Nemaline myopathy and distal arthrogryposis associated with an autosomal recessive *TNNT3* splice variant

Authors: Sarah A Sandaradura*[1,2] and Adam Bournazos*[1], Amali Mallawaarachchi[3], Beryl B Cummings[4,5], Leigh B Waddell[1,2], Kristi J Jones[1,2], Christopher Troedson[6], Annapurna Sudarsanam[6], Benjamin M Nash[7], Gregory B Peters[7], Elizabeth M Algar[8], Daniel G MacArthur[4,5], Kathryn N North[1,9,10], Susan Brammah[11], Amanda Charlton[12], Nigel G Laing[13,14], Meredith J Wilson[3], Mark R Davis[13], Sandra T Cooper [1,2].

* These authors contributed equally to the manuscript

[1]Institute for Neuroscience and Muscle Research, The Children's Hospital at Westmead, Sydney, New South Wales, Australia.

[2]Discipline of Paediatrics and Child Health, University of Sydney, Sydney, New South Wales, Australia.

[3]Department of Clinical Genetics, Children's Hospital at Westmead, Sydney, New South Wales, Australia.

[4]Analytic and Translational Genetics Unit, Massachusetts General Hospital, Boston, Massachusetts,

USA.

[5]Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, Massachusetts, USA.

[6]Department of Neurology, Children's Hospital at Westmead, Sydney, New South Wales, Australia.

[7]Sydney Genome Diagnostics, Children's Hospital at Westmead, Sydney, New South Wales, Australia.

[8]Centre for Cancer Research, Hudson Institute of Medical Research, Clayton, Victoria, Australia. Department of Molecular and Translational Science, Monash University, Clayton, Victoria, Australia.

[9]Murdoch Children's Research Institute, Melbourne, Victoria, Australia

[10]Department of Paediatrics, Faculty of Medicine, University of Melbourne, Melbourne, Victoria, Australia

[11]Electron Microscope Unit, Concord Repatriation General Hospital, Sydney, New South Wales, Australia.

[12]Department of Anatomical Pathology, Middlemore Hospital, Auckland, New Zealand.

[13]Department of Diagnostic Genomics, PathWest Laboratory Medicine, QEII Medical Centre, Perth, Western Australia, Australia

[14]Centre for Medical Research University of Western Australia; Harry Perkins Institute of Medical Research, Perth, Western Australia, Australia

Supplemental Data: TnT_{fast} protein products predicted from abnormal splicing events, Figure 1; Supplemental materials and methods.

Corresponding Author:

A/Prof. Sandra Cooper, Institute for Neuroscience and Muscle Research, Children's Hospital at Westmead, Sydney, NSW 2145, Australia; Discipline of Child and Adolescent Health, Faculty of Medicine, University of Sydney, Sydney, Australia; Telephone: (+61 2) 9845 1456; E-mail: sandra.cooper@sydney.edu.au

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Abstract: 150 words

A male neonate presented with severe weakness, hypotonia, contractures and congenital scoliosis. Skeletal muscle specimens showed marked atrophy and degeneration of fast fibres with striking nemaline rods and hypertrophy of slow fibres that were ultrastructurally normal. A neuromuscular gene panel identified a homozygous essential splice variant in *TNNT3* (chr11:1956150G>A, NM_006757.3:c.681+1G>A). *TNNT3* encodes skeletal troponin-T_{fast} and is associated with autosomal dominant distal arthrogryposis. *TNNT3* has not previously been associated with nemaline myopathy, a rare congenital myopathy linked to defects in proteins associated with thin filament structure and regulation. cDNA studies confirmed pathogenic consequences of the splice variant, eliciting exon-skipping and intron retention events leading to a frameshift. Western blot showed deficiency of troponin-T_{fast} protein with secondary loss of troponin-I_{fast}. We establish a homozygous splice variant in *TNNT3* as the likely cause of severe congenital nemaline myopathy with distal arthrogryposis (NM-DA), characterised by specific involvement of Type-2 fibres and deficiency of troponin-T_{fast}.

Main Text:

TNNT3 (MIM# 600692) encodes troponin-T_{fast} (TnT_{fast}), the fast skeletal muscle isoform of TnT (~30 kDa). The troponin complex (TnC, TnI and TnT) binds to tropomyosin and regulates calcium-triggered contraction and relaxation of muscle fibres. Autosomal dominant inherited or de novo variants in *TNNT3* have been identified in individuals from multiple families with DA type 1A and 2B (MIM# 108120 and #601680). All patients described with DA due to variants in *TNNT3* have missense variants affecting Arg63 of TnT_{fast} (NM_006757.3); Arg63His, Arg63Cys and Arg63Ser (Beck et al., 2013; Sung et al., 2003; Zhao et al., 2011).

Nemaline myopathy (NM) is a rare congenital myopathy characterised by early-onset muscle weakness, hypotonia, absent or reduced deep tendon reflexes and the finding of protein-dense inclusions, known as nemaline bodies or rods, on skeletal muscle biopsy. The spectrum of NM

clinical severity ranges from onset in utero with lethality in the neonatal period, to mild non-progressive weakness with onset in childhood or adulthood (North, Laing, & Wallgren-Pettersson, 1997). NM is associated with variants in 12 genes that encode components of the sarcomeric thin filament or proteins associated with thin filament organisation, alignment and regulation (Alazami et al., 2015; Miyatake et al., 2017; Romero, Sandaradura, & Clarke, 2013; Yuen et al., 2014).

TNNT3 has not previously been identified as a cause of nemaline myopathy. However, autosomal recessive variants in *TNNT1* (MIM# 191041; encoding TnT_{slow}) cause severe congenital NM (9). Developmental switching of TnT isoforms in skeletal muscle corresponds to the onset of *TNNT1*-NM (MIM# 605355) in the Amish population, whereby affected infants have normal skeletal muscle function at birth, but rapidly develop severe muscle dysfunction and contractures, typically leading to death within the first 2 years of life (Johnston et al., 2000). Here we report a novel homozygous essential donor splice variant in intron 14 of *TNNT3* (chr11:1956150G>A, NM_006757.3:c.681+1G>A) that ablates normal splicing and induces deficiency of TnT_{fast} , as the likely cause of a severe congenital form of nemaline myopathy with arthrogryposis.

The male proband was the third child to non-consanguineous parents of Aboriginal/Caucasian and Caucasian ethnicity. There was a history of polyhydramnios during the pregnancy with antenatal ultrasounds identifying overlapping digits, bilateral talipes and a “rocker-bottom” appearance to the feet. There was no relevant family history. He was born at 31 weeks gestation by normal vaginal delivery following spontaneous onset of premature labour. His birthweight was 1.5 kg (50^{th} percentile for gestation), length 31 cm (90^{th} percentile) and head circumference 31 cm (90^{th} percentile). On examination he had severe weakness of facial and limb muscles, hypotonia, a high arched palate, contractures (bilateral vertical talus, knee contractures, finger contractures), bilateral hip dislocation

and a thoracic scoliosis (Figure 1A). He had respiratory muscle weakness, requiring invasive respiratory support for the first two weeks of life, followed by non-invasive ventilation. He had bulbar weakness, requiring nasogastric tube feeding followed by laparoscopic fundoplication and gastrostomy. Serum creatine kinase was normal. The strength of the proband improved over time and he developed antigravity movement of the upper and lower limbs. He remained on non-invasive respiratory support. He died at a corrected age of 8 months due to an acute event, presumed respiratory in nature. Autopsy was not performed.

Targeted massively parallel sequencing of 277 genes associated with neurogenetic conditions did not identify likely pathogenic variants in known congenital myopathy genes (PathWest NMD Gene panel, see supplemental methods). However, a homozygous essential donor splice variant in *TNNT3* (chr 11:1956150 G>A, NM_006757.3:c.681+1G>A) was identified. The gnomAD database identifies two heterozygous individuals with this *TNNT3* variant, with no homozygotes. Sanger sequencing of the mother and two unaffected siblings confirmed all three were heterozygous for this variant. Paternal DNA was not available. Analysis of copy number variation on SNP array did not support a microdeletion within either *TNNT3* allele. Loss of heterozygosity was detected over a region of ~1 Mb within chromosome band 11p15.5, encompassing the *TNNT3* variant. Methylation studies were performed, as this segment includes known imprinted loci, but were not suggestive of uniparental isodisomy. The segment of homozygosity included 466 consecutive SNP probes.

Histology on skeletal muscle biopsy showed selective and marked atrophy of Type 2 (fast) fibres which, on modified Gomori trichrome stain, contained numerous nemaline rods (Figure 1B). Rods were restricted to Type 2 fibres and there were no intranuclear rods. There was hypertrophy of Type 1 (slow) fibres, which appeared structurally normal. There was an increase in interstitial fibrosis and

evidence for fibre degeneration (Figure 1B, acid phosphatase). There were no regenerating fibres. Electron microscopy of skeletal muscle showed numerous electron-dense rods within the small fibres (Figure 1B, *v - viii*), with normal sarcomeric structure in the large fibres. No caps, cores or minicores were seen on light or electron microscopy. Immunohistochemistry of skeletal muscle samples confirmed hypertrophy of slow fibres with small atrophied fast fibres (Figure 1C, fast myosin, TnI_{fast}). Small atrophied fast fibres showed marked reduction/near deficiency of skeletal fast troponin T (TnT_{fast}, green; results confirmed using two validated antibodies, see Figure 1C).

Analysis of cDNA derived from muscle biopsy specimens from the proband demonstrated the essential donor splice site variant in *TNNT3* induced skipping of exon 14 and increased levels of intron 14 retention (Figure 2A and see schematic Figure 2D). Normally spliced transcripts were not detected on the gel. Although low levels of intron 14 retention is observed in controls (see Figure 2B), semi-quantitative RT-PCR suggests levels of intron 14 retention are elevated in the proband. There is an increasing body of evidence documenting intron retention as a natural means to regulate protein expression levels (Ge & Porse, 2014). Unfortunately, RNA sequencing failed for the *TNNT3* muscle specimen, and relative levels of exon 14 skipping and intron 14 retention could not be further quantified. Overall, mRNA studies show the homozygous *TNNT3* c.681+1G>A splice variant results in aberrant splicing, with both detected outcomes effecting a frameshift and premature stop codon or inclusion of ectopic sequences at the troponin-T_{fast} C-terminus (see schematic Figure 2D). *TNNT3* has developmentally regulated and alternatively-spliced exons (Figures 2B and 2D, exons 6A, 7A and 15A denoted in orange) (Stefancsik, Randall, Mao, & Sarkar, 2003; Wei & Jin, 2011; Wu et al., 1994). The *TNNT3* splice variant identified in this patient affects splicing of constitutively expressed exon 14 (of NM_006757.3), which encodes residues within the conserved troponin core domain (see Figure 2D).

Normalizing for fast myosin content, western blotting established a marked reduction of TnT_{fast} protein (<5% levels in age-matched control muscle) that was detected as a faint doublet (32-34 kDa) (Figure 2C). In controls, the predominant species migrated at 32 kDa, with a minor doublet band at ~34 kDa, previously proposed to correspond to differently phosphorylated forms of TnT_{fast} (Wei & Jin, 2011). We could not find convincing evidence for truncated, or elongated TnT_{fast} protein products predicted from abnormal splicing events (see Supp. Figure 1), suggesting they may be unstable and degraded. Western analyses revealed a marked secondary reduction in levels of TnI_{fast} (detected predominantly as the higher migrating phosphorylated form) (Wei & Jin, 2011). Collective data suggests that expression of mutant forms of TnT_{fast} that lack the coiled-coil tropomyosin interaction domain that tethers the TnC/TnI/TnT complex to the actin filament (Figure 2) (Takeda, Yamashita, Maeda & Maeda, 2003; Wei & Jin, 2011), results in destablisation of the entire troponin complex in fast fibres, and a secondary reduction in levels of TnI_{fast}.

In summary, we identify a homozygous splice variant in *TNNT3* as the likely cause of severe congenital nemaline myopathy with distal arthrogryposis and striking NM pathology restricted to Type 2 fibres. Importantly, the proband had a congenital scoliosis which is not common in NM, and did not have the facial features associated with autosomal dominant DA2B, such as a triangular face, down-slanting palpebral fissures and small mouth. Evidence suggest the NM pathology is due to deficiency of TnI_{fast} in fast fibres. Cardiac TnT and slow TnT isoforms may partially compensate for loss of functional TnT_{fast} during development. Although speculation, it is also possible that conversion to slow fibres in the proband, a feature common to many myopathies, may have contributed to the postnatal improvement in muscle function (autopsy specimens were not available). The gradual clinical improvement in the proband with recessive *TNNT3* myopathy represents a contrasting clinical course to recessive *TNNT1*-NM.

A mouse model with targeted knock-out of murine *Tnnt3* with a LacZ-reporter cassette recapitulates features of *TNNT3* NM, resulting in neonatal lethality of pups with decreased fetal weight (Ju et al., 2013). Interestingly, mice heterozygous for *Tnnt3*$^{lacz/+}$ are mildly affected, smaller than their wildtype littermates and manifest minor skeletal defects (Ju et al., 2013). In contrast, the mother and siblings heterozygous for the *TNNT3* splice donor variant were clinically normal. Pathological consequences of a heterozygous null allele in the murine model may relate to the 'faster' characteristics of murine muscles. Interestingly, mRNA expression levels of all fast troponins (*tnnt3, tnnc2 and tnni2*) were significantly downregulated in heterozygous *Tnnt3*$^{lacz/+}$ embryos at E18.5, suggesting co-regulation of the troponin genes during development (Ju et al., 2013). Congenital contractures are thought to occur due to abnormal function of skeletal muscle during fetal development (Beck et al., 2013; Sung et al., 2003). This homozygous *TNNT3* splice variant may therefore exert effects related both to troponin-T_{fast} deficiency, and disruption of normal transcriptional co-regulation of fast troponin isoforms, during development and postnatally.

TNNT3 should be considered as a candidate gene for patients with congenital myopathy, particularly if patients have distal arthrogryposis or histopathology shows preferential involvement of Type-2 fibres.

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Figure 1. Clinical and pathological features in recessive TNNT3 myopathy with deficiency of troponin T_{fast} protein demonstrated by immunohistochemistry. A) Clinical photographs at 2 weeks (*i*), 1 day (*ii* and *iii*) and 9 weeks (*iv*) of age. The proband showed severe weakness of facial and limb muscles, congenital contractures, overlapping digits (*i, iii, iv*) and congenital vertical talus (*ii*). **B)** Immunohistochemistry (IHC) with fast myosin (*i*) shows atrophic fast fibres, and hypertrophic slow fibres. Average diameter of hypertrophied slow fibres was 40 μm; usual mean diameter of type 1 fibres at 3 months of age on frozen section is 12-13 μm. Acid phosphatase (*ii*) shows evidence for degeneration of small atrophic fast fibres. Gomori trichrome (*iii-iv*) and electron microscopy (*v-viii*) identifies numerous nemaline bodies within atrophic small fibres. *v*) Lower power electron microscopy image shows one fibre of normal diameter with normal sarcomeric register (top right of field), immune cells (arrows) and numerous atrophied small fibres (asterisks) bearing small electron dense bodies. vi-vii) Electron microscopy images of increasing magnification show numerous nemaline bodies within atrophied fibres. **C)** IHC of skeletal muscle cryosections from the *TNNT3*-myopathy proband, reveals atrophied Type 2 fibres positive for fast-myosin (red) that show deficiency/markedly reduced levels of TnT_{fast} (green; data using the Sigma antibody shown). A field with oblique atrophied fast fibres is shown in C, to highlight the absence of detectable labelling for TnT_{fast} (green). Scale bar 25 microns.

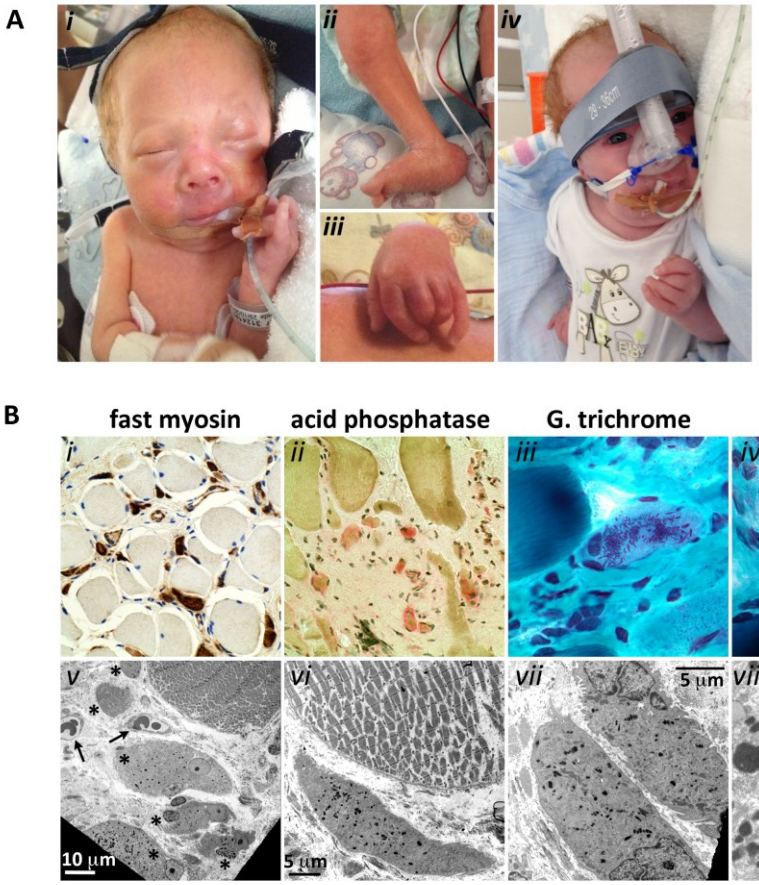
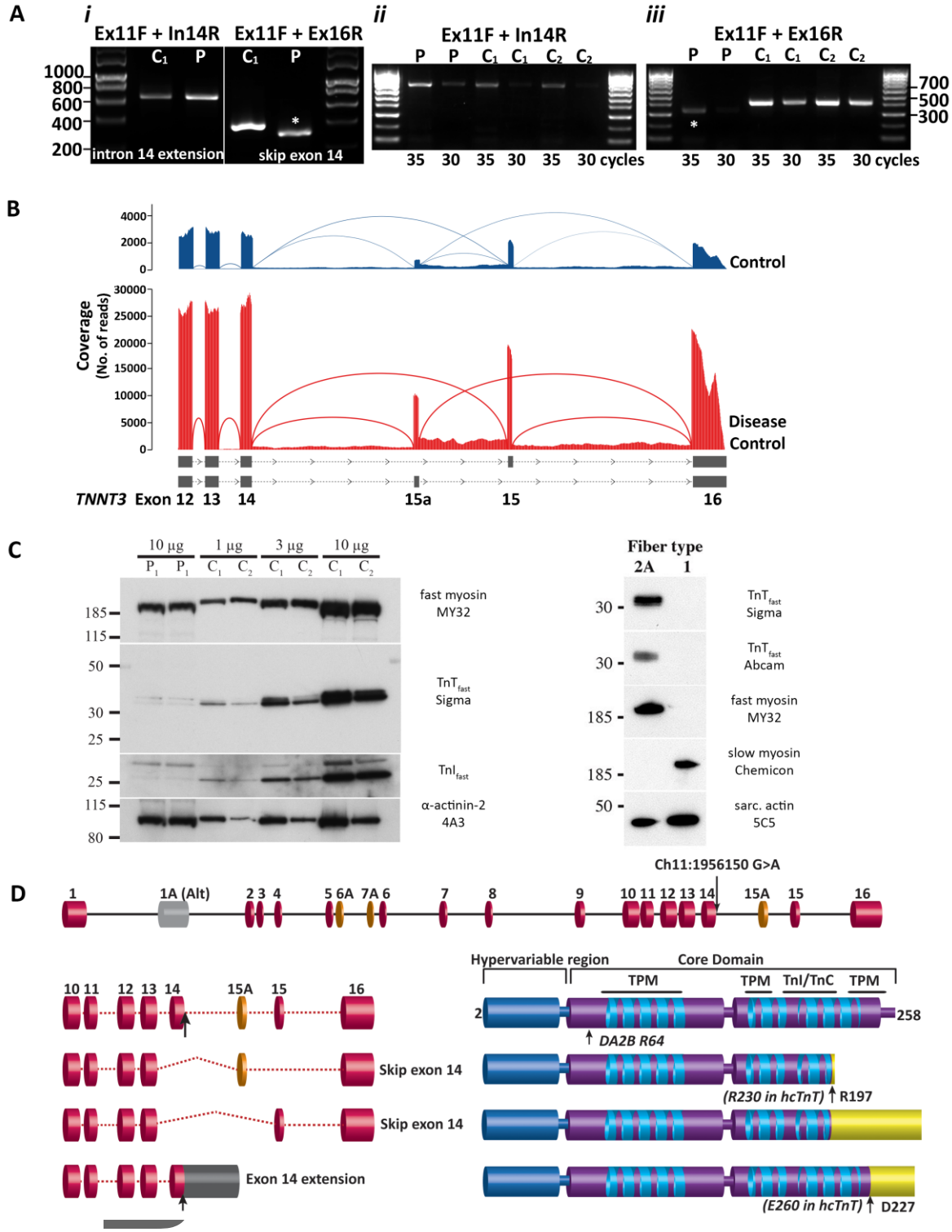


Figure 2. mRNA studies confirm pathogenic outcomes arising from the TNNT3 essential splice donor variant. A) ***i)*** RT-PCR of muscle cDNA from the *TNNT3* proband. *Lane 1*: Exon 11 forward primer and intron 14 reverse primer show higher levels of intron 14 retention in the patient, relative to an age-matched disease control (male, 4 months, quadricep, mitochondrial myopathy). *Lane 2*: Exon 11 forward primer and Exon 16 reverse primer amplified a smaller band in the proband, relative to the control. Sanger sequencing showed skipping of exon 14, and roughly equal levels of transcripts splicing from exon 13 – exon 15a – exon 16, or exon 13 – exon 15 – exon 16. RNA sequencing failed for the *TNNT3* sample. Thus, relative levels of intron 14 retention versus exon 14 skipping were unable to be determined. ***ii-iii)*** Repeat of RT-PCR described in (i), using 30 and 35 cycles to control for saturation, and a second disease control (male, 4 months, quadricep, myotubular myopathy). **B)** Sashimi plots showing RNA sequencing coverage across *TNNT3* exons 12 – 16 (www.gtexportal.org) in controls, showing alternative splicing of either exon 15a or 15. The functional significance of inclusion of either exon is not understood. Sashimi plots also demonstrate low levels of intron 14 retention in controls. **Control 1**: female, age 33 yrs, deceased tissue donor, left gastrocnemius, cause of death cardiovascular. **Disease Control 2:** male, age 18 yrs, right quadricep, mild Becker muscular dystrophy (due to *DMD* splice variant). **C)** *Left:* Western blot normalizing for fast myosin content showed marked reduction to near deficiency of TnT_{fast}, and secondary reduction in TnI_{fast}, in the *TNNT3*-myopathy proband (P1, quadriceps femoris, corrected age 1 month) relative to two age-matched controls (C1: male, vastus lateralis, 4 months; C2: male, vastus lateralis, 4 months). No evidence for a truncated TnT_{fast} protein, or higher molecular weight TnT_{fast} protein, was observed. *Right:* Specificity of TnT_{fast} and TnI_{fast} antibodies for fast troponin isoforms was validated via western blot of single fibres (fast Type 2A or slow Type 1) dissected from human quadriceps. **D)** Schematic of *TNNT3* exon structure and consequences of the *TNNT3* splicing variant on the encoded troponin-T_{fast} protein. *Upper:* Exon structure of the TNNT3 gene based on collective information from (Stefancsik et al., 2003), https://genome.ucsc.edu/, www.gtexportal.org/. Alternately spliced exons shown in orange. Alternate exon 1 shown in greyscale, as it is reported as a very minor species in

www.gtexportal.org/. Exon sizes are relative and to scale of 1 pixel per 10 bases. Intronic distances are relative but not to scale. *Lower:* Schematic of consequences of splicing aberrations on troponin-T_{fast}. Blue coils correspond to predicted alpha-helical domains (Takeda et al., 2003; Wei & Jin, 2011). *Yellow* corresponds to missense sequences encoded due to a frameshift. *hcTnT* - Human cardiac troponin-T.



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