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Systemic ablation of RyR3 alters Ca²⁺ spark signaling in adult skeletal muscle

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Abstract

Ca²⁺ sparks are localized intracellular Ca²⁺ release events from the sarcoplasmic reticulum in muscle cells that result from synchronized opening of ryanodine receptors (RyR). In mammalian skeletal muscle, RyR1 is the predominant isoform present in adult skeletal fibers, while some RyR3 is expressed during development. Functional studies have revealed a differential role for RyR1 and RyR3 in the overall Ca²⁺ signaling in skeletal muscle, but the contribution of these two isoforms to Ca²⁺ sparks in adult mammalian skeletal muscle has not been fully examined. When enzyme-disassociated, individual adult skeletal muscle fibers are exposed to an osmotic shock, the resting fiber converts from a quiescent to a highly active Ca²⁺ release state where Ca²⁺ sparks appear proximal to the sarcolemmal membrane. These osmotic shock-induced Ca²⁺ sparks occur in *ryr3* (−/−) muscle with a spatial distribution similar to that seen in wild type muscle. Kinetic analysis reveals that systemic ablation of RyR3 results in significant changes to the initiation, duration and amplitude of individual Ca²⁺ sparks in muscle fibers. These changes may reflect the adaptation of the muscle Ca²⁺ signaling or contractile machinery due to the loss of RyR3 expression in distal tissues, as biochemical assays identify significant changes in expression of myosin heavy chain protein in *ryr3* (−/−) muscle.

Introduction

Ca²⁺ acts as a signal to regulate myriad cell functions, including the contraction of muscle fibers. Effective control of Ca²⁺ release from Ca²⁺-sequestering organelles within cells is essential to maintain internal Ca²⁺ homeostasis. Loss of control over intracellular Ca²⁺ levels can result in many pathologic states, particularly within excitable cells such as neurons and muscle cells[1]. One excitable cell type where regulation of Ca²⁺ release must be maintained at the highest level is the adult skeletal muscle fiber, where Ca²⁺ release from the sarcoplasmic reticulum (SR) must be tightly repressed in resting conditions, and capable of both rapid activation and prompt termination when required to initiate or end contraction.

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In muscle cells, rapid release of Ca^{2+} from the SR is mediated by opening of the ryanodine receptor (RyR). Gene cloning and biochemical studies have identified two RyR isoforms present in skeletal muscle, RyR1 and RyR3[2–4]. It is known that adult skeletal muscle predominantly expresses RyR1, whereas RyR3 is primarily associated with muscle development since the RyR3 protein is undetectable in some adult muscle fibers[5,6]. The function of RyR1 is tightly under the control of the voltage-sensor in the sarcolemmal membrane, while RyR3 acts as an ancillary component that amplifies RyR1-mediated Ca^{2+} release in neonatal skeletal muscle[7]. Homozygous ablation of RyR1 leads to post-natal lethality resulting from defective excitation-contraction coupling[8], while ablation of RyR3 produces viable animals whose muscle cell Ca^{2+} signaling machinery does not appear to be significantly affected[9–11]. Both isoforms are expressed in other tissues types, particular within the nervous system[12–14].

Confocal microscopy imaging has resolved the elemental intracellular Ca^{2+} release process in muscle cells as discrete events known as Ca^{2+} sparks. These Ca^{2+} spark signals are generated by paracrystalline arrays of RyR channels on the SR surface[15,16]. Spontaneous Ca^{2+} sparks are common in cardiomyocytes or amphibian skeletal muscle, however they are rare or absent in resting mammalian skeletal fibers[17]. Permeabilization of the cell membrane can produce significant numbers of spontaneous Ca^{2+} sparks in skeletal muscle[18]. Our recent findings reveal that transient disruption of the membrane structures of skeletal muscle triad junctions can eliminate the repression of Ca^{2+} spark activity in enzyme-disassociated, intact adult mammalian muscle fibers[19,20]. This activates the silent Ca^{2+} release machinery and produces robust Ca^{2+} spark activity.

Using the viable *ryr3*($-/-$) mouse model[9], here we show that RyR1 is sufficient to generate the dynamic Ca^{2+} spark signaling observed in adult skeletal muscle fibers following osmotic shock. Through comparative studies with wild type (*wt*) and *ryr3*($-/-$) muscle fibers, we identified a distinct phenotype in *ryr3*($-/-$) muscle where the characteristics of individual Ca^{2+} sparks and Ca^{2+} bursts (a longer duration Ca^{2+} release signal; see Results) appear to be significantly altered. This may be the result of adaptive changes in muscle fiber type due to ablation of RyR3 expression from adult tissues.

Materials and Methods

Skeletal muscle fiber isolation

RyR3 knockout male mice and age-matched *wt* control mice bred from the same genetic background (129S4/SvJ, 8–12 weeks old) were used in the studies. Mice were sacrificed by cervical dislocation and hind limbs were surgically removed and placed in Tyrode, a balanced salt solution consisting of (in mM) 140 NaCl, 5 KCl, 10 HEPES, 2.5 CaCl_2 , 2 MgCl_2 (pH 7.2), with a measured osmolarity of 290 mOsm. Flexor digitorum brevis (FDB) muscles were excised and transferred into Tyrode supplemented with 0.2 % type I collagenase (Sigma C-0310), for 60 min at 37 °C. Muscle fibers were gently dissociated by several passages through a small diameter pipette. Isolated FDB muscle fibers were plated onto TC glass-bottomed dishes (Biotechs, Inc) in 1 ml of Tyrode buffer.

Confocal Ca^{2+} imaging and spark analysis

Within 10 minutes of isolation, FDB muscle fibers were loaded in the dark with the Ca^{2+} indicator Fluo-4-AM (10 μM) for 60 min at room temperature. After the loading period, fibers were washed four times by replacement of 50% of the dish volume with Tyrode buffer. Measurements of SR Ca^{2+} release were performed on a BioRad Radiance-2100 confocal microscope equipped with an argon laser (488 nm) and a 60x, 1.3NA oil immersion objective. An acquisition rate of 2 ms per line was used for line-scan imaging while serial x-y images

were acquired at 3.08 sec per frame (512×512 pixels). At a resting state, fibers attached to glass-bottomed dishes were bathed with Tyrode solution. Fibers were selected for experimentation based on observation of intact sarcolemmal membranes and clear striation patterns. For Ca^{2+} spark measurements, fibers were perfused with a 170 mOsm hypotonic solution containing (in mM) 70 NaCl, 5 KCl, 10 HEPES, 2.5 CaCl_2 , 2 MgCl_2 (pH 7.2) for 60 to 180 sec to induce swelling before perfusion was switched back to Tyrode solution. Six animals of each genotype were used for fiber isolation. For each animal, 6–9 fibers were treated by osmotic shock, and from each fiber 2–4 images (comprised of 30,000 2 ms linescans of 512 pixels) were recorded. Image analysis and normalization of linescan intensities to the calculated resting fluorescence (F_0) and determination of the amplitude and duration of individual events was performed using custom routines for IDL software [19,21].

Measurement of muscle protein expression

FDB and soleus muscle from *wt* and *ryr3*($-/-$) mice were dissected and whole muscle extracts were generated by a handheld motorized rotary homogenizer (Kontes) using a lysis buffer containing 150 mM NaCl, 1% Triton-X, 0.1% SDS, 50 mM Tris-HCl (pH 8.0). Protein concentrations were determined by Dc protein assay (BioRad) and 30 μg per sample was separated by SDS-polyacrylamide gel electrophoresis at 4°C on 4% gels for 24 hours at 20 volts. Coomassie staining was conducted at room temperature. Gels were fixed for 1 hour in 50% methanol, 10% glacial acetic acid and then stained for 20 min in 0.1% Coomassie Brilliant Blue R-250 (Sigma), 50% methanol and 10% glacial acetic acid. Stained gels were de-stained three times in 40% methanol and 10% glacial acetic acid for 30 min, then once more overnight before observation. Western blotting was conducted on 5 μg of protein per sample using standard techniques with a concentrated primary mouse monoclonal antibody against sarcomeric myosin heavy chain (MF20, University of Iowa Hybridoma Bank) at a dilution of 1:5000. Equivalent loading was confirmed using Ponceau S (Sigma) staining of membranes after transfer. Results were visualized with an ECL+ kit (GE Healthcare) using the manufacturer's directions. Densitometry evaluation was conducted with Image/J (NIH) software.

Results

Similar spatial distribution of osmotic-shock induced Ca^{2+} sparks in *wt* and *ryr3*($-/-$) muscle fibers

Skeletal muscle fibers are thought to have tightly controlled SR Ca^{2+} release machinery at a resting state. As a result, spontaneous Ca^{2+} sparks are rarely observed in enzyme-disassociated, intact adult mammalian skeletal muscle[17]. Our recent study revealed that transient exposure of mouse FDB muscle fibers to osmotic shocks produces a robust Ca^{2+} spark response under resting conditions[19]. Confocal microscopy imaging revealed that the induced Ca^{2+} sparks are confined to the peripheral region of the muscle fiber obtained from adult *wt* muscles (Fig. 1a).

Recent studies have suggested that the frequency and morphology of Ca^{2+} sparks in skeletal muscle may be correlated with the presence of parajunctional RyR3 in skeletal muscle fibers [22,23]. The frequent appearance of Ca^{2+} sparks following osmotic shock in FDB fibers, where expression of RyR3 is undetectable[24], argues against the importance of RyR3 in induction of these Ca^{2+} release events. However, there is a possibility that residual RyR3 may contribute to Ca^{2+} sparks under these conditions, especially if concentrated at the sub-sarcolemmal area where Ca^{2+} sparks are observed following osmotic shock. To test this possibility, we isolated FDB muscle fibers from *ryr3*($-/-$) mice and subjected these fibers to similar osmotic shock protocols. Similar to the *wt* muscle, Ca^{2+} spark signaling also appears in the peripheral region of the *ryr3*($-/-$) muscle fiber (Fig. 1b).

Osmotic shock-induced Ca^{2+} spark activity is transient and returns to a level that is slightly elevated above baseline (Fig. 2a). Such properties are similar between *wt* and *ryr3(-/-)* muscle fibers (Fig. 2b). To examine the spatial localization of these Ca^{2+} sparks in the fiber, 5 minutes of spark counts on x-y scan images were submitted to bin analysis and plotted in a binary manner. Clearly, both *wt* (Fig. 2c) and *ryr3(-/-)* (Fig. 2d) Ca^{2+} sparks are peripherally localized within the skeletal muscle fiber after osmotic shock.

The nearly identical spatial localization of osmotic-shock induced Ca^{2+} sparks in *wt* and *ryr3(-/-)* fibers indicates that RyR1 alone is sufficient to generate these Ca^{2+} signals in mammalian skeletal fibers. It also suggests that the *ryr3(-/-)* condition does not greatly alter the intracellular Ca^{2+} release machinery in adult skeletal muscle.

Distinct kinetic properties of Ca^{2+} sparks and Ca^{2+} bursts in *wt* and *ryr3(-/-)* muscle fibers

Kinetic analysis reveals that there two patterns of Ca^{2+} signals in adult skeletal muscle under these conditions, one that resembles the brief Ca^{2+} sparks observed in cardiomyocytes and permeabilized skeletal muscle preparations, and the other that displays prolonged Ca^{2+} transients that we term Ca^{2+} bursts[19]. Line-scan images showed that many Ca^{2+} sparks and Ca^{2+} bursts could originate from the same position within the *wt* fiber (Fig. 3a). Therefore, these events suggest that a single cluster of RyR channels is capable of Ca^{2+} release with different kinetic properties in skeletal muscle, as suggested by previous results from other investigators[18].

To quantify the kinetic properties of these Ca^{2+} signaling events, the rising and decaying phases of individual events were separately fitted with an exponential function (Fig. 3b). This analysis provides the following four parameters: a) D_{50} , which represents the time between the half rising time and the corresponding decay time; b) TTP (total time to peak), which defines the time required to reach the peak of Ca^{2+} sparks; c) $\Delta F/F_0$, which defines the peak amplitude of the Ca^{2+} signal; d) FWHM (full width at half maximum), which defines the spatial width of release events at the half maximal position determined by these fitting parameters.

As shown in Fig. 3c, a combination of two-exponential functions is required to best fit the D_{50} histogram. The resulting time constants are $t_{01} = 70.9 \pm 3.5$ ms, corresponding to Ca^{2+} sparks, and $t_{02} = 456 \pm 63$ ms, corresponding to Ca^{2+} bursts, for the *wt* muscle fibers (Fig. 3c, left). We find that the time constant for Ca^{2+} bursts in *ryr3(-/-)* fibers ($t_{01} = 45.7 \pm 1.9$ ms; $t_{02} = 835 \pm 296$, Fig. 3c, right) is different from that in *wt* fibers.

To compare the different properties of Ca^{2+} sparks and Ca^{2+} bursts between *wt* and *ryr3(-/-)* fibers, individual events were divided into two groups separated by a D_{50} value of 150 ms. Events with a D_{50} less than 150 ms were considered to be sparks, while those of longer duration were considered to be bursts. Using this criteria, we found a relative increase in the quantity of Ca^{2+} sparks ($D_{50} < 150$ ms) in *ryr3(-/-)* muscle fibers, which comprise ~49% (610 sparks in 1245 events) of the total number of Ca^{2+} release events in *ryr3(-/-)* fibers compared to ~22% (208 sparks in 945 events) in *wt* fibers.

FWHM is a representation of the spatial restriction of individual Ca^{2+} sparks. Histogram analysis indicates that Ca^{2+} sparks in *ryr3(-/-)* fibers are more spatially restricted than those seen in *wt* fibers (Fig. 3d). A Gaussian function can best fit the histogram with a mean value of 2.39 ± 0.03 μm in *wt*, and 1.46 ± 0.03 μm in *ryr3(-/-)* fibers ($p < 0.005$). These FWHM values are similar to those reported previously in *wt* mammalian skeletal muscle (2.0 ± 0.5 or 1.97 ± 0.01)[17,25]. Our data are also consistent with a previous report demonstrating that embryonic *ryr3(-/-)* muscle displays a shift to the left in FWHM distribution[17].

Using the criteria for separation of Ca^{2+} sparks and Ca^{2+} bursts, we found that the mean TTP of Ca^{2+} sparks in *wt* (23.6 ± 1.8 ms) is not that different from that in *ryr3(-/-)* fibers (20.3 ± 0.8 ms) (Fig. 3e). These values are in a similar range with those for Ca^{2+} sparks measured in intact and permeabilized skeletal muscle fibers (16.4 ± 14 or 16.8 ± 0.27) [17,25]. In contrast, we found that the TTP for Ca^{2+} bursts is significantly altered in *ryr3(-/-)* fibers (80.7 ± 6 ms versus 220 ± 9 ms, $p < 0.005$) (Fig. 3e).

In addition to the different kinetic properties, the amplitude distribution of Ca^{2+} sparks and Ca^{2+} bursts also appears to be different in the *ryr3(-/-)* FDB muscle. The mean amplitudes of both Ca^{2+} sparks and Ca^{2+} bursts in *ryr3(-/-)* fibers are significantly smaller than those in *wt* fibers (Fig. 4a). Correlation of the amplitude and D_{50} of individual Ca^{2+} release events clearly reveals that a group of high amplitude, short duration Ca^{2+} sparks that appear in *wt* fibers (Fig. 4b) are not present in *ryr3(-/-)* fibers (upper left quadrant in Fig. 4c).

Fiber type alteration could contribute to altered Ca^{2+} spark characteristics

The differences observed in Ca^{2+} spark characteristics between *wt* and *ryr3(-/-)* are more dramatic than would be expected considering the minimal expression of RyR3 in many adult mammalian skeletal muscles. To evaluate if an adaptive change due to the absence of RyR3 from mammalian skeletal muscle during development contributes to the observed changes in the kinetic properties of Ca^{2+} sparks in *ryr3(-/-)* fibers, we examined the protein expression profile of *wt* and *ryr3(-/-)* muscles (Fig. 5a). While the overall protein expression profiles between *wt* and *ryr3(-/-)* skeletal muscle remains relatively similar, there are some significant differences visible in the abundance of several proteins. One of the most striking differences is the marked decrease in expression of myosin heavy chain (MHC) in *ryr3(-/-)* FDB fast twitch muscle, but not in slow twitch soleus muscles (Fig. 5a). On average, the expression of MHC in *ryr3(-/-)* FDB decreased $79 \pm 7\%$ compared to *wt* muscle ($n = 6$ pairs). This suggests that changes in fiber type or contractile protein expression occur in the fast twitch muscles of *ryr3(-/-)* mice, which may account for some of the alterations to Ca^{2+} sparks characteristics observed in this study.

Discussion

Our study reveals that RyR1 is sufficient to produce Ca^{2+} sparks and Ca^{2+} bursts following osmotic shock in mammalian skeletal muscle, since similar events are observed in both *wt* and *ryr3(-/-)* muscle fibers. Although RyR3 is not essential for induction of Ca^{2+} sparks in skeletal muscle, the absence of RyR3 in mice leads to significant changes in the elemental properties of Ca^{2+} spark signaling. It is unlikely that these decreases are simply due to a diminished SR Ca^{2+} store, as the store in *ryr3(-/-)* myotubes is equivalent to *wt* [26,27].

Previous studies demonstrate that RyR3 acts to amplify RyR1-mediated Ca^{2+} release in neonatal skeletal muscle [6,7]. RyR3 itself can produce Ca^{2+} sparks in homologous myotubes null for other RyR isoforms [28,29] and also in non-excitable cells [30]. While RyR3 is expressed preferentially during embryonic development [12], RyR3 is not expressed at detectable levels in the adult FDB muscle studied here [24]. Therefore, the Ca^{2+} spark alterations observed in *ryr3(-/-)* adult fibers likely represent a compensatory response to the absence of RyR3 during development of muscle fibers. Since RyR3 is normally present in adult neuronal cells, germ-line ablation of RyR3 will likely affect the motor neuron activity and contribute to the impaired synaptic plasticity and altered spatial learning observed in *ryr3(-/-)* mice [10,11]. One potential mechanism that could result in the altered Ca^{2+} spark characteristics observed in *ryr3(-/-)* fibers may result from the disruption of neurons innervating skeletal muscle fibers. Our biochemical study reveals that the expression of MHC protein is significantly reduced in the fast-twitch FDB muscle of the *ryr3(-/-)* mice. This suggests that fiber type switching is one potential mechanism for the observed changes in

Ca²⁺ spark signaling. The changes in the abundance of additional proteins in *ryr3*(^{-/-}) FDB muscle could also reflect switching of muscle fiber type, or they could also represent other adaptive responses that could contribute to the alterations observed in Ca²⁺ spark signaling.

Considering the magnitude of alterations to Ca²⁺ sparks characteristics observed in *ryr3*(^{-/-}), one would expect there would be physiologic or pathologic consequences to muscle function. Since previous studies report no major abnormalities in adult *ryr3*(^{-/-}) muscle function[9–11], it may be possible that stress conditions, such as strenuous exercise, are necessary to fully uncover any pathologic changes in *ryr3*(^{-/-}) mouse muscle function. It should be noted that our studies were performed under conditions of strenuous osmotic shock, thus future experiments will help to resolve to what degree, if any, changes in sensitivity to the intensity of the osmotic stress affects *wt* and *ryr3*(^{-/-}) muscle fibers.

We found that many of the characteristic properties of Ca²⁺ sparks are maintained in the *ryr3*(^{-/-}) muscle fiber, including the induction and localization of events following osmotic shock. These results suggest that the absence of RyR3 in the FDB muscle does not impact significantly the global Ca²⁺ release machinery. Studies from other investigators have shown that osmotic shock results in the swelling of the TT system and alteration of the triad junction structure throughout the entire muscle fiber[31–33]. This is in contrast to the localization of Ca²⁺ sparks in the periphery of *wt* and *ryr3*(^{-/-}) fiber observed in our study, suggesting that some sarcolemmal-restricted signal may be involved in the initiation or amplification of the robust Ca²⁺ spark response in stressed skeletal muscle. This signal may supplement a physical rearrangement of the triad junction complex due to mechanical swelling of the TT system to initiate a spark response. Further experimentation will be necessary to determine the identity of this membrane-delimited signal and examine how this signal can participate in the formation of Ca²⁺ sparks. Moreover, elucidation of the physiological function of RyR3 in adult skeletal muscle will require comparative studies in a muscle tissue where RyR3 expression is preserved. It is known that diaphragm muscle contains measurable amounts of RyR3 in the adult stage [34]. It will be interesting to determine if the properties of Ca²⁺ spark signaling are dependent on the presence of RyR3 in different muscle tissues.

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Figure 1. Osmotic shock-induced Ca²⁺ sparks and Ca²⁺ bursts in *wt* and *ryr3*(-/-) muscle fibers
 An enzyme-disassociated, intact mouse FDB muscle fiber subjected to transient osmotic shock exhibits a robust Ca²⁺ spark response that includes prolonged Ca²⁺ release events termed Ca²⁺ bursts. Transversal line-scans from *wt* (a) and *ryr3*(-/-) (b) fibers following an osmotic shock. Note the appearance of the Ca²⁺ sparks response at the periphery of both *wt* and *ryr3*(-/-) fibers. Fibers were initially perfused with isotonic solution before hypotonic solution was applied for one minute. When the perfusion solution was returned to isotonic solution, Ca²⁺ sparks can be immediately observed at the cell periphery.

Figure 2. Similar spatial characteristics of the Ca^{2+} sparks response in *wt* and *ryr3(-/-)* muscle fibers

Diary plots of hypo-osmotic shock induced Ca^{2+} spark response in *wt* (a) and *ryr3(-/-)* (b) muscle fibers. Blue numbers and lines represent osmolarity of the solution in mOsm. The green line indicates the area of the confocal image as a qualitative measurement of the cell volume. Binary plotting of the events reveals a similar spatial distribution pattern for *wt* (c) and *ryr3(-/-)* (d) fibers. Sparks over the span of 5 minutes were analyzed to generate bin results with a user-controlled automated spark detector IDL software routine. Pseudo color scale represents the average number of Ca^{2+} release events at each point location within the fiber, with the scale of 0.00 to 0.06 in sparks/s.

Figure 3. Altered kinetic properties of Ca^{2+} sparks and Ca^{2+} bursts in *ryr3(-/-)* fibers
 (a) Representative line-scan image illustrating two populations of Ca^{2+} release events, Ca^{2+} sparks and Ca^{2+} bursts, that originate from the same position in a *wt* fiber. Black trace represents the mean amplitude ($\Delta F/F_0$) averaged from 15 spatial pixels for each time point along the linescan image. (b) The rising and decaying phases were resolved from amplitude ($\Delta F/F_0$) traces (black) for individual Ca^{2+} release events by fitting with single exponential functions (red) to allow determination of various characteristics, including the half duration (D_{50}) (green) and total time to peak (TTP) (blue). (c) Histograms of D_{50} distributions for Ca^{2+} release events in *wt* (black) and *ryr3(-/-)* (red) fibers. These distributions were best fit by the following equation: $y = A1 \cdot \exp(-x/t_{01}) + A2 \cdot \exp(-x/t_{02})$. The best fit time constants are $t_{01} = 70.9 \pm 3.5$

ms and $t_{02} = 456 \pm 63$ ms for *wt*; and $t_{01} = 45.7 \pm 1.9$ ms and $t_{02} = 835 \pm 296$ ms for *ryr3(-/-)*. Values for A1 and A2 are 62.8 ± 9.3 and 429.5 ± 10.4 for *wt*; and 30.0 ± 4.5 and 585 ± 13.2 for *ryr3(-/-)*. A bin size of 35 was used. **(d)** Histograms of full width at half maximum (FWHM) distributions for Ca^{2+} release events in *wt* (black) and *ryr3(-/-)* (red) fibers. Solid line represents the best-fit Gaussian distribution function (green). **(e)** Average data from individual Ca^{2+} release events for the total time to peak (TTP). The two exponential components obtained in **b** were used to separate events into groupings for Ca^{2+} sparks and Ca^{2+} bursts ($D_{50} > 150$ ms) in *wt* (black) and in *ryr3(-/-)* (red) muscles. Mean $\pm$ SE tested by ANOVA. * $p < 0.005$.

Figure 4. Decreased amplitude of Ca^{2+} sparks and Ca^{2+} bursts in *ryr3*(-/-) fibers

(a) Average amplitude ($\Delta F/F_0$) data from individual Ca^{2+} release events. Events were divided into Ca^{2+} sparks and Ca^{2+} bursts (>150 ms) in wt (black) and in *ryr3*(-/-) (red) muscles. Mean $\pm$ SE tested by ANOVA. * $p < 0.005$. (b) The $\Delta F/F_0$ for all Ca^{2+} release events with a $\Delta F/F_0 > 0.3$ were correlated to their respective D_{50} and plotted for wt (n=945) muscle. (c) Correlation of $\Delta F/F_0$ and D_{50} in *ryr3*(-/-) fibers (n=1245) (b). Green lines indicate a $\Delta F/F_0$ of 1.5 and a D_{50} of 150ms.

Figure 5. Reduced expression of myosin heavy chain protein in *ryr3*($-/-$) fast twitch muscle
(a) Coomassie blue stained gel of whole muscle extracts with FDB shown on lanes 1–4 and soleus on lanes 5–8. Lane 1 is from *wt*, lanes 2–4 are from three *ryr3*($-/-$) mice. Lanes 5–6 are from two *wt* mice, lanes 7–8 are from two *ryr3*($-/-$) mice. A total of 6 mice from each genotype were analyzed and representative samples are provided. Molecular weights are indicated on the left. **(b)** Enlarged view of area boxed in **a**. **(c)** Western blot for myosin heavy chain from a separate gel to confirm identity of the protein. Equal loading was confirmed with Ponceau S staining (not shown).