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## ***TNNI1*, *TNNI2* and *TNNI3*: Evolution, Regulation, and Protein Structure-Function Relationships**

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### **Abstract**

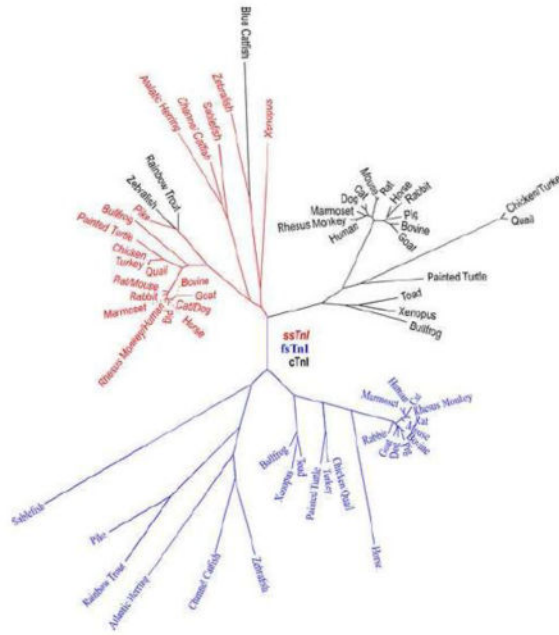
Troponin I (TnI) is the inhibitory subunit of the troponin complex in the sarcomeric thin filament of striated muscle and plays a central role in the calcium regulation of contraction and relaxation. Vertebrate TnI has evolved into three isoforms encoded by three homologous genes: *TNNI1* for slow skeletal muscle TnI, *TNNI2* for fast skeletal muscle TnI and *TNNI3* for cardiac TnI, which are expressed under muscle type-specific and developmental regulations. To summarize the current knowledge on the TnI isoform genes and products, this review focuses on the evolution, gene regulation, posttranslational modifications, and structure-function relationship of TnI isoform proteins. Their physiological and medical significances are also discussed.

### **Graphical Abstract**

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## Keywords

troponin I; cardiac and skeletal muscle; evolution; gene regulation; isoform; posttranslational modification

## 1. Introduction

Troponin I (TnI) is the inhibitory subunit of the troponin complex in striated muscle (skeletal and cardiac muscles). The basic contractile machinery of striated muscles is the sarcomere that consists of overlapping myosin thick filaments and actin thin filaments. Contraction is produced by sliding between the thick and thin filaments, a process that is powered by actin-activated myosin ATPase and regulated by cytosolic  $\text{Ca}^{2+}$  via the troponin complex associated with the sarcomeric thin filament (Jin et al., 2008). Troponin I functions along with the other two subunits of troponin, troponin T (TnT) (Wei and Jin, 2011) and troponin C (TnC) (Li and Hwang, 2015), to govern muscle contraction and relaxation. To summarize the current knowledge on the TnI isoform genes and products, this review focuses on the evolution, gene regulation, posttranslational modifications, and structure-function relationship of TnI isoform proteins. Their physiological and medical significances are also discussed.

## 2. Evolution of TnI Isoform Genes

Three homologous genes have evolved in higher vertebrates, encoding the three muscle fiber type-specific isoforms of TnI (Hastings, 1997; Perry, 1999; Chong and Jin, 2009) (Table 1). In the human chromosomal genome, *TNNI1* is located at 1q31.3, encoding the slow skeletal muscle isoform of TnI (ssTnI); *TNNI2* is located at 11p15.5, encoding the fast skeletal muscle isoform of TnI (fsTnI); and *TNNI3* is located at 19q13.4, encoding the cardiac

isoform of TnI (cTnI). The three TnI isoform genes are structurally diverged, differentially expressed under fiber type-specific transcriptional control, and regulated during embryonic and postnatal development (Jin et al., 2008).

A comparison of the amino acid sequences of rabbit cardiac, fast and slow skeletal muscle TnI isoforms suggested that the three TnI genes arose by gene duplication (Baldwin et al., 1985). Sequence analysis and co-evolutionary relationship with TnT isoforms established via an epitope similarity and conformational modulation study indicated that the original TnI gene was fast skeletal muscle TnI-like (Chong and Jin, 2009).

Diverged from the fsTnI gene lineage, the present-day ssTnI and cTnI sequences exhibit higher degree of similarity among the three isoforms. Their co-evolutionary relationship with the closely linked TnT isoform genes (Huang and Jin, 1999) indicates that they evolved from a slow skeletal muscle TnI-like common ancestor gene (Chong and Jin, 2009). Therefore, the first gene duplication event generated a slow skeletal muscle TnI-like gene that was further duplicated into the present-day ssTnI and cTnI genes.

The direct evolutionary relationship between ssTnI and cTnI genes is supported by the facts that a) cTnI in lower vertebrates, such as zebra fish, aligned with the ssTnI monophyletic group (Fu et al., 2009) (Fig. 1), b) the unique N-terminal extension of mammalian and avian cTnI, which is absent in fast and slow skeletal muscle isoforms of TnI, is an adult heart specific structure added during evolution as it is absent in fish cTnI (Fig. 2), and c) ssTnI is expressed as the sole TnI isoform in embryonic hearts (Jin, 1996).

Phylogenetic analysis of TnI isoform sequences also demonstrated that each of the muscle type-specific isoforms is conserved among species while the three TnI isoforms in a given species are significantly diverged (Jin et al., 1998). This observation suggests that the TnI isoform genes were diverged early on during vertebrate evolution (Chong & Jin, 2009). The phylogenetic tree in Fig. 1 further demonstrates that the divergence of cTnI genes is greater than that of fsTnI genes, whereas the ssTnI genes are the most conserved among vertebrate species. This pattern implicates different rates of the evolution of TnI isoform genes, possibly reflecting different functional demands in the three types of muscle fibers.

### 3. Muscle Type-Specific Expression and Developmental Regulation of TnI Isoform Genes

The expression of TnI isoform genes is under muscle-specific and developmental controls (Jin et al., 2008). ssTnI is expressed in both skeletal muscle and heart in embryos (Sasse et al., 1993). The expression of ssTnI in the heart is switched to cTnI during perinatal development (Saggin et al., 1989; Sasse et al., 1993). The presence of solely ssTnI in fetal hearts demonstrates the functional conservation and exchangeability of TnI isoforms in sustaining muscle contraction. Consistently, transgenic mice with cTnI replaced by ssTnI in the heart were viable and fertile, exhibiting no cardiac hypertrophy or failure (Fentzke et al., 1999).

fsTnI is restrictively expressed in fast twitch skeletal muscle fibers early on during myogenesis and continues expression through adulthood (Hastings and Emerson, 1982). A switch from slow to fast skeletal muscle TnI isoforms concurs with the switch of type I to type II fibers during skeletal muscle adaptation to simulated microgravity (Stevens et al., 2002). cTnI is exclusively expressed in the heart (Bodor et al., 1995), becoming predominant in postnatal cardiac muscle (Sasse et al., 1993). The adult heart expresses cTnI as the sole isoform with no change found in pathological conditions such as ischemic heart disease, dilated cardiomyopathy, and end-stage heart failure (Sasse et al., 1993).

Whereas cardiac TnT is expressed in developing fetal (Anderson et al., 1991) and regenerating adult (Bodor et al., 1997b; Rittoo et al., 2014) skeletal muscles, cTnI was exclusively expressed in cardiac muscle and not detected at protein level in skeletal muscle (Bodor et al., 1995; Rittoo et al., 2014). cTnI mRNA was controversially reported positive (Messner et al., 2000) or negative (Ricchiuti and Apple, 1999) in skeletal muscle of patients with Duchenne muscular dystrophy. No cTnI mRNA was detected in embryonic or adult skeletal muscle or in embryonic skeletal muscle cell cultures (Hastings et al., 1991).

Interestingly, ssTnI is not expressed in the embryonic hearts of *Xenopus* and zebra fish, while it is expressed in the somites and skeletal muscles (Warkman and Atkinson, 2002; Fu et al., 2009). In the meantime, the expression of zebra fish cTnI is not restricted to cardiac muscle and is detectable in craniofacial muscles (Fu et al., 2009). These observations indicate the high degree of similarity between lower vertebrate ssTnI and cTnI that lacks the N-terminal extension (Fig. 2). Therefore, the apparently high diversity of cTnI across vertebrate species (Fig. 1) mainly reflects the evolutionary addition of the adult heart-specific N-terminal extension in cTnI of higher vertebrates.

The different TnI isoforms expressed in different types of muscle fibers and regulated during heart and skeletal muscle development may function in fine tuning myofilament  $\text{Ca}^{2+}$ -sensitivity, cooperativity and pH tolerance as an adaptation to the cellular environment and contractile performance (Westfall et al., 1997; Westfall et al., 1999). Over-expression of ssTnI in the cardiac muscle of adult transgenic mice altered relaxation and diastolic function by increasing myofilament  $\text{Ca}^{2+}$  sensitivity (Fentzke et al., 1999) and the tolerance to acidosis-induced decrease in myofilament  $\text{Ca}^{2+}$  sensitivity in cardiomyocytes (Westfall et al., 1997; Westfall et al., 2000). These findings indicate that ssTnI produces a higher  $\text{Ca}^{2+}$  affinity for the troponin complex than that of cTnI, which may sustain  $\text{Ca}^{2+}$  sensitivity of myofilaments at the lower pH (6.5 versus 7.0) in embryonic cardiomyocytes (Solaro et al., 1988).

Although TnI is generally considered a striated muscle-specific protein, recent studies reported that fsTnI, as well as fsTnT and fsTnC were expressed at significant levels in smooth muscle cells in mouse blood vessels (Moran et al., 2008), while fsTnT was found in smooth muscle cells in aorta, bladder and bronchus (Ju et al., 2013). Expression of fsTnI was also found in non-muscle cells, such as human corneal epithelium (Kinoshita et al., 2001) and cartilage (Moses et al., 1999; Li et al., 2003b). fsTnI was localized to the nuclei of breast cancer cells and may be a co-activator of estrogen receptor related-receptor  $\alpha$  (Li et al., 2008).

#### 4. Structure-Function Relationship of TnI Isoforms

TnI is a protein of 21 – 24 kDa in size with an alkaline isoelectric point (Table 1). Of the exons of the TnI genes, exons 4 – 8 are conserved among the isoforms and across species. In contrast, exons 1, 2 and 3 encode as few as 5 amino acids in fsTnI and ssTnI but as many as 30 amino acids in cTnI corresponding to the unique N-terminal extension (Fig. 2) (Jin et al., 2008). The last exon (exon 9, Table 1) of human *TNNI1* gene is rather big (5,484 base pairs) and contains only a non-coding sequence (Corin et al., 1994). The last exon of mouse *TNNI1* gene also contains only non-coding sequence. This additional non-coding exon is only found in *TNNI1* genes and its functional significance is unknown.

Troponin I interacts with all known regulatory proteins in the thin filament: TnC, TnT, actin, and tropomyosin, reflecting its key position in the Ca<sup>2+</sup> regulation of striated muscle contraction (Perry, 1999). Crystallography of the troponin complex determined the high resolution structure of human cTnI from amino acid 31 to 191 except for the inhibitory peptide (residues 137–148) (Takeda et al., 2003) (Fig. 3). Crystallography of chicken fast skeletal muscle troponin showed similar overall organization to that of cardiac troponin (Vinogradova et al., 2005). However, the inhibitory region of fsTnI is well ordered in skeletal muscle troponin whereas it is flexible and not visible in the crystal structure of cardiac troponin (Takeda et al., 2003) (Fig. 3).

Based on *in vitro* structure-function relationship studies and interactions with other thin filament proteins, the structure of TnI can be divided into six functional segments (Li et al., 2004) (Fig 3): 1) the cardiac-specific N-terminal extension (residues 1–30 in cTnI); 2) the N-terminal conserved region (residues 42–65 in cTnI and 1–40 in fsTnI) that is the amphiphilic portion of H1  $\alpha$ -helix and binds the C domain of TnC; 3) the TnT-binding region (residues 66–136 in cTnI and 50–106 in fsTnI) from the C-terminal portion of H1 to H2  $\alpha$ -helices, which forms a coiled-coil interface with TnT; 4) the inhibitory region (residue 137–148 in cTnI and 107–115 in fsTnI) that interacts with TnC and actin–tropomyosin filament; 5) the switch or triggering region (residue 148–163 in cTnI and 115–131 in fsTnI) that is an  $\alpha$ -helix (H3) and binds the N domain of TnC via its N-terminal segment; and 6) the C-terminal mobile domain (residue 164–210 in cTnI and 132–180 in fsTnI) that consists of a protruding  $\alpha$ -helix (H4) (cTnI 164–188).

Recent studies demonstrated that the last 20 amino acids of the C-terminal end segment of TnI (residues 191–210 in cTnI) encoded by exon 8 with a highly conserved sequence is a Ca<sup>2+</sup>-modulated allosteric structure and interacts with tropomyosin (Zhang et al., 2011; Akhter et al., 2014). While the N-terminal extension of cTnI does not have definitive interaction with other known myofilament proteins, it plays a role in modulating the conformation of other regions of the cTnI molecule (Akhter et al., 2012).

#### 5. Posttranslational Modifications

No alternative RNA splicing has been reported for the transcripts of TnI isoform genes. On the other hand, posttranslational modifications play a major role in regulating structure and function of TnI (Solaro et al., 2008). The mechanisms include amino acid side chain

modifications and cleavages of the polypeptide chain, which induce conformational changes to modify troponin function and muscle contractility (Pi et al., 2003; Layland et al., 2005; Westfall et al., 2005; Solaro and van der Velden, 2010).

### 5.1. Phosphorylation

Whereas there is very limited information for the phosphorylation of ssTnI and fsTnI, phosphorylation of cTnI plays significant but sometimes controversial roles in cardiac muscle function (Solaro and Kobayashi, 2011). The 210 amino acid human cTnI polypeptide chain contains 12 serine, 8 threonine, and 3 tyrosine residues. Eighteen of these residues are predicted to be potentially phosphorylatable and 16 of them were experimentally demonstrated (Zhang et al., 2012). The known phosphorylation sites, corresponding kinases and function of these sites are summarized in Table 2.

Discussed above, cTnI of higher vertebrates differs from the two skeletal muscle TnI isoforms mainly by its N-terminal extension of ~30 amino acids, which contains a characteristic protein kinase A (PKA) substrate motif RRRSS (residues 20–24 in human cTnI) (Fig. 2). PKA phosphorylation of Ser<sub>23</sub> and Ser<sub>24</sub> under the regulation of adrenergic signaling cascades (Quirk et al., 1995; Solaro et al., 2008; Solaro and Kobayashi, 2011; Rao et al., 2012) enhances the diastolic function of cardiac muscle (Zhang et al., 1995a; Stelzer et al., 2007; Li et al., 2010) by reducing the Ca<sup>2+</sup>-binding affinity of the N domain regulatory site of cardiac TnC (Zhang et al., 1995b) and weakened TnC-TnI interaction in the presence of Ca<sup>2+</sup> (Rao et al., 2014). These two serine residues have also been reported to be phosphorylated *in vitro* by PKC-β, PKC-ε (Kobayashi et al., 2005), PKD (previously named PKCμ) (Haworth et al., 2004; Cuello et al., 2007; Bardswell et al., 2010) and PKG (Layland et al., 2002).

Four other sites (Ser<sub>5</sub>, Ser<sub>6</sub>, Tyr<sub>26</sub> and Thr<sub>31</sub>) in the N-terminal extension of cTnI are proposed with potential phosphorylations. Thr<sub>31</sub> is a substrate of mammalian sterile 20-like kinase 1 (Mst1) (You et al., 2009). Tyr<sub>26</sub> phosphorylation was shown to function similarly to that of Ser<sub>23/24</sub> in decreasing myofilament Ca<sup>2+</sup> sensitivity and increasing cardiac muscle relaxation (Salhi et al., 2014). Physiological significance and regulatory mechanisms of these phosphorylation sites remain to be further investigated.

The absence of the cardiac specific N-terminal extension in fish cTnI (Fig. 2) indicates its nature as an evolutionarily added regulatory structure in TnI of higher vertebrates. Troponin complex containing trout cTnI that lacks the N-terminal extension showed greater Ca<sup>2+</sup> affinity than that containing human cTnI (Kirkpatrick et al., 2011). Although trout cTnI lacks the two N-terminal Ser residues, myofilament Ca<sup>2+</sup> affinity decreased upon PKA treatment similar to the response of mammalian cTnI control (Kirkpatrick et al., 2011). This observation is worth further investigation.

Phosphorylation at Ser<sub>42</sub> and Ser<sub>44</sub> by PKC produces opposite effects to that of PKA at Ser<sub>23</sub> and Ser<sub>24</sub> by slowing down cardiac muscle relaxation and increasing the durations of Ca<sup>2+</sup> transient and twitch contraction (MacGowan et al., 2001; Pi et al., 2002; Burkart et al., 2003; Westfall et al., 2005).

The TnT binding region, *i.e.*, residues 66–136 in cTnI, contains several potentially phosphorylatable residues, Ser<sub>77</sub>, Thr<sub>78</sub> and Thr<sub>129</sub> (Zhang et al., 2012) with unknown function. Thr<sub>143</sub> in the inhibitory region is a cTnI-specific phosphorylation site. In mouse heart, phosphorylation of Thr<sub>143</sub> of cTnI by PKC-βII increased myofilament Ca<sup>2+</sup> sensitivity (Wang et al., 2006). However, another study observed that Thr<sub>143</sub> phosphorylation did not alter Ca<sup>2+</sup> sensitivity, but depressed cooperative activation of the thin filaments (Lu et al., 2010). Replacing Thr<sub>143</sub> with Pro to mimic that in ssTnI resulted in delayed relaxation of cardiomyocytes (Westfall et al., 2005). PKC phosphorylation of cTnI at Thr<sub>143</sub> also impaired the interaction between the inhibitory region and TnC, leading to depressed actomyosin ATPase and contractility (Lindhout et al., 2002; Li et al., 2003a). The physiological function of Thr<sub>143</sub> phosphorylation requires more investigation.

Ser<sub>150</sub> of cTnI in the TnC binding region has been shown as a phosphorylation site with opposite effect to that of PKA phosphorylation at Ser<sub>23/24</sub> (Nixon et al., 2014). Ser<sub>150</sub> can be phosphorylated by P21-activated kinase (Pak) (Buscemi et al., 2002; Ke et al., 2004) and AMP-activated protein kinase (AMPK), resulting in increased Ca<sup>2+</sup> sensitivity of cardiac myofibrils, prolonged relaxation (Oliveira et al., 2012), and increased development of adrenergic-induced myocardial hypertrophy (Taglieri et al., 2011). Equivalent to Ser<sub>150</sub> in cTnI, Ser<sub>118</sub> in fsTnI was also reported to be phosphorylated by AMPK (Sancho Solis et al., 2011). As AMPK is a key regulator of cellular energetics, phosphorylation of Ser<sub>150</sub> may suggest an adaptive mechanism in energy deprivation of both cardiac and skeletal muscles.

Phosphorylation at Ser<sub>166</sub> in cTnI reduced binding affinity for cardiac TnC (Zhang et al., 2012). Phosphorylation at Ser<sub>199</sub> in the actin-binding region of cTnI by PKC increased Ca<sup>2+</sup> sensitivity of troponin with decreased affinity for actin-tropomyosin thin filament (Wijnker et al., 2015). Thr<sub>181</sub> in this region was phosphorylated with a level higher than that of Ser<sub>166</sub> and Ser<sub>199</sub> (Zhang et al., 2012), but the responsible kinase and physiological function are unknown.

The phosphorylation of cTnI changes in cardiomyopathy and heart failure. In human end-stage dilated cardiomyopathy, the baseline phosphorylation of cTnI was diminished with increased myofilament Ca<sup>2+</sup> affinity (Zakhary et al., 1999). In failing human heart, PKA phosphorylation of Ser<sub>23/24</sub> in cTnI was decreased (Bodor et al., 1997a; Messer et al., 2007) and PKC phosphorylation of Ser<sub>42</sub>/Ser<sub>44</sub>/Thr<sub>143</sub> increased (Zhang et al., 2012), resulting in ventricular diastolic dysfunction. In post-infarct myocardium under remodeling, the expression of PKA and PKA-mediated phosphorylation of cTnI were decreased (Van der Velden et al., 2004).

## 5.2. O-linked GlcNAc modification

O-Linked N-acetylglucosaminylation (O-GlcNAc) is a dynamic cytosolic and nuclear mechanism of glycosylation, which is a ubiquitous post-translational modification and plays a role in regulating protein functions. Studies of isolated cardiomyocytes suggested increased levels of O-GlcNAcylation of cardiac muscle proteins in hearts with diabetic cardiac dysfunction (Fulop et al., 2007). High O-GlcNAc level would decrease Ca<sup>2+</sup> sensitivity and affinity of myofibrils (Hedou et al., 2007). Mass spectrometry identified Ser<sub>150</sub> of mouse cTnI as one of the O-GlcNAcylation sites (Ramirez-Correa et al., 2008).

Interestingly, Ser<sub>150</sub> is also a phosphorylation site by Pak and AMPK (discussed above), increasing Ca<sup>2+</sup> sensitivity. O-GlcNAc of cTnI at Ser<sub>150</sub> may compete for this site with phosphorylation to down-regulate protein phosphorylation. Conversely, it is conceivable that decreased phosphorylation may be a result of increased O-GlcNAc levels. The balance between phosphorylation and O-GlcNAc is worth further investigation.

### 5.3. S-glutathionylation

Reactive oxygen and nitrogen species are generated in skeletal muscle with normal activity and also in pathological conditions, and acutely or chronically affect muscle function (Lamb and Westerblad, 2011). H<sub>2</sub>O<sub>2</sub> treatment decreased Ca<sup>2+</sup> sensitivity of intact muscle fibers, which was reversed by glutathione pretreatment in only fast-twitch fibers, indicating specific S-glutathionylation target proteins in fast-twitch fibers (Murphy et al., 2008). It was found that fsTnI in rodent skeletal muscle was S-glutathionylated at Cys<sub>134</sub>, which increased the Ca<sup>2+</sup> activation of contraction. Similarly, fsTnI in human type II muscle fibers was S-glutathionylated with increased Ca<sup>2+</sup> sensitivity in physically active individuals, which may benefit skeletal muscle performance (Mollica et al., 2012). fsTnI of chicken and toad, both of which lack cysteine 133, showed no S-glutathionylation effects when the fast-twitch fiber went through the same treatment as that for rodent muscles (Mollica et al., 2012). This observation suggests a protective role of fsTnI S-glutathionylation against oxidative stress.

### 5.4. Proteolytic modifications

Cardiac TnI is a substrate of intracellular proteases, with a demonstrated sensitivity to  $\mu$ -calpain and m-calpain (Di Lisa et al., 1995). Its degradation by  $\mu$ -calpain was regulated by phosphorylation of cTnI, in which phosphorylation by PKA reduced the sensitivity whereas phosphorylation by PKC increased the sensitivity to  $\mu$ -calpain proteolysis (Di Lisa et al., 1995). Phosphorylation of cTnI by PKC at Ser<sub>199</sub> increased myofilament Ca<sup>2+</sup> sensitivity and cTnI's susceptibility to calpain-mediated proteolysis (Wijnker et al., 2015).

In addition to the overall degradation, C-terminal or N-terminal truncations have been reported in cTnI with distinct effects on cardiac function.

**5.4.1. C-terminal truncation**—The C-terminal region of TnI binds and stabilizes tropomyosin on the actin filament in the absence of Ca<sup>2+</sup> to inhibit muscle contraction (Galisker et al., 2010; Zhang et al., 2011). The C-terminal end segment (192–210) is the most conserved region of the TnI polypeptide chain (Jin et al., 2001) and a Ca<sup>2+</sup>-modulated allosteric structure in the troponin complex (Jin et al., 2001; Zhang et al., 2011; Wang et al., 2012a). R192H and R204H mutations in the C-terminal end segment of human cTnI cause restrictive cardiomyopathy (Mogensen et al., 2003; Gambarin et al., 2008) with alterations in the conformation and function of the TnI-TnT interface and increased binding affinity of cTnI for TnT (Akhter et al., 2014).

A truncation of the C-terminal 19 amino acids of cTnI was found during myocardial ischemia-reperfusion injury in Langendorff perfused rat hearts (McDonough et al., 1999). It was also seen in myocardial stunning in coronary bypass grafted patients (McDonough et al., 2001). Over-expression of the C-terminal truncated cTnI (cTnI<sub>1–193</sub>) in transgenic mouse



heart reproduced myocardial stunning with systolic and diastolic dysfunctions (Murphy et al., 2000). Partial replacement of intact cTnI with cTnI<sub>1-192</sub> in myofibrils *in vitro* and in cardiomyocytes *ex vivo* did not affect maximal tension development but hindered the rates of force redevelopment and relaxation (Narolska et al., 2006). Troponin complex containing cTnI<sub>1-192</sub> exhibited increased activity of Ca<sup>2+</sup>-activated actomyosin ATPase and faster sliding velocity as compared with that of troponin containing intact cTnI (Foster et al., 2003).

However, the pathological significance of the C-terminal truncation of cTnI is controversial. No C-terminal truncated cTnI was detectable in swine hearts after *in vivo* regional ischemia-reperfusion (Thomas et al., 1999). Myocardial stunning in pigs induced by regional ischemia was found with dephosphorylation of phospholamban without degradation of cTnI (Kim et al., 2001). No degradation of cTnI was detected in the hearts of conscious dogs after reversible ischemia (Lüss et al., 2000; Sherman et al., 2000).

**5.4.2. Restrictive N-terminal truncation**—The adult heart-specific N-terminal extension of cTnI is a regulatory structure (Sheng and Jin, 2014). The N-terminal extension contains the key PKA phosphorylation sites and plays a role in modulating the overall molecular conformation and function of cTnI (Akhter et al., 2012). Different from the C-terminal truncation, a selective removal of the N-terminal extension of cTnI through restrictive proteolysis occurs as a regulatory mechanism in cardiac adaptation in physiological and pathological stress conditions.

The restrictive N-terminal truncation of cTnI is at low levels in normal hearts of all species examined including human, and is significantly increased in response to hemodynamic stress (Yu et al., 2001) and  $\beta$ -adrenergic deficiency-caused failing mouse hearts (Feng et al., 2008). The N-terminal truncated cardiac TnI (cTnI-ND) remains in the myofibrils with a function in increasing myocardial relaxation to improve ventricular filling, similar to the effect of PKA phosphorylation (Barbato et al., 2005).

Transgenic mouse hearts expressing cTnI-ND exhibit improved diastolic function (Feng et al., 2008) and a better preservation of cardiac function in aging (Biesiadecki et al., 2010). Co-expression of cTnI-ND corrected the diastolic dysfunction of restrictive cardiomyopathy mouse hearts caused by cTnI-R193H mutation (Li et al., 2010). Isolated cTnI-ND mouse cardiomyocytes exhibited increased diastolic and systolic functions (Wei and Jin, 2013). These findings indicate that the N-terminal extension of cTnI is a potential site for targeted treatment for the clinically challenging condition of diastolic heart failure (Zile and Brutsaert, 2002).

## 6. Pathogenic Mutations

### 6.1. Patient phenotypes

To date, no human disease has been reported with mutations in ssTnI. Mutations in the fsTnI gene have been found to cause myopathy and distal arthrogryposis (DA). A missense mutation R174Q, a nonsense mutation (premature stop codon R156X), and three in-frame deletion mutations E167, K175 and K176 have been reported in DA patients (Sung et

al., 2003; Jiang et al., 2006; Kimber et al., 2006; Robinson et al., 2007). The mutations associated with DA are all in the C-terminal actin-tropomyosin binding domain.

Many mutations of cTnI have been found to cause cardiomyopathies (Seidman and Seidman, 2001; Curila et al., 2012). Comparing with pathogenic mutations in other myofilament proteins, cTnI mutations are often associated with more severe clinical courses (Doolan et al., 2005). R21C mutation in the N-terminal extension (Wang et al., 2012b), R141Q, L144P, R145Q, R145G in the inhibition region, A157V, R162W, R162Q, R162P in the switch region (Willott et al., 2010), and S166F, K177, K178del, K183E, K183, R186Q, I195M, D196N, L198V, L198P, S199N, E202G, G203R, G203S, R204C, R204H and K206Q in the C-terminal region (Willott et al., 2010) were found in hypertrophic cardiomyopathy patients. A116G mutation in the  $\alpha$ -helix in TnI at the interface with TnT was found in human dilated cardiomyopathy (Millat et al., 2011). A171T, K178E, D190G and R192H in the C-terminal region of cTnI were found in restrictive cardiomyopathy patients (Willott et al., 2010).

Most of these disease-causing single nucleotide mutations in cTnI are located in the C-terminal half of the polypeptide chain (residues 128–210) (Palpant et al., 2010), demonstrating the critical role of the C-terminal domain of TnI in muscle relaxation and diastolic function of the heart (Davis et al., 2007). This observation may indicate more stringent structure-function relationships in this region, or on the other hand reflect that this region of TnI has a high tolerance to structural variations to avoid embryonic lethality, allowing mutations to remain in the population. Both hypotheses are worth investigating.

## 6.2. Experimental studies

The pathophysiologic mechanism underlying the cTnI mutation-caused cardiomyopathies is a current research topic and much data have been elucidated from molecular to animal level studies.

R21C, which is the only in cTnI mutation found in the N-terminal extension, alters the PKA substrate motif and abolishes *in vivo* phosphorylation of Ser<sub>23</sub> and Ser<sub>24</sub>. cTnI-R21C knock-in mice showed diastolic dysfunction with delayed Ca<sup>2+</sup> re-sequestration (Dweck et al., 2014), a phenotype that supports the role of the N-terminal extension of cTnI in the regulation of diastolic function of the heart.

Mouse cTnI-A117G, the corresponding site of human A116G, exhibits faster mobility in SDS-PAGE as compared with wild type control, indicating a significant change in overall protein conformation (Akhter and Jin, 2015). Interestingly, an adjacent mutation K118C produces slower SDS-gel mobility than wild type control (Wei et al., 2010; Akhter and Jin, 2015), further demonstrating the potent and apparently complex effects of the TnI-TnT interface structure of TnI on molecular conformation and function. The K118C mutation decreases binding affinity of cTnI for TnC at pCa 4, which can be reversed by the restrictive N-terminal truncation (Akhter and Jin, 2015), reflecting a dominant effect of the N-terminal extension of cTnI on modulating the function of TnI-TnT interface.

R145G mutation in the inhibitory region of cTnI alters the interaction of cTnI with cardiac TnC, reduces the inhibition of actomyosin ATPase, and thus increases  $\text{Ca}^{2+}$  sensitivity (Kimura et al., 1997; Lindhout et al., 2002).

Different mutations of the same residue in cTnI may produce different phenotypes. For example, R204P mutation showed weakened interaction with TnT and TnC, R204C showed mild impairment of affinity for TnT and greater impairment of affinity for TnC (Cui et al., 2013), and R204W produced increased  $\text{Ca}^{2+}$  sensitivity.

Transgenic mouse models of cTnI mutation-caused cardiomyopathy are valuable tools to study the pathogenesis and pathophysiology of the human diseases, as well as to better understand the structure-function relationship of TnI. Some extensively characterized representative mouse models are summarized in Table 3.

## 7. Applications in Clinical Diagnosis of Acute Myocardial Infarction and Muscle Injuries

Cardiac troponin has been widely used as an indicator for cardiac muscle injuries. Testing of plasma cardiac TnI and cardiac TnT is uniformly performed in the diagnosis of patients with ischemic heart diseases (Januzzi et al., 2012). cTnI is exclusively expressed in the adult cardiac muscle cells and is a more specific diagnostic marker for myocardial infarction (Bodor et al., 1995) over cardiac TnT that is expressed in the heart as well as in fetal and regenerating skeletal muscles with elevations in patients with neuromuscular diseases (Rittoo et al., 2014). cTnT is also a sensitive and specific marker for even minor myocardial injuries. In addition, an increased level of serum cTnI independently predicts poor prognosis of critically ill patients in the absence of acute coronary syndrome (Reynolds et al., 2012; Lee et al., 2015). When skeletal muscle diseases are ruled out, elevation of cTnI together with cTnT in plasma could predict poor prognosis due to cardiomyocyte injury secondary to non-cardiac diseases (Giannitsis et al., 2000).

Skeletal muscle TnI has been proposed as a muscle fiber specific and sensitive marker of skeletal muscle injuries (Simpson et al., 2005; Chapman et al., 2013). fsTnI concentration in serum increased more than that of ssTnI after eccentric exercise of elbow flexors, indicating more damages of fast twitch fibers (Chapman et al., 2013).

## 7. Summary

An essential component of the thin filament  $\text{Ca}^{2+}$  regulatory system of vertebrate striated muscles, TnI has evolved with three muscle fiber type-specific isoform genes and diverse posttranslational modifications. Understanding the regulation and structure-function relationships of TnI isoforms, posttranslational modifications, and pathogenic mutations has broad biological and medical significance and applications.

Based on the current knowledge, many important questions regarding the gene evolution, regulation, structure-function relationship of TnI and its interactions with other myofilament proteins remain to be answered. For example, what are the key functional differences that

prevent the isoforms from substituting for each other? How does the cardiac specific N-terminal extension regulate the molecular conformation and function of cTnI? What is the molecular mechanism that governs the restrictive N-terminal truncation of cTnI in physiological adaptations? How does the phosphorylation of TnI regulate its functions? And what is the function of TnI found in smooth muscle and non-muscle cells? Much future work is needed to address these questions, which will enrich our knowledge on muscle contraction in health and diseases.

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## Abbreviations

<b>AMPK</b>	AMP-activated protein kinase
<b>cTnI</b>	cardiac isoform of troponin I
<b>cTnI-ND</b>	N-terminal truncated cardiac troponin I
<b>DA</b>	distal arthrogyrosis
<b>fsTnI</b>	fast skeletal muscle isoform of troponin I
<b>Mst1</b>	mammalian sterile 20-like kinase 1
<b>O-GlcNAc</b>	O-linked $\beta$ -N-acetyl-D-glucosamine
<b>PKA</b>	protein kinase A
<b>PKC</b>	protein kinase C
<b>PKD</b>	protein kinase D
<b>PKG</b>	protein kinase G
<b>ssTnI</b>	slow skeletal muscle isoform of troponin I
<b>TnI</b>	troponin I
<b>TnT</b>	troponin T
<b>TnC</b>	troponin C

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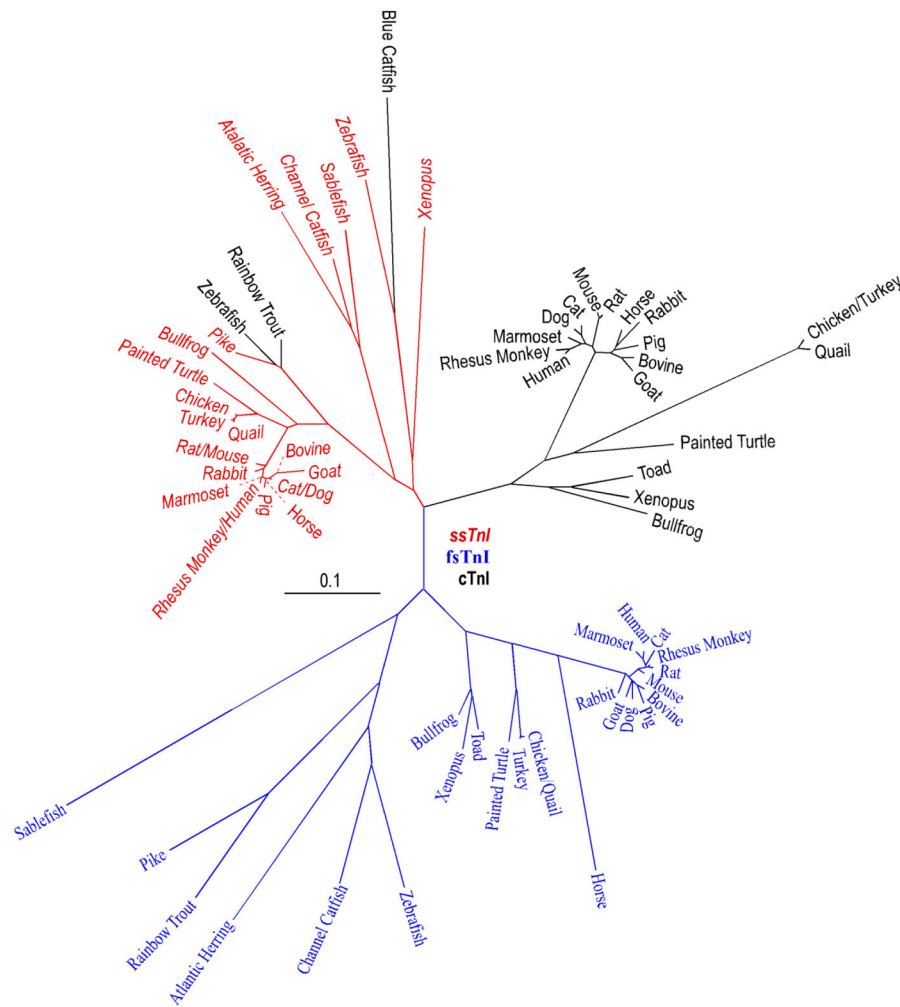
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### Highlights

- Human and higher vertebrate genomes contain three homologous troponin I isoform genes, *TNNI1*, *TNNI2* and *TNNI3*.
- The muscle fiber type-specific troponin I isoforms are evolutionarily diverged whereas each isoform is conserved across species.
- The N-terminal extension of cardiac troponin I is an adult heart-specific structure, and its phosphorylation by PKA and restrictive deletion regulate cardiac function.
- Mutations of troponin I cause myopathies.



**Figure 1.**

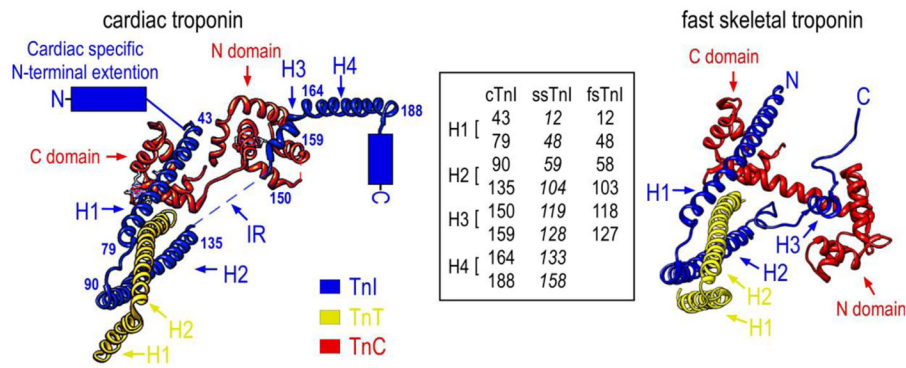
A phylogenetic tree of vertebrate TnI isoform genes is derived from protein sequence alignment. ssTnI, fsTnI and cTnI are marked in red, blue and black fonts, respectively. The NCBI database accession numbers for the sequences analyzed are: Sablefish ssTnI, ACQ58112.1; Sablefish fsTnI, ACQ58096.1; Atlantic Herring slow-like TnI, XP\_012674152.1; Atlantic Herring fsTnI, AAB05825.1; Zebra fish ssTnI, NP\_001002101.1; Zebra fish fsTnI, NP\_991138.2; Zebra fish cTnI, NP\_001008613.1; Channel Catfish ssTnI, NP\_001187788.1; Channel Catfish fsTnI, NP\_001187493.1; Blue Catfish cTnI, ADO28353.1; Pike ssTnI, NP\_001291009.1; Pike fsTnI, XP\_010900246.1; Rainbow Trout fsTnI, NP\_001123462.1; Rainbow Trout cTnI, NP\_001171957.1; Painted Turtle ssTnI, XP\_005310684.1; Painted Turtle fsTnI, XP\_005307820.1; Painted Turtle cTnI, XP\_008176331.1; Xenopus ssTnI, NP\_001079781.1; Xenopus fsTnI, NP\_001079556.1; Xenopus cTnI, NP\_001088122.1; Toad fsTnI, AEZ53888.1; Toad cTnI, AAX69047.1; Bullfrog ssTnI, AAO33938.1; Bullfrog fsTnI, AAW73073.1; Bullfrog cTnI, AAO33937.1; Turkey ssTnI, XP\_010722333.1; Turkey fsTnI, XP\_003206336.1; Turkey cTnI, NP\_001290153.1; Quail ssTnI, AAC59937.1; Quail fsTnI, AAB00122.1; Quail cTnI, AAA49513.1; Chicken ssTnI, XP\_004934896.1; Chicken fsTnI, NP\_990748.1; Chicken

cTnI, NP\_998735.1; Bovine ssTnI, NP\_001290364.1; Bovine fsTnI, NP\_001179023.1; Bovine cTnI, NP\_001035607; Dog ssTnI, XP\_003639192.1; Dog fsTnI, XP\_851068.1; Dog cTnI, NP\_001003041.1; Goat ssTnI, AFN20331.1; Goat fsTnI, AHK12864.1; Goat cTnI, AFN20332.1; Horse ssTnI, XP\_005608116.1; Horse fsTnI, XP\_005613620.1; Horse cTnI, NP\_001075373; Cat ssTnI, XP\_006942912.1; Cat fsTnI, XP\_011285371.1; Cat cTnI, Q863B6.3; Rabbit ssTnI, XP\_008266820.1; Rabbit fsTnI, NP\_001076252.1; Rabbit cTnI, P02646.2; Pig ssTnI, AAP37479.1; Pig fsTnI, NP\_001027530.1; Pig cTnI, NP\_001092069.1; Mouse ssTnI, NP\_001106173.1; Mouse fsTnI, NP\_033431.1; Mouse cTnI, NP\_033432.1; Rat ssTnI, NP\_058880.1; Rat fsTnI, NP\_058881.1; Rat cTnI, NP\_058840.1; Marmoset (New World Monkey) ssTnI, NP\_001230000.1; Marmoset fsTnI, JAB14438.1; Marmoset cTnI, XP\_002762542.2; Rhesus monkey (Old World Monkey) ssTnI, NP\_001252569.1; Rhesus monkey fsTnI, XP\_001117040.1; Rhesus monkey cTnI, XP\_001085820.1; Human ssTnI, NP\_003272.3; Human fsTnI, NP\_003273.1; Human cTnI, P19429.3.



**Figure 2.** Amino acid sequence alignment of the N-terminal extension of vertebrate cTnI was performed with the MegAlign computer program (Lasergene; DNASTAR, Inc, Madison, WI) using the Clustal V method. The sequence alignment demonstrated that the N-terminal extension of mammalian cTnI is highly conserved, whereas it shows notable sequence variations in avian, reptile and amphibian species, and is absent in fish cTnI. The three arrows indicate the exon boundaries based on the structure of human *TNNI3* gene. The two arrowheads indicate the two PKA phosphorylated Ser residues. The NCBI database accession numbers for the protein sequences analyzed are the same as that for Fig. 1.





**Figure 3.**

The left schematic structure illustrates the position of cTnI in the crystal structure of the troponin complex and its interactions with TnC and the partial structure of TnT in the  $\text{Ca}^{2+}$ -saturated state (PDB 1J1E) (Takeda *et al.*, 2003). The four  $\alpha$ -helices are indicated: H1 (43–79), H2 (90–135), H3 (150–159), H4 (164–188). The counterpart residues in ssTnI and fsTnI were shown in the inset box to denote the helix boundaries. As there is no crystal structure available for ssTnI, the helix boundaries (in *Italic font*) in ssTnI are deduced from protein sequence alignment with cardiac and fast skeletal muscle TnI. No high resolution structure of the N-terminal extension, the C-terminal end segment and the inhibitory region (IR) were resolved for human cardiac troponin. The right schematic crystal structure of chicken fast skeletal troponin (PDB 1Y TZ) (Vinogradova *et al.*, 2005) showed only three  $\alpha$ -helices (H1, H2 and H3) in fsTnI but included the inhibitory region. The C-terminal end segment of fsTnI is disordered in the crystal structure of chicken fast skeletal muscle troponin complex when  $\text{Ca}^{2+}$  is bound.

**Table 1**

Human TnI isoform genes and tissue-specific expression

<b>Protein isoforms</b>	<b>Slow Skeletal Muscle TnI</b>	<b>Fast Skeletal Muscle TnI</b>	<b>Cardiac TnI</b>
<b>Gene</b>	<i>TNNI1</i>	<i>TNNI2</i>	<i>TNNI3</i>
<b>Chromosomal Location</b>	1q31.3	11p15.5	19q13.4
<b>Number of Exons</b>	9	8	8
<b>Number of Amino Acids</b>	187	182	210
<b>Molecular Weight (kDa)</b>	21.7	21.3	24.0
<b>Isoelectric Point</b>	9.59	8.74	9.87
<b>Tissue Specificity</b>	Slow-twitch fibers Embryonic heart	Fast-twitch fibers	Adult heart

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Table 2

## Phosphorylation sites in troponin I

	Phosphorylation Site		Kinases	Function	Reference
	ssTnI	fsTnI			
cTnI					
S5,S6	NA	NA	Unknown	Unknown	(Zhang et al., 2012)
S23,S24	NA	NA	PKA PKC-β PKC-ε PKD PKG	Enhancing diastolic function	(Solaro et al., 2008) (Kobayashi et al., 2005) (Haworth et al., 2004) (Layland et al., 2002)
Y26	NA	NA	Unknown	Enhancing diastolic function	(Zhang et al., 2012) (Salhi et al., 2014)
T31	NA	NA	Mst1	Increasing affinity for TnC; Decreasing affinity for TnT	(You et al., 2009)
S39	(P8)	(N8)	PKA	Decreasing affinity for TnC	(Ward et al., 2001)
S42,S44	T11,S13	(I11,A13)	PKC	Slowing cardiac relaxation	(Kooij et al., 2011)
T51	S20	S20	Mst1	Unknown	(You et al., 2009)
S77,T78	(A46,E47)	(A46,E47)	Unknown	Unknown	(Zhang et al., 2012)
T129	(K98)	(N97)	Mst1	Unknown	(You et al., 2009)
T143	(I12P)	(I11P)	PKC PKC-βII Mst1	Decreasing relaxation and contraction	(Westfall et al., 2005) (You et al., 2009)
S150	S119	S118	Pak AMPK	Slowing cardiac relaxation	(Buscemi et al., 2002) (Oliveira et al., 2012) (Sancho Solis et al., 2011)
S166	S135	(C134)	PKA	Decreasing affinity to TnC	(Ward et al., 2001)
T181	T150	T149	Unknown	Unknown	(Zhang et al., 2012)
S199	S169	S169	PKC	Decreasing affinity to actin-tropomyosin	(Wijnker et al., 2015)

The amino acid residues in cTnI with known phosphorylation are listed. The residue numbers refer to that in human TnI isoforms with the first methionine included. NA, not applicable. Some of the residues are conserved between cardiac and skeletal muscle TnI, suggesting possible phosphorylations in skeletal muscle TnI. Some of the sites have been studied with engineered substitutions in cTnI with the counterpart amino acids in ssTnI or fsTnI (in brackets), suggesting that the phosphorylation modification of these sites in cTnI is a mechanism of tuning the function toward a skeletal muscle-like state. Phosphorylation of the sites in cTnI has been experimentally identified. Among the potential phosphorylation sites in ssTnI and fsTnI (in *italic* font), only S118 in fsTnI was experimentally demonstrated.

**Table 3**

Transgenic mouse models of cTnI mutations and truncations

Mouse Line	Phenotype	Reference
R21C knock-in	Hypertrophic cardiomyopathy	(Wang et al., 2012b)
K118C over-expression	Diastolic dysfunction	(Wei et al., 2010)
R145G over-expression *	Hypertrophic cardiomyopathy	(Wen et al., 2008)
R145W over-expression *	Restrictive cardiomyopathy	(Wen et al., 2009)
K178E over-expression	Restrictive cardiomyopathy	(Jean-Charles et al., 2012)
K179E over-expression	Restrictive cardiomyopathy	(Jean-Charles et al., 2012)
K184deletion over-expression	Diastolic dysfunction	(Iorga et al., 2008)
R193H over-expression	Restrictive cardiomyopathy	(Du et al., 2008)
C-terminal truncation over-expression	Stunning heart	(Murphy et al., 2000)
cTnI-ND over-expression	Enhanced diastolic function	(Feng et al., 2008)

Several gene-targeted or transgenic overexpression mouse models of cTnI are listed with their major phenotypes. The mutation sites in mouse cTnI are indicated with residue # counting from the first methionine.

\* The R145G and R145W mouse lines over-express human cTnI in the heart.