Non- Ca^{2+} -conducting Ca^{2+} channels in fish skeletal muscle excitation-contraction coupling

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During skeletal muscle excitation-contraction (EC) coupling, membrane depolarizations activate the sarcolemmal voltage-gated L-type Ca^{2+} channel ($\text{Ca}_v1.1$). $\text{Ca}_v1.1$ in turn triggers opening of the sarcoplasmic Ca^{2+} release channel (RyR1) via interchannel protein-protein interaction to release Ca^{2+} for myofibril contraction. Simultaneously to this EC coupling process, a small and slowly activating Ca^{2+} inward current through $\text{Ca}_v1.1$ is found in mammalian skeletal myotubes. The role of this Ca^{2+} influx, which is not immediately required for EC coupling, is still enigmatic. Interestingly, whole-cell patch clamp experiments on freshly dissociated skeletal muscle myotubes from zebrafish larvae revealed the lack of such Ca^{2+} currents. We identified two distinct isoforms of the pore-forming $\text{Ca}_v1.1\alpha_{15}$ subunit in zebrafish that are differentially expressed in superficial slow and deep fast musculature. Both do not conduct Ca^{2+} but merely act as voltage sensors to trigger opening of two likewise tissue-specific isoforms of RyR1. We further show that non- Ca^{2+} conductivity of both $\text{Ca}_v1.1\alpha_{15}$ isoforms is a common trait of all higher teleosts. This non- Ca^{2+} conductivity of $\text{Ca}_v1.1$ positions teleosts at the most-derived position of an evolutionary trajectory. Though EC coupling in early chordate muscles is activated by the influx of extracellular Ca^{2+} , it evolved toward $\text{Ca}_v1.1$ -RyR1 protein-protein interaction with a relatively small and slow influx of external Ca^{2+} in tetrapods. Finally, the $\text{Ca}_v1.1$ Ca^{2+} influx was completely eliminated in higher teleost fishes.

calcium conductivity | evolution | ion channels | slow and fast muscle | zebrafish

Excitation-contraction (EC) coupling is understood as the mechanism linking membrane depolarization to the contraction of a muscle cell. Depolarization-induced activation of the sarcolemmal voltage-gated Ca^{2+} channel (Ca_v) triggers opening of the sarcoplasmic Ca^{2+} release channel (ryanodine receptor, RyR) to release from SR stores Ca^{2+} that is needed for myofibril contraction. Though in cardiac muscle the homologous isoform RyR2 is activated by the Ca^{2+} influx through $\text{Ca}_v1.2$, in skeletal muscle, conformational changes of $\text{Ca}_v1.1$ directly activate RyR1 via protein-protein interaction (1, 2). However, a simultaneously occurring Ca^{2+} current through $\text{Ca}_v1.1$ is observed in mammalian skeletal myotubes. In contrast to the large and rapidly activating currents that are needed for efficient RyR2 activation in cardiac myocytes (3, 4), the skeletal muscle $\text{Ca}_v1.1$ Ca^{2+} current is relatively small and slowly activating (5). Phylogenetically interesting is that EC coupling in skeletal muscle of very early chordates, such as *amphioxus* or tunicates, is activated by Ca^{2+} influx, and in lamprey muscles, maximum-force generation is dependent on Ca^{2+} currents, although direct $\text{Ca}_v1.1$ -RyR coupling is already established (6). However, in cultured mammalian skeletal muscle cells the absence of extracellular Ca^{2+} or pharmacological blocking of $\text{Ca}_v1.1$ does not immediately affect intracellular Ca^{2+} release and contractions (7, 8). Despite the outcome of these short-term in vitro experiments, it remains unclear if the $\text{Ca}_v1.1$ Ca^{2+} influx into the intact muscle plays an essential role on the long-term.

Here we show that in skeletal muscle of teleost fishes the voltage-gated Ca^{2+} channel $\text{Ca}_v1.1$ is unable to conduct Ca^{2+} and,

thus, turned from an ion channel to a pure voltage sensor to trigger RyR1 opening. Therefore, our findings suggest that on the level of the whole organism, Ca^{2+} influx via $\text{Ca}_v1.1$ is not required in teleost fishes. Furthermore, we discuss that this $\text{Ca}_v1.1$ non-conductivity stands at the topmost position of an evolutionary trajectory from Ca^{2+} influx-dependent, cardiac muscle-like EC coupling in skeletal muscles of early chordates to Ca^{2+} influx-independent EC coupling with small Ca^{2+} currents in mammals, and finally to the complete elimination of $\text{Ca}_v1.1$ Ca^{2+} influx in teleost fishes.

Results

Non- Ca^{2+} -conducting $\text{Ca}_v1.1$ Channels Perform EC Coupling in Zebrafish Skeletal Muscle. Whole-cell patch clamp analysis of myotubes isolated from wild-type zebrafish larvae revealed the lack of voltage-dependent inward Ca^{2+} currents at physiologically relevant potentials between -40 and $+70$ mV (Fig. 1A). Also, replacement of the charge carrier Ca^{2+} by Ba^{2+} did not evoke inward L-type currents (Fig. 1D). However, correct voltage-dependent activation of the $\text{Ca}_v1.1$ channels was indicated by robust $\text{Ca}_v1.1$ -specific gating currents (Fig. 1B) (9, 10) that reached the levels described for the Ca^{2+} -conducting $\text{Ca}_v1.1$ in mouse myotubes (11). Gating currents result from depolarization-induced displacements of charges in the channel's voltage sensing elements, resulting in a conformational change of the channel complex (12). This steric effect is not only inducing channel opening but is also responsible for the direct coupling of $\text{Ca}_v1.1$ with the Ca^{2+} release channel RyR1 in the SR (2). To test for functional $\text{Ca}_v1.1$ -RyR1 interaction, depolarization-induced intracellular Ca^{2+} release from the SR through RyR1 was recorded using the intracellular Ca^{2+} indicator dye Fluo-4. Robust Ca^{2+} transients indicated intact EC coupling in these myotubes (Fig. 1C). Thus, zebrafish $\text{Ca}_v1.1$ is able to undergo the voltage-dependent conformational change required for channel activation and to trigger EC coupling, but completely lost its function to conduct calcium ions.

Two $\text{Ca}_v1.1\alpha_{15}$ Isoforms Are Differentially Expressed in Zebrafish. The central α_{15} subunit forms the pore, the Ca^{2+} selectivity filter, and the voltage sensor of the skeletal muscle Ca^{2+} channel complex (12, 13), and thus primarily determines the channel's conductivity properties. Therefore, we performed a BLAST search within the ENSEMBL zebrafish genomic assembly Zv7 with the coding sequence of the rabbit α_{15} subunit (rb- α_{15}) as a template

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Data deposition: Sequences for zf- α_{15} -a and zf- α_{15} -b have been deposited in the GenBank database (accession nos. FJ769223 and AY495698).

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Fig. 1. Non- Ca^{2+} -conducting Ca^{2+} channels in larval zebrafish skeletal myotubes. (A) Depolarizing test pulses to potentials unto +70 mV were unable to elicit Ca^{2+} currents at any potential ($n = 14$). (B) Robust voltage-dependent α_{1S} gating is indicated by charge movements with a $Q_{\max}$ value of 10.45 ± 0.58 nC/pF and a half-maximal activation of -4.21 ± 1.36 mV ($n = 20$), assessed by integrating the "ON" component of α_{1S} gating currents (Q_{on}). (C) Depolarization-induced intracellular Ca^{2+} transients had a $(\Delta F/F_0)_{\max}$ value of 4.44 ± 0.42 with half-maximal activation at -10.39 ± 0.75 mV ($n = 17$). (D) Also, 10 mM Ba^{2+} instead of 10 mM Ca^{2+} as a charge carrier did not awake any current at any potential ($n = 6$).

(12). Surprisingly, two distinct genes coding for $\text{Ca}_v1.1\alpha_{1S}$ subunits were identified in the zebrafish genome and were located on chromosomes 8 and 22. Both genes code for intact α_{1S} subunits that contain all molecular characteristics, such as the interaction domains with the accessory β_{1A} subunit (14) and with RyR1 (15). One gene, identified on chromosome 22, consists of 43 exons and codes for an α_{1S} protein of 1,777 amino acids, hereafter named $\text{zf-}\alpha_{1S}\text{-a}$, with 66% sequence identity to $\text{rb-}\alpha_{1S}$. The second gene, identified on chromosome 8, is composed of 45 exons that code for the 1,847 amino acids of $\text{zf-}\alpha_{1S}\text{-b}$ with 64% sequence identity to $\text{rb-}\alpha_{1S}$. The two $\text{zf-}\alpha_{1S}$ isoforms share an amino acid homology of 73%. In a phylogenetic tree based on parsimony analysis of DNA sequences (Fig. 2A), both $\text{zf-}\alpha_{1S}$ subunits cluster with $\text{rb-}\alpha_{1S}$, clearly separated from the clade comprising the cardiac α_{1C} isoforms of rabbit and also zebrafish, consequently identifying them as α_{1S} subunits. Ancestral α_1 isoforms from the housefly *Musca domestica* (16) and the tunicate *Halocynthia roretzi* (17) were used as outgroups.

The question now arises, why—in contrast to e.g., mammals—two α_{1S} subunit isoforms are present in zebrafish, and whether they might follow specific expression patterns. Recently, two isoforms of the skeletal muscle RyR1 were identified in zebrafish (18), with RyR1a being expressed in superficial slow muscle (also called red muscle), which is aerobic and used for persistent swimming, and RyR1b as the predominant isoform in deep fast muscle (white muscle), the glycolytic musculature used for burst activity. To test if RyR1a and RyR1b might be explicit molecular interaction partners for $\text{zf-}\alpha_{1S}\text{-a}$ and $\text{zf-}\alpha_{1S}\text{-b}$, respectively, we performed quantitative RT-PCR on each of these muscle layers, isolated from adult zebrafish. In deep fast muscle, $\text{zf-}\alpha_{1S}\text{-b}$ was by far the predominant isoform, whereas in superficial slow muscle

Fig. 2. Two distinct α_{1S} subunit isoforms are found in the zebrafish skeletal musculature. (A) Singlemost parsimonious tree of aligned DNA sequences groups both zebrafish α_{1S} subunit isoforms ($\text{zf-}\alpha_{1S}\text{-a}$ and $\text{zf-}\alpha_{1S}\text{-b}$) jointly with α_{1S} of the rabbit ($\text{rb-}\alpha_{1S}$) and clearly separated from the clade comprising cardiac α_{1C} subunits of both species. Bootstrap scores of 100% (1,000 replications) for all branches support evolutionary divergence. (B) Quantitative RT-PCR reveals different expression patterns for $\text{zf-}\alpha_{1S}\text{-a}$ and $\text{zf-}\alpha_{1S}\text{-b}$. Data represent mean $\pm$ SEM. In deep fast muscle we almost exclusively detect $\text{zf-}\alpha_{1S}\text{-b}$, whereas in superficial slow muscle, $\text{zf-}\alpha_{1S}\text{-a}$ is the predominant isoform ($***P < 0.001$ by Student's paired t test). (C) Data obtained for $\text{zf-}\alpha_{1S}$ (B) closely match with expression levels of RyR1. (D–F) Whereas control experiments with $\text{rb-}\alpha_{1S}$ (D) restored Ca^{2+} inward currents (Top and Middle) in murine α_{1S} -null myotubes with $I_{\max}$ of -2.42 ± 0.18 pA/pF ($n = 25$), both, $\text{zf-}\alpha_{1S}\text{-a}$ ($n = 14$) and $\text{zf-}\alpha_{1S}\text{-b}$ ($n = 12$) were unable to restore any Ca^{2+} current (E and F). Ca^{2+} transients (Bottom) obtained with $\text{zf-}\alpha_{1S}\text{-a}$ had $(\Delta F/F_0)_{\max}$ values of 0.84 ± 0.12 ($n = 15$), with a half-maximal activation at 9.91 ± 1.54 mV, and thus were smaller ($P < 0.001$) and shifted toward more positive potentials ($P < 0.01$) when compared with $\text{zf-}\alpha_{1S}\text{-b}$ [$(\Delta F/F_0)_{\max} = 1.76 \pm 0.2$, with a half-maximal activation at 4.13 ± 1.4 mV, $n = 17$], which was comparable ($P > 0.6$) to $\text{rb-}\alpha_{1S}$ [$(\Delta F/F_0)_{\max} = 1.95 \pm 0.24$, with a half-maximal activation at 3.52 ± 1 mV, $n = 30$].

the expression level of $\text{zf-}\alpha_{1S}\text{-a}$ was significantly higher compared with $\text{zf-}\alpha_{1S}\text{-b}$ (Fig. 2B). The considerable signal obtained for $\text{zf-}\alpha_{1S}\text{-b}$ in superficial slow muscle most likely results from a preparation artifact, because this superficial slow muscle layer is very thin in the relatively small zebrafish and thus hard to separate cleanly. Therefore, we performed the same experiments on the musculature of another species of the same superorder (Ostarioclupeomorpha), the sardine (*Sardina pilchardus*), in which the

tissue separation is much clearer due to a robust superficial slow muscle layer of this very active swimmer. Here, a perfect separation of the two α_{1S} and RyR1 isoforms in both muscle types was revealed (Fig. S1). As shown in Fig. 2 B and C, the expression patterns of zf- α_{1S} -a and zf- α_{1S} -b perfectly match with those of the two RyR1 isoforms, RyR1a and RyR1b.

Both zf- α_{1S} Isoforms Do Not Conduct Ca^{2+} . To test the two distinct isoforms for their ability to conduct Ca^{2+} , both zf- α_{1S} cDNAs were generated by RT-PCR from zebrafish skeletal muscle and cloned into expression vector pGFP37, allowing N-terminal GFP tagging (7). Constructs were heterologously expressed in murine α_{1S} -null myotubes (cell line GLT) (19) and patch-clamp analyzed. In stark contrast to rb- α_{1S} controls (Fig. 2D), Ca^{2+} inward currents could not be detected either through zf- α_{1S} -a (Fig. 2E) or zf- α_{1S} -b (Fig. 2F). However, like the rb- α_{1S} controls, both zf- α_{1S} isoforms restored EC coupling with robust intracellular Ca^{2+} transients (Fig. 2D–F). Notably, the Ca^{2+} transients recorded with zf- α_{1S} -a were smaller and shifted toward more positive potentials compared with those obtained with zf- α_{1S} -b (Table 1).

Non- Ca^{2+} -conducting $Ca_v1.1$ Channels Exist Also in Other Teleost Fishes. The question arises whether non- Ca^{2+} -conducting $Ca_v1.1$ channels are a common attribute of teleost fishes or are an exclusive trait of zebrafish or its order Cypriniformes. Therefore, we patch-clamp analyzed larval myotubes from a phylogenetically closely related fish, the pike characin (*Ctenolucius hujeta*, Characiformes) (Fig. 3A), and from ricefish medaka (*Oryzias latipes*, Beloniformes) (Fig. 3B), which is much higher in the phylogenetic scale. Results from both teleost species matched those from zebrafish. Again, no Ca^{2+} inward currents, but pronounced intracellular Ca^{2+} transients, were recorded.

Charged Pore Residues Are Correlated with Ca^{2+} Conductance in Both α_{1S} Isoforms. Surprisingly, zf- α_{1S} / rb- α_{1S} sequence alignments showed that at position 303 of zf- α_{1S} -a, glutamate is consistently exchanged by glutamine when compared with rb- α_{1S} (Fig. 4A and C). In a properly folded conducting Ca^{2+} channel, the glutamate at this position is the first of four glutamates located in the pore-forming loops (p-loops) of the four homologous repeats (Fig. 4A), thus forming the selectivity filter for Ca^{2+} ions (20). Consequently, Gln303 in zf- α_{1S} -a makes the formation of an intact Ca^{2+} selectivity filter doubtful. We tested the consequences of the hypothesized loss of an intact selectivity filter by exchanging the corresponding Glu292 together with the preceding Met291 to glutamine and threonine in the conducting rb- α_{1S} , according to zf- α_{1S} -a sequence. Construct rb- α_{1S} ME/TQ was unable to recover L-type Ca^{2+} currents in heterologous expression experiments (Fig. 4D). In contrast to the prominent exchange at position 303 of zf- α_{1S} -a, zf- α_{1S} -b shows a perfect conservation of the essential Ca^{2+} selectivity filter glutamates (Glu312/Glu633/Glu1034/Glu1348) (Fig. 4E). Instead, in homologous repeat II of zf- α_{1S} -b an additional negative charge (aspartate) in close proximity of the selectivity filter glutamate was

Fig. 3. Non- Ca^{2+} -conducting Ca^{2+} channels are found also in other teleost orders. (A and B) In skeletal myotubes from the pike characin *Ctenolucius hujeta* (A) and the ricefish medaka *Oryzias latipes* (B), no Ca^{2+} inward currents could be recorded upon test pulses unto +70 mV (Upper), despite the presence of pronounced intracellular Ca^{2+} transients with $(\Delta F/F_0)_{\max}$ values of 6.05 ± 0.37 (n = 10) for the pike characin and 3.7 ± 0.54 (n = 6) for medaka, respectively (Lower).

identified, and this was able to completely block Ca^{2+} conductance when introduced into rb- α_{1S} in point mutant rb- α_{1S} N617D (Fig. 4F). Interestingly, this asparagine to aspartate mutation was also found in α_{1S} -b of the pike characin, but not in α_{1S} -b of the more distantly related medaka (Fig. 4G). Furthermore, a chimera of rb- α_{1S} , containing the repeat II S5/p-loop/S6 region of medaka α_{1S} -b, continued to show Ca^{2+} inward currents (Fig. S2). Unlike the addition of a negative charge (aspartate) in the repeat II p-loop of zf- α_{1S} -b, in medaka- α_{1S} -b, a conversion of a negative to a positive charge (aspartate to lysine compared with rb- α_{1S}) was found in the repeat I p-loop, four residues C-terminal to the selectivity filter glutamate (Fig. 4G). Again, the corresponding point mutant rb- α_{1S} D296K was unable to conduct Ca^{2+} inward currents (Fig. 4H). Intact EC coupling of all chimeras and point mutants mentioned previously was indicated by robust intracellular Ca^{2+} transients with $(\Delta F/F_0)_{\max}$ values >1.4 (Table 1).

Non- Ca^{2+} -conducting $Ca_v1.1$ Channels Are a Common Trait of Higher Teleosts. We tested for the presence of one of three marker amino acids incompatible with $Ca_v1.1$ Ca^{2+} conductance, in a large selection of teleosts using sets of degenerated α_{1S} -specific primers (Table S1) that amplify short fragments from genomic DNA or cDNA coding for the p-loop regions of homologous repeats I and II of α_{1S} -a and α_{1S} -b. Sequence analysis of these fragments from 32 species of 17 different orders of teleost fishes (Figs. S3 and S4) revealed the presence of two α_{1S} isoforms in all teleosts from the ancestral branch Osteoglossomorpha (*Notopterus notopterus*) to the most recently branching Beloniformes (*Oryzias latipes*; Fig. 5). The marker amino acids threonine and glutamine in the selectivity filter of zf- α_{1S} -a were identified in α_{1S} -a of all tested higher-teleost fishes starting with the clade comprising the Cypriniformes. Interestingly, sequence analysis of α_{1S} -b from numerous teleosts revealed either the lysine at position +4 relative to the selectivity filter in repeat I (Fig. S3) or the aspartate at position +3 relative to the selectivity filter in repeat II (Fig. S4), strictly correlating with their position on the evolutionary tree (Fig. 5). Consequently, higher teleosts exclu-

Table 1. Intracellular Ca^{2+} transients recorded in expression experiments in GLT myotubes

Construct	$(\Delta F/F_0)_{\max}$	1/2V $(\Delta F/F_0)_{\max}$ (mV)	n
rb- α_{1S}	1.73 ± 0.13	2.28 ± 0.75	67
zf- α_{1S} -a	0.84 ± 0.12 *	9.91 ± 1.54 **	15
zf- α_{1S} -b	1.76 ± 0.2 *	4.13 ± 1.4 **	17
rb- α_{1S} ME/TQ	1.93 ± 0.22	1.15 ± 1.4	20
rb- α_{1S} N617D	1.46 ± 0.23	1.92 ± 1.71	12
rb- α_{1S} D296K	1.75 ± 0.3	1.38 ± 1.64	15
RmRR	1.96 ± 0.37	2.15 ± 2.3	5

Robust intracellular Ca^{2+} transients indicate intact EC coupling for all constructs * $P < 0.001$; ** $P < 0.01$.

Fig. 4. Amino acids incompatible with Ca^{2+} conductance. (A and B) In murine α_{1S} -null myotubes, expression of the wild-type α_{1S} from rabbit (rb- α_{1S}), with a typical amino acid arrangement of a conducting channel in the pore region of homologous repeats I–IV (RI–RIV), could restore Ca^{2+} inward currents of 1.79 ± 0.16 pA/pF ($n = 37$). (C) In zf- α_{1S} -a, a threonine and glutamine at position –1 and 0 relative to the selectivity filter (selectivity filter glutamates are indicated by gray bars) of homologous repeat I (RI) were identified and equally found in α_{1S} -a of pike characin and medaka. (D) Corresponding replacement of residues Met291 and Glu292 to threonine and glutamine in rb- α_{1S} (rb- α_{1S} ME/TQ) completely abolished Ca^{2+} inward currents ($n = 9$). Similar results were obtained with (F) rb- α_{1S} N617D ($n = 8$) in which Asn617 of rb- α_{1S} was exchanged to aspartate as found in α_{1S} -b of zebrafish (E) and pike characin, as well as with (H) rb- α_{1S} D296K ($n = 15$) in which Asp296 was exchanged to lysine as found in α_{1S} -b of medaka (G).

sively express non- Ca^{2+} -conducting $\text{Ca}_v1.1$ channels. However, none of the three marker amino acid positions were found in the ancestrally branching superorders Osteoglossomorpha and Elopomorpha, and thus it is feasible that basal teleosts, like nonteleost fishes and tetrapods, still have Ca^{2+} -conducting $\text{Ca}_v1.1$ channels.

Discussion

Ca^{2+} Influx-Independent EC Coupling Without Simultaneous $\text{Ca}_v1.1$ Ca^{2+} -Conductance Sets Teleosts at a Most-Derived Position Concerning the Evolution of Chordate Skeletal Muscle EC Coupling. Early chordates

such as tunicates or cephalochordates (*amphioxus*) were shown to activate RyR via influx of extracellular Ca^{2+} (Ca^{2+} influx-dependent EC coupling; see Fig. 6) (22, 23) with rapidly activating Ca^{2+} currents (24), rather resembling the kinetics of cardiac $\text{Ca}_v1.2$ currents. At this phylogenetic stage, Ca_v channels are clustered opposite to RyR but are ultrastructurally arbitrarily arranged (25). This cardiac-like Ca_v arrangement, together with the lack of an intact α_{1S} II–III loop critical domain (15) (Fig. S5), explains the inability for direct Ca_v -RyR coupling. The functional arrangement of $\text{Ca}_v1.1$ in groups of four (tetrads), a prerequisite for direct $\text{Ca}_v1.1$ -RyR1 interaction, can first be observed in hagfish and lamprey (25). In these species, blocking of extracellular Ca^{2+} influx could no more fully obstruct EC coupling (24, 26, 27), even though $\text{Ca}_v1.1$ Ca^{2+} influx was found to still contribute to maximum force generation in EC coupling in lampreys (6). In tetrapods, from frog to human, the tetrads changed their arrangement inside the orthogonal arrays, and skeletal-muscle EC coupling became independent of Ca^{2+} influx (29), although a small and slowly activating $\text{Ca}_v1.1$ Ca^{2+} current can be observed, whose role is largely unknown. In higher-teleost fishes, the $\text{Ca}_v1.1$ Ca^{2+} current completely vanished, in part due to substantial sequence exchanges in or close to the selectivity filter of both $\text{Ca}_v1.1\alpha_{1S}$ isoforms, and consequently, RyR1 activation is solely achieved by direct $\text{Ca}_v1.1$ -RyR1 interaction. This clear evolutionary trajectory points to an evolutionary pressure on the reduction of $\text{Ca}_v1.1$ Ca^{2+} currents.

Fig. 5. Non- Ca^{2+} -conducting $\text{Ca}_v1.1$ channels are a common trait of all higher teleost fishes. Sequencing of a phylogenetic wide range of teleost fishes revealed that two distinct α_{1S} isoforms (α_{1S} -a and α_{1S} -b) are present in all teleosts. Marker amino acids indicating non- Ca^{2+} conductivity (boxed in black) were identified in α_{1S} -a and α_{1S} -b of all higher teleosts starting with the clade comprising the order Cypriniformes. The positions of the selectivity filter glutamates are indicated by gray bars. Phylogenetic tree is drawn after the Tree of Life web project (21).

Organ Differentiation in Teleost Skeletal Muscle. We identified two different non- Ca^{2+} -conducting isoforms of the pore-forming $\text{Ca}_v1.1\alpha_{1S}$ subunit in teleost fishes. $\text{Ca}_v1.1$ α_{1S} -a and α_{1S} -b are differentially expressed in superficial slow and deep fast musculature, respectively, which have repeatedly been shown to be very diverse musculature types regarding their transcriptome and physiology (30–32). We further showed that the expression pattern of the two different α_{1S} subunits nicely matches the expression pattern of the two RyR1 isoforms of teleost skeletal muscle, suggesting the presence of two distinct musculature-type specific $\text{Ca}_v1.1$ -RyR1 couplings. This two-coupling adaptation is an intriguing example of how the whole-genome duplication at the basis of the teleost lineage (*WGD*; see Fig. 6) continues into isoform formation during phylogenetic organ differentiation. Future studies will investigate the putative physiological and biophysical differences of EC coupling in these two different skeletal musculatures.

Fig. 6. Non- Ca^{2+} -conducting $\text{Ca}_V1.1$ channels position higher teleost fishes as most derived group concerning the evolution of skeletal muscle EC coupling. In Ca^{2+} influx-dependent EC coupling (22, 23) of early prochordates, such as tunicates or *amphioxus*, RyR opening is induced via rapidly activating Ca^{2+} currents (Ca^{2+} current) (24), and Ca_V channels are arbitrarily arranged in clusters (Ca_V clusters) opposite RyR (25). Later, hagfish and lampreys developed direct $\text{Ca}_V1.1$ -RyR1 coupling (24, 26, 27) with $\text{Ca}_V1.1$ arranged in groups of four (tetrads) (25). In tetrapods, the tetrads changed their disposition within the array, and a slowly activating Ca^{2+} current is still observed, despite that EC coupling is independent of the Ca^{2+} influx. The Ca^{2+} inward current completely vanished in teleost fishes, and EC coupling is solely induced via $\text{Ca}_V1.1$ -RyR1 protein–protein interaction. This is true for both distinct $\text{Ca}_V1.1\alpha_{1S}$ isoforms (α_{1S} -a and α_{1S} -b) that are present in higher teleosts, concordant with the hypothesis of a whole-genome duplication (WGD) at the basis of the teleost lineage (28). Selectivity filter glutamates are boxed in gray, marker amino acids indicating non- Ca^{2+} conductivity in black. Phylogenetic tree is drawn after the Tree of Life web project (21).

Ca^{2+} Influx in Mammalian Skeletal Muscle—A Tolerated Remnant or a Physiological Necessity? Apparently, all aspects of teleost fish skeletal muscle work appropriately without Ca^{2+} influx through $\text{Ca}_V1.1$ —a fact that also sheds light on the question about the role of the $\text{Ca}_V1.1$ Ca^{2+} influx into mammalian skeletal muscle. Together with the findings that pharmacological blocking of $\text{Ca}_V1.1$ or elimination of extracellular Ca^{2+} does not inhibit EC coupling (7, 8, 29), it is manifested that the Ca^{2+} influx in tetrapods is largely dispensable and could be seen as a tolerated remnant of the ancestral Ca^{2+} -influx-dependent skeletal muscle EC coupling. However, Ca^{2+} influx in mammalian skeletal muscle seems to be variably regulated, e.g., by the accessory $\text{Ca}_V1.1$ subunits $\alpha_{2\delta}$ -1 (33) and γ (34) or by retrograde current amplification, induced by RyR1- $\text{Ca}_V1.1$ interaction (35). Ca^{2+} current density is also massively up-regulated in developing skeletal muscle by expression of an alternatively spliced α_{1S} subunit (36). A possible role of this regulated Ca^{2+} influx might be in Ca^{2+} homeostasis by, e.g., SR store filling. However, experimental attempts to evaluate the importance of this Ca^{2+} influx for muscle function *in vivo* have not yet been realizable due to the lack of an appropriate system. The presence of non- Ca^{2+} -conducting Ca^{2+} channels in teleost fishes now provides evidence that on the organism level, skeletal muscle is completely functional without any $\text{Ca}_V1.1$ Ca^{2+} influx even on the long term. However, it is still feasible that principal differences in the physiology of Ca^{2+} handling in mammalian and teleost skeletal muscle might exist, and that in mammalian muscle the Ca^{2+} influx is indeed indispensable. To address this question, a knock-in mouse model carrying a nonconducting $\text{Ca}_V1.1$ and a transgenic zebrafish strain that expresses a conducting $\text{Ca}_V1.1$ in skeletal muscle will be valuable tools in further studies.

Taken together, this report might serve as the starting point for a diverse range of further studies in the fields of skeletal

muscle physiology, biophysics of ion channel pores, and evolutionary biology of skeletal muscle function.

Materials and Methods

Cell Culture. Myotubes of zebrafish, pike characin, and medaka were isolated and cultured exactly as described in detail (9). The murine α_{1S} -null line GLT was cultured and transfected as described (19, 37).

Biophysical Analysis. Myotubes were analyzed for L-type Ca^{2+} currents and gating currents by using the whole-cell patch-clamp technique in combination with intracellular Ca^{2+} recordings by loading the cells with the Ca^{2+} -sensitive dye Fluo-4 as described for GLT (38) and zebrafish myotubes (39). Charge carrier was 10 mM Ca^{2+} , or in one control experiment, 10 mM Ba^{2+} (Fig. 1D).

Quantitative RT-PCR. RNA samples of deep fast and superficial slow muscle tissue from adult zebrafish or from sardine were isolated using the RNeasy Kit (Qiagen) and reverse-transcribed using random primers and M-MLV reverse transcriptase (Promega). All qPCR assays were designed with at least one primer spanning an exon–exon boundary. Primer sequences are found in Table S2. To generate absolute standard curves for all targets DNA fragments of 400–500 bp containing the qPCR primer annealing regions were cloned into the pGEM-T Easy vector (Promega) and sequence fidelity checked (Eurofins MWG Operon). PCR fragments generated from the pGEM constructs were quantified using the Quant-iT PicoGreen dsDNA Quantitation Kit (Invitrogen) and used at concentrations of 1×10^2 – 1×10^5 molecules/PCR as templates for the site-specific standard curves. All qPCRs were performed on a Stratagene MX3000P using MESA GREEN qPCR Mastermix Plus for SYBR Assay (Eurogentec). Absolute copy numbers of samples were calculated using MxPro 4.0 software (Stratagene). All expression values are given as copy number of target gene divided by the copy number of the housekeeping gene succinate dehydrogenase complex, subunit A (SDHA) (40).

Molecular Cloning. zf- α_{1S} -a and zf- α_{1S} -b cDNA fragments were PCR generated from reverse-transcribed RNA isolated from zebrafish skeletal muscle. Generation of zf- α_{1S} constructs and of point mutations into rb- α_{1S} cDNA, all contained in expression plasmid pGFP37 (7), is described in SI Materials and Methods.

Genomic and cDNA Sequencing. Tissue samples of various species were collected from alive (tail fin clip) or freshly killed fish. gDNA was isolated immediately or after transportation in 100% alcohol. RNA was isolated and reverse transcribed as described previously for quantitative RT-PCR. α_{15} -specific but species-unspecific degenerate primers were used to PCR-generate fragments coding for the α_{15} subunit pore regions of homologous repeats I and II carrying marker amino acids for nonconductivity (Table S1).

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