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Review

How does α-actinin-3 deficiency alter muscle function? Mechanistic insights into *ACTN3*, the 'gene for speed'

Fiona X.Z. Lee [a,b], Peter J. Houweling [c], Kathryn N. North [a,c], Kate G.R. Quinlan [b,d,*]

[a] The Institute for Neuroscience and Muscle Research, The Children's Hospital at Westmead, NSW 2145, Australia
[b] Discipline of Paediatrics and Child Health, Faculty of Medicine, The University of Sydney, NSW 2006, Australia
[c] Murdoch Childrens Research Institute, the Royal Children's Hospital, VIC 3052, Australia
[d] School of Biotechnology and Biomolecular Sciences, University of New South Wales, NSW 2052, Australia

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ABSTRACT

An estimated 1.5 billion people worldwide are deficient in the skeletal muscle protein α-actinin-3 due to homozygosity for the common *ACTN3* R577X polymorphism. α-Actinin-3 deficiency influences muscle performance in elite athletes and the general population. The sarcomeric α-actinins were originally characterised as scaffold proteins at the muscle Z-line. Through studying the *Actn3* knockout mouse and α-actinin-3 deficient humans, significant progress has been made in understanding how *ACTN3* genotype alters muscle function, leading to an appreciation of the diverse roles that α-actinins play in muscle. The α-actinins interact with a number of partner proteins, which broadly fall into three biological pathways—structural, metabolic and signalling. Differences in functioning of these pathways have been identified in α-actinin-3 deficient muscle that together contributes to altered muscle performance in mice and humans. Here we discuss new insights that have been made in understanding the molecular mechanisms that underlie the consequences of α-actinin-3 deficiency.

1. Introduction

The α-actinins belong to the spectrin protein family, which also includes dystrophin [1]. In mouse and human skeletal muscle, the two sarcomeric α-actinin isoforms, α-actinin-2 and α-actinin-3 (encoded by the genes *ACTN2* and *ACTN3* respectively), form major components of the contractile apparatus at the Z-line [1,2] where they cross-link and anchor actin filaments [3]. The two isoforms are highly conserved (80% identical and 90% similar in humans) [2] and are considered products of gene duplication [4]. They share a common domain topology, consisting of a highly conserved N-terminal actin-binding domain, a central rod domain consisting of four spectrin-like repeats, and a C-terminal EF hand region [5]. The expression of α-actinin-2 is ubiquitous in human muscle fibres, while α-actinin-3 is restricted to fast Type 2 fibres [6]. This difference in expression pattern raises the possibility that α-actinin-3 has a specific function in fast muscle fibres that is independent from the function of α-actinin-2 [4].

A common stop codon polymorphism in the *ACTN3* gene, R577X, was discovered serendipitously while searching for possible causative genes for muscular dystrophy [7,8]. Individuals who are homozygous for the 577X allele are completely deficient in α-actinin-3. Interestingly, homozygosity for the null polymorphism is very common in humans. The frequency of this null polymorphism varies between human populations, with the 577X allelic frequency being around 0.093 in African populations to 0.764 in the Americas and an average worldwide frequency of 45% [9]. Overall, it is estimated that 20% of the world population, or approximately 1.5 billion people, are *ACTN3* 577XX genotype and thus deficient in α-actinin-3 [7,9].

α-Actinin-3 deficiency does not cause muscle disease, but has been shown to alter muscle function. The consequences of the *ACTN3* R577X genotype in humans were first investigated in elite athletes. In an Australian cohort of 439 elite Caucasian athletes and 436 unrelated Caucasian controls, the frequency of the 577X allele was significantly lower in both male ($P < 0.001$) and female ($P < 0.01$) sprint athletes while the frequency of 577XX genotype was higher in female endurance athletes ($P < 0.05$) [10]. The under-representation of *ACTN3* 577XX genotype in sprint and power athletes has been replicated in over 15 studies (reviewed in Eynon *et al.* [11]). A meta-analysis conducted on ten

Abbreviations: 2D, two-dimensional; ALP, actinin-associated LIM protein; ATP, adenosine triphosphate; BHAD, 3-hydroxyacyl-CoA dehydrogenase; CCO, cytochrome c oxidase; CS, citrate synthase; CSA, cross-sectional area; DHPR, dihydropyridine-receptor voltage sensor; EDL, extensor digitorum longus; EM, electron microscopy; FBP1, fructose-1, 6-bisphosphatase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPh, glycogen phosphorylase; GS, glycogen synthase; HK, hexokinase; KO, knockout; LDH, lactate dehydrogenase; LoF, loss of function; MCAD, medium chain acyl-CoA dehydrogenase; MyHC, myosin heavy chain; NADH-TR, nicotinamide adenine dinucleotide tetrazolium reductase; NFAT, nuclear factor of activated T-cell; RCAN1-4, regulator of calcineurin 1 isoform 4; RYR1, ryanodine-receptor Ca^{2+}-release channel; SDH, succinate dehydrogenase; SERCA1, sarcoplasmic reticulum calcium ATPase1; SR, sarcoplasmic reticulum; WT, wild type; ZASP, Z-band alternatively spliced PDZ motif containing protein.

* Corresponding author at: School of Biotechnology and Biomolecular Sciences, University of NSW, Kensington, NSW 2052, Australia.
E-mail address: kate.quinlan@unsw.edu.au (K.G.R. Quinlan).

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athlete cohorts also strongly confirmed this association [12]. Two studies also report a higher frequency of the 577XX genotype in endurance athletes [13,14]. Overall, the athlete studies suggest a strong association between *ACTN3* genotype and athletic performance with α-actinin-3 deficiency being detrimental for power and sprint activities and potentially beneficial for endurance sports.

The *ACTN3* R577X polymorphism has also been found to influence muscle performance in the general population. α-Actinin-3 deficient women produced significantly less elbow flexor isometric strength than women homozygous for the *ACTN3 577R* allele who express α-actinin-3 [15] and young *ACTN3* 577RR men had significantly higher knee extension strength compared to *ACTN3* 577XX men [16]. Compared to their *ACTN3* 577RR and 577RX counterparts, adolescent boys deficient in α-actinin-3 took significantly longer to complete a 40 m sprint [17] while older males were slower to complete a 400 m walk [18]. These findings are consistent with the athlete data and suggest that α-actinin-3 deficiency has a detrimental effect on power performance in the general population.

The *ACTN3* 577X allele is estimated to be over one million years old [19], however, the genomic region surrounding the allele shows low levels of genetic variation and recombination suggestive of recent positive selection [19]. The loss of α-actinin-3 appears to be beneficial in certain situations. Combined with evidence suggesting the *ACTN3* 577X allele may be beneficial for endurance [10,13,14] and the varied frequency of the allele among different ethnic groups [7,20], the *ACTN3* 577X allele has been postulated to have conferred a selective advantage for modern humans as they moved out of Africa into a Eurasian environment [9,21]. We have shown that the X-allele frequency is at its highest in places with reduced mean annual temperature and species diversity, and this increase occurs along a global latitudinal gradient [21]. This has led us to postulate that the *ACTN3* X-allele may have conferred a resistance to famine or enhanced cold tolerance [19]. There are only two known examples in the human genome of a loss of function (LoF) variant resulting in a clear selection advantage (*ACTN3* [22] and *CASP12* [23]). As such, there is significant interest in understanding how α-actinin-3 deficiency was advantageous during human evolution and why the absence of this protein alters human muscle function today.

2. Insight from the *Actn3* knockout (KO) mouse model

In order to study the normal functions of α-actinin-3 and the consequences of α-actinin-3 deficiency an *Actn3* knockout (KO) mouse was generated [19]. Both the *Actn3* KO mouse model and *ACTN3* 577XX humans express no α-actinin-3 with the closely related protein α-actinin-2 expressed across all fibre types. The expression of α-actinin-2 is also up-regulated in the *Actn3* KO mouse. The higher expression of α-actinin-2 is likely to partially compensate for the loss of α-actinin-3. Morphologically, *Actn3* KO mice are indistinguishable from their wild type (WT) littermates [24]. However, differences in muscle performance were observed compared to WT littermates. *Actn3* KO mice produce lower grip strength, a measure of muscle power, [24] but are able to run further on a motorized treadmill at increasing speeds before reaching exhaustion [19], a measure of endurance performance. These findings mirror those from the human genetic association studies—homozygosity for the 577X allele is detrimental to power activities but may be beneficial for endurance performance. As the *Actn3* KO mouse recapitulates the human phenotype it represents an ideal model in which to molecularly dissect human α-actinin-3 deficiency.

Mouse skeletal muscle is made up of four fibre types defined by the expression of myosin heavy chain (MyHC) isoforms: Type 1, 2A, 2X and 2B [25]. Type 1 fibres are the slowest but most resistant to fatigue due to their ability to use oxidative metabolism for energy production. The fast Type 2 fibres are responsible for rapid forceful contraction, fatigue readily and rely on energy generated through anaerobic metabolism, with 2B being the fastest and 2A being the slowest [26]. In mice, α-actinin-3 is expressed in 2B muscle fibres. As such, it was hypothesised that α-actinin-3 deficiency would specifically result in consequences in 2B muscle fibres and in muscles rich in 2B fibres in the mouse. *Actn3* KO mice show a reduction in muscle mass in muscles that consist predominantly of fast Type 2 fibres. When individual fibres were examined it was found that the reduction in muscle mass could be accounted for by a specific reduction in 2B fibre diameter. Both the soleus muscle (which contains very few 2B fibres), and the heart (in which α-actinin-3 is not expressed in WT mice) are unaffected by the absence of α-actinin-3 [24].

At a functional level *Actn3* KO mice display enhanced adaptive response to endurance running, which is evident after a 4 week treadmill training protocol. Trained WT mice ran twice the distance of untrained WT while *Actn3* KO mice ran 3.3 times farther than untrained *Actn3* KO mice [27]. The training protocol resulted in a more marked fibre-shift in *Actn3* KO mice with significant decrease in the proportion and total cross-sectional area (CSA) of 2B fibres and increase in 2X fibres in trained *Actn3* KO mice, compared to no significant change in the proportions or CSA of WT fibres as a result of training. In response to muscle disuse, in typical WT muscle fibres, denervation results in a shift towards a slow MyHC profile; this shift is more marked in denervated *Actn3* KO muscles. Immobilisation typically results in a shift in the MyHC profile towards a faster phenotype; this change is less striking in immobilised *Actn3* KO muscle with the MyHC profile resembling that of control *Actn3* KO muscle [28]. There is growing evidence from human association studies suggesting that *ACTN3* genotype alters response to exercise training in both athletes and individuals with disease [10,29], as well as progression of, and susceptibility to muscle disease [30] and spinal cord injury [31]. Understanding how *Actn3* genotype influences muscle adaptation to endurance training and muscle disuse in mice will no doubt improve our knowledge of human muscle adaptation.

3. α-Actinin-3 deficiency influences structural, metabolic, signalling and calcium handling pathways in skeletal muscle

So, what changes occur in muscle to cause altered muscle performance in the α-actinin-3 deficient state? As the α-actinins have been considered first and foremost ***structural*** proteins, responsible for attaching and cross-linking actin filaments and coordinating muscle contraction, contractile properties were examined in *Actn3* KO mice. Studies on isolated extensor digitorum longus (EDL) muscle revealed lower force generation in *Actn3* KO but improved resistance to contraction-induced fatigue [24]. This is consistent with a shift in the fast fibre properties of *Actn3* KO muscles towards those of slower fibres, without a change in MyHC fibre type. This shift in the KO fast fibres led to an examination of the ***metabolic*** properties of α-actinin-3 deficient muscle. Generally, fast glycolytic muscle fibres (Type 2B in mice and Type 2X in humans) where α-actinin-3 is usually expressed, rely on the anaerobic pathway to produce the ATP required for muscle contraction. In the anaerobic pathway glucose is converted to lactate, with the final step being catalysed by the enzyme lactate dehydrogenase (LDH). *Actn3* KO muscles show a lower LDH activity [19]. In slower muscle fibres (Type 1 and 2A fibres), the pyruvate from glucose is preferentially fully oxidized within the mitochondria. Activities of key enzymes in oxidative metabolic pathways including the citric acid cycle, the mitochondrial electron transport chain and fatty acid oxidation pathways were consistently higher in the *Actn3* KO muscle [19,24]. No difference in mitochondrial copy number was observed [24], suggesting that the higher activity of oxidative metabolic enzymes is a consequence of the shift towards a slower metabolic phenotype in *Actn3* KO muscle.

Fast muscle fibres also rely on a readily available source of intramuscular glycogen to supply the energy necessary for muscle contraction. A significant level of glycogen accumulation was observed in 577XX humans and *Actn3* KO mice compared to controls [32]. The key enzyme in glycogen breakdown, glycogen phosphorylase (GPh) was

significantly lower in *Actn3* KO compared to WT muscle. There was also a trend towards lower GPh activity in 577XX humans compared to 577RR and 577RX humans, but due to small numbers the data did not reach significance. The lower GPh activity is likely to reduce the ability of muscle to catabolise glycogen, leading to the accumulation of glycogen in *Actn3* KO muscles [32]. It has been hypothesised that the reduced ability of α-actinin-3 deficient muscle to catabolise glycogen may result in the compensatory shift towards oxidative metabolism seen in the *Actn3* KO mice.

The difference in structural and metabolic properties in α-actinin-3 deficient muscle is consistent with a shift towards slower muscle properties within fast muscle fibres without a shift in MyHC fibre-type. As such, muscle ***signalling*** pathways responsible for determining fibre properties were examined in *Actn3* KO muscle. Interestingly, a calsarcin-2 KO mouse model exhibits a similar phenotype to the *Actn3* KO mouse including greater endurance capacity, and a shift in fast fibre metabolism towards a slower phenotype [33]. The phenotype in the calsarcin-2 KO mouse is due to activation of calcineurin signalling, as measured by higher expression of regulator of calcineurin 1 isoform 4 (RCAN1-4) [34]. Calcineurin activity was shown to be higher in *Actn3* KO mouse and ACTN3 577XX humans compared to that of controls [27].

The presence or absence of α-actinin-3 and differences in baseline calcineurin activity have been shown to influence muscle adaptation to changing physical demands, such as exercise [27] and muscle atrophy [28]. Overall, the higher baseline calcineurin activity explains why *Actn3* KO muscle has a lower threshold for adaptation to stimuli that cause the slowing of muscle properties (endurance training and immobilisation) and a higher threshold for adaptation to stimuli that lead to faster muscle properties (denervation), compared to WT mice [27,28].

The changes in contractile properties in α-actinin-3 muscle also led to an examination of the ***calcium handling*** properties of the sarcoplasmic reticulum (SR) in the *Actn3* KO fibres. There is a higher rate of calcium release and absorption by the SR in *Actn3* KO fibres, with the fibres being more resistant to fatigue due to the slower rate of decline in calcium release following repeated muscle stimulation [35]. The higher rate of calcium release can be explained by the higher levels of the sarcoplasmic reticulum calcium ATPase1 (SERCA1), while higher levels of the calcium-binding proteins calsequestrin and sarcalumenin, along with SERCA1, facilitate higher calcium absorption by keeping intraluminal free calcium concentrations at low levels. The higher energy expenditure as a consequence of higher calcium pumping is proposed to be a key contributor in the muscle fibre metabolism shift towards the more efficient aerobic pathway. This generates greater basal heat, potentially resulting in improved cold acclimatisation in α-actinin-3 deficient humans, which would be a strong evolutionary advantage [35].

Now that we understand **what** α-actinin-3 deficiency does to muscle function it is very important to uncover the **why.** Based on our current understanding of skeletal muscle function in the *Actn3* KO and *ACTN3* 577XX humans, we will explore what is known about the complex interactions between structural, metabolic, signalling and calcium handling pathways that combine to alter muscle function in the absence of α-actinin-3.

4. Functional differences between α-actinin-2 and α-actinin-3 begin to explain the consequences of human α-actinin-3 deficiency

The differential functions of α-actinin-2 and α-actinin-3 are not well characterised, as historically studies have predominantly focused on α-actinin-2 or purified sarcomeric α-actinin, which is a mixture of α-actinin-2 and α-actinin-3. Although α-actinin-2 is able to partially compensate for the absence of α-actinin-3 (α-actinin-3 deficiency does not result in muscle disease), the different patterns of sarcomeric α-actinin expression in muscle fibre types raises the possibility that α-actinin-2 and α-actinin-3 have specialised roles. As the total sarcomeric α-actinin pool is maintained in α-actinin-3 deficient muscle by up-regulating α-actinin-2 [36], the changes in muscle function in the absence of α-actinin-3 are more likely due to the differential functions of the α-actinin(s) being expressed rather than overall amount. Therefore recent studies have focused on the unique roles of α-actinin-2 and α-actinin-3 to identify the molecular mechanisms underlying the skeletal muscle phenotypes associated with α-actinin-3 deficiency.

The sarcomeric α-actinins interact with a diverse network of proteins at the Z-line of skeletal muscles [5,37]. Mapping the interaction partners of α-actinin-2 on STRING [38] reveals a complex interaction web with the binding partners broadly falling into three biological pathways—structural, metabolic and signalling (Fig. 1). α-Actinin-2 and α-actinin-3 share high sequence homology and one would expect the interaction map for α-actinin-3 to be similar to that of α-actinin-2. However, the program could not build a map for α-actinin-3 due to paucity of known interactions. The interaction network of the sarcomeric α-actinins is highly intricate. It is therefore possible for small changes to protein interactions to have significant impact on the overall properties of α-actinin-containing complexes, both at the Z-line and throughout the sarcomere.

The functional differences between α-actinin-2 and α-actinin-3 are therefore likely to be defined by their respective abilities to interact with different proteins. We will now explore each of the three pathways from the interaction map in more detail, discussing the key proteins involved and the biological processes altered in response to α-actinin-3 deficiency.

5. Structural phenotypes associated with α-actinin-3 deficiency are attributable to changes in interactions with Z-line proteins

Differences in the contractile properties in *Actn3* KO muscles are likely attributable to altered protein structures at the Z-line. Indeed a host of Z-line proteins are up-regulated at the transcript and protein levels in α-actinin-3 deficient muscle, including the Z-band alternatively spliced PDZ motif containing protein (ZASP), myotilin, desmin and γ-filamin (Table 1) [36]. Myotilin is involved in regulating sarcomere assembly and acts in concert with the sarcomeric α-actinins to cross-link actin [39], while desmin connects the Z-lines of adjacent myofibrils to the sub-sarcolemmal cytoskeleton [40,41]. The expression pattern of both myotilin and desmin shifted from being predominately found in non-type 2B fibres in WT muscle to being localized uniformly across all muscle fibres in *Actn3* KO muscle [36]. Accumulations of myotilin and desmin and a greater number of internalised nuclei were also detected in a subset of *Actn3* KO muscles [36]. Protein accumulations and internalised nuclei are hallmarks of muscle remodelling and regeneration, suggesting that *Actn3* KO muscles are more susceptible to ongoing damage.

Interestingly, dominant missense mutations in many of the proteins up-regulated in *Actn3* KO muscle including myotilin [42], desmin [43], αβ-crystallin [44], γ-filamin [45], and ZASP [46] are associated with myofibrillar myopathy. This is an inherited myopathy characterized by the disintegration of the Z-disk leading to accumulation of myofibrillar degradation products and ectopic expression of multiple Z-line proteins [47]. In addition, mutations in many genes known to interact with sarcomeric α-actinins have been implicated in muscle disorders including nemaline myopathy, intranuclear rod myopathy, dilated cardiomyopathy and Duchenne muscular dystrophy (reviewed by Houweling et al. [48].) The up-regulation and accumulation of Z-line proteins observed in *Actn3* KO muscle may reflect greater muscle remodelling in the absence of α-actinin-3 [36].

The stability and rigidity of the sarcomeric Z-line are likely to be largely determined by protein–protein interactions. As the sarcomeric α-actinins are major components of the Z-line, differences between the two α-actinin isoforms are likely to affect their ability to bind Z-line proteins, thereby resulting in altered Z-line protein composition. As outlined above, ZASP and myotilin are up-regulated in *Actn3* KO muscle. ZASP is a small Z-line protein found in skeletal and cardiac

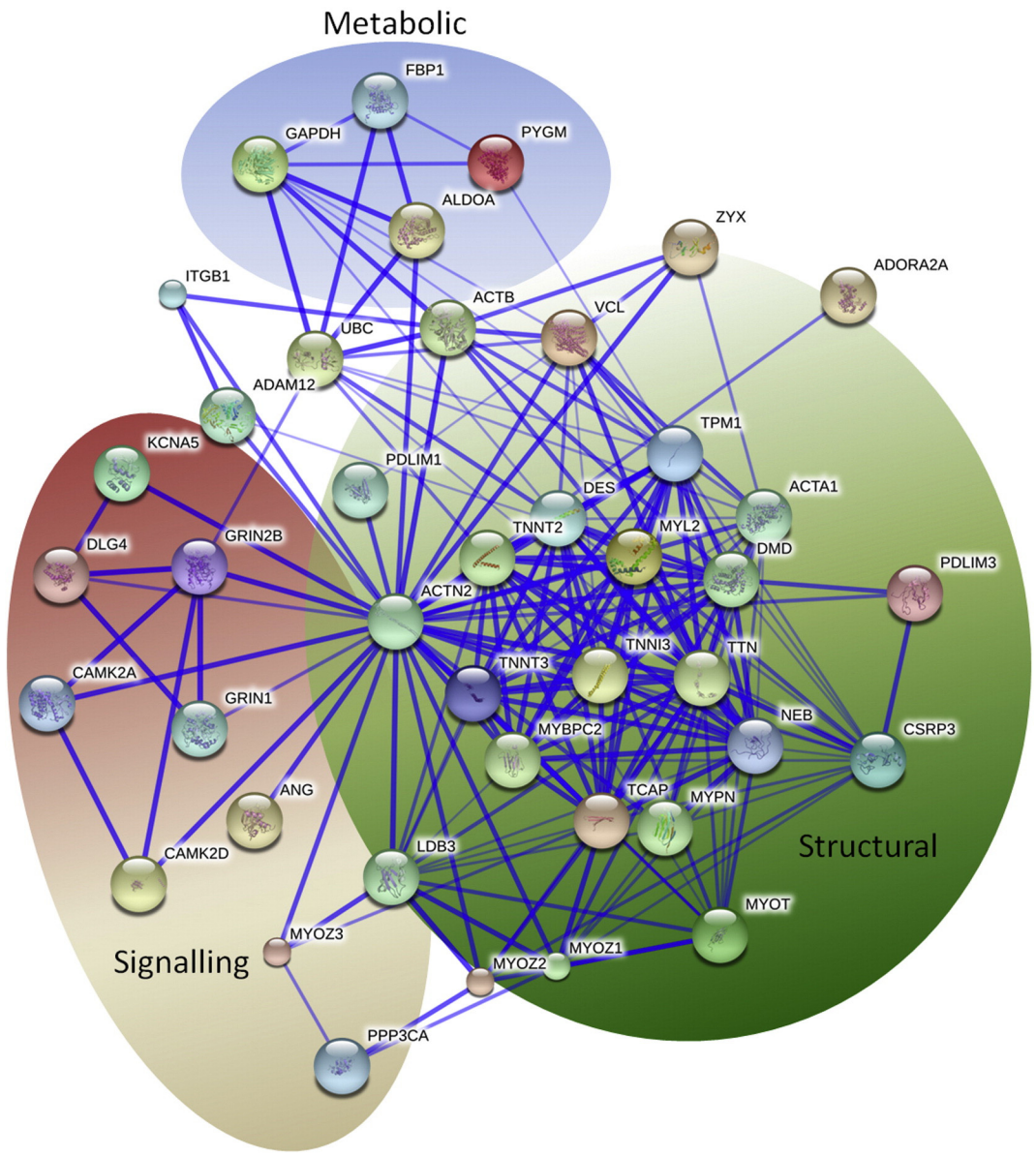

Fig. 1. Interaction map of α-actinin-2. Interaction map of human α-actinin-2 generated using STRING 9.1 [38] showing known and predicted interaction partners of α-actinin-2. These interaction partners broadly fall into three biological pathways—structural, metabolic and signaling. The confidence score of an interaction is depicted by the thickness of the line connecting two proteins.

Table 1
Expression of Z-line proteins in *Actn3* KO mice and their binding affinities to α-actinin-2 and -3. The over-expression of Z-line proteins in *Actn3* KO mice and the binding affinities of ZASP, myotilin, titin and vinculin to α-actinin-2 and -3 as determined by yeast two-hybrid [36]. The number of '+' indicates strength of growth which correlates to strength of protein interaction. The expression of titin and vinculin in *Actn3* KO muscle and the binding affinities of desmin, γ-filamin, and actinin-associated LIM protein (ALP) are yet to be determined.

	Over-expression in *Actn3* KO mice	Binding to α-actinin-2	Binding to α-actinin-3
α-Actinin-2	√	TBD	TBD
ZASP	√	+++	+
Myotilin	√	+++	+++
Desmin	√	TBD	TBD
γ-Filamin	√	TBD	TBD
ALP	x	TBD	TBD
Titin	TBD	+++	+
Vinculin	TBD	+++	+

muscles [49] and is important for sarcomere maintenance and stabilizing the Z-line during muscle stress [50]. The ZASP KO mouse displays a severe form of congenital myopathy [46]. ZASP and myotilin were assessed for their ability to interact with α-actinin-2 and α-actinin-3 in a yeast two-hybrid system [36]. ZASP preferentially interacts with α-actinin-2, while myotilin displays no preference for either α-actinin isoform. Furthermore, two additional Z-line proteins, titin and vinculin, preferentially bind α-actinin-2 (Table 1) [36]. Titin spans the whole length of the sarcomere, organizing the actin cytoskeleton via interactions with sarcomeric proteins including α-actinin-2 [51]. Both titin and vinculin are also involved in the modulation of Z-line width [52,53].

The notion of preferential binding is not unprecedented and has been explored in a study of two histone deacetylases from yeast [54]. Hst1p and Sir2p, like α-actinin-2 and α-actinin-3, are products of gene duplication, belonging to a family of NAD^+ dependent deacetylases important in transcriptional silencing. The two genes have different modes of silencing due to preferential interaction with different substrates and normally

act in independent pathways. However when one gene is absent, the other becomes a substitute, contributing to silencing a pathway in which it does not normally participate, a scenario which echoes the up-regulation of α-actinin-2 to compensate for the absence of α-actinin-3. However the substitute gene is less efficient in performing transcriptional silencing due to weaker interaction with the substrates involved, a situation similar to the inability of α-actinin-2 to completely compensate for the effects of α-actinin-3 deficiency.

The up-regulation of Z-line proteins including α-actinin-2 in response to α-actinin-3 deficiency, coupled with the higher binding affinity of Z-line proteins to α-actinin-2, is likely to lead to disruption of normal protein complexes at the Z-line, altering the structural properties of the *Actn3* KO muscle [36]. The altered Z-line protein composition likely results in the greater susceptibility to the contraction-induced damage associated with α-actinin-3 deficiency [36].

The discovery of proteins that preferentially interact with α-actinin-2 over α-actinin-3 raised the possibility that other proteins share this property; conversely are there proteins that preferentially interact with α-actinin-3? Identifying these proteins and characterising the consequences of their altered binding affinity will be crucial in deciphering the functional differences between α-actinin-2 and α-actinin-3 and, as such, the molecular consequences of α-actinin-3 deficiency.

6. Metabolic changes associated with α-actinin-3 deficiency may relate to altered interactions with glycogen phosphorylase

Glycogen phosphorylase (GPh) is a key regulatory enzyme involved in glycogen breakdown and has previously been reported to interact with sarcomeric α-actinins [55]. Interestingly, cytoplasmic inclusions of GPh were detected in fast-Type 2B fibres of *Actn3* KO muscle and concentric ring-like structures surrounded and filled by glycogen particles were detected by electron microscopy (EM) in *Actn3* KO muscle. GPh and sarcomeric α-actinins co-localise at the Z-lines of both WT and *Actn3* KO muscles but no differences in co-localisation were observed by confocal microscopy in the absence of α-actinin-3 [32].

GPh was one of several proteins identified by a global proteomic analysis to be differentially expressed between WT and *Actn3* KO mouse muscle [32]. By two-dimensional (2D) gel electrophoresis, GPh appears as a chain of seven to eight proteins spots, likely to represent differently post-translationally modified forms of GPh. The pattern of spots observed is consistent with multiple phosphorylation sites. To date, the only validated phosphorylation site for GPh is serine 14, which is phosphorylated by phosphorylase kinase to activate GPh [56]. *In silico* examination of GPh using NetPhos 2.0 predicts eight high-confidence phosphorylation sites which correspond well with the

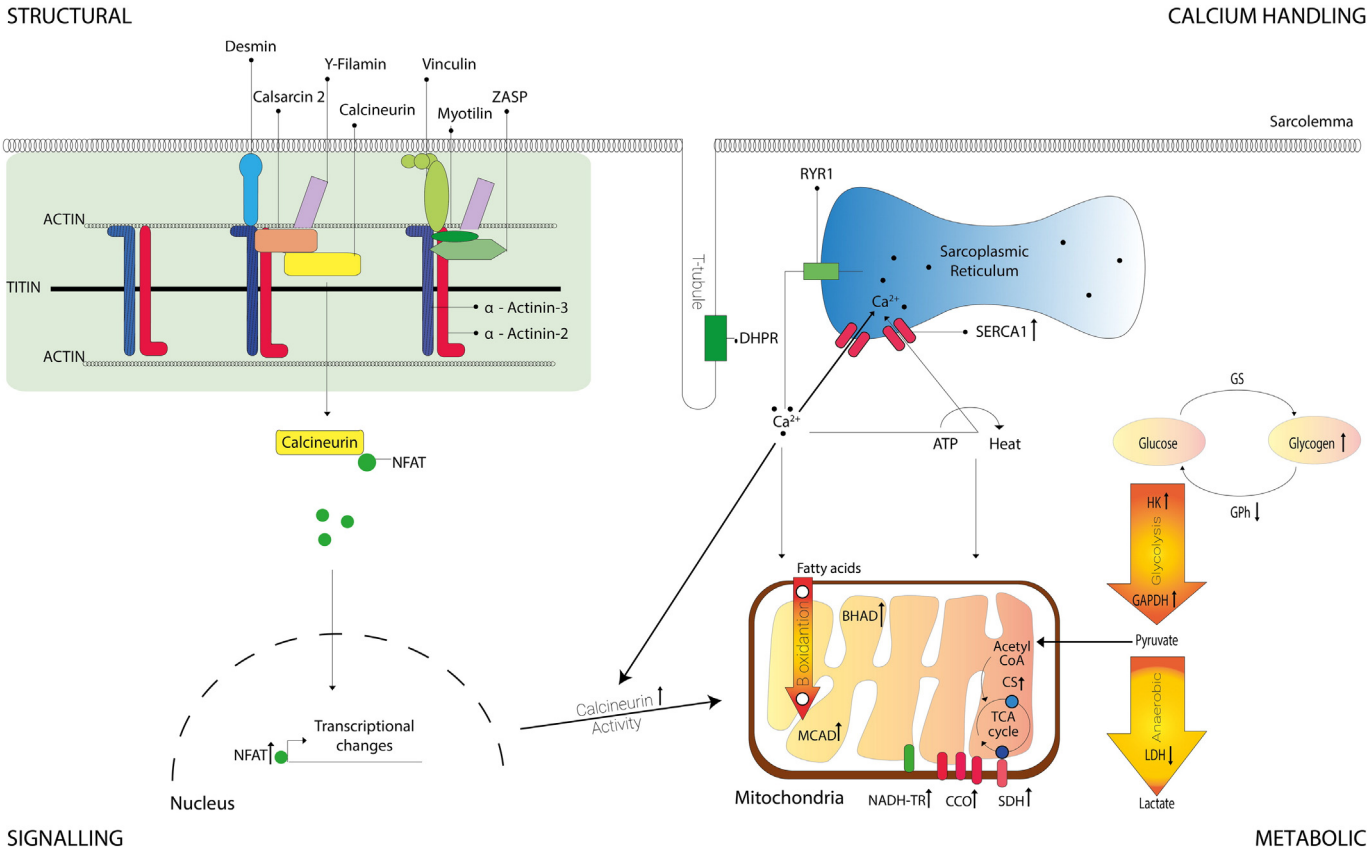

Fig. 2. Schematic of the downstream effects of α-actinin-3 deficiency on four biological pathways. Structural, metabolic, signalling and calcium handling changes seen in the *Actn3* KO mouse muscle begin to provide a mechanistic explanation for how α-actinin-3 deficiency alters muscle function. **Structural**—The sarcomeric α-actinins are actin binding proteins and exist as homo- and heterodimers. Several other structural proteins including desmin, γ-filamin, myotilin and ZASP are up-regulated in Actn3 KO muscle. In addition, ZASP, vinculin and titin also preferentially bind α-actinin-2 over α-actinin-3. **Signalling**—The preferential binding of calsarcin 2 to α-actinin-2 releases inhibition on calcineurin activity resulting in enhanced calcineurin signalling in *Actn3* KO muscle. The nuclear factor of activated T-cell (NFAT) is activated as a result which then translocates into the nucleus where it up-regulate the expression of genes involved in oxidative metabolism and the slow myogenic program. **Metabolic**—Enzyme analysis from *Actn3* KO quadriceps muscles revealed shift in muscle metabolism with lower activity of anaerobic metabolism enzyme lactate dehydrogenase (LDH) and higher activity of enzymes involved in glycolysis [hexokinase (HK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] as well as various enzymes relating to mitochondrial oxidative metabolism and fatty acid oxidation [3-hydroxyacyl-CoA dehydrogenase (BHAD), medium chain acyl-CoA dehydrogenase (MCAD), citrate synthase (CS), succinate dehydrogenase (SDH), cytochrome c oxidase (CCO) and NADH tetrazoleum reductase (NADH-TR)]. Lower activity of glycogen phosphorylase (GPh) along with higher glycogen content was also observed in *Actn3* KO muscle. Glycogen synthase (GS) activity was not different. **Calcium handling**—Higher rate of calcium (Ca^{2+}) release and absorption by the sarcoplasmic reticulum can be explained by higher levels of the sarcoplasmic reticulum calcium ATPase1 (SERCA1) in *Actn3* KO muscle which consumes ATP and generates heat. The expression of the dihydropyridine-receptor voltage sensor (DHPR) and the ryanodine-receptor Ca^{2+}-release channel (RYR1) are not different between WT and *Actn3* KO muscle.

seven to eight GPh spots observed above [32]. However there is currently no experimental data to confirm the modifications on GPh is indeed phosphorylation. Nevertheless, it is intriguing to speculate that α-actinin-3 may play a role in the post-transcriptional regulation of GPh [32].

A lower activity of GPh in α-actinin-3 deficient individuals is hypothesised to result in a reduced ability to utilise muscle glycogen as a source of energy for muscle contraction. Consequently, this may lower the ability of α-actinin-3 deficient human and *Actn3* KO mouse to generate the energy necessary for sprint and power sports. The muscle could adapt to this change by producing energy through alternative pathways. Hence the shift to oxidative metabolism in *Actn3* KO muscles is likely a consequence of altered glycogen metabolism.

7. Enhanced calcineurin signalling and altered fibre-type properties in the absence of fibre-type shift result from changes in interactions with calsarcin-2

The calsarcin family, which includes calsarcin-1, -2 and -3, directly interacts with the α-actinins as well as an array of other Z-line protein including ZASP, telethonin, γ-filamin and myotilin [33]. Like α-actinin-3, calsarcin-2 (also known as myozenin and FATZ) is exclusively expressed in the fast-twitch fibres of skeletal muscle [57–59]. The calsarcins are calcineurin-binding proteins [57]; calcineurin is a calcium and calmodulin-dependent protein phosphatase [60] that when active promotes slow-fibre-specific myosin heavy-chain expression conversion [61,62]. Calsarcin-2 is an inhibitor of calcineurin activity [33].

The expression of calsarcin-2 is not altered in the *Actn3* KO muscle suggesting that the differential activity of calcineurin is independent of calsarcin-2 levels. Calsarcin-2 binds preferentially to α-actinin-2 over α-actinin-3 [27]. Calsarcin-2 also binds calcineurin and this binding is inversely proportional to the level of α-actinin-2 present. The higher α-actinin-2 concentrations, as seen in α-actinin-3 deficient mice, prevent binding of calcineurin to calsarcin-2, which effectively relieves inhibition on calcineurin activity resulting in enhanced calcineurin signalling [27].

Furthermore, RCAN1-4 expression was unchanged in mouse models over-expressing α-actinin-3 while significantly induced in mice over-expressing α-actinin-2 [27]. The results strongly suggest the enhanced calcineurin signalling is a consequence of α-actinin-2 up-regulation in the *Actn3* KO muscle, which, via interactions with calsarcin-2, subsequently leads to a shift in fast fibre metabolism towards those of slower fibres [27].

8. Overlap and cross-talk between distinct pathways complete the picture

Homozygosity for the R577X polymorphism in *ACTN3* influences muscle performance and function in approximately 1.5 billion people worldwide. Loss of α-actinin-3, leads to alterations in the expression of α-actinin-2 and downstream effects on four biological pathways (Fig. 2). Individually we have examined the structural, metabolic and signalling pathways which contribute mechanistic insights into the consequences of α-actinin-3 deficiency. The reality is much more complex with much overlap and cross-talk between the pathways, summarised

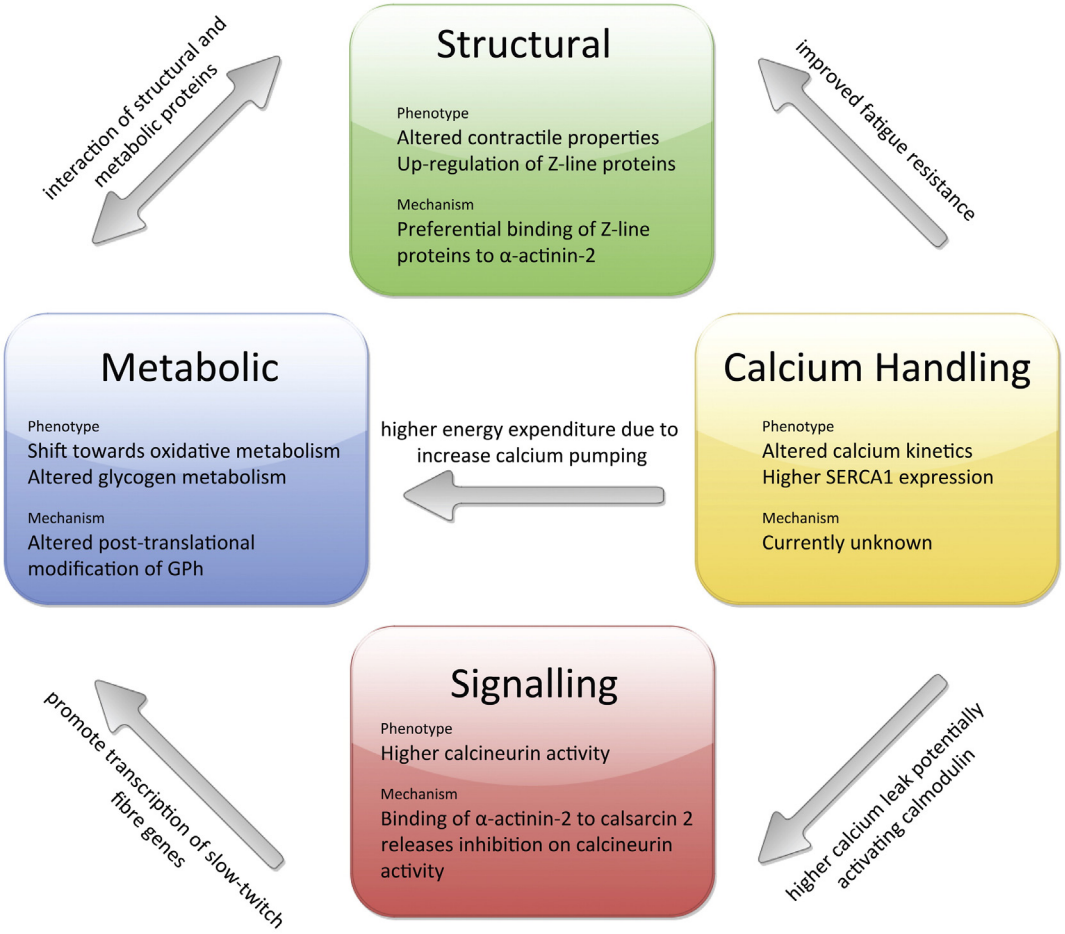

Fig. 3. Summary of the overlap and cross-talk between the four biological pathways. Summary of the structural, metabolic, signalling and calcium handling consequences of α-actinin-3 deficiency, the mechanisms that underlie these phenotypes and the cross-talk between the pathways.

in Fig. 3. α-Actinin-3 deficient muscles are phenotypically characterised by altered contractile properties and up-regulation of Z-line proteins (structural) [36], shift towards slower muscle fibre properties [24] and altered glycogen metabolism [32] (metabolic) with higher calcineurin signalling [27] and altered calcium kinetics [35]. The up-regulation of α-actinin-2 lifts inhibition on calcineurin signalling through competitive binding of calsarcin 2 providing a mechanism for the enhanced calcineurin signalling observed in α-actinin-3 deficient muscles [27]. When activated, calcineurin promotes transcription of genes involved in oxidative metabolism and promoting slow twitch characteristics in these muscle fibres [61,62].

The altered calcium kinetics in the form of higher calcium leak may be sufficient to activate Ca^{2+}-calmodulin which can in turn activate calcineurin, providing another mechanism for the enhanced calcineurin activity [35]. α-Actinin-3 deficient fibres are more fatigue resistant likely due to enhanced maintenance of titanic Ca^{2+} following repeated muscle stimulation. Additionally, the higher energy expenditure due to higher calcium pumping and consequent adenosine triphosphate (ATP) consumption is likely a factor which contributes to the muscle fibre metabolism shift towards the more efficient oxidative pathway [35].

We are also beginning to uncover the complex interactions between structural and metabolic proteins with a number of metabolic proteins already known to interact with α-actinin including GPh [55], fructose-1, 6-bisphosphatase 1 (FBP1) and aldolase [63] and many more localising to the Z-line [64]. The calsarcin protein family can also interact with multiple Z-line proteins including ZASP, γ-filamin and telethonin [65]. Both ZASP and γ-filamin are up-regulated in *Actn3* KO muscle [27] and the implication of their interactions with calsarcin proteins remains to be explored.

9. Conclusion

The ***structural, metabolic, signalling and calcium handling*** changes seen in the *Actn3* KO mouse muscle begin to provide a mechanistic explanation for the selective advantage of the *ACTN3* 577X allele during human evolution and the association between *ACTN3* genotype and muscle performance in humans today. α-Actinin-3 deficient humans, with a shift towards slower muscle properties in the absence of a shift in fibre-type, may have been at a selective advantage due to resistance to cold or famine. The shift towards slower fibre properties and altered adaptation to physical demands also explains the association with athletic performance and muscle function. Powerful muscle contractions required for sprint and strength activities rely on fast muscle fibres, which do not function optimally in the absence of α-actinin-3. Continued advances in understanding the interplay between the structural, metabolic and signalling pathways will present a clearer picture on why α-actinin-3 deficiency affects muscle function and how this normal human variation continues to influence skeletal muscle both in health and disease.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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