Overexpression of troponin T in *Drosophila* muscles causes a decrease in the levels of thin-filament proteins

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Formation of the contractile apparatus in muscle cells requires co-ordinated activation of several genes and the proper assembly of their products. To investigate the role of TnT (troponin T) in the mechanisms that control and co-ordinate thin-filament formation, we generated transgenic *Drosophila* lines that overexpress TnT in their indirect flight muscles. All flies that overexpress TnT were unable to fly, and the loss of thin filaments themselves was coupled with ultrastructural perturbations of the sarcomere. In contrast, thick filaments remained largely unaffected. Biochemical analysis of these lines revealed that the increase in TnT levels could be detected only during the early stages of adult muscle formation and was followed by a profound decrease in the amount of this protein as well as that of other thin-filament proteins such as tropomyosin, troponin I and actin. The decrease in thin-filament

proteins is not only due to degradation but also due to a decrease in their synthesis, since accumulation of their mRNA transcripts was also severely diminished. This decrease in expression levels of the distinct thin-filament components led us to postulate that any change in the amount of TnT transcripts might trigger the down-regulation of other co-regulated thin-filament components. Taken together, these results suggest the existence of a mechanism that tightly co-ordinates the expression of thin-filament genes and controls the correct stoichiometry of these proteins. We propose that the high levels of unassembled protein might act as a sensor in this process.

Key words: muscle protein, overexpression, protein stoichiometry, thin filament, transgenic *Drosophila*, troponin T.

INTRODUCTION

The role of the highly asymmetric TnT (troponin T) protein in the regulation of muscle contraction is still unclear. However, studies of vertebrate muscles indicate that it is a key component in co-ordinating all the components of the troponin-Tm (tropomyosin) complex, i.e. Tm, TnI (troponin I) and TnC (troponin C) [1]. The troponin tail plays a key role in anchoring troponin-Tm to actin, both in the Ca²⁺ conformation of the thin filament and in the inhibitory conformation found in the absence of Ca²⁺. A complex pattern of TnT protein isoforms can be observed, generated both through differential splicing at the transcript level and through post-translational modification at the protein level [1–4].

The correlation of changes in cardiac TnT isoform expression with heart disease has further augmented research interest in TnT [1]. Indeed, mutations of the vertebrate TnT gene (TNNT2), which encodes the cardiac thin-filament protein TnT, are associated with familial hypertrophic cardiomyopathy, dilated cardiomyopathy or distal arthrogryposis [5–7]. Mutant proteins are supposed to act as dominant-negatives that impair the function of heart muscles [8]. In *Drosophila*, TnT mutants show severe muscle abnormalities, indicating a possible effect of this protein on myofibrillar assembly and/or stability [9]. Furthermore, *Caenorhabditis elegans* TnT mutants have defects in the contraction of the embryonic body wall muscle cells, in sarcomere organization and in cell positioning [10]. However, the mechanisms underlying these defects remain poorly understood.

Sarcomeres, the functional units of myofibrils, are highly complex structures in which all components must be present in the correct amount. Changes in protein stoichiometry result in severe

muscle defects, as has been shown not only in TnT mutants but also in mutants of other muscle proteins such as TnI, actin, myosin heavy chain and Tm [6,11–15]. To maintain this stoichiometry, cells have developed a series of control mechanisms, which are only now beginning to be understood. At the transcriptional level, we and others have recently shown that *Drosophila* thin-filament genes sharing the same spatiotemporal pattern of expression are regulated by enhancer elements with a very similar organization and localization in each locus [16,17]. In other words, co-regulated thin-filament genes share a conserved set of transcriptional regulators that act simultaneously in all of them. Additional systems must also act to control the stoichiometry of muscle proteins at the cellular protein level, although nothing or very little is known about those systems.

We have taken advantage of the simplicity of the *Drosophila* musculature to study several aspects of TnT regulation in the context of the whole organism. In contrast with mammals, there are only a few specialized muscle types generated in *Drosophila* and each muscle type is composed of only one type of fibre [18,19]. These two features provide us with an opportunity to investigate the mechanisms that control and co-ordinate muscle formation. The IFM (indirect flight muscles) of *Drosophila* provide an excellent system for exploring muscle protein function since these muscles are dispensable for viability. Disrupting their structure and function simply impedes flight, providing an easy assay for functional changes [20,21]. Moreover, the insect IFM is similar to vertebrate cardiac muscles in that it displays stretch activation [22]. The sensitivity of IFM function to a decrease or increase in muscle proteins has been studied through different approaches, such as the analysis of mutants or through changes

Abbreviations used: APF, after puparium formation; IFM, indirect flight muscles; ORF, open reading frame; RT, reverse transcriptase; TDT, tergal depressor of trochanter; Tm, tropomyosin; TnC, troponin C; TnI, troponin I; TnT, troponin T; yw, yellow white; UTR, untranslated region.

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Figure 1 Structure of the *Drosophila* construct used for germline transformation

P[w+, Act88F-TnT] was constructed to express TnT under the control of the *Act88F* gene promoter. In the construct, the 5'-UTR of the *Act88F* actin gene was fused to the ORF of the thoracic TnT cDNA.

in gene dosages [9,14,23–25]. Despite the importance of TnT in human disease, the effects of its overabundance have yet to be described. Hence, we have studied the function of TnT by linking the TnT cDNA to a promoter that drives high levels of expression in *Drosophila* IFM, and we have tested the effects of overexpressing TnT on the structure and function of these muscles. We have seen that the excess of TnT causes a secondary decrease of the endogenous TnT and of other thin-filament components: Tm, TnI and actin. Nevertheless, this alteration did not affect the thick-filament proteins, the levels of which remained unaffected. These results suggest that a feedback mechanism exists in muscle cells that co-ordinate the expression of thin-filament proteins and controls stoichiometric imbalances.

EXPERIMENTAL

TnT gene constructs and germline transformation

The vector P[w+, Act88F-TnT] was constructed to express TnT from the *Act88F* gene promoter (Figure 1). The PCR-amplified coding regions for the IFM-specific TnT, including the initiator methionine and the translational stop codon, were cloned into the *Drosophila* P element vector CaSpeR4 [26] using appropriate restriction sites in the primers. In the construct, the 5'-UTR (5'-untranslated region) of the *Act88F* actin gene was fused to the ORF (open reading frame) of the TnT cDNA and the *Act88F* gene promoter was used to drive high expression of TnT in the IFM. All cloning junctions and DNA insertions were sequenced to ensure the proper fidelity and orientation for expression.

The construct was cloned into a CaSpeR vector for P element-mediated transformation. The *white* gene was used as a marker in the CaSpeR vector and germline transformation was performed as described by Spradling and Rubin [27]. The P element helper plasmid was Δ2-3 [28] and the concentrations of purified DNA injected were 0.6 µg/ml for the P element construct and 0.2 µg/ml for Δ2-3. Constructs were injected into embryos of the genotype *yw* (*yellow white*) and the transformants were identified in the G1 generation as orange-eyed individuals. Homozygous lines were obtained by standard genetic techniques using the stock *yw*; *CyO/If*; *TM2/MKRS* from Dr S. Campuzano (Consejo Superior de Investigaciones Científicas, Madrid).

Drosophila maintenance

Flies were grown on Carpenter's medium at 25 °C [29].

Flight and jump testing

Flight testing was performed as described previously on 1-day-old flies [13]. For each line, 100 flies were tested for their ability to fly up towards a light source.

RNA purification, Northern hybridization and RT (reverse transcriptase)-PCR

Total RNA was prepared using the guanidine/phenol method [30]. Total RNA from pupae of the transformed lines was purified and Northern-blot analysis was performed as described previously [4]. RT-PCR using RNA from distinct transformed lines was performed by standard methods [30]. Sequencing was carried out by automatic sequencing following the manufacturer's instructions (Applied Biosystems, Warrington, Cheshire, U.K.) and the sequences were analysed using the GCG software (version 7.1) [31].

Electron microscopy

Transmission electron microscopy was performed using red-eye pupae and 1-day-old adult flies as described by O'Donnell and Bernstein [32]. The dissected flies were fixed overnight with 2 % (w/v) glutaraldehyde, 3 % (w/v) paraformaldehyde and 0.2 % tannic acid in 0.1 M phosphate buffer (pH 7.2) at 4 °C. Samples were washed with 0.1 M phosphate buffer and post-fixed in 1 % osmium tetroxide in 0.1 M phosphate buffer for 2 h on ice. After washing with double-distilled water, the samples were dehydrated in acetone and then infiltrated with Epon 812. Samples were polymerized for 48 h at 60 °C under vacuum and thin sections (90 nm) were obtained using a Reichert Ultracut S ultramicrotome. These sections were double-stained with 2 % uranyl acetate and lead citrate and then viewed with a Zeiss EM10 transmission electron microscope at 60 kV.

Protein gel electrophoresis and immunoblot analysis

The thoraxes from acetone-freeze-dried 24-h-old flies were micro-dissected [33], homogenized and boiled in sample buffer [34]. Electrophoresis and immunoblot analyses were performed essentially as described previously [35]. The amount of protein loaded for each sample was approx. 10 µg. The polyclonal rabbit antisera to *Drosophila* TnT and paramyosin used have been described previously [3,36]. Polyclonal rabbit antiserum to *Drosophila* *Act88F* actin was raised against the specific N-terminal region of the protein (M. C. Lebart and J. C. Sparrow, personal communication) and the secondary antibodies used were from Nordic Immunological Laboratories. Antibody detection was performed with the ECL[®] system (Amersham Biosciences, Little Chalfont, Bucks., U.K.) and the protein bands visualised in Western blots were scanned and normalized to the major band present in a Coomassie Blue-stained protein gel run in parallel. Protein transfer was controlled by staining the membrane with Ponceau Red and the time of ECL[®] development was approximately calibrated to establish the linear range for the amount of protein used. To adjust the data, several gel electrophoresis analyses were performed with distinct amounts of protein in proportion to the protein levels present in the *yw* flies. The experiment was repeated several times to obtain information on variability and we found the results to be very consistent.

RESULTS

TnT protein and mRNA expression in flight muscles of transgenic flies

The *Drosophila* TnT gene is transcribed into four muscle-type-specific products that are generated by developmentally regulated alternative splicing. Exons 3, 4 and 5 are absent from the transcripts found in the jump (TDT, tergal depressor of trochanter) and IFM, the major muscles present in the thorax. All 11 exons

Table 1 Transgenic lines transformed with the P[w+, Act88F-TnT] construct

Construct	Size including CaSpeR vector	Transgenic lines	Chromosomal location
P[w+, Act88F-TnT]	12.169 kb	OTnT48	3rd
		OTnT37	2nd
		OTnT32	2nd
		OTnT30	3rd
		OTnT19	2nd
		OTnT15	Not determined
		OTnT5	2nd

are incorporated into the adult hypodermic muscle transcripts, whereas microexon 4 is absent from the larval hypodermic musculature [4].

The effect of overexpressing TnT in transgenic *Drosophila* flies was studied by employing a strong promoter to drive the high levels of expression in the IFM. Hence, 1 kb of the 5'-flanking sequence and the 5'-UTR of the *Drosophila* Act88F actin gene were fused to the ORF of the IFM-specific TnT cDNA, this construct being named P[w+, Act88F-TnT] (Figure 1). The promoter region used is sufficient to drive high levels of IFM-specific expression, since the *Drosophila* Act88F actin gene is almost exclusively expressed in these muscles [14,23,37–39].

The resulting construct was used to transform *yw* flies. In these transgenic studies, TnT was expressed in a background of endogenous TnT. Several different transgenic lines were identified after microinjection of the P element construct and these lines were named OTnT (Table 1). The presence of the transgenic TnT transcript was confirmed by RT-PCR (results not shown). No significant differences were observed between the independent lines in terms of the characteristics described below. The TnT protein levels of four representative OTnT lines carrying the P[w+, Act88F-TnT] construct were estimated by analysing the thorax of newly emerged flies by immunoblotting (Figure 2). Unexpectedly, the level of TnT varied slightly between different transgenic lines, although in all the lines a decrease in the amount of TnT protein was detected when compared with *yw* control animals. The protein recognized by the TnT antibodies corresponded to the IFM and TDT muscle-specific isoform [4]. The levels of thoracic TnT in the OTnT lines were decreased approx. 2-fold in late pupae and newly emerged flies. This decrease probably corresponded to the TnT present in IFM. Surprisingly, we also observed a profound decrease in the levels of the IFM-specific Act88F actin in the same membranes. In contrast, the levels of paramyosin, a component of the thick filament, were apparently normal. Hence, whereas components of the IFM thin filaments, actin and TnT were markedly decreased in these animals, thick-filament components may not have been affected.

The Act88F gene promoter is activated 14–16 h after the formation of the puparium [40,41]. When we tested the levels of TnT at this stage of development, in six independent lines, an approx. 2.0–2.1-fold increase of TnT protein levels was observed with respect to *yw* controls. In Figure 3, four representative lines are shown. The level of actin Act88F, the IFM-specific isoform, in the six lines is similar to the *yw* control (Figure 3). Thus it appears that the amount of TnT protein increases in the earlier puparium stages, yet degradation or diminution of the protein levels seems to occur at later stages of development.

To determine whether this decrease in TnT in the OTnT lines was due to either diminished synthesis or protein degradation, we measured the relative levels of the transcripts of thin- and thick-filament components from newly emerged flies in Northern blots. Transgenic and endogenous TnT, Act88F actin, TnI, Tm2 and

Figure 2 Expression of TnT in newly emerged flies of the OTnT lines

(A) Thoracic protein homogenates from the transgenic flies were separated by PAGE and stained with Coomassie Blue. The OTnT transgenic lines analysed were OTnT32, OTnT48, OTnT30 and OTnT5; *yw* indicates the *yw* control flies. (B) Western-blot analysis of the TnT, Act88F actin and paramyosin protein expression. The TnT thoracic band corresponds to IFM and TDT isoforms. In contrast, the Act88F actin band corresponds exclusively to IFM isoform.

paramyosin mRNA from red-eye pupae were tested in all lines (Figure 4). Since the IFM and TDT TnT isoforms differ from the others owing to the absence of exons 3, 4 and 5, it proved impossible to make an IFM-specific TnT probe. The TnT probe hybridized with all TnT transcripts, and we found a decrease in the endogenous levels of TnT mRNA in all the OTnT lines tested, with no significant variation among the different lines (Figure 4B). The expected size of the transgenic TnT transcript containing the 5'-UTR of the Act88F mRNA was slightly larger than that of the corresponding endogenous TnT mRNAs (Figures 4B and 4C). However, this transgenic TnT transcript could only be detected in the overexpressing lines after longer exposure of the Northern blots (Figure 4C), being observed in the RNA from late pupae from all OTnT lines analysed. The marked decrease in the Act88F actin, Tm2 and TnI mRNAs – all components of the thin filament – should be emphasized. Indeed, when Act88F actin and TnI probes were hybridized to IFM-specific mRNAs, the transcripts were almost undetectable (Figures 4D–4F). On the other hand, paramyosin mRNA levels were normal, confirming the data obtained from Western blotting (results not shown).

Overexpression of TnT causes muscle dysfunction and myofibril degeneration

To examine if changes in the degree of expression of TnT in flight muscles impaired muscle function, we tested adults from each transgenic line for their capacity to fly (Figure 5). Flies from all OTnT lines were completely unable to fly, although their skeletal and smooth muscles remained functional as evident from their

Figure 3 Expression of TnT and Act88F at 14 and 16 h APF

Protein extracts of the OTnT37, OTnT48, OTnT32 and OTnT30 lines obtained from pupae at 14 and 16 h APF were separated by PAGE and stained with Coomassie Blue. **(A)** In the upper part, a Western blot of the IFM- and TDT-specific TnT isoforms is presented. In the lower part, a densitometric analysis of bands in the Western blot is shown. **(B)** In the upper part, a Western blot of the IFM-specific Act88F isoform is presented. In the lower part, a densitometric analysis of bands in the Western blot is shown. The relative amounts of TnT and Act88F were normalized to the total protein in each sample and to the TnT protein in yw control pupae.

ability to hatch and survive. Our results indicated that changes in the levels of TnT generate an important impairment of flight.

We analysed the ultrastructure of the IFM from representative lines using transmission electron microscopy to determine whether the defects in muscle function were translated into structural abnormalities in the muscles. Samples from late pupae and newly emerged flies from representative lines were used to ensure that any defects observed were due to abnormal assembly rather than to use- or time-dependent degeneration, as seen in some other mutants [23,42]. The IFM ultrastructure from wild-type flies is extremely regular (Figures 6A, 6B and 6G). Myofibrils are circular in cross-section (Figure 6B) and contain regular sarcomeric units aligned in parallel between adjacent myofibrils in longitudinal section (Figure 6A). Furthermore, in transverse sections, the thin and thick filaments form a regular hexagonal lattice array (Figure 6G). Pupae and 1-day-old adult flies from the OTnT lines exhibited severe abnormalities in the ultrastructure of their IFM myofibrils as seen in Figure 6. There was virtually a complete absence of thin filaments from these muscles, although in longitudinal sections, thick filaments associated into myofibril-like

Figure 4 Changes in the mRNA levels of thin-filament proteins in OTnT transgenic lines

Total RNA from red-eye pupae of the lines was separated in a denaturing gel and, after Northern blotting, hybridized to distinct probes: total endogenous TnT **(B, C)**, Act88F actin **(D)**, TnI **(E)** and Tm2 **(F)**. In the upper part, rRNAs of the lines analysed in **(A)** are shown. yw corresponds to the yw control flies. On longer exposure of the Northern blot, the transgenic TnT transcript in the OTnT lines could be observed **(C)**.

structures with Z-discs and occasional M-lines were observed. However, the sarcomere length was variable and myofibril diameters were decreased. Most of the muscles were severely disrupted and a high percentage of myofibrils had incomplete Z-discs and M-lines. This muscle degeneration varied between the six muscles that comprise the dorsal longitudinal IFM, from a moderate disruption to severely affected dorsal longitudinal IFMs, even within the same fly (Figure 6F). Swollen sarcoplasmic reticulum-like structures were frequently observed. These results suggest that changes in TnT levels influence the assembly and stability of thin filaments, which are required for sarcomere assembly. The

Figure 5 Differences in the capacity of the transgenic lines to fly

Flight performance was evaluated by scoring the number of staged flies that could fly upwards (white) or glide (grey) or that were unable to fly (black) when released in a cage. The wild-type response is to fly up towards a light source and the level of flight impairment can be noted as the percentage of flies in other categories. Nearly 65% of 1-day-old yw control flies can fly up (reaching 80–85% in older flies), whereas this ability was severely impaired in the transgenic fly lines. The flies from the OTnT37, OTnT48, OTnT30, OTnT32 and OTnT5 lines are completely flightless.

severe degeneration of the myofibrillar ultrastructure in OTnT flies correlates with their flight impairment.

DISCUSSION

In the present study, we have analysed the effects of overexpressing a thin-filament protein, TnT, in highly specialized muscles such as the IFM of *Drosophila melanogaster*. We have seen that increasing the levels of TnT in these muscles during early stages of pupal development ultimately provokes a decrease in its levels, as well as that of all other thin-filament components (Figures 2–4). TnT accumulation can be detected at 14–16 h APF (after puparium formation) when the *Act88F* gene promoter used to drive the expression of transgenic TnT becomes active [40,41] (Figure 3). Surprisingly, when the muscles should be completely formed, by the end of pupation, much lower levels of this protein are detected when compared with the wild-type. Interestingly, this decrease in protein quantity affects not only TnT, but rather all the thin-filament proteins. Moreover, this decrease not only affects protein accumulation but also its synthesis. The mRNA levels for all the thin-filament proteins are significantly lower when compared with control flies (Figure 4). However, these alterations do not appear to affect thick-filament proteins, the levels of which appear to remain normal. The decrease in thin-filament components unavoidably leads to a loss of thin filaments themselves and, therefore, to alterations in myofibril assembly and function (Figures 5 and 6).

This effect on the accumulation of TnT protein and mRNA cannot be attributed to the promoter used to drive the expression of TnT specifically in IFM. The *Act88F* gene promoter has been successfully used to overexpress other sarcomeric proteins in these muscles, such as the thick-filament proteins miniparamyosin and the myosin heavy chain, or the *Act88F* actin itself [14,23,25]. In none of these studies was a decrease in the levels of these proteins

detected, indicating that the effect observed in the IFM of the OTnT lines is specific to TnT.

In addition to a decrease in TnT, in OTnT lines there is also a substantial decrease in the levels of its partners in the thin filaments. The decrease in accumulation of the associated thin-filament proteins has been reported in several *Drosophila* IFM thin-filament null mutants: *TnI*, *TnT*, *Tm2* and *Act88F* actin [9,11,15,43,44]. Moreover, these secondary effects are not restricted to *Drosophila*. In zebra fish, a mutation of the *TNNT2* gene (silent heart, *sih*) has been found to decrease severely not only the expression of TnT but also TnI3 and Tm- α [45]. In several of these mutants, a decrease in the mRNA levels of thin-filament genes has been reported, suggesting that they are transcriptionally down-regulated [11,45]. However, the levels of thick-filament components seem to be unaffected. Taken together, these results suggest the existence of some form of communication between the thin-filament genes that co-ordinate their expression. In every muscle cell, these genes are expressed concomitantly throughout the *Drosophila* life cycle [46,47]. Since they contribute to the same structure, their expression must be co-ordinately activated to form the filaments and therefore the myofibrils and muscles during myogenesis. Recent studies have demonstrated that the expression of these genes is regulated by enhancers that show a very similar structure and localization in the gene, suggesting a common mechanism of transcriptional regulation [16,17] (R. Marco-Ferreres, J. J. Arredondo, B. Fraile and M. Cervera, unpublished work).

Surprisingly, for OTnT lines the decrease in thin filament components is triggered by an initial overexpression in TnT protein. This decrease might be easily explained by an increase in TnT degradation. Some studies on the slow isoform of vertebrate TnC have shown that the overexpression of its mRNA in C2 cells did not produce a corresponding increase at the polypeptide level. The protein is accumulated in the cytoplasm and its degradation was significantly augmented [48]. However, this does not seem to be the only explanation since we detected a notable decrease in the mRNA levels. It is probable that the increase of TnT in the muscle cell involves an increase in unassembled TnT in the cytoplasm. We propose that this unassembled TnT is somehow detected, causing the activation of the degradation machinery and triggering a signal to the nucleus that leads to a decrease in the transcription of TnT. In this way, the accumulation of TnT will be impaired. Since the transcription of thin-filament genes is co-ordinated, this signal would affect the transcriptional regulation of all of them to avoid a stoichiometric imbalance. In the absence of one of the thin-filament proteins, such as in the null mutants, the cytoplasmic accumulation of soluble thin-filament partners would trigger the same effect.

Alternatively, our results might indicate that an increase in TnT might favour the formation of inappropriate complexes with the other thin-filament proteins, generating aberrant and instable filaments that would be quickly degraded. This increased rate of degradation would be transmitted to the nucleus to stop the transcription of thin-filament genes. It has been shown that the accumulation of unfolded proteins in the endoplasmic reticulum activates the 'unfolded protein response pathway' [49]. The cell responds by eliminating the unfolded proteins through activation of the degradation machinery and decreasing the transcription [49]. A similar undefined mechanism may explain the effect of increased TnT levels.

Previous studies on thin-filament protein mutants showed that the lack of one component causes the down-regulation of the co-regulated thin-filament proteins. This effect is conserved across evolution since it has also been observed in zebra fish TnT mutants [45]. The lack of any of the thin-filament components will lead to

Figure 6 Analysis of the ultrastructure of the IFM of OTnT transgenic lines revealed severe myofibril degeneration

(A, B) Longitudinal and transverse sections of adult IFM yw control. (C, D) Longitudinal sections of IFM of newly emerged flies from the OTnT19 transgenic line, where the arrows indicate the presence of swollen sarcoplasmic reticulum. (E, F) Longitudinal sections of the IFM of newly emerged flies from the OTnT32 transgenic lines. The stars indicate the presence of unknown circular electron-dense material. (G, H, I) Transverse sections of adult IFM yw control, OTnT19 and OTnT32 transgenic lines respectively. Scale bars, 2 μm (A–F) and 0.25 μm (G–I).

the accumulation of the others, relative to the missing one. This is analogous to the situation in our studies with *Drosophila* where the overexpression of one component leads to its accumulation in the cytoplasm. Taken together, the results suggest that, in response to the accumulation of any unassembled thin-filament protein in the cytoplasm and to keep the required stoichiometry, a signalling pathway is activated to stop the accumulation of thin-filament proteins. We think that this pathway might be the same

that works to stop the production of thin-filament proteins when myofibrils are completely formed. Indeed, this mechanism would be independent of that controlling the expression of thick-filament components, in keeping with the failure to detect any effect on the expression of thick-filament proteins in our OTnT flies.

We have demonstrated here that an excess of TnT is as harmful as a deficit, since both produce a loss of thin filaments and, hence, profound alterations in muscle formation. We propose that

a regulatory mechanism must exist to co-ordinate tightly the levels of thin-filament proteins. The OTnT lines could provide us with an ideal system to study the components of this regulatory system *in vivo*.

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