Ozz-E3 Ubiquitin Ligase Targets Sarcomeric Embryonic Myosin Heavy Chain during Muscle Development

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Abstract

Muscle contractile proteins are expressed as a series of developmental isoforms that are in constant dynamic remodeling during embryogenesis, but how obsolete molecules are recognized and removed is not known. Ozz is a developmentally regulated protein that functions as the adaptor component of a RING-type ubiquitin ligase complex specific to striated muscle. Ozz⁻/⁻ mutants exhibit defects in myofibrillogenesis and myofiber differentiation. Here we show that Ozz targets the rod portion of embryonic myosin heavy chain and preferentially recognizes the sarcomeric rather than the soluble pool of myosin. We present evidence that Ozz binding to the embryonic myosin isoform within sarcomeric thick filaments marks it for ubiquitination and proteolytic degradation, allowing its replacement with neonatal or adult isoforms. This unique function positions Ozz within a system that facilitates sarcomeric myosin remodeling during muscle maturation and regeneration. Our findings identify Ozz-E3 as the ubiquitin ligase complex that interacts with and regulates myosin within its fully assembled cytoskeletal structure.

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Introduction

Striated muscle cells exhibit the paradoxical association of a rigidly ordered fine structure with the ability to adapt their size and contractile properties during growth and development, or in response to changes in their patterns of use. Many sarcomeric proteins are developmentally expressed as a series of isoforms leading at maturation to patterns appropriate for slow or fast contraction, and aerobic or anaerobic metabolism. Accordingly, mechanisms must exist to enable replacement of isoforms while maintaining an almost crystalline regularity of structural pattern. The classic suggestion of how such mechanisms may operate is based on in vitro experiments where myosin monomers spontaneously polymerize to reach a dynamic equilibrium between fully polymerized myosin and a small pool of soluble monomers [1]. However, in a theoretical study, Davis concluded that a model based on kinetic parameters could not account for the rapid replacement of one myosin isoform by another that is seen in vitro [2].

The ubiquitin-proteasome system [3–5] is the prime candidate for targeted degradation of most soluble and myofibrillar proteins. In skeletal muscles, ubiquitination of muscle proteins to target them for proteolysis is an important component of cachexia and muscle atrophy [6,7]. Evidence for ubiquitin-mediated degradation of myosin is mostly indirect, but the E3 ubiquitin ligases MuRF1, which is induced during muscle atrophy, and MuRF3 mediate the ubiquitination of soluble myosin in vitro [8,9], binding to multiple sites near the head region of MyHC molecules. Ubiquitination by MuRF1 has recently been shown to regulate the disassembly and degradation of the myofibrillar proteins MyBP-C, MLC1, and MLC2; however, MyHC is not ubiquitinated by MuRF1 in vitro when associated in the actomyosin complex or in the intact myofibrils [10]. Interestingly, ubiquitin-dependent degradation has also been indirectly implicated in the regulation of myosin folding and assembly [11].

Ozz, also known as Neurl2 (Neutralized-like protein 2), is the substrate-binding component of a RING (Really Interesting New Gene)-type ubiquitin ligase complex, which comprises Elongin B/C (Elo B/C), Rbx1 and Cullin 5 (Cul5) [12]. The protein primary structure contains two Neutralized Homologous Repeats (NHR1 and NHR2) that serve as protein-protein interaction domains and a SOCS (Suppressor of Cytokine Signaling) box at the C-terminus for recognition by the Elo B/C subcomplex. Ozz expression is muscle-specific and upregulated during muscle fiber differentiation, but we show here that it is downregulated in muscle atrophy. To form an active E3 ligase, Ozz must assemble with the other components of the complex, a process that adds an extra tier to regulation of substrate recognition and ubiquitination by this ligase [12]. This is in contrast to the MuRF family of ubiquitin ligases, which are monomeric and can initiate ubiquitination immediately upon binding their substrates [8,9,13].
We have established that sarcomembran-associated β-catenin is a substrate for Ozz-E3 and that ozz−/− mice develop overt sarcomeric defects, which we have attributed in part to the impaired turnover of β-catenin at the membrane of differentiating myofibers [12].

We report here that the sarcomeric embryonic myosin heavy chain (MyHCemb/Myh3) is a novel substrate of Ozz, which specifically recognizes the rod domain or tail region of this protein. MyHCemb expression is associated with initiation of sarcomere formation [14], leading to the idea that it is optimized for self-assembly into new thick filaments followed by a sequence of subunit changes to give rise to adult myofilaments [15]. Embryonic muscles form in two stages: a small number of primary myotubes form a scaffold to direct the later formation of secondary myotubes, which give rise to the majority of adult muscle fibers [16–18]. MyHCemb together with MyHCslow, is expressed during secondary myotube formation [19,20]. Also it is the first myosin isoform to be expressed when new myotubes form in regenerating adult muscle [21]; in intact adult muscles during hypertrophy induced by passive stretch [22]; or during recovery of post-injury myofiber regeneration [23].

Our present evidence that the Ozz-E3 ligase, by binding to the rod domain of a fully assembled MyHCemb, marks it for ubiquitination and degradation, probably facilitating the subsequent assembly of new isoforms. These observations lead to the idea that in muscle tissue the ubiquitin-proteasome system, in addition to its involvement in atrophy, removal of misfolded/malfunctional proteins, may also facilitate exchange of isoforms within large polymeric assemblies to regulate tissue development, remodeling and regeneration.

Results

Ozz is Downregulated in Muscle Atrophy

Up-regulation of ubiquitin ligases and protein ubiquitination are common correlates of muscle atrophy. We have shown earlier that the expression of ozz mRNA and Ozz protein increases during muscle development from embryonic day E12.5 onward [12] (Fig. 1A). We now wished to test if there was a similar response with muscle atrophy. Ozz levels progressively fell following denervation (Fig. 1B), suggesting the involvement of Ozz in muscle growth rather than atrophy. The latter conclusion was further supported by the pattern of Ozz expression upon injury of adult muscles with local injection of cardiotoxin. This procedure provokes initial profound muscle degeneration, followed by regeneration [21,25]. Ozz expression was downregulated during the phase of degeneration, but was upregulated during the regeneration phase (Fig. 1C), confirming that Ozz expression is associated with muscle development and growth.

Ozz Targets Embryonic Myosin Heavy Chain

In a yeast 2-hybrid screen of an E14.5 mouse cDNA library we identified MyHCemb as a novel interaction partner of Ozz. Either full-length Ozz or the N-terminal half of the protein (residues 1–229), including the entire NHR1 domain (residues 14–104) and most of the NHR2 domain (residues 208–242) were used as baits. Both screens yielded 3 clones with 93% homology to MyHCemb spanning residues 1042–1941 of the tail domain (Fig. 1D). To confirm Ozz′ interaction with the tail of MyHCemb and to identify the minimal regions of the tail needed for this interaction, we performed a series of 2-hybrid experiments using as preys either the full length tail domain (residues 1040–1941), the full length head/neck domain (residues 1–1040), or several deletion mutants of the tail region (Fig. 1E and Fig. S1A). These mutant peptides either included or excluded a 29 amino acids (1873–1901 aa) assembly competence domain (ACD) near the C-terminus of the myosin tail, which is responsible for proper myosin assembly into thick filaments [26]. We found that Ozz interacted strongly with the full-length tail, but not with the head/neck domain. Two deletion fragments of the myosin tail encompassing either the N-terminal amino acids 1041–1353, or the C-terminal amino acids 1536 to 1941 interacted differently with the full length Ozz: the former bound weakly, while the latter maintained a strong interaction (Fig. 1E and Fig. S1A). By further deleting the latter fragment at either its C-terminus (1738–1941 aa) or N-terminus (1536–1871 aa) we completely abolished Ozz binding. Similarly, two truncated fragments spanning amino acids 1872–1941, encompassing the ACD domain, also showed no interaction with Ozz. These results identified at least two regions of the MyHCemb tail crucial for Ozz binding, which likely depends on the 3D folding of the MyHCemb tail.

To verify whether the Ozz-MyHCemb interaction occurred in vivo, crude lysates of proliferating (day 0), differentiating (day 2) and terminally differentiated (day 4) primary myoblast cultures were immunoprecipitated with anti-MyHCemb antibody or an isotype matching control IgG, and probed on immunoblots with anti-Ozz antibody. The results showed that Ozz was specifically co-immunoprecipitated with MyHCemb tail, indicating that the two endogenous proteins were linked (Fig. 2A).

We next tested if Ozz could exert its ubiquitin ligase activity towards MyHCemb. For this purpose we performed in vitro ubiquitination assays using a purified, reconstituted Ozz-E3 complex [12] and a GST-tagged MyHCemb fragment spanning the tail portion of the protein (residues 1041–1942). The ubiquitinated products were then immunoprecipitated with anti-MyHCemb and the immunoblots probed with anti-ubiquitin antibody or anti-GST antibody, used as control (Fig. 2B). Reconstituent MyHCemb tail and its proteolytic fragments of smaller molecular weight were ubiquitinated only in the presence of the Ozz-E3 complex (Fig. 2B, compare lanes 4 and 5). Furthermore, if the assay was performed using the ubiquitin mutant K48R to avoid the formation of a conjugated ubiquitin chain at this residue, ubiquitination was reduced to background levels (Fig. 2B, lane 6), demonstrating that Ozz-E3 polyubiquitinated the MyHCemb tail. Five other GST fusion proteins, used as internal controls, were not ubiquitinated in this assay (data not shown), confirming the specificity of Ozz-E3 activity towards its substrate.

Ozz Segregates with Fully Assembled Sarcomeric Myosin during Myofibrillogenesis

In a chaperone-mediated process [27–30] newly synthesized myosin isoforms are serially polymerized as monomers; dimers plus 4 myosin light chains (MLC) to form hexamers; and assembled thick filaments. To determine which pool of myosin is targeted by Ozz in vivo we first tested the chromatographic profiles of Ozz and MyHCemb, after gel filtration of muscle extracts from embryos of different stages (E14.5–E18.5). Myosin preparations conventionally employ high ionic strength extraction buffers to solubilize fibrillar myosin. Here we used differential centrifugation of muscle lysates of wild-type embryos in a buffer close to physiological ionic strength to obtain a supernatant containing mostly soluble myosin (S) and an insoluble high-speed pellet including sarcofilamentous myosin (P). Examination of the P
myosin preparations with immunofluorescence microscopy confirmed the presence of fragments of sarcomeres stabilizing for MyHCemb and α-actinin (data not shown), which validated our extraction procedure. We then separated the P and S myosin preparations on gel filtration columns and assessed the levels and distribution of MyHCemb and Ozz on immunoblots of the eluted fractions probed with anti-MyHCemb, anti-Ozz and anti-MLC antibodies. The profiles shown in Fig. 3 (upper panels) were generated by densitometric analyses of band intensities.

Throughout myofibrillogenesis (E14.5–E18.5) full-length myosin eluted from the column mainly in two groups of fractions in the size ranges ~1500–900 kDa and ~800–500 kDa. These fractions from both sarcomeric (Fig. 3) and soluble myosin preparations (data not shown) contained MyHCemb and MLC, indicating the presence of multimeric myosin (~1500–900), myosin hexamers (2 heavy chains and 4 light chains, MW 520 kDa), as well as lower mw fragments. A third group of fractions, corresponding to sizes < ~200 kDa, contained reproducible anti-MyHCemb, +ve bands of ~50 kDa, 30 kDa and 25 kDa, evidently myosin peptides (Fig. 3). Their size distribution indicated that some passed through the column as dimers. The separation pattern of the different MyHCemb bands varied only slightly among embryos of different ages.

In the same high molecular weight column fractions of sarcomeric preparations (P), a portion of Ozz consistently co-eluted with filamentous myosin, suggesting that Ozz is already bound to sarcomeric myosin during the early stages of myofibrillogenesis (E14.5, Fig. 3). In contrast, Ozz was totally absent from all fractions in the size range ~1500–550 kDa from the S preparations (data not shown). Free Ozz eluted from the column in size fragments near its monomeric molecular weight of ~51 kDa and was detected at comparable levels in both the P and S preparations.

The finding that Ozz co-elutes with the high molecular weight pool of MyHCemb, on size exclusion columns, was further supported by immunofluorescence labeling of differentiated primary myotubes (day 4), treated and not treated with the MG-132 proteasome inhibitor, using anti-Ozz and anti-MyHCemb antibodies (Fig. 4). Confocal microscopy and computational analyses of the two fluorescent signals indicated that a selected pool of sarcomeric MyHCemb co-localized with Ozz in the untreated myotubes (Fig. 4A and C). Given that the co-localization of the two proteins increased substantially in fibers treated with the proteasome inhibitor, as determined by the co-localization coefficient (Fig. 4B and D), we can infer that Ozz regulates the proteasomal degradation of a selected pool of sarcomeric MyHCemb during myofiber differentiation.

Ozz Interacts and Ubiquitinates Fully Assembled Sarcomeric Myosin

Having established that a portion of the Ozz protein segregates with assembled MyHCemb, we wanted to ascertain whether Ozz was detectable in a classical preparation of muscle thin-thick filaments from E16.5 embryos [31,32]. We chose E16.5 embryos because this embryonal stage coincides with the onset of secondary myogenesis. Western blot analysis of these preparations demonstrated the co-purification of Ozz and its direct interacting partner Elo C with the thin-thick filaments, indicating an association of the entire Ozz-E3 complex with fully assembled myosin (Fig. 5A). To further validate these results, we checked whether all components of the Ozz-E3 complex were bound to assembled myosin in the insoluble preparations (P) from muscles of E16.5 embryos. Direct interaction of Ozz with myofilamentous MyHCemb was proven by co-immunoprecipitation of Ozz with anti-MyHCemb antibody only from the insoluble preparations (P), but not from the soluble preparations (S), albeit the amount of Ozz was greater in the latter (Fig. 5B, panels 2 and 6). Together these data show that Ozz is bound in a stable form to assembled sarcomeric myosin since the early stages of myofibrillogenesis, but at an untraceable level to soluble myosin.

Fractions from both S and P preparations were also probed on immunoblots with antibodies against each component of the Ozz-E3 complex (Elo B/C, Cul5, and Rbx1). Notably, all 4 proteins were detected together with Ozz not only in the soluble pool (S) of extracted muscle proteins but also in the insoluble sarcomeric preparations from wild-type embryos (Fig. 5B, panels 7–10). Furthermore and in agreement with the observed presence of both Ozz and Elo C in purified thin-thick filaments (Fig. 5A), we found a portion of Ozz bound to Elo C within the myofibrils (P fraction) but not in the soluble (S) fraction (Fig. 5B, panel 4). To ascertain whether the other components of the Ozz-E3 complex associated with the Ozzylated sarcomeric MyHCemb, extracted muscle proteins were immunoprecipitated with anti-Elo C from the S and P fractions and probed on immunoblots with both anti-MyHCemb and antibodies against the remaining E3 components. We found that MyHCemb was effectively co-immunoprecipitated with anti-Elo C (Fig. 5B, panel 5). However, under these stringent experimental conditions (high salt concentration), while we were able to demonstrate Ozz binding with its direct interacting partners, MyHCemb and Elo C, we could not co-immunoprecipitate the remaining components of the Ozz-E3 complex (not shown). Nonetheless, the presence in the sarcomeric, insoluble fractions of all Ozz partners and the demonstrated interaction of Ozz, Elo C and MyHCemb strongly support the notion that the Ozz-E3 complex is assembled within the myofilaments.

Finally, to test whether MyHCemb was efficiently ubiquitinated by Ozz-E3 when associated with the myofilibril, we used sarcofilamentous myosin, purified from wild-type newborn muscle [33,34], as substrate in an in vitro ubiquitination assay. We found that Ozz-E3 efficiently ubiquitinated the endogenous, assembled MyHCemb (Fig. 5C). The specificity of the reaction was confirmed by the lack of ubiquitinated products in the absence of either the substrate (lane 2) or the Ozz-E3 complex (lane 3).

MyHCemb Expression Persists in Ozz−/− Muscle

Mouse myoblasts in primary culture multiply and then quickly fuse into multinucleated myotubes expressing MyHCemb, and other MyHC isoforms. Immunoblots of lysates of primary myoblasts induced to differentiate in vitro confirmed that Ozz expression was
MyHCemb fragment with anti-MyHCemb antibody, followed by immunoblotting with anti-MyHCemb. (Lane 3 and 5) Ozz-E3 + ubiquitin + MyHCemb (lane 6) Ozz-E3 + ubiquitin mutant K48R + MyHCemb. doi:10.1371/journal.pone.0009866.g002
The total number of MyHCemb+ve fibers present throughout the length of the limbs is shown in Fig. 7B.

Discussion

We have previously described the identification of Ozz as the substrate-recognition component of a striated muscle-specific RING-type E3 ubiquitin ligase complex, involved in myofiber differentiation [12]. Here, we present evidence that Ozz plays a critical role in muscle development and regeneration, but not in muscle atrophy. In addition, we found that during muscle differentiation Ozz recognizes the developmental isoform MyHCemb as one of its substrates, marks it for ubiquitination, and is both necessary and sufficient for its ubiquitination in an in vitro assay. Ozz binding to MyHCemb differs from that of other E3 ligases in that it targets the tail portion of assembled sarcomeric myosin rather than the head portion of soluble myosin, and we suggest that these properties are fundamental to its role in muscle development as opposed to muscle atrophy.

MyHCemb is the majority myosin isoform in embryonic and neonatal muscle fibers and its expression declines after birth to become undetectable around 3 weeks postnatal [20]. This postnatal decline is a robust process, not affected in null mutants of other myosin isoforms [20] or in animals where development of adult isoforms is retarded by undernutrition [36]. However, we found that the decline is slowed in a model system of differentiating ozz+/+ primary myoblasts and in postnatal ozz+/2 muscles.

During myofibrillogenesis, the developmental exchange of MyHC isoforms requires a myosin molecule to be released from its complex insertion into a sarcomeric thick filament, in order to be replaced by a subsequently expressed isoform.Davis, in a model of this process [2], concluded that core myosin molecules within a myofilament are essentially inaccessible to exchange by mass action and that a "facilitated exchange" process must exist in order to account for the rapid and complete change of isoforms observed in vivo. Without an additional regulatory process, exchange at equilibrium would be limited to the exchange of subunits away from the center of the filament.

We found that Ozz is associated with sarcomeric but not soluble MyHCemb from the earliest stages of muscle formation. The fact that Ozz and its direct partner Elo C could be co-immunoprecipitated from sarcomeric but not soluble myosin extracts of E16.5 embryonic muscle indicates that at this age a proportion of Ozz molecules bound to MyHCemb is assembled into the E3 ligase complex. We also demonstrated that formation of such a complex is sufficient for in vitro ubiquitination of sarcom filamentous MyHCemb. From these findings, we can infer that the orderly removal of assembled MyHCemb is achieved by tagging it with the Ozz-E3 ubiquitin ligase (Fig. 8). This refined mechanism, exchanging single molecules within a macromolecular assembly, would enable isoform exchange without any necessity for demolition and reconstruction within the cell, and is expected to represent a principle, which may be exploited by other subcellular systems. Moreover, our findings complement recent work on myosin assembly, which requires the coordinated action of

![Figure 3. Ozz Segregates with Myofilamentous MyHCemb from the Earliest Stages of Myofibrillogenesis.](https://www.plosone.org/doi/fig/10.1371/journal.pone.0009866.g003)

In Figure 3, Ozz segregates with myofilamentous MyHCemb from the earliest stages of myofibrillogenesis. Sarcomeric (P) fractions from E14.5 (top), E-16.5 (middle), and E18.5 (bottom) embryonic muscles were separated according to their molecular size (horizontal axis) by gel filtration chromatography. Aliquots of each eluted fraction were separated by SDS-PAGE and then immunoblotted with anti-MyHCemb (green), anti-Ozz (red), and anti-MLC (blue). The average intensities from three experiments were measured and plotted, as indicated. The densitometric analysis of the protein profile shows that the high molecular weight fractions (≈1500 kDa - ≈900 kDa) of sarcomeric (P) preparations contained polymeric myofilamentous MyHCemb as confirmed by the presence of MLC. Ozz co-eluted with the high molecular weight myofilamentous MyHCemb only in the sarcomeric (P) fractions, but was conspicuously absent from the cytoplasmic fractions (data not shown). Ozz and MLC also eluted in size fractions corresponding to their monomeric molecular weights, in both sarcomeric and cytoplasmic subcellular fractions (data not shown). doi:10.1371/journal.pone.0009866.g003
chaperones and ubiquitin ligases [28,37] to construct multimeric myosin ready for insertion into sarcomeric thick filaments.

Myosin assembly into thick filaments depends on a 29 amino acid assembly competence domain (ACD) near the C-terminal end of the myosin rod domain [26]. We found that Ozz recognizes the rod portion of MyHCemb, which forms the core of sarcomeric thick filaments where it is not easily accessed for binding or exchange by soluble cytoplasmic molecules. This is in contrast to the other known myosin E3 ligases, which target the head region of myosin [9], the portion of myosin exposed for interaction with actin and ATP. Furthermore, they appear able to access only sarcoplasmic, not sarcomeric myosin [9,38], and their E3 ligase activity does not require further activation once they have recognized their substrate.

We hypothesize that during muscle fiber differentiation sarcomeric MyHCemb becomes ubiquitinated by the Ozz-E3, and dissociates from the rest of the sarcomere. We showed that Ozz and Elo C are present together with sarcomeric myosin from the earliest stages of myofibrillogenesis, but we saw no obvious increase in Ozz/MyHCemb ratio in association with the onset of peak periods of MyHCemb degradation during embryogenesis [e.g. Fig. 3C vs. Fig. 3A, 3B]. On the basis of these results, we conclude that Ozz-E3 ligase promotes the ubiquitination and the degradation of sarcomeric MyHCemb (Fig. 8).

However, we cannot exclude that this process may occur in two phases. This alternative model implies that, in contrast with the action of one-chain ligases, Ozz does not immediately initiate ubiquitination and proteolytic degradation but may have a chaperone-like function in a preassembled form and only subsequently may gather the rest of the complex and become an active ligase. This two-step mode of action of the Ozz-E3 may explain the timely and regulated replacement/exchange of myofilamentous myosin during muscle differentiation and regeneration. This model of regulated assembly and disassembly of MyHC could be envisaged also for the adult muscle, where Ozz is expressed at basal levels [12]. In the adult muscle the Ozz-E3 activity would ensure the maintenance of myofiber integrity and the regulated exchange of isoforms under stress fiber conditions.

Modulation of cell structure by disaggregation and reassembly of cytoskeletal subunits is common to all cells, as is ubiquitination of many of the signaling proteins that control this process. The ubiquitin system also regulates signaling proteins controlling cell metabolism, cell cycle, and ion channel turnover, and is involved in chaperone-mediated myosin assembly [27–30]. It has a

Figure 4. Co-localization of Ozz with Sarcomeric MyHCemb during Myofiber Differentiation. (A) and (B) Confocal microscopy images of differentiating myotubes (day 4) untreated (A) and treated (B) with MG-132. Cells treated with the proteasome inhibitor showed a clear increase of co-localization of MyHCemb and Ozz compared to untreated myotubes. (C) and (D) Computational analyses of confocal images of differentiated myotubes untreated (C) and treated (D) with MG132 confirmed the visualization of the co-localized fluorochromes. Pearson’s correlation coefficient (Rr), Manders overlap (R), and Manders overlap coefficients k1 and k2 were employed to evaluate the extent of colocalization of the two fluorescent dyes.

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prominent role in recognizing misplaced or damaged proteins and promoting their degradation, and has a particular association with muscle atrophy. However, Ozz is upregulated during development, growth and regeneration, and downregulated in atrophy, and represents to our knowledge the first E3 ligase to be described as having a role in targeting myosin in its fully assembled sarcomeric structure during muscle remodeling. The Ozz model may be a paradigm for other developmental systems requiring protein isoform exchange within complex cytoskeletal structures.

Materials and Methods

Yeast Two-Hybrid Screening

Screening of an E14.5 mouse cDNA library (gift of P. McKinnon) for putative Ozz-binding partners was performed as previously described [12]. Three cDNA clones encoding the tail portion of MyHCemb were isolated. To confirm the absence of interaction with the head and neck portion of MyHCemb, the corresponding prey construct (1–1047 aa) cDNA was amplified using a 10 μl reverse transcription reaction with primers 5′-GAGGGTGGGTCGACCATGAGTAGCGACACCGAGATGG-3′ and 5′-TACTTAGCGGCCGCTCATTGTTCGAGGAGCTCTCCAG-3′. The RT-PCR product was cut with Sall and NotI and inserted into the prey vector pEXP-AD502 in frame with the GAL4 activation domain. For yeast two hybrid assays, the bait and prey constructs were co-transformed into yeast strain Mav203. Two reporter genes (HIS3 and LacZ) were employed to study the protein (Invitrogen).
Figure 6. MyHCemb Expression is Abnormally Prolonged in Ozz−/− Mutants. (A) MyHCemb expression is prolonged during in vitro differentiation of ozz−/− primary myoblasts. Western blots showing the time course of MyHCemb and Ozz expression in undifferentiated myoblasts (day 0) and differentiating or differentiated multinucleated myotubes (day 1-5) from null mutants and wild-type controls. (B) Quantification of MyHCemb expression in the soluble fractions shown in (A), normalized against Hsp70 levels. Data are expressed as mean ± SD of three independent experiments. Groups were compared by the Student t-test for two samples assuming equal variance. Mean differences were considered statistically significant when P values were less than 0.05 (*). (C) Western blot analyses of the sarcofilamentous, insoluble fractions of muscle isolated from E18.5 mutants. Ozz deletion–regeneration was induced by injecting 10 mM isofluorane, and following surgery received ibuprofen, 7.5 mg/kg daily, for pain relief. Muscles of the lower hind limbs were denervated by sectioning the sciatic nerve in the lower thigh, just above the common peroneal–tibial nerve junction. Alternatively, muscles of the right limbs, with the left used as controls. Mice were sacrificed by CO2 inhalation before dissection.

Isolation of Embryonic Muscles

Dated pregnant mice were sacrificed by CO2 inhalation, and the uterus quickly removed and placed on ice. Individual embryos were pinned on a Sylgard dish and dissected under ice-cold saline solution. The viscera, skin and spinal cord were removed and the embryo decapitated, leaving muscles of all four limbs, ribcage and back, with their associated cartilage. Tissues were snap frozen in liquid nitrogen.

Surgical Procedures

All animal experiments were performed according to animal protocols approved by our Institutional Animal Care and Use Committee and National Institutes of Health guidelines. C37Bl/6 wild-type and ozz-null mice [12] were anaesthetized with isoflurane, and following surgery received ibuprofen, 7.5 mg/kg daily, for pain relief. Muscles of the lower hind limb were denervated by sectioning the sciatic nerve in the lower thigh, just above the common peroneal–tibial nerve junction. Alternatively, they were tenotomised by sectioning the Achille’s tendon. Muscle degeneration–regeneration was induced by injecting 10 mM isofluorane into the gastrocnemius (100 µl) or tibialis anterior (30 µl) muscles of 1–3 month-old mice. All experimental muscles were in the right limbs, with the left used as controls. Mice were sacrificed by CO2 inhalation before dissection.

Cell Cultures

Myoblast cultures were established as described previously [12,39,40].

Antibodies and Reagents

Rabbit anti-Ozz antibody was prepared as described [12]. The antibody was diluted 1:500 for Western blotting and 1:10 for immunofluorescence. Mouse monoclonal antibodies anti-MyHCemb (F1.652) 1:400, anti-MLC (T14) 1:500, and anti-pan myosin (MF20) 1:500 were purchased from the Developmental Studies Hybridoma Bank. Anti-MyHCemb (2B6) 1:500, was a gift from Dr. N. Rubinstein. Other commercial antibodies included mouse anti-GST 1:500 (UPSTATE), mouse anti-ubiquitin 1:500 (Zymed), anti-Elo C 1:300 (BD Biosciences), anti-GAPDH 1:5000 (Mili-pore), rabbit anti-Elo B 1:300 (Santa Cruz), anti-Rbx-1 1:500 (Thermo Scientific), anti-Cul5 1:200 (Santa Cruz), anti-pan actin (1:5000; Sigma) and Alexa Fluor 488-conjugated anti-mouse 1:500 (Invitrogen), FITC-Phalloidin 1:500 (SIGMA-ALDRICH) and MG-132 (Enzo Life Sciences).

Co-immunoprecipitation

Ozz-MyHCemb complexes were detected in crude lysates of myoblast by immunoprecipitation with anti-MyHCemb antibody, followed by Western blotting of the immunoprecipitates with the anti-Ozz antibody. Cultured myoblasts were lysed (lysis buffer: 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, protease inhibitors, phosphatase inhibitors), incubated at 4°C for 30 min, and cellular debris were pelleted by 20 min centrifugation at 12000 x g. The lysate was precleared by 1 hr incubation at room temperature with 30 µl of Gamma Binds Plus Sepharose beads (Amersham Biosciences) and then spun at 1500 x g for 30 min. Anti-MyHCemb (2B6) or anti-Ozz antibody was added to the supernatant and incubated for 1 hr at room temperature, followed by immunoprecipitation with Gamma Binds Plus Sepharose beads and overnight incubation at 4°C. The beads were washed 3x with lysis buffer, and 1x with lysis buffer without detergents, and the bound proteins detected and run on SDS-polyacrylamide gels under denaturing conditions.

Fractionation of Muscle Tissues and Co-immunoprecipitation

Muscles from E14.5, E16.5, and E18.5 embryos were homogenized for 60s in a Dounce homogenizer in 4 volumes of lysis buffer [28] (Tris-HCl, 20 mM, pH 8.0; NaCl, 200 mM; MgCl2, 5 mM; DTT, 5 mM) and centrifuged at 16000 x g for 5 min. The supernatant was then spun at 100,000 x g for 2.5 hr at 4°C. The resulting supernatant was kept as the S fraction.
cytoplasmic) and the pellet as the P fraction (sarcomeric). The P fraction was resuspended in 600 µl of lysis buffer.

S and P fractions prepared from E16.5 embryos were immunoprecipitated with anti-Elo C or anti-MyHCemb using Protein G Dynabeads (Invitrogen), as described above.

Gel filtration Columns of S and P Fractions

S and P fractions were separated on a Superose 6 gel filtration column (GE Healthcare). Aliquots from the gel filtration column were then denatured and run on SDS-polyacrylamide gels, followed by Western blotting with the appropriate antibodies. For calculation of the molecular weight the column was calibrated with the following proteins: thyroglobulin, 669 kDa; apoferritin, 443 kDa; β-amylase, 200 kDa; carbonic anhydrase, 29 kDa (SIGMA). At least 54 aliquots were analyzed, giving a size resolution from ≈2000 kDa to <10 kDa.

In vitro Ubiquitination of GST-MyHCemb Fragment

Four μg of a bacterially expressed GST-MyHCemb fragment (1041–1941 aa) was incubated with 150 ng of purified recombinant E1 (Calbiochem), 200 ng UbcH5b (a gift of Dr. B. Schultman), 1.0 μg Ozz-E3 ubiquitin ligase and 7.5 μg of ubiquitin (Calbiochem) or K48R ubiquitin (Calbiochem) in a final volume of 30 μl of ubiquitination buffer (0.05M Tris-HCl, pH 7.6; 0.01M MgCl2, 0.004M ATP) at 30°C for 60 min.
To analyze the ubiquitinated products, 4.0 mg of GST-MyHCemb fragment (used as control) or the ubiquination reaction mixtures were diluted in 500 ml of RIPA buffer (50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 1 mM EDTA, protease inhibitors and phosphatase inhibitors), immunoprecipitated with anti-MyHCemb (2B6), resolved on a 7.5% SDS-gels, and immunoblotted with either anti-MyHCemb, anti-ubiquitin, or anti-GST antibodies (Zymed).

Purification and In Vitro Ubiquitination of Native MyHC

Sarcomeric MyHC from P1 muscle was purified as described [33,34]. Purified MyHC (3 μg) was subjected to in vitro ubiquitination following the conditions described above. The reaction mixtures were resolved on a 7.5% SDS-polyacrylamide gel and immunoblotted with anti-ubiquitin antibody (Zymed).

Thin-Thick Filaments

Muscle sarcomeric thin-thick filaments were purified according to the procedure of Trinick et al. [31].

Western Blotting

Protein concentrations were determined as OD 595, using BSA as standard. 25 μg of soluble protein or 6.25 μg of insoluble protein were electrophoresed (100 V, 60 min) on SDS-gradient gels (NuPAGE 4–12% Bis-Tris Gel, Invitrogen), and wet-blotted overnight at 30 mA. Membranes were probed with specific antibodies at the dilutions listed above, followed by HRP conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories). Signals were detected with a West Femto maximum sensitivity substrate kit (Thermo Scientific) on a molecular imager (ChemiDoc XRS, BioRad). Each of the immunoblots included in the Figures was representative of results obtained in at least three independent experiments.

Immunoblots were photographed in a BioRad Chemidoc XRS Molecular Imager, and, where appropriate, band densities measured using BioRad Quantity One software. Montages were assembled using Adobe Illustrator, and then converted to TIFF files.

Figure 8. Model of the Sequence of Events Involved in Ozz-Mediated MyHCemb Degradation from Sarcomeric Thick Filaments. (A) Ozz is bound to the rod portion of sarcomeric MyHCemb in the vicinity of residue 1535, a possible region for lateral binding with the ACD residues on adjacent myosin rods [43]. Exchange between sarcomeric MyHCemb and newly synthesized neonatal MyHC may be initiated by full Ozz-E3 ligase assembly and initiation of ubiquitination of sarcomeric MyHCemb, as demonstrated by co-immunoprecipitation of Ozz-Elo C from sarcomeric myosin, and the presence of ubiquitinated MyHCemb in sarcomeric myosin preparations. (B) Isoform exchange, releasing soluble MyHCemb by now having a full Ozz-E3 ligase assembled (C), leading to its prompt ubiquitination and proteolytic cleavage, resulting in the absence of Ozzylated MyHCemb in the soluble myosin pool. (D) Further proteolytic degradation, involving specific proteases and ubiquitination of soluble fragments mediated by monomeric ubiquitin ligases, e.g. MuRF family members.

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Immunofluorescence and Imaging

Immunofluorescence analyses were performed on cultured myotubes (day 4) treated and not treated with proteasome inhibitor. MG-132 was added to the culture medium at a final concentration of 5 μM for 6 hr at 37°C. Myotubes were fixed in 3% PFA and immunostained with anti-Ozz and anti MyHCemb antibodies. Cy3 anti-rabbit IgG (Jackson Laboratories) and Alexa Fluor 488 anti-mouse IgG (Invitrogen) were used as secondary antibodies. Analysis of the fluorescence intensity of MyHCemb was performed on cultured myotubes (day 4). In this experiment Cy3 anti-mouse IgG (Jackson Laboratories) was used as secondary antibody.

Immunofluorescence analyses of MyHCemb expression in muscle tissue were performed on hind limb muscles from wild-type and ozz−/− P7.5 pups. Muscles were embedded in OTC freezing solution and sectioned sequentially from the distal tendinous insertion towards the mid belly region. Cross sections were labeled with anti-MyHCemb and FITC-Phalloidin (SIGMA) followed by incubation with Cy3-conjugated secondary antibody (Jackson ImmunoResearch) prior to confocal microscopy imaging. Images were acquired on a Nikon C1si confocal microscope, with a Plan Apo 40X, NA 1.3 and/or Plan Apo 60X, NA 1.45 objective (Melville, NY).

Calculation of Co-localization Coefficients

Computational analyses of confocal images were performed with the NES-Elements AR 3.1 (Melville, NY). Pearson’s correlation coefficient (Rr), Manders overlap (R), and Manders overlap coefficients k1 and k2 were employed to evaluate the extent of co-localization of the two fluorescent dyes. Pearson’s correlation coefficient (Rr) is one of the standard measures in pattern recognition. It is used for describing the correlation of the intensity distributions between channels. It takes into consideration only similarity between shapes while ignoring the intensities of signals. Its values range is between -1.0 and 1.0, where 1.0 indicates no overlap and 1.0 is a complete co-localization. Manders overlap coefficient is a generally accepted measure of co-localization. It indicates an overlap of the signals and thus represents the true degree of co-localization. Values of the R are defined from 0 to 1.0. If an image has an overlap coefficient equal to 0.5, it implies that 50% of both its components overlap with the other part of the image. A value of zero means that there are no any overlapping objects Overlap coefficients k1 and k2 split the value of co-localization into two separate parameters. k1 and k2 coefficients depend on the sum of the products of the intensities of two channels. Thus, they are sensitive to the differences in the intensity of two signals and should be used accordingly [41,42].

Statistical Analysis

Data were expressed as mean ± SD and evaluated using Student’s t-test for comparison with wild-type samples. Mean differences were considered statistically significant when P values were less than 0.05 (*)

Supporting Information

Figure S1 (A) Yeast 2-Hybrid Screen Demonstrating that Ozz Interacts with the Tail Portion of MyHCemb (1047-1941 aa) but not the Head and Neck (1-1041 aa). Found at: doi:10.1371/journal.pone.0009866.s001 (8.67 MB TIF)

Figure S2 (A) Immunofluorescence analyses of the expression of MyHCemb in ozz knock-out and wild-type differentiated myoblast (day 4). (B) Quantification of intensity of the expression of MyHCemb in the differentiated myoblast. Found at: doi:10.1371/journal.pone.0009866.s002 (5.45 MB TIF)

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Author Contributions

Conceived and designed the experiments: YC AJH AdA. Performed the experiments: YC XQ EZ SM. Analyzed the data: YC XQ EZ SM AB AHH AdA. Contributed reagents/materials/analysis tools: NV. Wrote the paper: YC AJH AdA. Contributed reagents/materials/analysis tools: NV. The authors thank G. Grosveld for useful discussion and suggestions; T. Nastasi, L. Fink and C. Saint Martin for their contributions in the initial phase of this project; B. Schulman and N. Rubinstein for their generous gift of reagents for the ubiquitination reactions and the anti-MyHCemb monoclonal antibody; E. Bonten for help with chromatography and useful discussion; E. Gomez for invaluable help in maintaining the mouse colonies; Samuel Connell and Jennifer Peters of the Cell and Tissue Imaging Shared Resource of SJCRH for help with confocal imaging; Charlotte Hill for outstanding secretarial assistance. A. d’Azzo holds an endowed chair in Genetics and Gene Therapy from the Jewelry Charity Fund.

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