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A Tropomyosin-2 Mutation Suppresses a Troponin I Myopathy in *Drosophila*

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A suppressor mutation, *D53,* of the *held-up*^{2} allele of the *Drosophila melanogaster Troponin I* (*wupA*) gene is described. *D53*, a missense mutation, S185F, of the *tropomyosin-2*, *Tm2,* gene fully suppresses all the phenotypic effects of *held-up*^{2}, including the destructive hypercontraction of the indirect flight muscles (IFMs), a lack of jumping, the progressive myopathy of the walking muscles, and reductions in larval crawling and feeding behavior. The suppressor restores normal function of the IFMs, but flight ability decreases with age and correlates with an unusual, progressive structural collapse of the myofibrillar lattice starting at the center. The S185F substitution in *Tm2* is close to a troponin T binding site on tropomyosin. Models to explain suppression by *D53*, derived from current knowledge of the vertebrate troponin-tropomyosin complex structure and functions, are discussed. The effects of S185F are compared with those of two mutations in residues 175 and 180 of human α-tropomyosin 1 which cause familial hypertrophic cardiomyopathy (HCM).

INTRODUCTION

Muscle contraction is usually activated by a neurally stimulated intracellular release of Ca^{2+}. In vertebrate striated muscle, released Ca^{2+} binds to the thin filament troponin-tropomyosin (Tn-Tm) complex, which consists of tropomyosin (Tm) and the troponins T, I and C (TnT, TnI, and TnC). Current models (see Farah and Reinach, 1995; Geeves and Lehrer, 1998; Squire and Morris, 1998) agree that Ca^{2+} binding to TnC changes the conformation of the Tn complex, releasing the inhibitory binding of TnI to F-actin and allowing Tm to move across the actin surface (Vibert *et al.*, 1997). This movement cooperatively increases myosin accessibility to F-actin, activating the cross-bridge cycle.

Muscle protein interactions are generally well characterized, but the pathway from Ca^{2+} binding to contraction cannot yet be described in molecular detail. The amino acid sequences of vertebrate thin filament proteins (actin, Tm and Tn) are known and their general location in the Tn complex has been determined by low resolution structural studies (White *et al.*, 1987; Al Khayat *et al.*, 1995; Cabral-Lilly *et al.*, 1997) and biochemical analyses. The atomic structure of part of the complex was recently determined (Vassylyev *et al.*, 1998) and functional information derives from considerable biochemical research (see Farah and Reinach, 1995; Geeves and Lehrer, 1998).

Thin filament protein mutants with phenotypic effects are important in identifying residues with significant in vivo roles. Such mutants are known in humans through familial hypertrophic cardiomyopathies (HCM) (Redwood *et al.*, 1999), in the nematode *Caenorhabditis elegans* (Kagawa *et al.*, 1997; McArdle *et al.*, 1998), and *Drosophila melanogaster,* where mutations affecting the indirect flight muscles (IFM) have been recovered (see Bernstein *et al.*, 1993). The *D. melanogaster* IFMs are striated muscles and contain a Ca^{2+}-regulated thin filament system but activation also requires applied strain (Peckham *et al.*, 1990), as also found in vertebrate cardiac muscle.

We aim to study the function of the *Drosophila* Tm-Tn complex by isolating mutations that suppress the phenotypes of selected TnI mutants. *held-up*^{2} (*hdp*^{2}) a missense mutation of the TnI gene, produces a fully penetrant raised wing phenotype. Six dominant suppressors of *hdp*^{2}, including *D53,* were recovered (Prado *et al.*, 1995). Here we show that *D53* is a missense mutation, S185F, of the *Tm2* tropomyosin gene and characterize the functional and structural effects of the suppression. We discuss these results with respect to known interactions within the vertebrate Tn-Tm complex. We observed that S185F occurs within a region of the *Drosophila* Tm which is homologous to that containing

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two human cardiac α-tropomyosin mutations which cause HCM and compare the effects of *D53* with these mutations.

MATERIALS AND METHODS

Stocks and Fly Strains

Flies were maintained at 25°C on a yeast-sugar-agar medium. Unless otherwise stated, strains were obtained from the European Stock Center (Umeå) or the MidAmerica Drosophila Stock Center (Bowling Green). All chromosome and gene symbols unless specifically described are as in Flybase on http://flybase.bio.indiana.edu/. For the two Tm genes, the symbols *Tm1* (was *TmII* - Bautch *et al.*, 1982) and *Tm2* (was *TmI*) are used as proposed in Karlik *et al.* (1984). The *hdp* and *up* alleles are in genes which encode respectively TnI, *wupA* (*wings-up A*) (Beall and Fyrberg, 1991; Barbas *et al.*, 1991) and TnT, *up* (*upheld*) (Fyrberg *et al.*, 1990). The *wupA* gene is the sole TnI-encoding gene in *Drosophila* (Barbas *et al.*, 1991). The hdp^2 mutation is in constitutive exon 5 (Beall and Fyrberg, 1991; Prado *et al.*, 1995) where it causes a substitution of alanine116 by valine, A116V (using the numbering system of Prado *et al.*, 1995 for *Drosophila* TnI residues which includes the IFM-specific exon 3 which introduces a 61-residue N-terminal extension).

Microscopy Procedures

For polarised light photomicroscopy thoraces were prepared and mounted as described in Nongthomba and Ramachandra (1999). Fly half-thoraces were prepared for transmission electron microscopy (TEM) as described in Kronert *et al.* (1995).

PCR and Sequencing

A 3334 bp fragment including the complete *Act88F* coding sequence was PCR amplified with *Taq* DNA polymerase from genomic DNA of hdp^2 and hdp^2; *D53* flies using the 5′TGTAGGTGGAGCTAACCGTGTGC (sense) and 5′GCTGCCTTTGAAGAGCTTTCGG (antisense) primers. The amplified product was gel-purified (Geneclean, BIO101), ligated into the pGEM-T vector system (Promega), and transformed into TG1 rec^o cells. DNA from three separate clones was extracted, purified, sequenced with internal oligonucleotide primers using an ABI Prism Dye Cycle Sequencing Kit containing *AmpliTaq* DNA polymerase (Perkin Elmer-Cetus), and analyzed using an in-house automated sequencer.

RT-PCR was used to amplify the *Tm1* gene exons that are spliced to form the 'mTm' (muscle tropomyosin) mRNA. This mRNA is derived by splicing together exons 1–3, 5, 7, 8, 10, 11, 13 and 17 (Karlik and Fyrberg, 1986; Hanke and Storti, 1988). Total RNA was isolated from newly emerged hdp^2 and hdp^2; D53 flies and reverse transcribed using random nucleotide hexamers and Moloney murine leukemia virus reverse transcriptase (Life Technologies). The cDNA was used in PCR reactions with *Pfu* DNA polymerase (Stratagene) using 5′GTTCAAGTCGCGGATAACTCCGAATAAAAGTT (sense) and 5′CAGTGCGCCTACGATTATGC (antisense) primers to amplify specifically the coding region. The PCR products were gel purified, ligated into pGEM-T, and transformed into TG1 rec^o cells. Three clones from two separate RT-PCR reactions were sequenced as above.

DNA extracted from *Oregon-R*, hdp^2, and *D53* flies was used for PCR amplifications with *Pfu* DNA polymerase (either native or cloned) of overlapping *Tm2* genomic fragments containing exons 2, 3 and the coding sequence of exon 4. The primers used were 5′AGGATTCAGTTATTCAGCATAC, 5′TCCTCAATCTGTTGCACCTT, 5′TTGGTATCGGCATCCTCAGC, 5′GGAGGAGGAGCTGAAGGTGG, 5′GGGAGTTGCCGACAACCTAGT, 5′GGGTGGTCAAGGGCATTGTGTG, and 5′CAAACTGAACGAGGAGGTGC. Amplified products from a minimum of two PCR reactions for each fragment were purified and sequenced as described above.

Adult and Larval Muscle Performance Tests

Flight testing was done as previously described (Drummond *et al.*, 1991). Individual flies were scored for flight upwards (U), horizontal (H), downwards (D), or not at all (N). The mean flight score, [U + H]/Total flies, was arcsine transformed to give a Normal distribution. Five samples, > 50 flies, were scored. Wing beat records were obtained and analyzed as described in Schmitz *et al.* (2000) using 6 flies per genotype, each sampled 3 times.

Adult walking ability was measured in a vertical 100-ml glass measuring cylinder, internal diameter 30 mm, with a line marked 80 mm above the base. For each test 8–30 flies were introduced into the cylinder. Flies were knocked to the cylinder bottom and the time taken for 50% of the flies to walk past the marked line was scored.

Late third instar larval crawling was measured on 1.5% (wt/vol) agar-sucrose medium in plates marked with a 0.5-cm grid, as total gridlines crossed in 5 min. Larval feeding movements were quantified by counting nonlocomotory, pharyngeal movements in a 2-min period on agar plates thinly coated with yeast. Larvae which did not feed continuously were discarded.

RESULTS

Suppressor D53 Is a Tropomyosin 2 Mutation

In hdp^2 flies the IFMs hypercontract (Figure 1C) so that myofibrillar material remains (arrows) only near the muscle attachment sites. This effect is completely suppressed in hdp^2; *D53*/+ (Figure 1D) and hdp^2; *D53/53* flies (not shown), which are indistinguishable from wild-type (Figure 1A). Even in pupae at 78 h APF (after puparium formation), ~ 20 h before adult emergence, the hdp^2 muscles (Figure 1B) begin to hypercontract. IFMs cannot be imaged by birefringence before 78 h, but using a *lacZ* gene promoter-reporter construct and β-galactosidase staining we have shown that developing hdp^2 IFMs have a normal structure at 75 h APF (Nongthomba and Sparrow, in preparation). *D53* suppression mapped to a region on chromosome 3 (Prado *et al.*, 1995) that contains the IFM-specific actin gene, *Act88F*, and two tropomyosin genes, *Tm1* and *Tm2*. Due to the very tight linkage of these genes *D53* was located by sequencing the relevant coding regions of all three genes. The *Act88F* sequence was identical to the GenBank sequence (M18830) except for seven silent codon changes.

The *Tm1* gene produces four alternatively spliced products (Hanke and Storti, 1988). Only one, the mTm mRNA, has an expression pattern coincident with the muscles in which hdp^2 phenotypes are suppressed by *D53* (see below). The *Tm1* coding sequences from hdp^2 and hdp^2; *D53* flies, obtained by RT-PCR, and the wild-type mTm isoform sequence (Swissprot ID:TPM1__DROME) generated by splicing appropriate exons from EMBL sequences L00355-L00363 (Hanke and Storti, 1986, 1988) were identical.

Larval muscles express a *Tm2* mRNA, containing exons 1–4; IFM and leg muscles express a *Tm2* mRNA consisting of exons 1–3 and 5 (Basi *et al.*, 1986; Karlik and Fyrberg, 1986). As *D53* suppresses hdp^2 effects in IFMs, legs, and larval muscles (see below), the common coding exons (2 and 3) were sequenced by PCR of genomic DNA from hdp^2 and hdp^2; *D53* flies. In hdp^2 flies *Tm2* exons 2 and 3 were identical to the wild-type sequence (EMBL AC: K03277; Swissprot TPM2__DROME; Basi and Storti, 1986). However, exon 2 in hdp^2; *D53* flies contained a TCC to TTC mutation in codon 185, which will replace serine by phenylalanine (S185F); exon 3 was wild-type.

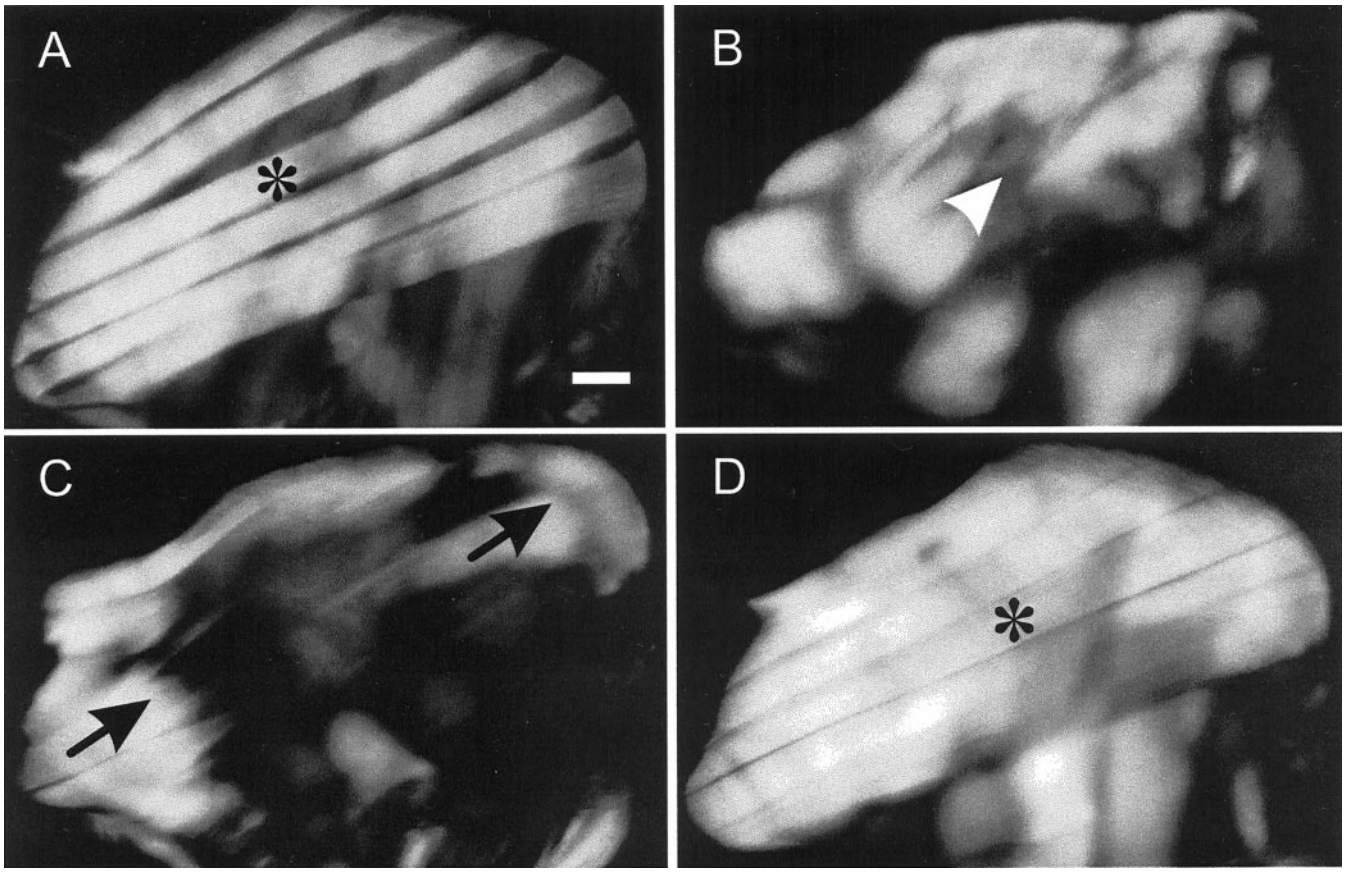

Figure 1. Polarized light micrographs of dissected thoraces to show the progressive hypercontraction of *hdp*^{2} IFMs and suppression of this phenotype by *D53*. Each thorax is shown with the anterior-posterior axis running right to left. (A) Wild-type fly dorso-longitudinal IFMs (asterisk). (B) *hdp*^{2} pupa at 78 h APF to show the initiation of hypercontraction (arrowhead). (C) *hdp*^{2} fly just after emergence showing IFM remnants (arrowheads) after hypercontraction and (D) a *hdp*^{2}*; D53*/+ fly showing complete suppression of *hdp*^{2} hypercontraction. Bar = 0.120 mm.

Suppression of IFM Defects

Flies hemi- or homozygous for *hdp*^{2} hold their wings vertically, but when *D53* was present in one (*+/D53*) or two (*D53/D53*) gene doses, their wings were in the resting position. Young (2–3 days) *hdp*^{2}; *+/D53* or *hdp*^{2}; *D53/D53* flies flew, but less well than wild-type (Table 1A). By 6–7 days old the flight ability of *hdp*^{2}; *D53*/+ flies, but not *hdp*^{2}; *D53/D53*, had been dramatically reduced. These data show that *D53* suppression of the *hdp*^{2} flightless phenotype is dominant and almost complete. The age effect suggests that incomplete suppression in *hdp*^{2}; *D53*/+ heterozygotes causes a progressive deterioration in muscle function. Does this correlate with structural changes to the myofibrils?

Electron micrographs of IFMs from 2–3 d-old *hdp*^{2} flies showed (Figure 2B) an almost complete absence of wild-type myofibrillar structure (Figure 2A). The fibers contained disorganized thick and thin filaments with only small islands of myofibrillar lattice (asterisk) remaining. IFMs of 2–3 d-old *hdp*^{2}; D53/+ flies (Figure 2C) showed an almost complete restoration of wild-type myofibrillar structure. However, some myofibrils showed small areas of disorganized filament lattice at their centers which in longitudinal sections (Figure 2F) were visible as Z-disk dislocations. By 6–7 days, all the myofibrils of *hdp*^{2}; *D53*/+ flies (Figure 2D) contain extensive disorganized centers, an effect never seen in *hdp*^{2}; *D53* (Figure 2E) or *D53* homozygotes (not shown). These structural changes correlate with the reduced flight ability of *hdp*^{2}; *D53*/+ flies with increasing age (Table 1). Since neither occur in *D53* homozygotes, complete *D53* suppression is a recessive character.

Suppression in Adult, Non-IFM Muscles

Since the *hdp*^{2} mutation occurs in a constitutive exon of the sole *Drosophila* TnI gene (Beall and Fyrberg, 1991), its dysfunction in non-IFM muscles should affect behavior. *hdp*^{2} flies cannot jump (Deak *et al.*, 1982) but *hdp*^{2}; *D53*/+ and *hdp*^{2}; *D53/D53* flies jumped readily. Since jumping is powered by the tergal depressor of trochanter (TDT) muscles, *D53* must be expressed there. The walking speed of *hdp*^{2} flies was reduced even in young flies (Figure 3A) compared with wild-type, as reported previously (Deak, 1977) and was further reduced as flies aged (Figure 3A). Older flies appeared crippled, continually fell over, or showed a propen-

Table 1. Flight abilities of adult flies of *hdp*^{2} and *D53* genotypes aged 2 to 3 (A) and 6 to 7 (B) days post-eclosion

	X-chromosome		
Chromosome III	+/+	+/*hdp*^{2}	*hdp*^{2}/*hdp*^{2}
A) 2–3 d			
+/+	81.4 ± 5.0 *	64.9 ± 7.0 *	0.8 ± 0.3 *
+/*D53*	66.9 ± 3.5 (*)	75.6 ± 11.2	62.3 ± 16.4
D53/D53	68.1 ± 4.9	63.8 ± 10.1 *	55.2 ± 6.8
	X-chromosome		
Chromosome III	+/+	+/*hdp*^{2}	*hdp*^{2}/*hdp*^{2}
B) 6–7 d			
+/+	81.9 ± 6.9 *	74.5 ± 7.1 *	0.4 ± 0.03
+/*D53*	70.8 ± 6.5 (*)	60.2 ± 6.2 *	0.4 ± 0.07 *
D53/D53	62.8 ± 6.9	59.3 ± 7.2	42.7 ± 11.3

Figures are in degrees (± 1 SD) due to the arcsine transformation. Ninety degrees would indicate that all flies flew upwards. * between genotypes indicates a difference in flight ability at the 5% level in pairwise Student's *t*-tests; (*) indicates the same level of significance in difference between the *D53/D53* row and that of +/+. The +/+ and *hdp*^{2}/+ columns contain female data; the *hdp*^{2}/*hdp*^{2} data are from homozygous females and hemizygous males.

sity to fall off vertical surfaces. One or two gene doses of *D53* restored walking ability of *hdp*^{2} flies to almost wild-type levels, indicating that *D53* is expressed and suppresses the *hdp*^{2} phenotype in the muscle groups required for walking. Neither *hdp*^{2}; *D53/D53* nor *hdp*^{2}; *D53*/+ flies showed a reduced walking ability with age (Figure 3A). Leg muscles from 12-d wild-type flies (Figure 3B) had a normal fibrillar structure, but in *hdp*^{2} flies of similar age (Figure 3C), although a few muscles showed a normal muscle structure (arrow) most muscles (asterisk and arrowhead) lacked myofilaments and exhibited large intracellular vacuoles (asterisk) and electron dense staining of cells and nuclei (arrowhead). One muscle (arrowhead) has detached from one of its apodemes. These effects correlate with the behavioral studies. Aged *hdp*^{2}; *D53*/+ flies showed (Figure 3D) an intermediate phenotype in which some muscles were clearly damaged (arrowhead) but most appeared normal (arrow). Although the behavioral studies suggested a complete dominant suppression of *hdp*^{2} by *D53*, these structural data indicate that dominant suppression is incomplete.

Suppression of Larval Muscle Defects

As *wupA* must be expressed in larval muscles (see above), *hdp*^{2} should affect larval behaviors. Crawling of *hdp*^{2} larvae was very different from wild-type (Table 2). Many appeared paralyzed in their posterior segments; frequently the posterior was raised, reminiscent of the 'hook' phenotype of kinesin or kinesin-related mutants (Hurd and Saxton, 1996). As *hdp*^{2} larvae crawl, they 'roll'. Locomotory and feeding behaviors of *hdp*^{2}; *D53* larvae were not significantly different from those of wild-type confirming a) that the *Tm2* gene is expressed in larval muscles and, b) that *D53* suppresses the effects of *hdp*^{2} (Table 2) in these muscles.

Muscle Effects due to D53 Mutation Per Se

Often genetic suppressors have their own mutant phenotype. IFMs from *D53* homozygotes have a wild-type structure (Figure 2E). To look for more subtle effects the flight ability of *D53*/+ and *D53/D53* flies was measured (Table 1). Both genotypes showed a small, significant reduction in flight ability that was age-independent. *Oregon-R* flies gave a wing beat frequency of 240.6 ± 23.4 Hz (n = 6 flies) while *D53* homozygotes produced 214.7 ± 18.6 Hz, a modest but significant reduction (Student's *t* test p = 0.041). The walking speed of *D53* and wild-type flies was very similar and did not change with age (data not shown). Thus, *D53* has a mild IFM phenotype, which is not detected in leg muscles.

Specificity of the D53 Suppression

Suppression could result from altered stoichiometry or from specific amino acid alterations affecting protein interactions. Gene dosage studies should reproduce the former type of suppression, whereas the latter should be allele-specific. *Df(3R)ea*^{5022rx1} lacks both the *Tm1* and *Tm2* genes and *Tm2*^{C10} lacks a functional *Tm2* copy (Kreuz *et al*., 1996). Males that had *hdp*^{2}/Y; *Df(3R)ea*^{5022rxl}/+ or *hdp*^{2}/Y; *Tm2*^{C10}/+ genotypes showed partial suppression of IFM hypercontraction but did not fly; *hdp*^{2}/Y; *Df(3R)ea*^{5022rxl}/+ flies had the wings-up phenotype (Table 3). We define partial IFM suppression as thoraces in which muscle fibers often span the complete thorax, but where some do not show continuous birefringence (cf. Figure 4B) from one attachment site to the other. The *hdp*^{2}/Y; *Df(3R)ea*^{5022rxl}/+ and *Tm2*^{C10}/+ results suggest that the reduction in *Tm1* gene copy number did not contribute to suppression. This was confirmed by a lack of *hdp*^{2} dominant suppression (data not shown) by recessive lethal P-element insertion *Tm1* alleles (gift of Dr. C. O'Kane). Other recessive lethal *Tm2* mutations partially suppressed IFM hypercontraction, but all the flies were flightless (Table 3).

Flies hemi- or homozygous for the *hdp*^{3} and *hdp*^{5} mutations of *wupA* also show the wings-up phenotype but in these cases the IFMs fail to develop. These IFM-specific mutations affect alternative transcript splicing of IFM mRNA and produce no TnI protein in the IFMs (Barbas *et al*., 1993). *D53* did not suppress the muscle phenotype of either mutation suggesting that suppression requires the presence of functional TnI protein. The myosin heavy chain "rod" domain mutation *Mhc*^{13}, which causes a recessive hypercontraction of IFMs (Kronert *et al*., 1995), is also not suppressed by *D53*. Thus *D53* is not a general suppressor of IFM hypercontraction. Its lack of effect on *hdp*^{3} and *hdp*^{5} and its more effective suppression of *hdp*^{2} than the *Tm2* null mutations suggest that it is an allele-specific suppressor.

up^{101} is a missense mutation of the troponin T gene (Fyrberg *et al*., 1990). It produces a recessive wings-up phenotype (~ 90% penetrant) and IFM hypercontraction (100% penetrant) indistinguishable from that of *hdp*^{2} (compare Figures 4A and 1C). Homozygous *D53* completely suppressed both phenotypes but *up*^{101}/Y; *D53*/+ flies showed a range

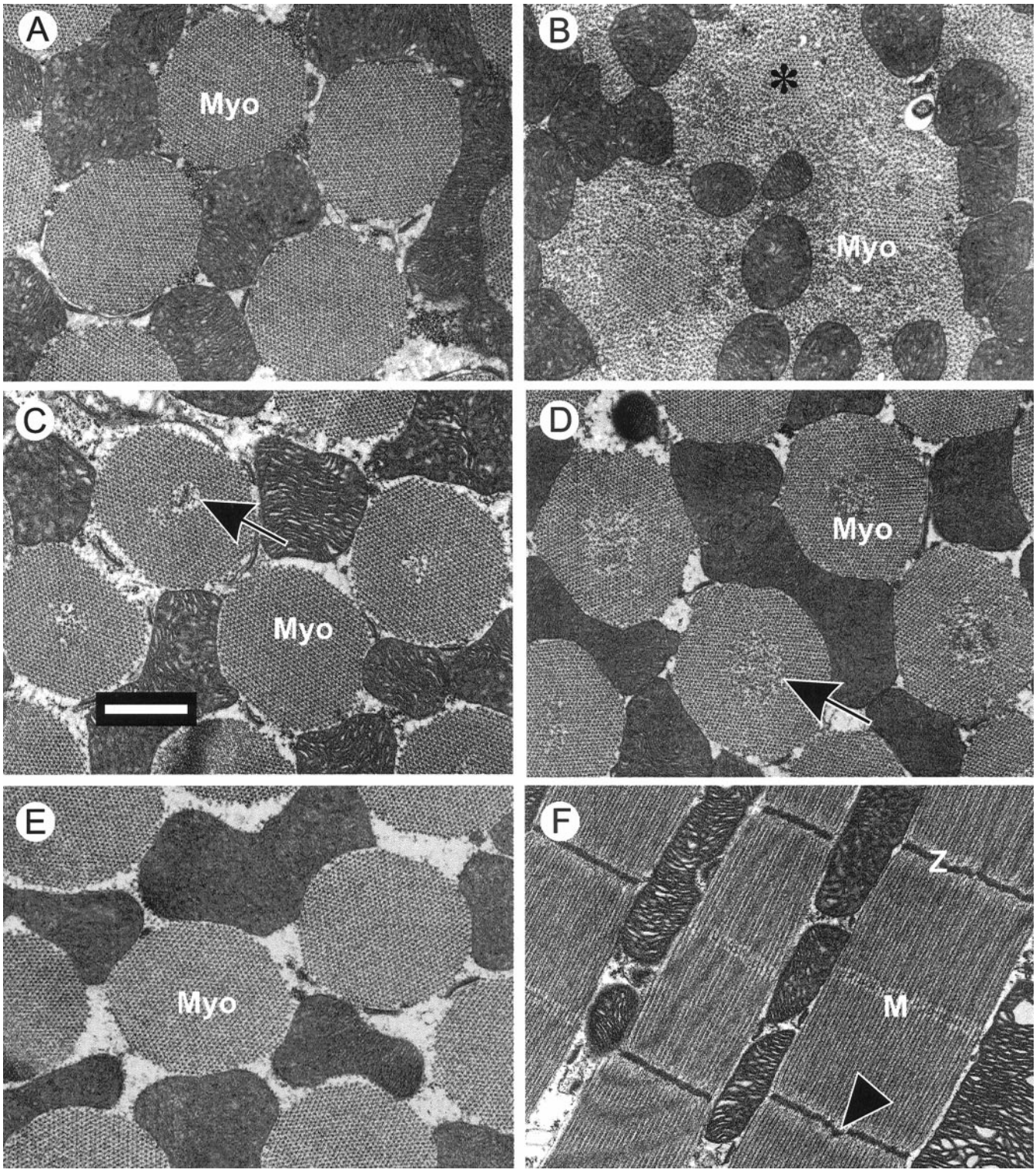

Figure 2. Transmission electron micrographs of *hdp*^{2}*; D53/+* IFMs to show age effects. (A-C) Transverse sections of IFM myofibrils (Myo) from 2- to 3-day-old flies: (A) Wild-type. (B) *hdp*^{2} showing the almost complete disruption of the myofibrils as a result of hypercontraction and (C) *hdp*^{2}*; D53/+* showing suppression but with some myofibrillar lattice disorder at the centers. (D, E) Transverse sections of myofibrils from 6- to 7-day-old flies: (D) *hdp*^{2}*; D53/+* flies demonstrating the increased central disruption of all myofibrils and (E) *hdp*^{2}*; D53/D53* myofibrils showing the complete suppression of *hdp*^{2} myofibrillar defects. (F) Longitudinal section of 6- to 7-day-old *hdp*^{2}*; D53/+* myofibrils, with disruptions at some of the Z-disk centers. Bar = 1 μm.

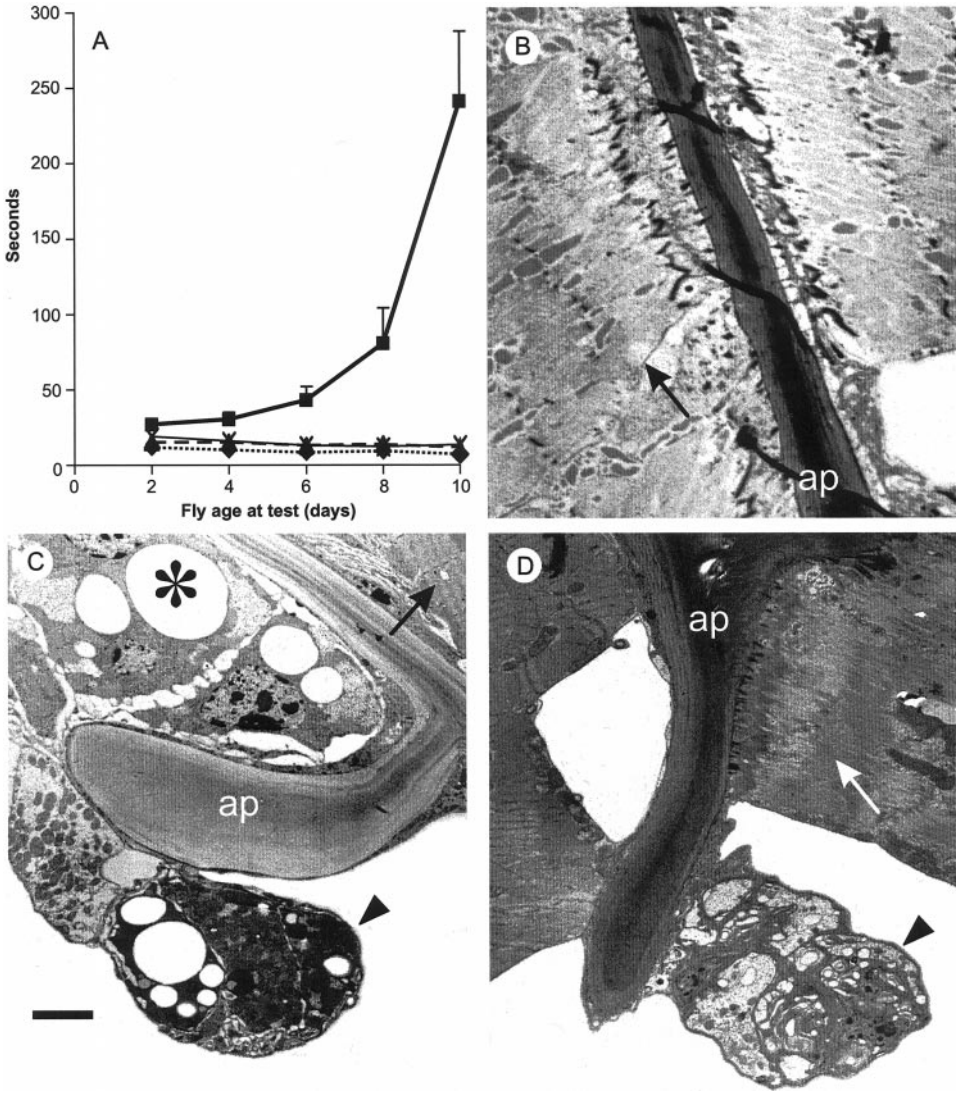

Figure 3. The effects of age on wild-type and *hdp*^{2} leg muscles. (A) Walking ability of *hdp*^{2} (■), *Oregon-R* (♦), *hdp*^{2}; *D53*/+ (×) and *hdp*^{2}; *D53*/*D53* (▲). 'Seconds' is mean time (10 tests/sample) for 50% of flies to walk upwards >80 mm. Error bars $\pm$ 1 SD. (B-D) Electron micrographs of the proximal apodeme (ap) of femur from 12-day-old (B) wild-type, (C) *hdp*^{2}, and (D) *hdp*^{2}; *D53*/+ males. In *hdp*^{2} flies a few muscles were structurally normal (compare arrows in B and C), but some were detached (arrowhead) and with most of the others showed degenerative changes with intracellular vacuoles (*) and electron dense staining of cells and nuclei (arrowhead); in *hdp*^{2}; *D53*/+ flies (D) although some muscles were collapsed (arrowhead), others were attached and had normal structure (compare arrows B and D). Bar = 2 μm.

of partially (Figure 4B) or completely suppressed IFM phenotypes. The wing position phenotype was also partially suppressed by *D53*/+; 39% of the flies had wings in the normal position compared with 10% of control *up*^{101}/Y flies. No *up*^{101}; *D53*/+ or *up*^{101}; *D53/D53* flies could fly.

Adult *up*^{101} flies walked more slowly than wild-type (Student's *t*, P $\ll$0.001), a difference that increased with age (Figure 4C). This effect was smaller than in *hdp*^{2} flies and became extreme only in much older flies (cf. Figure 3A). *D53* suppression of *up*^{101} was apparently complete in walking

Table 2. Larval behavior

		Genotype		
Behavior		*hdp*^{2}	*hdp*^{2}; *D53*	Wild-type
Crawling (grid squares/5 min)		7.7 $\pm$ 2.1	12.1 $\pm$ 2.6**	13.3 $\pm$ 5.7**
	n=	19	19	19
Feeding (movements/min)		100.5 $\pm$ 11.1	155.4 $\pm$ 23.2**	148.4 $\pm$ 27.2**
	n=	113	116	119

The behavior of wild type (Oregon-R) and *hdp*^{2}; *D53* larvae were not significantly different. ** indicates significant difference from *hdp*^{2} (P $<$ 0.01).

Table 3. Effects of *Tm2* null alleles on the hdp^2 phenotype

		Wing position (%)			Muscle fiber phenotype (%)		
Genotype	n	U	D	N	H	P	N
hdp^2/Y; +/+	25	100	0	0	100	0	0
hdp^2/Y; $ea^{5022rxl}$/+	26	100	0	0	50	50	0
hdp^2/Y; *DL2*/+	12	42	58	0	58	25	17
hdp^2/Y; *C10*/+	36	11	89	0	50	50	0
hdp^2/Y; *J8*/+	26	100	0	0	58	35	7
hdp^2/Y; *L2*/+	22	100	0	0	100	0	0

n = number of flies examined. Wing position: 'N' is wild type (normal), 'U' is wings raised (up) position typical of hdp^2, and 'D' is wings held alongside (down) the abdomen. Muscle fiber phenotypes were assessed by polarized light microscopy of bisected thoraces. H, hypercontracted' (*cf.* Figure 1C); P, partially suppressed (see text and Figure 4B); N, wild type/fully suppressed (Figure 1A/1D). *Df(3R)* $ea^{5022rxl}$ is a deficiency that completely deletes *easter, Tm1* (Erdelyi *et al.*, 1995), and *Tm2* (Kreuz *et al.*, 1996); *DL2* is a γ-ray induced dominant flightless, recessive lethal mutation of *Tm2* associated with a translocation breakpoint within 88F (Sparrow, unpublished) and *C10, J8*, and *L2* are recessive lethal, null *Tm2* mutations (Kreuz *et al.*, 1996). All females of genotypes, $hdp^2/+$; $Tm2^x/+$ (not shown; *x* = different alleles) hold their wings in the normal position, are flightless, and show a normal IFM pattern.

muscles because up^{101}/Y; *D53*/+ flies of all ages walked at wild-type velocities (Figure 4C).

Thus, the suppressing effects of *D53* are clearly not gene-specific, but they are weaker in the IFMs with up^{101} compared with hdp^2 (compare Figures 1D and 4B); in walking behavior *D53* acts equally well in suppressing both mutations.

DISCUSSION

The *D53* suppressor is a missense mutation, S185F, of the *Tm2* gene. It suppresses all the effects of hdp^2 on muscle function, consistent with *D53* being a mutation in constitutively expressed exon 2, and *Tm2* expression occurring in the IFMs, TDT, leg muscles and supercontractile larval muscles (Basi *et al.*, 1984; Basi and Storti, 1986; Karlik and Fyrberg, 1986).

The dominant suppression of hdp^2 flightlessness in *D53*/+ heterozygotes, but not *D53* homozygotes, deteriorates with age. Clearly a mixture of mutant and wild-type Tm2 proteins suppresses in young flies but incompletely so as progressive degeneration occurs, unusually, from the myofibril centers outwards. A similar disruption pattern occurs in the *Drosophila* $Act88F^{G245D}$ mutant allele (Sakai *et al.*, 1990) and the intragenic *D3* suppressor of hdp^2 (Prado *et al.*, 1995). These observations suggest that the myofibrillar center and periphery either have different protein constitutions or experience different physiological conditions. Myofibrillogenesis begins at the center (Reedy and Beall, 1993) and different isoforms could be assembled in the myofibril center compared with the periphery but there is no evidence for this. Calcium diffusion into and out from the myofibril upon activation and relaxation will produce temporal differences of Ca^{2+} concentration across the myofibril. If hdp^2 and *D53* affect the response to Ca^{2+} a trans-myofibrillar calcium gradient could generate damaging forces.

Tm2 null mutant heterozygotes produce myofibrils with a normal central lattice but considerable peripheral disruption (Kreuz *et al.*, 1996). Why do they partially suppress hdp^2? Fiber destruction in the hdp^2 hypercontraction likely involves actomyosin generated forces. Kreuz *et al.* (1996) showed that $Tm2^{C10}$/+ fibers produced significantly less work than wild-type. This would explain the partial suppression by extreme *Tm2* alleles and why flight is not restored. A similar argument may explain the effects of the myosin heavy chain gene suppressors of hdp^2 (Kronert *et al.*, 1995) though other explanations including direct interactions between the myosin head and the Tn-Tm complex cannot be excluded.

Structural Implications

The vertebrate inhibitory, actin-binding TnI and the calcium-binding TnC form a globular domain (Herzberg and James, 1985) which sits on TnT, an elongated molecule (see Figure 6A). TnT is the major structural link between TnI + TnC and Tm binding (see Farah and Reinach, 1995) and extends along the carboxy-terminal third of the Tm coiled-coil to overlap with the N-terminus of the adjacent Tm dimer. Two regions of Tm bind TnT one of which, the Ca^{2+}-sensitive binding region, includes residues 170–190.

Residue 185, phenylalanine in *D53,* is indicated in the alignment (Figure 5) of Tm sequences. The *Drosophila Tm2* IFM isoform (TPM0_DROME) has considerable sequence homology with other Tm sequences in this region. TPM0_DROME and TPM1_DROME (the standard muscle Tm encoded by the *Tm1* gene) show many residue differences. Vertebrate Tm sequences are more similar to each other than to invertebrate sequences and residue type conservation occurs at many positions (Figure 5). In part, this reflects the α-helical coiled-coil heptad repeat requirement for hydrophobic residues at most 'a' and 'd' positions. Residue 185 is predicted to be a 'c' residue, implying that the phenylalanine side chain of *D53* will point away from the dimer axis and not affect coiled-coil stability. More probably it affects interactions between Tm and TnT or F-actin.

Our interpretations assume that current models for the vertebrate complex are relevant. This seems likely as *Drosophila* thin filament proteins show substantial sequence homology to their vertebrate counterparts although TnI (Barbas *et al.*, 1991; Beall and Fyrberg, 1991), TnT (Fyrberg *et al.*,

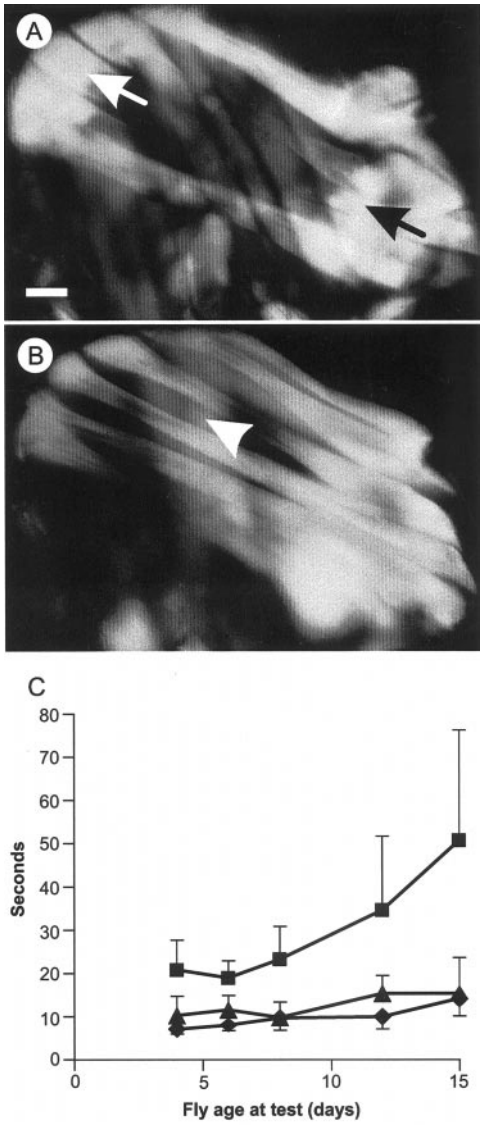

Figure 4. Suppression of up^{101} by *D53*. (A-C) Polarized light microscopy of IFMs. Each thorax is shown with anterior-posterior axis running left to right. Bar = 0.125 mm. (A) In up^{101}/Y birefringence is seen only at the muscle ends. The dorso-longitudinal (DLM) flight muscles are most readily seen, but the opposing dorso-ventral (DVM) muscles are visible. (B) The more complete DLMs typical of partial suppression in up^{101}/Y; *D53*/+ males. (C) Age effects on walking ability of up^{101} (■), up^{101}; *D53*/+ (▲), and wild-type (♦) flies.

1990), and two IFM-specific Tm isoforms (Karlik *et al*., 1984; Hanke and Storti, 1988) have N- or C-terminal polypeptide additions. *Drosophila* and *Lethocerus* (waterbug) IFMs have a very similar physiology (Peckham *et al*., 1990) but in *Lethocerus* IFMs TnI is not detectable and is replaced by a unique 80 kDa molecule called TnH (Wendt *et al*., 1997). EM studies of *Lethocerus* Tn-Tm complexes (Wendt *et al*., 1997) suggest that the globular portion of the Tn complex lies not along the Tm as in vertebrates (Figure 6A) but at the Tm dimer overlap region (Figure 6B). While sequence homology strongly implies structural homology of the *Drosophila* Tn-Tm complex to that of vertebrates, common physiology argues for a structural similarity to *Lethocerus*. However, the orientation of the *Lethocerus* Tn complex with respect to the Tm N- and C-termini could not be determined from electron micrographs (Wendt *et al*., 1997). When their figure (Figure 6B) is inverted (Figure 6C) it shows that the Tm, TnT and F-actin in both insects and vertebrates could maintain the same relative position though the remainder of the troponin complex is located differently.

Functional Implications

Current models (Geeves and Lehrer, 1998; Squire and Morris, 1998) suggest that in the absence of Ca^{2+}, TnI binds actin maintaining the 'blocked' filament state (Geeves and Lehrer, 1998) with Tm occluding much of the myosin binding site on actin. Ca^{2+} binding to TnC changes TnC/TnI conformation, releasing the TnI inhibitory domain from actin, producing the 'closed' state, and Tm with its attached Tn complex, can now move across the F-actin surface. This exposes the complete myosin binding surface on actin ('open' state), activating the actomyosin cross-bridge cycle.

TnI sequence alignments (not shown) show that *Drosophila* TnI residue A116, (A116V in hdp^2) coincides with conserved residue A25 of vertebrate skeletal muscle isoforms. A25 contributes to a contact with the 'E' α-helix of TnC (Vassylyev *et al*., 1998). The hdp^2 A116V substitution increases residue size which must affect TnC-TnI binding by changing TnI α-helix and TnC 'E' α-helix interactions. These could result in hdp^2 either lowering the threshold for calcium activation or affecting the ability of the Tn-Tm complex to return to the relaxed state. We cannot yet distinguish which explanation is correct for hdp^2 but the latter one was previously proposed for a hypercontracting TnT mutant in *Caenorhabditis elegans* (McArdle *et al*., 1998) and would readily explain hypercontraction of hdp^2 IFMs. The less extreme effects on nonflight muscles argue strongly that hdp^2 can produce near-normal thin filament regulation.

The S185F substitution introduces a more bulky, hydrophobic residue. Its heptad 'c' position means that the phenylalanine ring will point outwards from the Tm axis. It could suppress hdp^2 either by altering Tn-Tm complex movement across the F-actin surface or by changing the Tn complex orientation on Tm through the Ca^{2+} sensitive TnT-Tm binding site. The former suggestion is supported by observations (Bing *et al*., 1998) that the *Act88F* gene *E93K* mutation reduces Tm movement across the F-actin surface in vitro and suppresses hdp^2 IFM hypercontraction in vivo (Nongthomba and Sparrow, unpublished). In the latter suggestion S185F would reverse the conformational effects of hdp^2 on the TnI/TnC interaction by altering structural relationships between Tm and the Tn complex. Reversal of the TnI/TnC conformational effects of hdp^2 could require very specific changes such as found with the intragenic *D3* suppressor, a TnI mutation (L188F) which suppresses hdp^2 (Prado *et al*., 1995) perhaps by changes in actin binding (L188 is close to an actin -binding domain). However, alterations in Tm mobility could affect any Tn mutant which causes slight regulatory changes. The partial suppression of up^{101} by *D53* might thus seem to favor this latter explanation, except for the observation that up^{101}, as *a TnT* mutation, is also located in the Ca^{2+}-sensitive TnT-Tm interaction site.

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a)                 150       160       170       180  F    190       200
                   |         |         |         |    |    |         |
TPM0_DROME         ARMLAEDADTKSDEVSRKLAFVEDELEVAEDRVRSGESKIMELEEELKVV
TPM1_DROME         ..F...E..K.Y...A....M..AD..R..E.AEQ..N..V......R..
TPM1_HUMAN         .KH......R.YE..A...VII.SD..R..E.AELS.G.CA.......T.
TPM3_HUMAN         .KH...E..R.YE..A...VII.GD..RT.E.AELA...CS.......N.
TPMA_BRARE         .KH...E..R.YE..A...VI..G...RT.E.AELN.G.CS.......T.
TPMA_COTJA         .KH...E..R.YE..A...VII.GD..R..E.AELS...CA.......T.
TPMA_RABIT         .KH......R.YE..A...VII.SD..R..E.AELS.G.CA.......T.
TPMA_RANTE         .KH...E..R.YE..A...VII.GD..R..E.AELS...CA.......T.
TPMA_RAT           .KH......R.YE..A...VII.SD..R..E.AELS.G.CA.......T.
TPMA_XENLA         .KH...E..R.YE..A...VII.GD..R..E.AELS...CA.......T.
TPMB_CHICK         .KH...E..R.YE..A...VVL.G...RS.E.AEVA...CGD......I.
TPMB_HUMAN         .KH....S.R.YE..A...VIL.G...RS.E.AEVA...CGD......I.
TPMB_RABIT         .KH....S.R.YE..A...VIL.G...RS.E.AEVA...CGD......I.
TPMC_PIG           .KH......R.YE..A...VII.SD..R..E.AELS.G.CA.......T.
TPMM_LOCMI         ..F...E..K.Y...A....M..AD..R..E.AEA.....V......R..
TPMM_TRICO         .Q....E..R.Y...A....M..AD..R..E.AEA..N..V......R..
b)                 AKHLAEEAD4KYEEVARKLV6VEGDLERAEERAE6SESKCAELEEELKTV
c)                   U   U  U U   U    U  U   U  U UUU     U
d)                    abcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcd
```

Figure 5. CLUSTALX multiple sequence alignment of muscle tropomyosin sequences corresponding to the TnT-2 binding site to show (a) position of S185F substitution in the protein sequence of the *Drosophila* Tm2 product expressed in the IFMs (TPM0_DROME); (b) consensus sequence (4 = KR, 6 = LIVM); (c) U = residues unique to the *Tm2* product; (d) Heptad repeat '*abcdefg* ' prediction generated with MacStripe (Dr. A. E. Knight, http://motility.york.ac.uk:85/) using the algorithm of Lupas *et al.* (1991). SwissProt Sequences: TPM0_DROME, *Drosophila Tm2* gene product (P09491); TPM1 DROME, *Drosophila Tm1* gene product (P06754); TPM1_HUMAN (P09493) and TPM3_HUMAN (P06753), human skeletal muscle α-chains; TPMA_BRARE (P13104), TPMA_COTJA (P18442), and TPMA_RABIT (P46902), zebrafish, quail, and rabbit skeletal/cardiac muscle α-chains; TPMA_RANTE (P13105), TPMA_RAT (P04692), TPMA_XENLA (Q01173), European common frog, rat, and South African claw toed frog skeletal muscle α-chains; TPMB_CHICK (P19352), TPMB_HUMAN (P07951), and TPMB_RABIT (P02560), chicken, human, and rabbit skeletal muscle β-chains; TPMC PIG (P42639), pig cardiac muscle α-chain; TPMM LOCMI (P31816) and TPMM_TRICO (P12324), locust and nematode muscle tropomyosins. Dots indicate residues identical to those in the TPM0 DROME sequence.

Clinical Implications

Human hypertrophic cardiomyopathies (HCM) include two dominant mutations, D175N and E180G, in highly conserved residues (Figure 5) of the *TPM1* gene (Thierfelder *et al.*, 1994). As with *D53*, these mutations map within the tropomyosin region which interacts with TnT in a Ca^{2+}-sensitive manner. In vitro motility experiments using reconstituted cardiac thin filaments showed that these HCM mutations affect the Ca^{2+} sensitivity of the fraction of filaments moving compared with wild-type, although in the absence of structural information on the Ca^{2+}-sensitive Tm binding site for TnT, it is not clear how these mutations could cause this (Bing *et al.*, 2000). There is evidence that HCM TnT mutations, I79N and R92Q, located within the same TnT-Tm interaction (residues 70–180) may affect Ca^{2+} sensitivity in tension versus force measurements (see Tobacman *et al.*, 1999 for discussion). Further biochemical studies of all these human and *Drosophila* Tm and TnT mutants are required to ascertain the role of this Ca^{2+}-sensitive contact between Tm and TnT in thin filament regulation.

Typically HCM symptoms do not manifest before adolescence, but penetrance and expressivity of HCM mutations are highly variable (Coonar and McKenna, 1997). This suggests that HCM mutations have relatively mild effects, but the functional deficits can induce cardiac changes, such as hypertrophy, leading to premature death. The variable expression of HCM mutations must be partly due to genetic background. Modifier genes may reduce or enhance HCM severity by affecting either the contractile machinery or the response to cardiac dysfunction. *D53* is similar to HCM mutations in that reduced IFM function is detectable only in older organisms. The *D53* suppression of the hdp^2 and up^{101} progressive myopathies suggests that *Drosophila* muscles

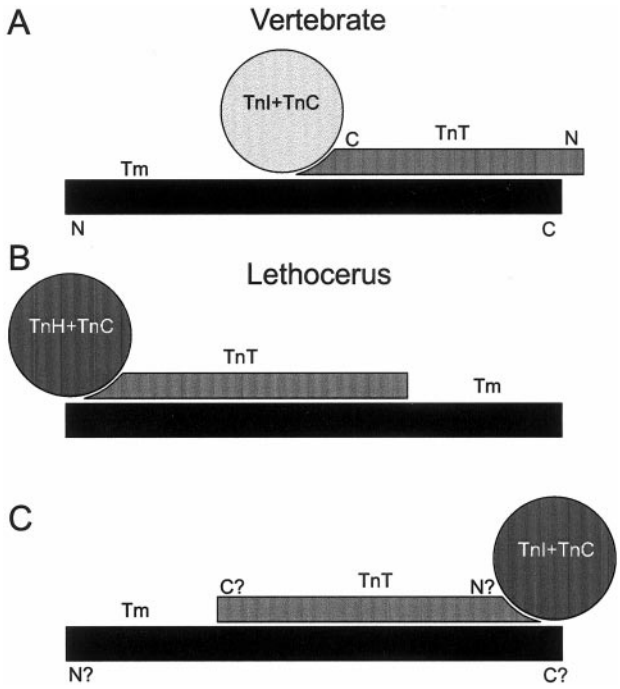

Figure 6. Cartoon of tropomyosin-troponin complex arrangements redrawn from Wendt *et al.*, (1997) to illustrate that an inversion of their diagram for *Lethocerus* would lead to a similar orientation between the Tm and TnT in vertebrate and invertebrate troponin-tropomyosin- complexes. (A) vertebrate, (B) *Lethocerus* redrawn from Wendt *et al.* (1997 and (C) proposed reorientation. The N- and C- termini labeling of Tm and TnT in (C) is based solely on the implied structural homology to the vertebrate structure.

may provide a useful genetic model with which to study HCM genes and their genetic modifiers.

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