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ORIGINAL PAPER

Calsequestrins in skeletal and cardiac muscle from adult *Danio rerio*

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Abstract Calsequestrin (Casq) is a high capacity, low affinity Ca^{2+}-binding protein, critical for Ca^{2+}-buffering in cardiac and skeletal muscle sarcoplasmic reticulum. All vertebrates have multiple genes encoding for different Casq isoforms. Increasing interest has been focused on mammalian and human Casq genes since mutations of both cardiac (Casq2) and skeletal muscle (Casq1) isoforms cause different, and sometime severe, human pathologies. *Danio rerio* (zebrafish) is a powerful model for studying function and mutations of human proteins. In this work, expression, biochemical properties cellular and sub-cellular localization of *D. rerio* native Casq isoforms are investigated. By quantitative PCR, three mRNAs were detected in skeletal muscle and heart with different abundances. Three zebrafish Casqs: Casq1a, Casq1b and Casq2 were identified by mass spectrometry (Data are available via ProteomeXchange with identifier PXD002455). Skeletal and cardiac zebrafish calsequestrins share properties with mammalian Casq1 and Casq2. Skeletal Casqs were found primarily, but not exclusively, at the sarcomere Z-line level where terminal cisternae of sarcoplasmic reticulum are located.

Keywords Ca^{2+}-binding proteins · Sarcoplasmic reticulum · Animal models · *Danio rerio*

Abbreviations

B2M	Beta-2 microglobulin
casq	Zebrafish calsequestrin genes
Casq	Zebrafish calsequestrin proteins
DTT	DL-Dithiothreitol
EF1a	Elongation factor 1-alpha
NDUFS3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
MOPS	4-Morpholinepropanesulfonic acid
PMSF	Phenylmethylsulfonyl fluoride
Ryr	Ryanodine receptor
SDS	Sodium dodecyl sulfate
SERCA1	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1
SR	Sarcoplasmic reticulum

Introduction

Excitation–contraction-coupling is the mechanism that converts electrical nervous impulses into Calcium (Ca^{2+}) release and muscle contraction. Releasable Ca^{2+} is stored

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into sarcoplasmic reticulum (SR) by a heterogeneous family of Ca^{2+}-binding proteins which display peculiar properties and multiple functions.

Calsequestrin (Casq) is a high capacity low affinity Ca^{2+}-binding protein, critical for Ca^{2+}-buffering in cardiac and skeletal muscle SR. Folding of the Casq monomer and successive oligomerization are both induced by Ca^{2+} (Wang et al. 1998; Park et al. 2004). Recent studies on Ca^{2+} complexed forms of human and rabbit Casq resolved by X-ray diffraction demonstrate that Ca^{2+} binding sites of Casq have different affinities (Sanchez et al. 2012).

All mammals have multiple genes encoding for different Casq isoforms. Increasing interest has been focused on mammalian and human Casq genes since mutations of both cardiac (Casq2) and skeletal (Casq1) isoforms cause different, and sometime severe, human pathologies (Ríos et al. 2015; MacLennan and Zvaritch 2011); much less is known on expression, structure and functions of Casq genes in fish and lower vertebrates. In *Solea senegalensis* and *Fundulus heteroclitus* (Infante et al. 2011; Whittington et al. 2012) different cDNAs have been cloned and biophysical properties of a deduced synthetic protein isoform have been studied.

In *Danio rerio* (zebrafish), three Casq genes have been identified by whole genome sequencing and mapped in distinct chromosomes: *casq2* (chromosome 9), *casq1a* (chromosome 2), *casq1b* (chromosome 7). Gene expression studies (Thisse and Thisse 2004) in high-peck to long-peck stage (48–72 h) show that *casq1a* is expressed predominantly at myotomes and *casq2* in myotomes, heart, pectoral fin and head muscles; however expression of *casq* genes in muscle and heart of adult individuals, is not known.

Mammal, avian and amphibian native proteins have been isolated from skeletal and cardiac muscles and from brain and characterized (Volpe et al. 1988; Damiani et al. 1986). In mammals, Casq1 is expressed in skeletal muscle, whereas Casq2 is expressed in cardiac, slow-twitch skeletal muscles and, at early stages of development, in fast-twitch skeletal muscles. Both Casqs are also expressed, although in low amount and at variable rates, in smooth muscle cells (Volpe et al. 1994). Association between expression of SR protein isoforms, including Casq, and fiber type has been studied in single fibers of rat and human skeletal muscle (Murphy et al. 2009; Lamboley et al. 2013; Klitgaard et al. 1989). These studies support the notion that specific functional properties of either fast- or slow-twitch fibers are accomplished by interplay between myosin isoforms and SR composition. Up to now in fish (*Cyprinus carpio)*, only one Casq-like protein, which exhibits biochemical properties similar to those of rabbit Casq, has been purified (Watabe et al. 1991). Zebrafish has become a powerful model for human heart and skeletal muscle diseases (see for example Sarparanta et al. 2012; Berger and Currie 2012; Asnani and Peterson 2014; Søndergaard et al. 2014) because the zebrafish model presents many methodological advantages and extensive collections of useful mutants. An important step to validate this model as a tool for studying function and mutations of human proteins, is evaluation of orthologue gene expression, functions and mutations. In adult *D. rerio* the distribution of slow- and fast-twitch fibers in axial muscles appears very clear in relation to expression of fast- and slow-twitch markers. In larvae and adult stages three layers of fibers have been identified (Devoto et al. 1996); a superficial layer of slow-twitch fibers surrounds an intermediate group of fibers and a deep layer of fast-twitch fibers. Expression of native Casq isoforms, their cellular and sub-cellular localization in different fiber types of *Danio rerio* and respective biochemical properties are unknown. Comparison of predicted zebrafish Casq genes and proteins with their mammalian counterparts shows significant similarity in the exon number, protein sequence and protein domains. In the present paper, we study *casq* mRNA, protein expression and localization in adult heart and skeletal muscle of *D. rerio* in order to improve the knowledge on the Ca^{2+} handling proteins of fish SR.

Results and discussion

Expression of calsequestrin genes and proteins in zebrafish skeletal muscles and heart

Information on *casqs* transcripts and proteins of *D. rerio* were collected from three databases: GENE (NCBI National Center for Biotechnology Information, US), ZFIN (The zebrafish Model Organism Database) and UniProt. Comparison of sequenced genes and protein predictions shows ambiguous results. In particular, the *casq1a* gene generates two transcripts (short and long); moreover several hypothetical proteins have been deposited at UNIPROT database raising the question as to how many mRNA are transcribed and which protein isoforms are effectively translated.

In order to quantify Casq mRNAs in skeletal and cardiac muscle, four primer sets, suitable for qPCR, were designed: Z_casq1a L + S specific for mRNA transcribed for *casq1a* short and long isoforms; Z_casq1b specific for *casq1b* and Z_casq2 specific for *casq2* gene and a primer set which maps in the region of casq1a specific for the long form only (Z_casq1a FL). Primer sequences are reported in "Methods" section. Expression level of mRNA identified by both couples of primers Z_casq1a L + S and Z_casq1a FL is identical indicating that in adult zebrafish the short casq1a derived transcript is either absent or present in trace amounts only (not shown), under control conditions.

As shown in Fig. 1a, in skeletal muscle, amplification of Casq1a, Casq1b and Casq2 mRNA was obtained; in heart, Casq1a and Casq1b mRNA were detected in small amounts whereas Casq2 mRNA was the main isoform. The comparison between skeletal and cardiac muscles shows a preferential expression of Casq1 genes in skeletal muscle and of Casq2 gene in heart. Moreover, these data suggest that multiple Casq proteins should be expressed in skeletal muscles and heart.

Comparison of deduced amino-acid sequences of zebrafish Casqs with those of mammals shows a significant homology level (*Danio rerio* Casq1a vs. *homo sapiens* Casq1, 66 % identity; *Danio rerio* Casq2 vs. *Homo sapiens* Casq2, 70 % identity). In the deduced amino-acid sequences of mature zebrafish Casqs, 50, 34 and 30 % of residues are acidic (for Casq1a, Casq1b and Casq2, respectively). For these proteins an aberrant mobility in SDS page at alkaline pH is expected. Moreover, zebrafish Casq2 sequence displays one putative N-glycosylation site that can account for an apparent molecular weight higher than the theoretical molecular weight.

In order to identify Casq isoforms in zebrafish, a panel of three antibodies was tested on total protein extracts of skeletal muscle and heart (See "Methods" section for antibody origin). In western blot of muscle total protein extracts, anti-canine cardiac Casq polyclonal antibodies PA1-913 ABR (CC) recognized several bands: a doublet with apparent MW of 83 kDa and two single bands (74, 58 kDa) (Fig. 1b). A second anti-rabbit Casq1 polyclonal antibody (Nori et al. 2006) produced in chicken (RS) showed higher affinity for the 83 kDa doublet and lower affinity for the 58 kDa protein, as compared to CC. The 74 kDa protein was similarly recognized by both antibodies. The anti-peptide polyclonal antibody (Sigma C3868, MC) almost exclusively recognized the 83 kDa protein. Such three bands are enriched in a muscle microsomal fraction (microsomes, b) compared to total homogenate. The apparent MW of proteins was calculated relative to

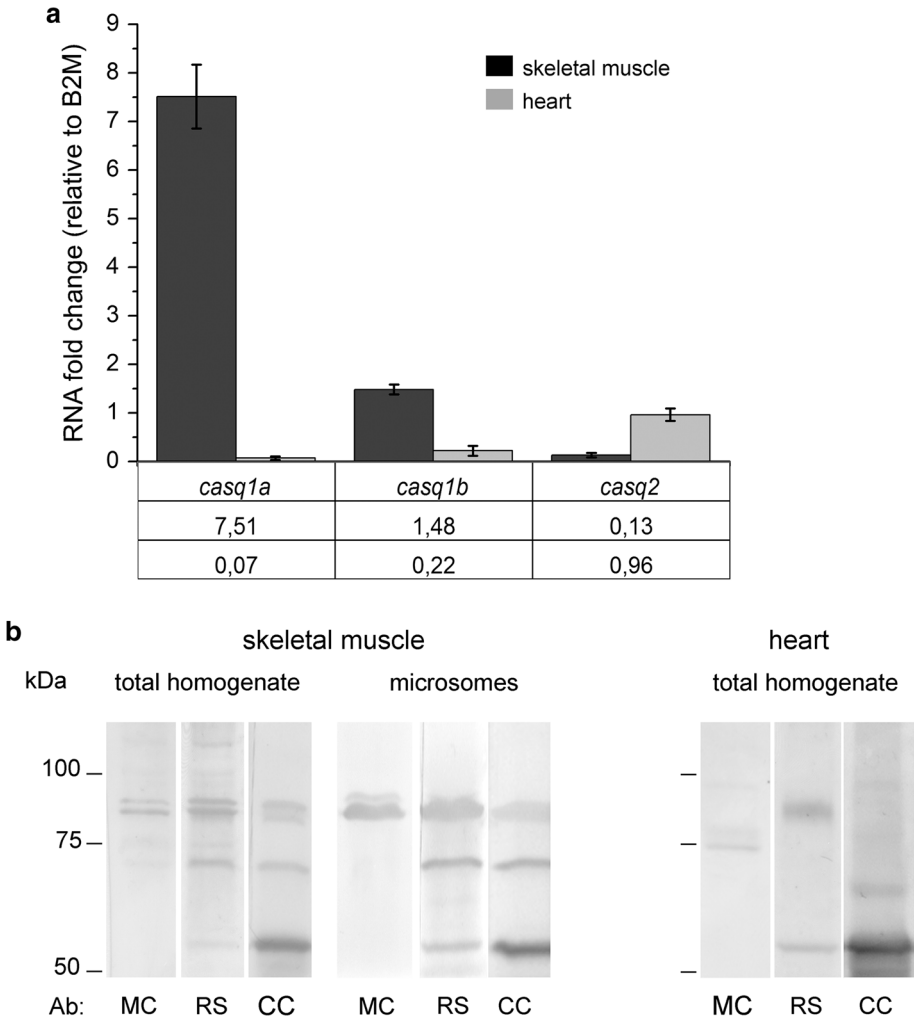

Fig. 1 mRNA analysis and immunodetection of calsequestrins in adult zebrafish skeletal muscle and heart. **a** Relative expression ratio of calsequestrins in skeletal and cardiac tissues. Values are expressed as mean (n = 2) ± SEM (*error bars*) mRNA levels normalized to B2M by the ΔCt method. **b** Representative Western blotting on total homogenates and microsomes from skeletal muscle and heart: immunodetection was carried out with a *panel* of anti-calsequestrin antibodies, as detailed in "Methods" section. Experiments were performed at least three times. Molecular mass markers: 250, 150, 100, 75, 37, 25 and 20 kDa

that of molecular weight markers, as described in "Methods" section. Total protein extract obtained from heart was probed with the same panel of antibodies (b, heart). The three antibodies recognized heterogeneous patterns except for a 58 kDa band which exactly co-migrated with the skeletal muscle protein. By correlating the immunoreactivity of anti Casq antibodies with mRNA expression, it would appear that proteins with MW compatible with the product of *casq1a, casq1b* and *casq2* genes are expressed in total protein extracts of either skeletal muscle or heart. Co-migration of the 58 kDa proteins of muscle and heart strongly indicate that such proteins are identical.

Whole-genome duplication is the basis of teleost radiation. Subsequent gene loss induced average duplicate retention rate across all genes of about 24 % (Braasch and Postlethwait 2012). One copy of duplicated genes is either not functional, acquires a new function, retains the original ancestral function or retains part of the original function (Babin et al. 2014). Our results demonstrate that *casq1a* and *casq1b* are both transcribed although at different rates in adult skeletal muscle of zebrafish, similarly to *S. senegalensis* (Infante et al. 2011). This finding suggests that the respective proteins, if expressed, could execute different functions. In heart, *Casq2* is prevalent, as reported in *S. senegalensis*. The cross-reactivity of zebrafish protein extracts with anti Casq antibodies indicates that a number of compatible proteins are expressed in skeletal muscle and heart.

Zebrafish calsequestrins co-fractionate with sarcoplasmic reticulum markers

In order to investigate the subcellular distribution of zebrafish Casqs, skeletal muscle was homogenized and fractionated into contractile, mitochondrial, cytosolic and microsomal fractions by differential centrifugation. After high-speed centrifugation of skeletal muscle homogenate, the supernatant contains almost exclusively cytosolic components, whereas the high-speed pellet contains microsomes including sarcoplasmic reticulum vesicles.

Ponceau staining of different sub-fractions (Fig. 2a) shows that protein composition of S4 (soluble fraction) and P4 (membrane fraction) is different: there are proteins enriched in P4 in comparison to S4 (molecular weight around 100, 50 and 25 kDa); other proteins are enriched in S4 (molecular weight around 37 kDa). P4 and P3 (membrane fractions) share a common electrophoretic pattern with exception of a 100 kDa protein that is enriched in P4. Fractions obtained by differential centrifugation were analyzed by western blotting using antibodies to either SERCA1 (for microsomes), α-actin (for myofibrils), GAPDH (for cytosol), NDUFS3 and TOM20 (for mitochondria), (c). In P4, SERCA1 is significantly enriched, as expected, whereas TOM20 and NDUFS3 are equally distributed in P3 and P4; moreover GAPDH is almost exclusively associated to S4. α-actin is mostly associated to P1-2. The overall distribution of these markers helps to identify two fractions: one is enriched in sarcoplasmic reticulum vesicles (P4) and depleted of myofibrillar proteins, the other fraction (S4) is enriched in cytoplasmic proteins. Mitochondria markers are partitioned almost equally in P3 and P4.

In order to ascertain whether proteins identified by anti Casq antibodies co-purify with the SR enriched fraction, as should be expected for Casq, Stains-All staining and immunodetection of individual sub-fractions were performed (d, e, f). The 83 kDa doublet is enriched in P4 as compared to P3 and homogenate, in parallel to SERCA1 staining, and is absent in S4, as shown in b. 74 and 58 kDa proteins (f) are also enriched in P4 and are absent in S4. In conclusion, the muscle fractionation analysis demonstrates that all proteins recognized by anti Casq antibodies belong to a microsomal fraction enriched in SR markers and are excluded from the cytoplasmic fraction.

A valuable tool for the identification of Ca^{2+} binding proteins is Stains-All staining (Campbell et al. 1983) of proteins separated on polyacrylamide gels. Different absorption spectra of the dye are expected for different proteins (Green et al. 1973). In general, metachromatic behavior of the dye indicates interaction with Ca^{2+} binding sites, sialoglycoproteins and phosphoproteins. Figure 2b shows the pattern of blue stained proteins in muscle sub-fractions. A group of blue stained proteins are distributed in muscle sub-fractions (b, e) in a heterogeneous fashion. The prominent blue protein running between 100 and 75 kDa markers is highly enriched in P4 fraction and almost absent in S4 whereas another faint blue band of lower MW is also visible both in P3 and P4 (arrow in e). Immunological analysis of the same muscle sub-fractions by MC and CC antibodies (d, f) shows that the 83, 74 and 58 kDa proteins are also enriched in P4 sub-fraction. Taken together these results strongly indicate that proteins enriched in P4 fraction are the same proteins recognized by anti-Casq antibodies. Based on compatibility between apparent molecular weight and length of deduced Casq1a mRNA product, intense blue staining, immunological identification in skeletal muscle and absence in heart protein extracts, our tentative conclusion is that the 83 kDa protein is a zebrafish skeletal muscle Casq, likely encoded by the *casq1a* gene.

The 74 kDa protein recognized in skeletal muscle extracts by two polyclonal antibodies and virtually absent in heart (Fig. 1b) which is enriched in P4 and stains blue with Stains-All (Fig. 2e arrow), is the second isoform of Casq specific for skeletal muscle. Due to the low quantity of 74 kDa blue stained protein and of *casq1b* mRNA, it

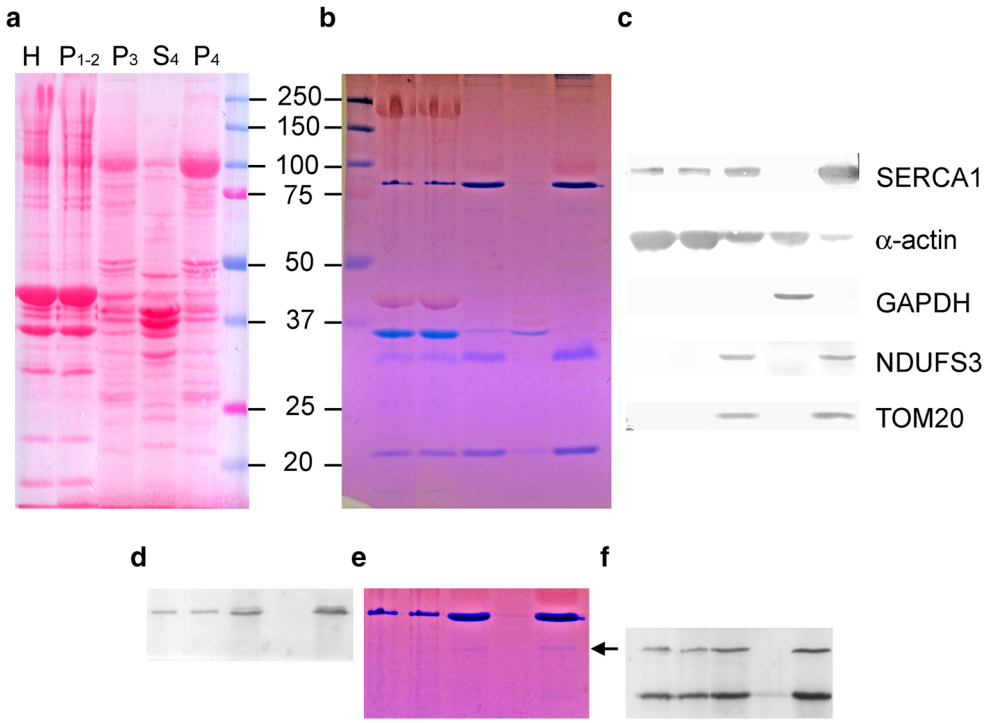

Fig. 2 Characterization of skeletal muscle sub-fractions obtained by differential centrifugation. Sub cellular fractionation of skeletal muscle was carried out as described in "Methods" section. Ponceau red staining (**a**), Stains-All staining (**b**, **e**) and Western blotting with specified antibodies (**c**), MC antibody (**d**) and CC antibody (**f**) of the sub fractions. In **e** the region from 100 to 50 kDa is shown. Key to *lanes*: H, total homogenate; P1-2, 830×*g* pellet; P3, 9000×*g* pellet; S4, 30,000×*g* supernatant; P4, 30,000×*g* pellet; molecular mass markers as in Fig. 1. (Color figure online)

could be supposed that the 74 kDa protein is the product of *Casq1b* gene (see "Identification of Calsequestrins in zebrafish by mass spectrometry"). The 58 kDa protein recognized by CC antibody in f is not visible in e (Stain's All staining). This is likely due to either the low quantity of the specific protein in the sample (under the detection level in e) and/or a different content of dye interaction sites in the protein.

Zebrafish calsquestrins change conformation in the presence of calcium

Two approaches were used to demonstrate that zebrafish Casqs share Ca^{2+}-related properties with mammalian Casqs, i.e., change of electrophoretic mobility in presence of Ca^{2+} and Ca^{2+}-induced elution of Casqs from phenyl-Sepharose. Two-dimensional electrophoresis of muscle microsomal fraction shows that two proteins (83 and 74 kDa) run faster in presence of Ca^{2+} (second dimension) than in absence of Ca^{2+} (first dimension) and stain blue with Stains-All at variance with the majority of other proteins (arrow and arrowhead in Fig. 3c).

In a matched gel, the three spots were recognized by CC antibody, two of them (arrow and arrowhead d) ran out of the protein diagonal whereas the third did not change its mobility in presence of Ca^{2+} (asterisk in d) and overlapped with a 50 kDa marker. Mobility shift in presence of Ca^{2+} indicates modification of protein conformation and acquisition of a more compact structure. The shift displayed by the 74 kDa protein is more pronounced than that of the 83 kDa protein suggesting that the Ca^{2+} induced conformation change is more profound in the 74 kDa protein. This observation agrees with the COOH-terminal of Casq1a being far more extended in comparison to that of Casq1b. Moreover, X-ray resolution of the human and rabbit Casq tetramer linked to Ca^{2+} (Kumar et al. 2013) and COOH-terminal biophysical studies (Bal et al. 2015) show that Casq monomer folding is not influenced by the COOH-terminal. Kumar et al. (2013) also found that eight high affinity Ca^{2+} binding sites and three intermediate affinity Ca^{2+} binding sites are responsible for Casq monomer compact structure. We performed Blast alignment of zebrafish skeletal muscle isoforms with human Casq1 and found that Casq1a predicted isoform displays only 7 conserved high affinity Ca^{2+} binding sites whereas Casq1b maintains 8 sites, thus suggesting a possible more profound structural change, in the presence of Ca^{2+}, and confirming the hypothesis that the 83 kDa protein is the product of the Casq1a gene. Total protein extracts from heart behave differently (e–g): in fact, no spot was

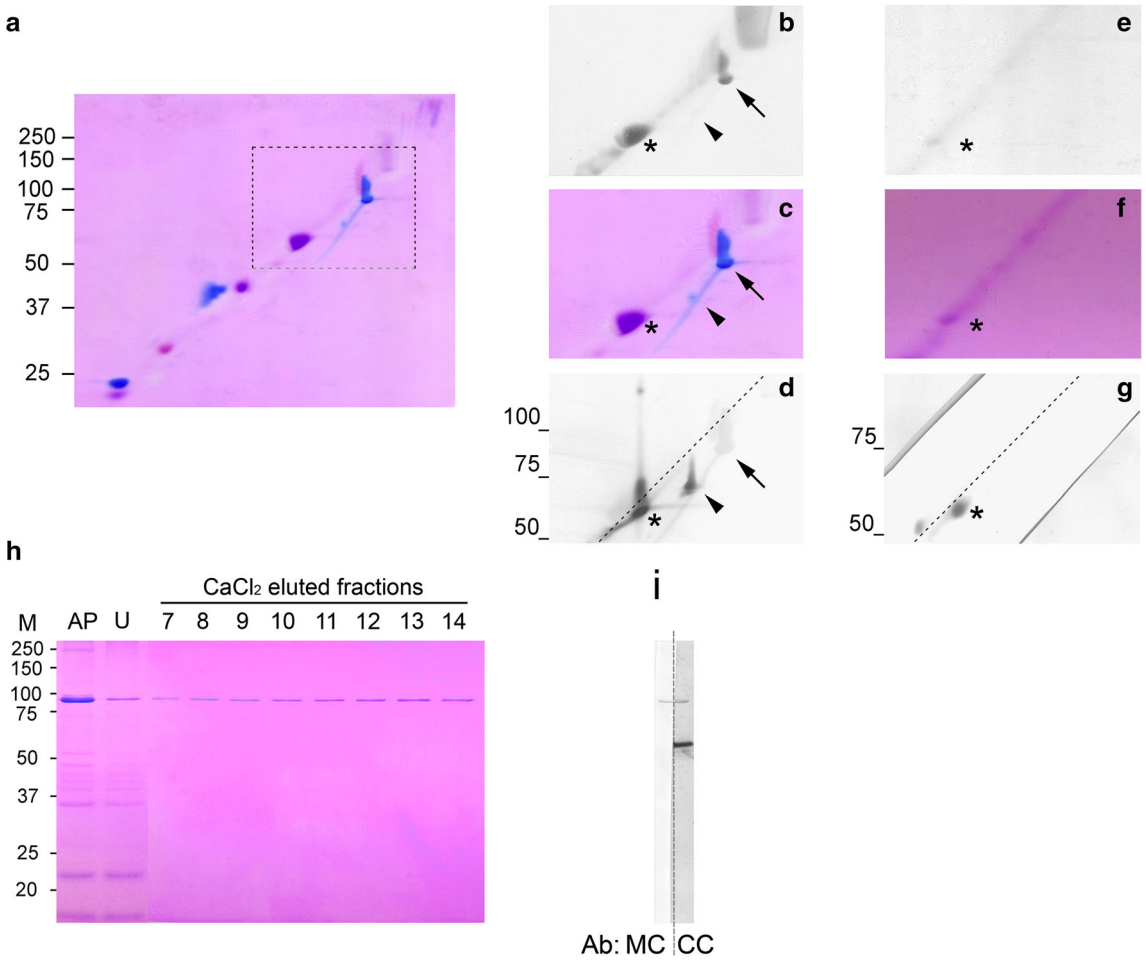

Fig. 3 Calcium dependent properties of zebrafish calsequestrins. Muscle microsomal membrane fraction (**a**–**d**) and heart total protein extract (**e**–**g**) were subjected to two-dimensional electrophoresis (SDS-gel containing 0.1 mM EGTA in the first dimension, 1 mM $CaCl_2$ in the second dimension). **a** Shows a complete gel while **b**–**g** a detail of the gel ranging from 150 to about 50 kDa. **a**, **c**, **f** Stains-All staining, **b**, **e** Ponceau red staining, **d**, **g** immunoblot with CC antibody. 83 kDa protein is indicated by *solid arrow*, 74 kDa protein by *arrowhead*, 58 kDa protein by *asterisk*. **h**, **i** purification of adult zebrafish skeletal muscle Casqs by Ca^{2+}-dependent elution from phenyl-sepharose. **h** Stains-All staining, **i** immunoblot with MC and CC antibodies. Key to *lanes*: *AP* acidic pellet, *U* unbound fraction, 7–14, Ca^{2+}-eluted sub-fractions, *M* molecular mass markers. (Color figure online)

detectable out of the protein diagonal in the Stains-All staining (f). Nevertheless the antibody CC identified one main spot (asterisk, g) running, in presence of Ca^{2+}, on the protein diagonal and staining purple with Stains-All (asterisk, f), thus indicating that Ca^{2+} does not influence significantly its migration. Considering that different mobility of vertebrate skeletal muscle Casqs, in presence and absence of Ca^{2+}, has been demonstrated (Damiani and Margreth 1991), we conclude that zebrafish 83 and 74 kDa proteins behave similarly to mammalian skeletal Casq1 and zebrafish 58 kDa protein behaves like a mammalian cardiac Casq2.

The second approach demonstrating that zebrafish Casqs change conformation in the presence of Ca^{2+} and consequently are real Ca^{2+} binding proteins, was their Ca^{2+}-dependent elution profiles from a phenyl-Sepharose column. Acidic proteins were enriched in the fraction obtained by an extraction procedure from whole tissue, followed by phenyl-Sepharose chromatography, that was originally developed for purification of skeletal and cardiac Casqs. (Slupsky et al. 1987; Cala and Jones 1983). In Fig. 3h is shown the protein composition of the final acidic pellet (AP) obtained by this procedure. A blue stained band migrating between 100 and 75 kDa is clearly visible and enriched in AP. After loading onto phenyl-Sepharose column, the blue band was significantly depleted from the unbound fraction (U). The 83 kDa blue protein was efficiently recovered in fractions 7 through 14. The 83 and 58 kDa proteins were identified in all fractions by two anti Casq antibodies (one representative fraction is shown in Fig. 3i). To compare directly the reactivity of CC with MC antibodies, one nitrocellulose lane containing 50 µl of

fraction 11 was cut longitudinally and the two pieces were developed separately by CC and MC antibodies. As shown in i in the Ca^{2+} eluted fraction were identified two proteins of 83 and 58 kDa. Densitometric analysis of all the different fractions also showed that peak elution of the 58 kDa protein came earlier than that of the 83 kDa protein (data not shown). We were not able to identify the 74 kDa isoform both in h and i, due to the low amount of the protein and low affinity of the antibodies.

Identification of calsequestrins in zebrafish by mass spectrometry

Identification of the 83, 74 and 54 kDa proteins as calsequestrins was further pursued by two experiments of mass spectrometry performed on Ca^{2+}-eluted fractions (fractions 11 and 12 in Fig. 3h). In the first experiment, fraction 11 was loaded on a polyacrylamide gel (as specified in "Methods" section) and all the proteins running between 100 and 50 kDa were subjected to Endoproteinase Lys-C in gel digestion. In the second experiment, the gel area ranging between 80 and 90 kDa containing the prominent protein band was isolated from the gel and processed separately from the lower MW (80–28 kDa) migrating proteins. Both analyses identified peptides belonging to Casq1a, Casq1b and Casq2. Figure 4 shows the sequence alignment of the predicted Casqs isoforms. The peptides identified by mass spectrometry covered the protein sequences highlighted in color. A list of the identified peptides is presented in Online Resources Table 1. The spectra of all identified peptides are shown in Online Resources 1a, b and c. Ten peptides spanning the region between aminoacids 23-255 in the sequence of Casq1a, three peptides scattered on Casq1b sequence and six peptides between aminoacids 23-202 of Casq2 were detected and quantified. These results confirm the presence of Casq1a, Cas1b and Casq2 in both phenyl-Sepharose-eluted fractions. From the first experiment the amount of Casq1b in the sample was very limited and its presence revealed by only one peptide. Despite the very low abundance in the sample, this protein was detected with very high accuracy (0.2 ppm, q value = 0.012) increasing the confidence of positive identification of the isoform. Casq1a and Casq2 were the most abundant proteins in the preparation, based on total protein intensity. Normalizing the intensity of the two proteins to the respective number of identified peptides, we estimate that Casq1a is approximately 10 times more abundant than Casq2 in this preparation. In the second experiment, quantitative peptide analysis from the two slices showed that in the higher part of the gel 86 % of the protein content was Casq1a, whereas Casq1b was absent and Casq2 was present in trace amounts (below 0.01 % of total). In the lower part of the gel, 10 % of the total protein was Casq2 while Casq1b was ranked in the lower 50 % of the fraction based on three peptides (one peptide was already identified in the first experiment). By this approach, all three calsequestrins were unambiguously identified by two criteria: the first one is the identification of ten unique peptides from Casq1a, three unique peptides from Casq1b and six unique peptides from Casq2, showing that the phenyl-Sepharose fractions contain the products of *Casq1a*, *Casq1b* and *Casq2* genes. The second criterion is quantitative: the 83 kDa protein was the most abundant in the phenyl-Sepharose fractions, as shown by Stain's All staining, (see Figs. 2b, e; 3a, c, h), accordingly, the most abundant protein of the preparative gel for mass spectrometry was Casq1a whereas Casq1b was undetectable. Thus, it appears that the 83 kDa protein is Casq1a.

The calculated molecular mass of zebrafish Casqs (64, 52 and 47 kDa for Casq1a, Casq1b and Casq2 respectively) seems discrepant with the apparent molecular weight on SDS-PAGE (83, 74, 58 kDa) but, conversely, it is a common feature of acidic Ca^{2+} binding proteins, which often migrate anomalously in Laemmli SDS-PAGE.

Localization of zebrafish calsequestrins in skeletal muscle

Longitudinal sections of zebrafish fast-twitch muscle were co-stained using Casq antibodies, α-actinin antibodies or Ryr antibodies, the latter two marking position of Z-lines and SR terminal cisternae, respectively. As shown in Fig. 5, the green fluorescence (indicating Casq) is organized in a regular cross striation pattern as expected for general SR proteins and for Casqs. Cross striations obtained with CC antibody are located mainly at the Z-line level but never co-localize perfectly with α-actinin (see for example inset of c). Perinuclear and sub-sarcolemmal areas were negative (not shown). The other antibodies (RS and MC), in addition to the Z-line overlapping striations, detected also a clear cross striation between Z-lines at the M band level (green striations in merge f, i). Cross striation at the M band was absent in rat soleus (Nori et al. 2006) and in mouse EDL and soleus muscles longitudinal sections stained with RS antibody. Since Casq expression at the level of M band is not documented in literature, the immunofluorescence experiment was repeated with different saturation buffers with similar results (not shown). Moreover, signal at the M band level was abolished when primary antibodies (RS or MC) were removed from the incubation buffer, strongly supporting the specificity of the fluorescence at M band level. These data suggest that further studies on localization of specific Casqs isoforms in zebrafish fast-twitch muscle could reveal new peculiar features of zebrafish SR. In order to locate terminal cisternae in longitudinal sections, a double-immunofluorescence was

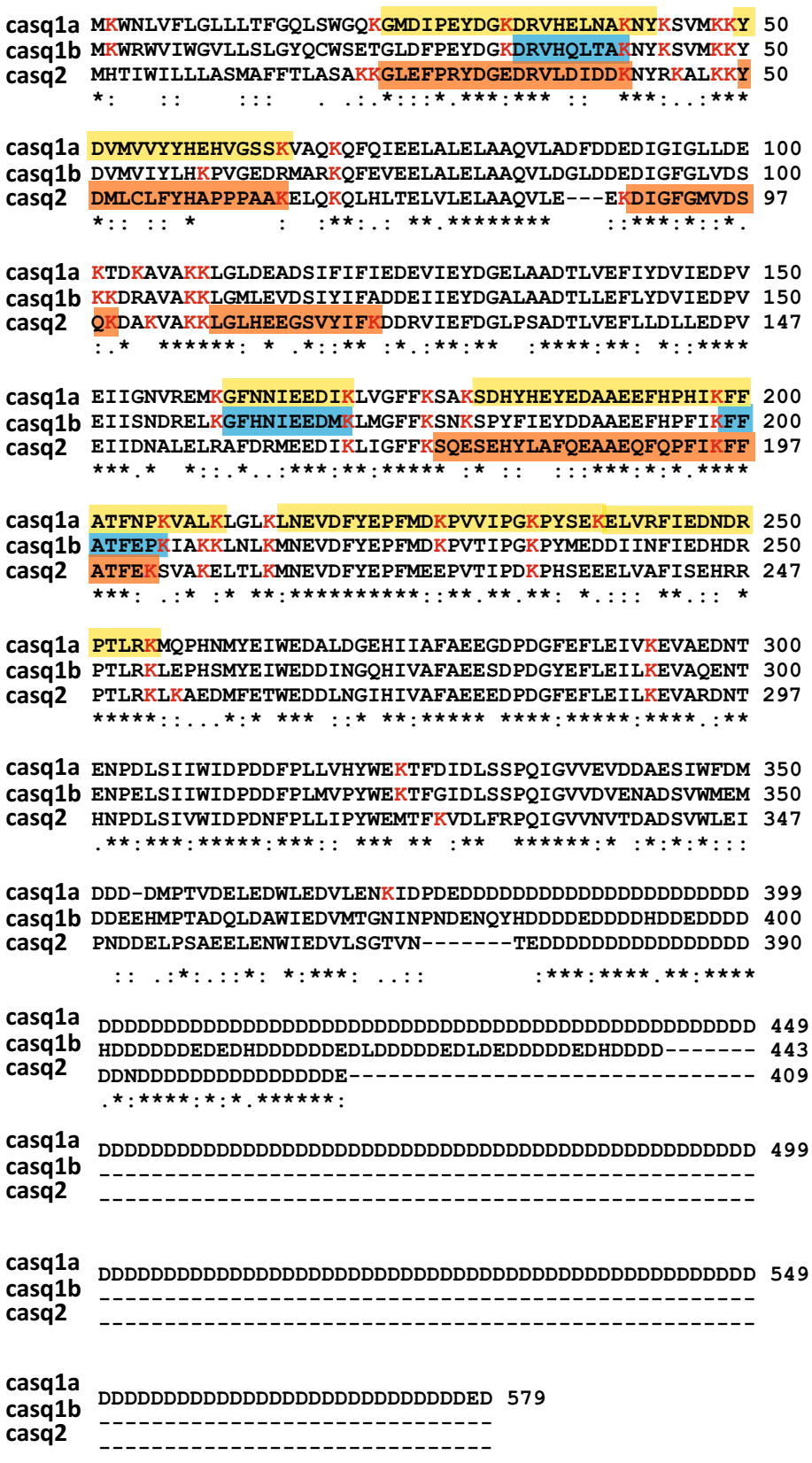

Fig. 4 Calsequestrin peptides identified by mass spectrometry analysis of zebrafish skeletal muscle. *Colored boxes* along the calsequestrin amino acid sequences (precursors) delimit the overall peptide coverage of Casq1a, Casq1b and Casq2 isoforms obtained by two mass spectrometry experiments. LysC cleavage sites are also indicated (*red*). (Color figure online)

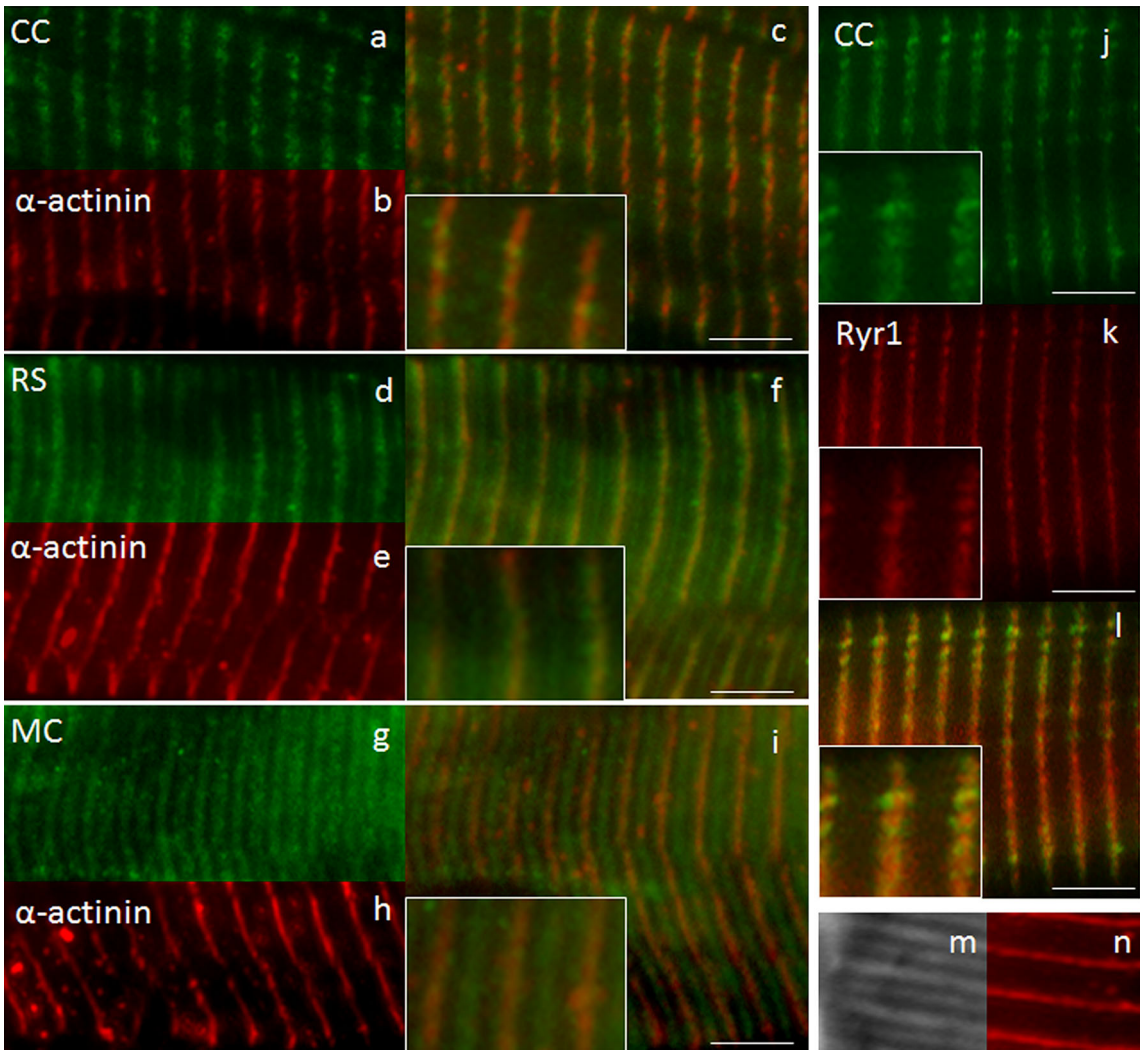

Fig. 5 Immunodetection of zebrafish calsequestrins in longitudinal skeletal muscle sections. Representative images obtained by immunofluorescence with antibodies against Casq *green* (**a**, **d**, **g**) and α-actinin *red* (b, e, h), marking the position of Z-lines. **a**, **d**, **g** Belong to the *upper part* of merge images (**c**, **f**, **i** respectively). **b**, **e**, **h** Belong to the *lower part* of merge images (**c**, **f**, **i** respectively). *Inset* magnification ×2 of merge pictures. **j**, **k**, **l** Show an immunocolocalization of Ryr and Casq while **m** and **n** show the alignment of α-actinin (**n**) with the Z-line (*light grey thick lane*) in a phase contrast image (**m**). *Bar* 5.0 µm. (Color figure online)

performed with anti Casq and anti Ryr antibodies (j–l). As expected, anti-Ryr antibodies stained zebrafish muscle (red) with a cross striation pattern similar to that observed by confocal analysis (Caprara et al. 2014) and showed good co-localization with anti-Casq antibodies (green, l). Taken together these results show that zebrafish Casqs are localized at the level of Z line where triads have been identified by electron microscopy (Perni et al. 2015; Raeker et al. 2014) and by co-localization with Ryr channel, as shown here. At the M band level, where fenestrated sarcoplasmic and endoplasmic reticulum have been described in mammals, frog and fish (Hayashi et al. 1987; Franzini-Armstrong et al. 1986; Ogata and Yamasaki 1987), the immunofluoresence experiment suggests that a Casq isoform is concentrated in the SR.

Whether zebrafish Casq isoforms are differently compartmentalized into either ER-SR or terminal cisternae is not revealed by these experiments due to the lack of antibodies specific for each isoform. We speculate that since MC and RS antibodies are more specific in Western Blot for the skeletal muscle Casqs, they could preferentially localize the skeletal muscle isoforms also in immunofluorescence, whereas CC antibodies could preferentially localize the cardiac isoform. If the previous speculations were true, the fluorescence patterns could suggest that cardiac Casq is more strictly anchored to terminal cisternae and the skeletal isoforms are more freely distributed between the junctional and longitudinal SR. It is interesting to note that Suzuki et al. (2004) found that Ca^{2+} during relaxation is accumulated in fenestrate SR of scorpion fish

muscle suggesting that a calcium binding protein located in this SR compartment might be involved in muscle relaxation. The present finding seems to confirm such a hypothesis.

In conclusion this paper provides the first identification of native zebrafish calsequestrins. Three isoforms are expressed in skeletal muscle and heart. Biochemical properties and localization of zebrafish calsequestrins in skeletal muscle are similar to mammal's orthologues.

These findings are the first step for validation of zebrafish as a model organism to examine skeletal and cardiac calsequestrin-linked diseases.

Methods

Tissue sources

Experiments were carried out on adult zebrafish (3–4 months old). Experimental protocols have been approved by University of Padua Review Boards.

Sequence analysis

Zebrafish DNA sequences encoding calsequestrin members were retrieved from GenBank database. For the identification of zebrafish calsequestrin isoforms a comparison was performed in GenBank, ZFIN and UniProt databank data.

Quantitative PCR (qPCR)

Total mRNA was obtained from skeletal muscle and heart of adult fish, using TRIzol® extraction method. 400 ng of mRNA were converted to cDNA by random hexamers and SuperScript® VILO™ (Invitrogen), following the manufacturer's instructions. Reverse transcription was performed in a thermal cycler: 25 °C for 10 min, 42 °C for 90 min, 85 °C for 5 min. Specific primers for qPCR were either designed using Primer3 software (http://bioinfo.ut.ee/primer3/) or already published (*) (McCurley and Callard 2008) and their thermodynamic specificity was determined using vector NTI® software (Invitrogen) and BLAST sequence alignment (NCBI). qPCR was performed as previously described (Salanova et al. 2011). β-actin, elongation factor 1-α (EF1a) and β-2 microglobulin (B2M) genes were considered and tested as candidate reference genes being the latter the most stable to normalize Ct values. Normalization was performed by ΔCt method. Values are expressed as mean (n = 2) ± SEM (error bars).

Primers sequences were as follows:

β-actin, forward primer 5′-GAGCAGGAGATGGGAACC-3′, reverse primer 5′-CAACGGAAACGCTCATTGC-3′; B2 M, forward primer 5′-GGAAAGTCTCCACTCCGAAA-3′, reverse primer (McCurley and Callard 2008) 5′-GCAGATCAGGGTGTTTGGTT-3′; EF1α, forward primer 5′-TCTACAAATGCGGTGGAATCG-3′, reverse primer 5′-GAGCAATGTCAATGGTGATACC-3′; Casq1a_L + S forward primer 5′-GTGGGCTTCTTCAAGAGTGC-3′, reverse primer 5′-ACGACGGGTTTATCCATGAA-3′; Casq1a_FL forward primer 5′-CCTGGGAAACCCTACAGTGA-3′, reverse primer 5′-ATCACCCTCCTCAGCAAATG-3′; Casq1b forward primer 5′-TGATCCCGACGACTTTCCAC-3′, reverse primer 5′-CACTGTCCGCATTTTCGACG-3′; Casq2 forward primer 5′-AGGAGGACCCTGATGGCTTT-3′, reverse primer 5′-GGTCGATCCACACAATGCTC-3′

Homogenates

Whole homogenates were prepared as previously described (Salvatori et al. 1997). Briefly, tissues were homogenized with a Teflon pestle equipped Potter–Elvehjem Tissue grinder in the presence of a medium containing 3 % (wt/vol) SDS, 0.1 mM EGTA, pH 7.0. Homogenates were then boiled for 5 min and clarified at 15,000 g for 10 min. Supernatants were used as whole protein extracts.

Total membrane isolation from skeletal muscle

Skeletal muscle was isolated from at least three fish and homogenized in the presence of ten volumes of homogenization buffer (0,3 M sucrose, 10 mM Hepes, pH 7.4, 100 μM PMSF, 0,5 μg/ml leupeptin, 1 mM benzamidine). The homogenate obtained was centrifuged at 830×*g* for 10 min, and the pellet re-extracted. Post-nuclear supernatants were combined and spun at 9000×*g* for 10 min to separate mitochondria. A crude membrane fraction was separated from the cytosolic fraction by centrifuging the last supernatant at 30,000×*g* for 1 h. The resulting pellet (crude microsomal membrane fraction) was resuspended in homogenization buffer. All fractions were stored at −80 °C, until use.

Partial purification of calsequestrins

Calsequestrins from skeletal muscle were isolated from 25 adult fish by selective ammonium sulfate precipitation at low pH, according to Slupsky et al. (1987) and Cala and Jones (1983), with slight modifications. Since zebrafish skeletal muscle possibly contains both skeletal and cardiac isoforms of Casq, 200 g/l of ammonium sulfate were added to the extract to fully precipitate both Casqs (Slupsky et al.

1987). After centrifugation at 13,000×*g* for 30 min, the acidic pellet was resuspended directly in phenyl-Sepharose binding buffer (50 mM MOPS, 500 mM NaCl, 1 mM DTT, pH 7.0) and then dialyzed in the same buffer overnight; the extract was then loaded onto phenyl-Sepharose resin, allowing hydrophobic proteins to interact with the resin for several hours, then the resin was washed extensively and calcium binding proteins were eluted by the same buffer containing 10 mM $CaCl_2$.

Protein analysis

Protein concentration was determined as described by Bradford (1976), using bovine serum albumin as standard. Tissue homogenates, skeletal and heart tissue fractions (50 µg/lane) and partially purified calsequestrin fractions were separated on either 7.5 or 10 % SDS-PAGE according to Laemmli (1970) and then electro blotted onto nitrocellulose membrane. In each gel, prestained protein markers (Prestained SDS-PAGE Standards, dual color; Bio-Rad) were also loaded. To check the transfer of the proteins the nitrocellulose membrane was analyzed by Red Ponceau staining. Destained membranes were blocked with 10 % (v/v) skimmed milk in Tris-buffered saline for 1 h and then incubated with primary antibody in Tris-buffered saline containing 2 % (v/v) skimmed milk for 2 h at 20 °C. Immunostained proteins were detected with an alkaline phosphatase-labelled secondary antibody and visualized by using the 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium system. Apparent Mr of the proteins were calculated from a graph of relative motilities versus log Mr of standard proteins.

List of tested anti-calsequestrin antibodies: anti Casq1 (rabbit) homemade in chicken (Nori et al. 2006) abbreviated RS in the text; anti Casq2 (mouse peptide 57–74, Sigma C3868) made in rabbit abbreviated MC in the text; anti Casq2 (canine, Thermo Scientific PA1-913) made in rabbit abbreviated CC in the text; other antibodies used: Serca1 (Thermo Scientific MA3-912), actin (Sigma A4700), α-actinin (Sigma A7811), GAPDH (Millipore MAB374), NDUFS3 (NADH dehydrogenase [ubiquinone] iron-sulfur protein 3) (Lifescience 459,130), Ryanodine Receptor (Affinity Bioreagents MA3-925), TOM20 (Santa Cruz Biotech SC11415).

In two-dimensional gels, 0,1 mM EGTA (1st dimension) or 1 mM $CaCl_2$ (2nd dimension) were added to the separating and stacking gel in order to ascertain whether the presence of Ca^{2+} changed the electrophoretic mobility of calsequestrins (le Maire et al. 1990; Burgess et al. 1980; Cox and Stein 1981). Seventy-five µg of muscle microsomal fraction or heart total extract were loaded on the first dimension, together with 5 µl of molecular mass markers to mark the diagonal line. After the run in the presence of EGTA, each lane containing the separated proteins was cut out and applied on top of a second gel containing 1 mM $CaCl_2$. The majority of the proteins runs along the gel diagonal, whereas calcium binding proteins (like calsequestrins) move faster in the presence of Ca^{2+}.

Peptide preparation by in-gel digestion

Purified fractions (100 µl) from phenyl-Sepharose were run on a 4–12 % Bis–Tris polyacrylamide gel (Invitrogen) under denaturing conditions. Staining was performed with colloidal blue (Invitrogen), followed by an overnight destaining. In the first mass spectrometry analysis the gel area comprising the 98 and 28 kDa MW markers was excised, whereas in the second experiment two gel slices (90–80 kDa and 80–28 kDa) were cut out and processed separately essentially as described in Shevchenko et al. (2006). We used Endoproteinase Lys-C as proteolytic enzyme, based on its potential ability to yield Casq isoform-specific peptides. The digestion was carried out overnight at 25 °C under continuous stirring. The extracted peptides were desalted on a SDB-XC matrix (purchased from 3 M) using Stop-and-go extraction tips (Rappsilber et al. 2003).

Liquid chromatography and mass spectrometry

Reverse-phase chromatography was performed on a Thermo Easy nLC 1000 system connected to a Q Exactive HF mass spectrometer (Thermo) through a nanoelectrospray ion source. Peptides were separated on a 50-cm column with an inner diameter of 75 µm packed in house with 1.9 µm C18 resin (Dr. Maisch GmbH). Peptides were eluted with a linear gradient of acetonitrile 0.1 % formic acid at a constant flow rate of 250 nl/min. The column temperature was kept at 50 °C by an oven (Sonation GmbH). Eluted peptides from the column were directly electrosprayed into the mass spectrometer. Mass spectra were acquired in a data dependent-mode to automatically switch between full scan MS and up to 15 data dependent MS/MS scans. The maximum injection time for full scans was 20 ms with a target value of 3e6 at a resolution of 60,000 at $m/z = 200$. Target values for MS/MS were set to 1e5 with a maximum injection time of 25 ms at a resolution of 15,000 at $m/z = 200$. To avoid repetitive sequencing, the dynamic exclusion of sequenced peptides was set to 20 s.

MS data analysis

The resulting MS and MS/MS spectra were analyzed using MaxQuant (version 1.5.1.2). Peak lists were searched against the UNIPROT databases for *D. rerio* (version of

February 2012) with common contaminants added. The search included carbamidomethylation of cysteines as fixed modification, and methionine oxidation and N-terminal acetylation as variable modifications. Maximum allowed mass deviation for MS peaks was set to 6 and 20 ppm for MS/MS peaks. Maximum missed cleavages were 2. The false discovery rate was determined by searching a reverse database. Maximum false discovery rates were 0.01 both on peptide and protein levels. Minimum required peptide length was 6 residues. Peptide identification was performed with an allowed initial precursor mass deviation up to 7 ppm and an allowed fragment mass deviation of 20 ppm. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaíno et al. 2014)via the PRIDE partner repository with the dataset identifier PXD002455. Bioinformatic analyses were performed with the Perseus software (version 1.5.1.2), part of the MaxQuant environment (http://www.perseus-framework.org).

Immunofluorescence of muscle cryo-sections

Adult zebrafish were deprived of head, eviscerated and then fixed, over-night in 4 % paraformaldehyde in PBS. Next morning they were washed in the same buffer, frozen and sectioned on a cryostat. The sections were rehydrated in PBS-Tw (PBS, 0.05 % Tween20), then blocked with PBS-Tw-N (PBS, 0.1 % Tween20, 5 % goat serum) for 30 min to avoid non-specific staining. Sections were then incubated in primary antibodies diluted in PBS-Tw-N for 2 h, washed 3 times in PBS-Tw for 5 min each, then blocked again in PBS-Tw-N for 5 min. Sections were then incubated for 1 h in fluorescently conjugated secondary antibody diluted in PBS-Tw-N. After washing as above, sections were mounted with ProLong Gold antifade reagent with DAPI (Life Technologies) and cover-slipped.

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