by biochemical modifications of the SR with a significant reduction in SERCA1 expression. Myotubes prepared from wild type, heterozygous and homozygous KO mice responded differently to depletion of intracellular Ca^{2+} stores, with myotubes from the latter almost completely failing to activate Ca^{2+} influx. Force measurements on muscles isolated from heterozygous KO mice confirm that lack of Stim1 causes diminished force development after tetanic contraction and increased susceptibility to fatigue [88]. It should be mentioned however, that in mouse muscles STIM1 expression peaks at embryonic day 8.5-15.5 and muscles from KO mice express low levels of MyoD, of MyHC and SERCA. Such a result is compatible with the hypothesis that this myopathy may be due to a defect of skeletal muscle development. In human myotubes, Stim1-mediated SOCE is required for cell differentiation [89], supporting the idea that the severe phenotype of STIM1 KO mice may be due to a maturation or developmental block, whereby muscle cell precursors (myoblasts/myotubes) are incapable of developing into mature muscle fibers.

Patients with Orai1 deficiency have a complex phenotype characterized by immunodeficiency, ectodermal dysplasia, a defect of dental enamel calcification and subtle histological features suggestive of a congenital myopathy [90]. Interestingly, a similar combination of symptoms was observed in a genetically unresolved patient with a histopathological diagnosis of MmD, reported before the identification of the Orai1 gene [91]. Cells isolated from patients carrying mutations causing reduced Orai1expression display impaired Ca^{2+} influx while in zebrafish lack of Orai1 leads to loss of skeletal (and cardiac) muscle integrity and myofibrillar disruption [92].

Dominant gain of function mutations in *STIM1* and *ORAI1* are more frequently encountered in patients with skeletal muscle involvement. Specifically, they have been identified in several patients with childhood or adult onset tubular aggregate myopathy [82-84, 93, 94] as well as patients with Stormorken syndrome [95,96] a rare disorder characterized by prolonged bleeding, thrombocytopenia/ thrombocytopathy, asplenia, intellectual disability, mild hypocalcemia, muscle fatigue and tubular aggregate myopathy. Muscle weakness is generally only mild in the adult-onset cases and some of the patients with an *ORAI1* mutation presented only with exertional cramps [97]. Similarly, the phenotype of patients with *STIM1* mutations includes cases with (post-exercise) myalgia, fatigability, and calf hypertrophy without muscle weakness [98]. Interestingly, most *STIM1* mutations identified in patients are located within the NH_2 terminal EF hand Ca^{2+} binding domain of Stim1, leading to a constitutively active molecule that oligomerizes and clusters at ER/PM junctions independently of Ca^{2+} store depletion. Theoretically, such Stim1 molecules should activate Ca^{2+} influx independently of how full the intracellular Ca^{2+} stores are, a hypothesis confirmed in cells carrying *STIM1* mutations, which exhibit dysregulation of Ca^{2+} homeostasis characterized by a high resting [Ca^{2+}] and Ca^{2+} entry independent of store depletion [84, 93, 94, 96].

In summary, recent experimental evidence unambiguously supports a role of SOCE in normal muscle development. Furthermore, dominant mutations in two players of SOCE lead to a myopathy characterized by the accumulation of tubular aggregates deriving from the SR. Nevertheless many questions remain unanswered, in particular the mechanism(s) linking *STIM1* and *ORAI1* mutations to accumulation of protein aggregates, how constitutively active Ca^{2+} influx is not compensated by increased activity of Ca^{2+} pumps and extrusion mechanisms and whether alteration of Ca^{2+} influx leads to a chronic activation of the Ca^{2+} dependent phosphatase calcineurin leading to nuclear localization of NFAT.

2.2.2 Brody's disease

Brody's disease is a rare inherited myopathy due to diminished SERCA1 activity caused by mutations in its encoding gene *ATP2A1* [99]. Since a number of patients do not have causative *ATP2A1* mutations however, it is now apparent that there is genetic heterogeneity in Brody's disease [100,101]. Consequently it has been

proposed to distinguish patients with *ATP2A1* mutations ("Brody disease") from patients with reduced SERCA1 activity without *ATP2A1* mutations ("Brody syndrome") [102,103]. Reduced SERCA1 activity results in delayed muscle relaxation due to the prolonged increase of the myoplasmic [Ca^{2+}] following muscle contraction [104]. Two types of SERCA1 are expressed in muscles: SERCA1 predominantly expressed in type 2 fast-twitch skeletal muscle and SERCA2 predominantly expressed in type 1 slow-twitch and cardiac muscles. Brody's disease is due to modifications of SERCA1 only, and accordingly, no cardiac complications have been reported. Since SERCA1 is only present in fast-twitch muscle fibres, impaired relaxation specifically occurs after phasic exercises (alternate contraction and relaxation). In contrast, tonic activity (such as maintaining posture) requires slow-twitch motor-unit activation, and remains unaffected as it predominantly recruits type 1 fibres.

Functional studies on muscle samples from humans with Brody's disease have demonstrated that some patients exhibit reduced levels of SERCA1 protein content [105,106], while in other cases the levels of SERCA1 protein are unaltered, but there is a significant reduction (up to 80%) in the Ca^{2+} pumping activity both in homogenates of muscle samples and in myotubes explanted from patients [99,106,107]. Chianina cattle pseudomyotonia, a disease similar to Brody's disease affecting cattle, is caused by the homozygous *ATP2A1* p.R164H mutation [108]. The mutation leads to protein instability and therefore reduced SERCA1 expression. Pharmacological inhibition of the ubiquitin/proteasomal system rescues the mutated SERCA1 protein as well as its enzymatic activity [109]. Such results suggest potential pharmacological treatments for patients with Brody's disease due to mutations leading to decreased protein stability.

Analysis of the behavior of zebrafish with a naturally occurring mutation in acc^{dta5} (the zebrafish homologue of *ATP2A1*) provides a useful and sensitive model of Brody's disease [110]. Accordion (*acc*) mutants fail to coil their tails normally after a touch-evoked stimulus. In contrast, its trunk muscles contract simultaneously to shorten the trunk resulting in a pronounced dorsal bend, which might be considered the correlate of a cramp. Subsequent muscle relaxation is significantly slower in *acc* than in wild type zebrafish larvae, corresponding to delayed relaxation in patients. Therefore the *acc* zebrafish mutant is considered a valuable model for the genetic and phenotypic evaluation of Brody's disease and related disorders [110].

2.3 ECC defects in congenital myopathies due to mutations in genes not directly involved in Ca^{2+} homeostasis

2.3.1 *SEPN1* related myopathy

Selenoproteins are a group of proteins mediating diverse cellular functions including the maintenance of the cellular redox status and scavenging reactive oxygen species. As their name indicates, members of this family of proteins contain the unusual amino acid selenocysteine. Recessive mutations in *SEPN1*, the gene encoding Selenoprotein N (SelN), are commonly associated with a subgroup of MmD, CFTD, a congenital form of desmin-related myopathy and/or Rigid Spine Muscular dystrophy (RSMD1), early-onset myopathies with a varying but usually severe axial involvement with scoliosis and respiratory failure. In contrast to *RYR1*-related MmD, extraocular involvement is not a typical feature. To date more than 40 *SEPN1* mutations have been identified, many of them predicted to result in a truncated protein or in diminished levels of SelN expression [111-113]. SelN is expressed in the endoplasmic reticulum of all cells, particularly in fetal tissue [114], making the mechanistic link between the pathophysiology of *SEPN1* mutations and neuromuscular disorders difficult to unravel. A first indication connecting *SEPN1* mutations to alterations of Ca^{2+} homeostasis was provided by Arbogast et al. [115] who demonstrated increased stress due to increased reactive oxygen and nitrogen species in myotubes from patients with *SEPN1*-related myopathies. More importantly, Ca^{2+} homeostasis was severely impaired as indicated by: (i) a 30% increase in the resting cytosolic [Ca^{2+}], (ii) a 50% reduction in the caffeine-induced peak Ca^{2+} response and (iii) a significant depletion of the SR intracellular Ca^{2+} stores. Such an effect was putatively attributed to a RyR1 dysfunction due to secondary modification by free radicals. These findings are supported by a study by Jurynec et al., who injected zebrafish eggs with morpholino oligonucleotides targeted to the exon intron junction of the *SEPN1* zebrafish homologue [116]. The resulting *Sepn1* morphant fish display reduced swimming activity, sarcomeric disorganization and importantly, decreased ryanodine binding capacity and a diminished responsiveness of RyR1 to the environmental redox potential, indicating that loss of SelN leads to a deficit of RyR1 regulation [117]. In order to understand in greater detail the role of SelN in muscle

physiology, a mouse *SEPN1* KO model was created (*Sepn1-/-*) [118]. Surprisingly, under normal housing conditions such mice displayed no overt phenotype, showed no alteration of muscle function, their muscles exhibited normal ultrastructural organization and there were no changes in the expression levels of RyR1 and $Ca_v1.1$ [119], though they did display a pronounced reduction of the satellite cell population [118]. Interestingly, following chronic challenging physical exercise, *Sepn1-/-* mice showed an obvious phenotype characterized by muscle rigidity and kyphosis indicating that in mice muscle use and/or environmental stressful conditions are necessary for the myopathy to develop [119]. In a recent study Marino et al. showed that the redox activity of SelN protects the ER from peroxides generated by the ER thiol oxidase protein ERO1. Furthermore, they identified SERCA2 as a redox target of SelN providing evidence of its role in regulating ER/SR Ca^{2+} levels [120].

Clearly much remains to be investigated concerning the pathophysiological mechanism(s) underlying *SEPN1*-related myopathies and in particular more mechanistic insight into its effects on Ca^{2+} homeostasis need to be provided. Studies aimed at investigating whether the absence of SelN leads to alterations of RyR1 function due to changes of its redox status, or secondary posttranslational modifications are particularly important as they may reveal mechanisms potentially amenable to pharmacological treatment.

2.3.2 *MTM1, BIN1 and DNM2* related disorders

Mutations in *MTM1*, the gene encoding the lipid phosphatase myotubularin 1, are the underlying cause of X-linked myotubular myopathy (XLMTM), a severe form of CNM characterized by generalized muscle weakness, with severe axial, respiratory and bulbar muscle involvement [121]. Typically symptoms are already evident at birth and affected males usually die within the first year of life without respiratory support. Myotubularin 1 specifically dephosphorylates phosphatidylinositol-3-phosphate (PI(3)P) and phosphatidinylinositol-3,5-bisphosphate ($PI(3,5)P_2$) to produce PI and PI(5)P respectively [122]. In humans 14 genes encoding for different myotubularin isoforms have been identified and most members of this family play important roles in membrane trafficking, movement of intracellular vesicles, autophagy and phagocytosis. Though myotubularin 1 is ubiquitously expressed, mutations in the *MTM1* gene result in a selective skeletal muscle phenotype. Studies

by Dowling et al. [123] on myotubularin knock down morphant zebrafish suggest that the specific muscle involvement is due to the lack of expression in muscle of myotubularin related proteins (MTMRs). Such proteins compensate for the lack of myotubularin's enzymatic activity in other tissues. Although a wide range of pathogenic mechanisms have been suggested in association with myotubularin 1 deficiency, in the present review we will only focus on mechanisms relevant to Ca^{2+} homeostasis and ECC.

Two experimental animal models have greatly advanced our understanding of how the absence of myotubularin 1 affects muscle function: the *Mtm1* KO mouse [124] and the *mtm* morpholino knock down zebrafish [123]. In both animal models the most obvious pathological changes occurring in muscle are the presence of centrally located nuclei with an abnormal appearance, fiber type 1 hypotrophy, abnormal T-tubules and atypical distribution of triads. Muscles from morphant zebrafish show reduced ECC with severely impaired muscle contraction evoked by stimuli above 10 Hz [123]. Interestingly, tibialis anterior muscles from asymptomatic *Mtm1* KO mice at three weeks of age show no changes in expression of proteins involved in Ca^{2+} homeostasis. However, the tibialis anterior muscles from *Mtm1* KO mice at five weeks of age show a significant decrease in the protein content of RyR1 and $Ca_v1.1$. Concomitantly there is a significant increase in the content of the ß1 subunit of the DHPR [124]. The latter protein is necessary for proper targeting of the $\alpha 1.1$ subunit onto T-tubules [125]. Detailed studies on intracellular Ca^{2+} homeostasis showed no difference in the resting $[Ca^{2+}]$, nor in the size if the intracellular Ca^{2+} stores, however the peak $[Ca^{2+}]$ evoked by depolarizing pulses was strongly reduced. These results support the hypothesis that *MTM1* mutations lead to changes in muscle function by affecting the expression of the two main Ca^{2+} channels involved in ECC, but shed no light on the mechanisms linking myotubularin to the observed changes.

An interesting hypothesis supported by experimental evidence is that the lipid composition of the membranes in which the DHPR and RyR1 are embedded is important for their function. Indeed, by confocal immunohistochemistry Dowling et al. showed that myotubularin localizes to T-tubules and its distribution overlaps with that of the DHPR [123]. Along the same lines, in an elegant report Berthier et al. [126] recently demonstrated that $PI(4,5)P_2$, another member of the PI family, is not only a constituent of T-tubules, but that its depletion represses electrically evoked Ca^{2+}

release. These results suggest that this lipid may either interact with the RyR1 or affect the activity of the voltage sensor. Although PI(4,5)P_2 is not a substrate of myotubularin 1, alterations in phosphoinositide metabolism due to the accumulation of some PIPs may alter the lipid composition of the membrane compartments involved in ECC. The absence of myotubularin 1 should theoretically cause an accumulation of membrane PI(3)P and PI(3,5)P_2 and increased levels of the latter lipids negatively affect muscle function by decreasing the size of intracellular Ca^{2+} stores [127]. While studying the effect of loss of MTMR14, a PI(3,5)P_2 lipid phosphatase expressed in heart and skeletal muscle, Shen et al. [127] showed that (i) PI(3)P, PI(5)P and PI(3,5)P_2 directly bind to the RyR1 protein; (ii) PI(3,5)P_2 activates the RyR1 and (iii) PI(3,5)P_2 promotes the open conformation of the RyR1 leading to Ca^{2+} induced Ca^{2+} release and depletion of Ca^{2+} from SR stores. While Rodríguez et al. [128] showed that microinjection of 100 µM PI(3,5)P_2 and PI(3) P but not PI(5)P significantly elevates the resting [Ca^{2+}], all three phospholipids decrease the frequency of spontaneous Ca^{2+} release events in skinned fibers, providing further evidence that RyR1 function is sensitive to its phospholipid environment. These results are interesting but not altogether compatible with the findings of Al Qusari et al. [124], who showed that the skeletal muscles from *Mtm1* KO mice have normal intracellular Ca^{2+} stores and normal resting Ca^{2+} levels and only show a severe reduction of electrically evoked Ca^{2+} transients.

Muscle function has also been extensively studied in a mouse model knocked in for *MTM1* p.R69C (c.205C>T) [129], a mutation associated with a less severe form of myotubular myopathy [130,131]. The *MTM1* p.R69C mutation putatively affects the binding domain of PI(3)P/PI(3,5)P_2 but not its catalytic domain and should therefore result in a phosphatase binding less substrate leading to a hypofunctional enzyme. Detailed genetic investigations provided evidence that exon skipping occurred in the mouse model since no full length myotubularin 1 protein was present; furthermore the muscle levels of PI(3)P were similar in *Mtm1* KO mice and mice knocked in for the mutation, making the interpretation of the less severe phenotype difficult to explain. These observations suggest that aside, its lipid phosphatase activity, myotubularin 1 may have other functions and may directly regulate the activity of proteins involved in ECC, thereby contributing to the pathogenesis of myotubular myopathy.

Milder forms of CNM have been associated with dominant and recessive mutations in *BIN1* and *DNM2*, encoding the functionally related proteins dynamin 2 and amphiphysin 2, respectively [132-135]. Dominant mutations in *DNM2* are associated with defective tubular membrane structures [134,135] most likely because they alter the function of amphiphysin 2 [134]. Amphiphysin 2 is ubiquitously expressed but the skeletal muscle isoform contains a PI binding domain and is localized to the T-tubules where it plays an important role in T- tubule biogenesis [136]. Acute down regulation of Bin1 by siRNA in mouse *flexor digitorum brevis* muscle fibers recapitulates T-tubule disruption as seen in the muscles of affected patients [132]. Furthermore, murine muscles knocked down for *Bin1* show profound alterations in Ca^{2+} homeostasis, including depletion of intracellular Ca^{2+} stores, reduced membrane current in response to depolarizing pulses leading to a reduced Ca^{2+} transient and fewer sparks. Not unexpectedly, these results confirm that changes in the organization of T-tubules lead to functional changes in ECC. Though patients with *BIN1* mutations are usually less severely affected than patients with *MTM1* mutations and may develop a child or adult onset congenital muscle diseases [133,137], it should be mentioned that the type of mutation plays an important role in the phenotype of the patient and that, contrary to patients who generally do not have heart disease, *Bin1* KO mice die shortly after birth because of a hypertrophic ventricular cardiomyopathy.

3 Conclusions and outlooks

The past decade has seen enormous progress in our knowledge concerning the pathophysiological mechanisms underlying congenital myopathies; the identification of the genetic causes and the functional characterization of mutations in genes involved directly or indirectly in Ca^{2+} regulation and ECC have greatly advanced our understanding of these diseases. The challenges for the upcoming years will be to improve the muscle function and thus the quality of life of affected patients either by specifically targeting the genetic defect, as is now being attempted in a preclinical model of MTM1, or by devising pharmacological strategies aimed at targeting alternative signaling pathways or pathological loops that are activated in the muscles of affected individuals. The increasing availability of different animal models with targeted mutations will considerably facilitate the development of experimental

therapies and the identification of biomarkers that could be used to monitor disease progression in affected patients.

Abbreviations: $Ca_v1.1$, alfa 1 subunit of the dihydropyridine receptor; CCD, central core disease; CNM, centronuclear myopathy; DHPR, dihydropyridine receptor; ECC, excitation contraction coupling; ECCE, excitation coupled calcium entry; ER, endoplasmic reticulum; HDAC, histone de-acetylase; MmD, Multi minicore disease; MH, malignant hyperthermia; MHS, malignant hyperthermia susceptibility; RyR1, ryanodine receptor; SERCA, sarcoplasmic(endoplasmic) reticulum Ca^{2+} ATPase; SelN, selenoprotein N; SOCE, store operated calcium entry; SR, sarcoplasmic reticulum; STIM1, stromal interacting molecule; T-tubules, transverse tubules.

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FIGURE LEGENDS

Figure 1: Schematic representation of the protein components of skeletal muscle sarcoplasmic reticulum (reproduced from [4]). The DHPRs (2) in the transverse tubules face the RyR1s (1) on the terminal cisternae junctional face membrane. The structural proteins triadin (TRISC, 5) and junctin (6) are shown to interact with the carboxy-terminal domain of the RyR1 and with the luminal calcium binding protein calsequestrin (CSQ, 3). The luminal domain of JP-45 (7) interacts with calsequestrin while its cytoplasmic domain interacts with the DHPR. The SERCA pumps (4) are on the longitudinal sarcoplasmic reticulum; the minor protein components junctate (8), mitsugumin- 29 (MG-29, 9) and junctophillin (11) are also indicated. Red spheres represent Ca^{2+} ions; yellow spheres represent K^{+} ions.

Figure 2: Prolonged depolarization induces nuclear translocation of NFAT and may be linked to increased level of circulating IL-6. A. Myotubes from a control and CCD individual were untreated or treated with 500 µM ryanodine and incubated with 60 mM KCl for 20 seconds and then with Krebs Ringer for a total of 5 minutes at 37°C. Cells were fixed and processed for immunofluorescence. Images (60X TIRF objective) show epifluorescence using anti-NFATc1 Ab (green) and DAPI (blue) for nuclear staining. Bar =30 µm. **B.** Bar histograms showing the nuclear to cytoplasmic NFATc1 fluorescence intensity in cells from 3 different control individuals (n ranged between 25-35 individual cells) and 2 different CCD individuals (n ranged between 32-38 individual cells) in untreated cells (white bars) and cells treated with 60 mM KCl plus 2 mM Ca^{2+} for 20 seconds and then incubated with Krebs Ringer for 5 min (light grey) or 30 min (dark grey). The area over the nucleus was selected using the DAPI image and transferred to the NFATc1 fluorescence image using the "transfer region option" of the Metamorph software package. Average fluorescent intensity in nuclei and region excluding nuclei were calculated with Metamorph. *$P<0.03$ compared to controls (from [53]). **C.** Plot of (logarithmic) serum concentrations of IL-6 in different groups of patients. Each symbol represents the mean triplicate IL-6 value obtained from a single individual. P values were statistically significant between controls and MHS individuals and control and CCD patients.

Figure 3 Cartoon depicting how mutations in *RYR1* may lead to a decrease in RyR1 content. Recessive mutations in the *RYR1* gene are accompanied by epigenetic modifications leading to DNA hyper-methylation and HDAC-4/HDAC-5 over-expression. This causes mef-2 sequestration thereby inhibiting transcription of genes regulated by mef-2, including the *RYR1* and muscle-specific miRs. A decrease in RyR1 would severely affect muscle excitation-contraction coupling since this calcium channel is a central player in this mechanism, releasing the calcium necessary for muscle contraction from the sarcoplasmic reticulum.

Figure 1

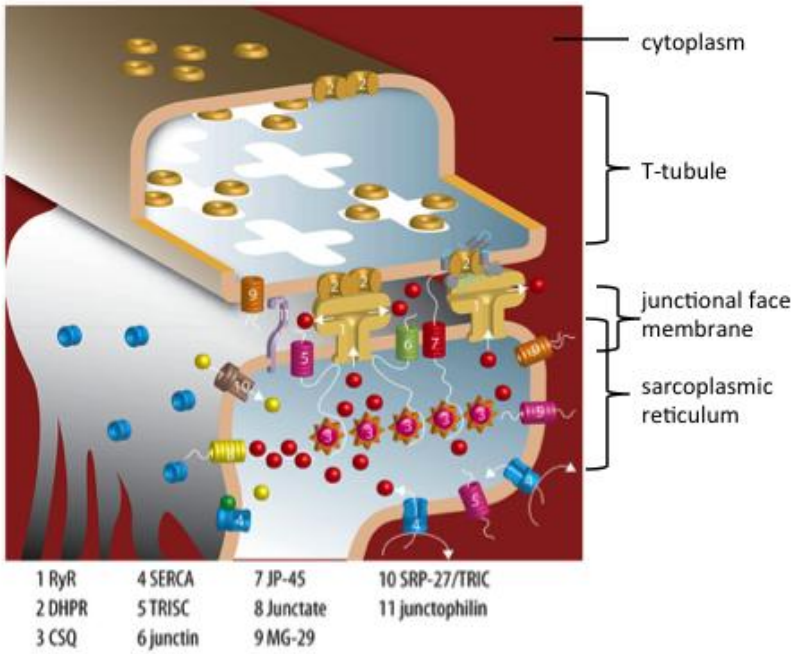

cytoplasm
T-tubule
junctional face membrane
sarcoplasmic reticulum
1 RyR
2 DHPR
3 CSQ
4 SERCA
5 TRISC
6 junctin
7 JP-45
8 Junctate
9 MG-29
10 SRP-27/TRIC
11 junctophilin

Figure 2

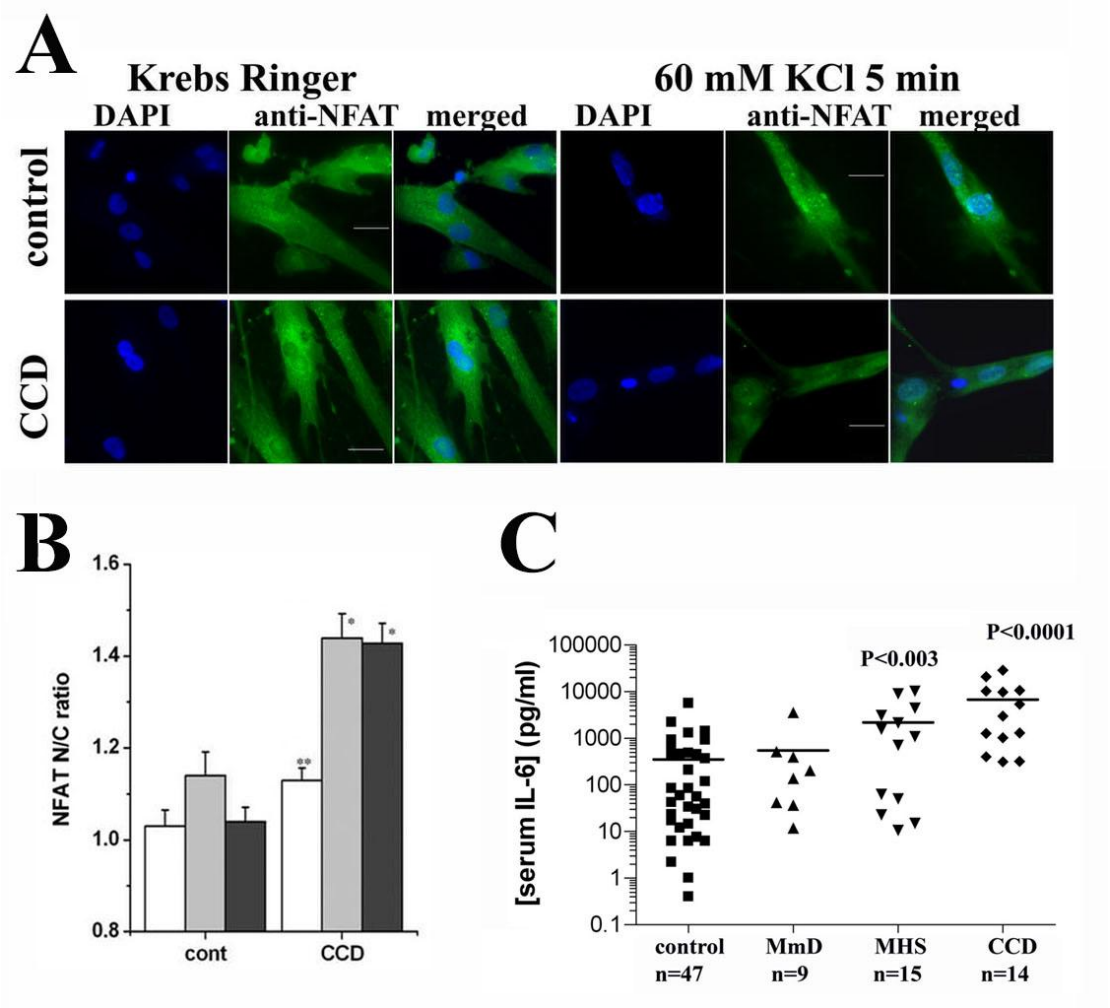
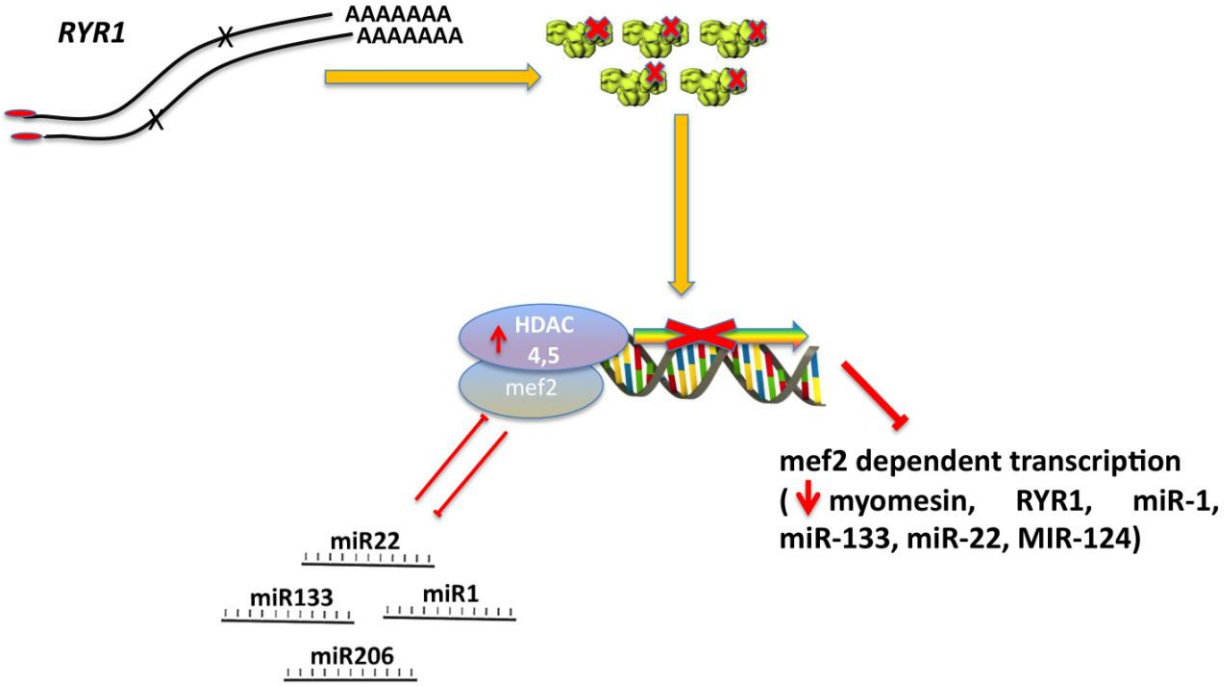

A
Krebs Ringer
60 mM KCl 5 min
DAPI
anti-NFAT
merged
DAPI
anti-NFAT
merged
control
CCD
B
NFAT N/C ratio
1.6
1.4
1.2
1.0
0.8
cont
CCD
C
[serum IL-6] (pg/ml)
100000
10000
1000
100
10
1
0.1
P<0.003
P<0.0001
control
n=47
MmD
n=9
MHS
n=15
CCD
n=14

Figure 3


RYR1
AAAAAAA
AAAAAAA
HDAC
4,5
mef2
mef2 dependent transcription
(myomesin, RYR1, miR-1,
miR-133, miR-22, MIR-124)
miR22
miR133
miR1
miR206