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A MUTATION IN THE TROPOMYOSIN GENE IS POSSIBLY RESPONSIBLE FOR SUPPRESSING THE EFFECTS OF TROPONIN-I MUTATION IN THE *DROSOPHILA MELANOGASTER* MUSCLES

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ABSTARCT

Striated muscles like the indirect flight muscles (IFM) of the Drosophila are made up of thick and thin filaments that slide pass each other to generate contraction. Many proteins are involved in the structure as well as the regulation of the muscles. The IFM of the Drosophila is used in many studies to understand the muscle regulation. The presences of IFM specific proteins/isoforms are thought to be of relevance to the specific structure and function of the IFM. *heldup2* (*hdp*^{2}) is a point mutation in the constitutive exon of troponin (Tn)-I gene, that shows effects in the structure and function of all the muscles including the IFM. We have created mutants in the lab, designated as *3A* and *3D* that were found to suppress the effects of *hdp*^{2}. In this research we are reporting the details of the molecular and behavioural analysis of the suppressor mutations and their affects on the TnI mutation *hdp*^{2}.

Key-words: Mutation, Tropomyosin, Troponin-I, Suppressor, *Drosophilla*

INTRODUCTION

Muscle cells are composed of thick and thin filaments. Myosin is the major constituent of the thick filament whereas actin, tropomyosin (Tm), and troponin complex (TnI, TnT and TnC) constitutes the thin filaments (reviewed in Gordon *et al*., 2000; Bernstein *et al*., 1993). Sliding of the thick and thin filaments causes the contraction.

Although there is some success in mapping precise interaction sites of the various contractile proteins through electron microscopy/image reconstruction etc. the *in vitro* approaches represent a trade off between structural resolution and biological significance of derived conclusions (Kronert *et al*., 1991). In order to avoid this problem, *Drosophila melanogaster* can be used as a powerful system for the *in vivo* study. Mutants which have biological consequences identify these residues and their interactions which are of significance in the functioning of the protein/protein complexes *in vivo*. Various reports are published about the use of mutants in *Drosophila* for identifying the important interaction sites among different contractile proteins.

The term hypercontraction (HC) is used for a condition in which the muscles begin to develop normally, and then auto-destruct due to mutations in *Drosophila* muscle proteins. The muscles rip themselves apart and bunch in the centre, or at either end of the thorax. There is a severe disruption in the regularity of sarcomere with the disruption of hexagonal packing of thick and thin filaments. The filaments are not integrated into the myofibril. The sarcomeres are found either completely destroyed or squeezed with the loss of Z-disc. The effect of this disruption often results in the abnormal wing position. The factor that makes IFM the ideal candidate for the study of mutations in muscle proteins is that IFM are dispensable for viability as well as fertility of the flies. Mutations in the muscle proteins expressed in the IFM gives the flightless phenotype and often a "wings up" phenotype that makes the screening of new mutations easy. Even the mutations that are present in the constitutive expressed proteins have a greater effect in the IFM than in other muscles (Karlik & Fyrberg, 1986; Mogami *et al*., 1986).

hdp^2 is a point mutation in the exon 5 of the *wupA* gene that changes alanine 116 to valine and appears to affect Ca^{++} regulation (Beall and Fyrberg, 1991, Nongthomba *et al*., 2003). As exon 5 is a constitutive exon of TnI, the effects of this mutation are observed as changes in behaviour like jumping, walking, larval crawling and age dependent myopathy of the legs, associated with ultrastructural defects (Naimi *et al*., 2001). Flies hemi- or homozygous hold their wings in vertical position due to the hypercontraction of the IFM (Beall and Fyrberg, 1991, Nongthomba *et al*., 2003). The IFM hypercontract in such a way that the myofibrillar material remains only near the muscle attachment sites (Naimi *et al*., 2001). We isolated two mutants in the lab that were able to suppress many of the affects of the *hdp*^{2} mutation. The aim of the research was to identify the protein in which the suppressors were present as well as to localize the mutation at the amino acid level. The site as well as the degree of suppression on the effect of *hdp*^{2} mutation was also studied in this research by monitoring the walking, jumping and flight abilities of the *hdp*^{2} flies with the suppressors.

MATERIALS AND METHODS

Fly strains: Flies were maintained at 25^{o} C on a yeast-agar medium. Stock of *hdp^{2}* was obtained from the Bloomington Stock Center. Other flies were used as described in the FlyBase (http://www.flybase.org). For wild-type controls, Texas and Canton-S flies were used.

Making of the *hdp^{2}*; *3A* and *hdp^{2}*; *3D* stocks: The mutation *3A* and *3D* were created by feeding the flies 0.025M ethyl methane sulfonate in 10% sucrose solution. The mutant flies obtained from the progeny, identified by normal wing position even with the *hdp^{2}* mutation, were selected and maintained initially along with the balancer chromosome *TM3* to prevent any recombination. Later the homozygous *forkedheldup2* (*fhdp^{2}*) stocks with the suppressors *3A* and *3D* were made.

Jumping test: Jumping test was performed by lightly touching the dorsal surface of the thorax with a paintbrush. 10 jumps were observed of each of the fly from each genotype and the distance jumped by them was recorded every alternate day for each fly for ten days or till the majority of genotypes were no longer able to jump. Mean was calculated and statistical analysis was performed on the data by analysis of variance (ANOVA) to see the difference if any in the walking abilities among different genotypes.

Walking test: This was performed as described by Naimi *et al*. (2001). Briefly a total of ten males and ten females were selected from each genotype and after cutting the wings the flies were allowed to recover for a day. Next day 10 flies of genotypes to be tested were transferred to a 100 ml measuring cylinder with a mark at 10.5 cm distance from the base. Flies were tapped down gently by knocking the cylinder on to the rubber pad and immediately the stopwatch was started to note down the time taken by 50% (5) of the flies to cross the 10.5 cm mark. This was repeated 5 times and the average was recorded. Record was taken till the sixteenth day after eclosion or if they stopped walking before that. Statistical analysis was performed on the data to see if there is a difference in the walking abilities among different genotypes.

Flight testing: 3-4 days old flies were separated on the basis of sex into different vials from each genotype and after giving them 2-3 hours to recover flight test was performed as described by Drummond *et al*. (1991) in a Perspex flight chamber. The flies were scored according to the zone in which they landed, up, horizontal, down, or none.

Polarized light microscopy of the IFMs: The method used was first described by Nongthomba and Ramachandra (1999). Briefly 2-3 drops of water were spread on a clean glass slide with the help of a thick paintbrush. Flies were anaesthetised with di-ethyl ether and a maximum of 5 flies at a time were placed on the slide with dorsal surface facing upwards, so that the fully stretched wings stick to the slide. The slide was dipped into the liquid nitrogen for 5 seconds and the frozen flies were immediately dissected into halves along the central midline of the thorax with a razor blade under the dissecting microscope. The halves were transferred to an eppendorf containing about 750 µl 50% ethanol. The half thoraces were dehydrated by transferring through serial dilutions of ethanol (70% - 100% in 10% increments, with 30 minutes minimum incubation in each dilution) then cleaned in methylsalicylate (100%) overnight. The legs and halters were removed from the hardened thoraces using the watchmaker's forceps and needle. The cleared thoraces were mounted on slides in dextrin pthalate xylene (DPX) and after drying were visualized on a photomicroscope with polarising light filters at 20x magnification.

Mapping of the suppressors *3A* and *3D*: Mapping was performed by crossing the homozygous *3A* and *3D* with the flies known as rucuca. They have the markers *ru h th st cu sr e^{s}*. i.e. rough eyes at position 0, hairy wings at 26.5, thread like probiscous at 43.2, scarlet eyes at 44, curl wings at 50, striped abdomen at 62 and ebony body colour at position 70.7 respectively on the third chromosome. The virgin female progeny obtained was crossed to ruprica males. They also have the same markers with the addition of another marker *p^{p}* i.e pink peach eye colour in between *st cu*. After recombination males with different markers were selected and were crossed to the *hdp^{2}* virgin females separately. Presence of the *3D* and *3A* were done on the basis of the flight ability. Flies with the alleles *3A* or *3D* were able to fly downwards whereas the ones without them were flightless.

Sequence analysis: As the mapping of both the suppressors came out around the region of 50 to 70.7 on third chromosome and the muscle regulatory genes *Tm1*(55), *Tm2* (54.2), and *Act88F*(57.1) are present very close to each other around the same position therefore it was thought easier to sequence all the three genes one by one. Genomic

DNA was extracted from 12-15 flies with the help of Qiagen DNA extraction kit. Specific primers were used to generate the *Act88F* and *Tm1* DNA fragments by PCR and were sequenced (data not shown).

As the gene for *Tm1* is relatively large, it was sequenced through making the cDNA from the mRNA obtained from the IFM of *3A* and *3D* flies. Total mRNA was extracted with the Qiagen Rneasy kit according to the manufacturer's instructions. First strand cDNA was preparedby oligo (dT) primers with the help of Stratagene RT-PCR kit according to the manufacturers protocol. Amplification of the first strand cDNA was done with Promega PCR mix with the primers Tm1-S 5`-CAT-ATGGCTAGCATCAAGAAGAAGA-3` and Tm1-AS 5`-AAGCTT-CGCTTATTCCTTGAGGATGA-3`. PCR conditions were denaturation 94^o for 1 minute, annealing 50^o for 1 minute, and extension 70^o for 2 minutes for 30 cycles. The DNA fragment was gel purified and cloned into pGEM-T vector and were sequenced from Oxford University DNA sequencing facility.

RESULTS

Making of the *hdp^2*; *3A* and *hdp^2*; *3D* stocks: Two types of stocks were made. One with the *3A* and the other with the *3D* mutation, also forked (*f*) marker was incorporated along with the *hdp^2* on the X chromosome to trace the *hdp^2* mutation. The scheme for the preparation of the *3A* stocks is given in Fig. 1.

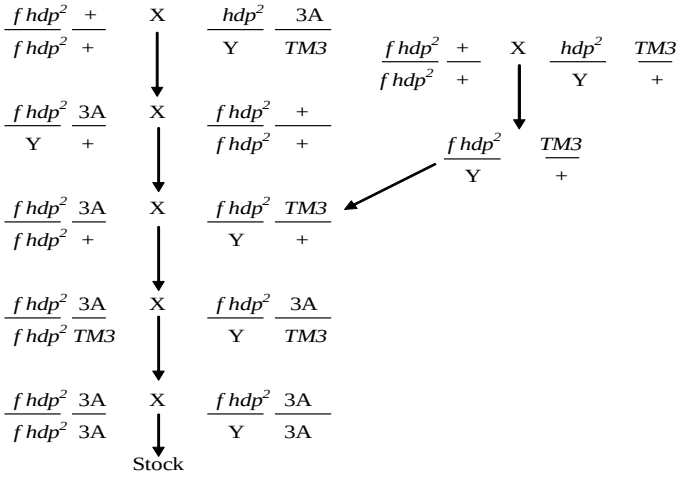

Fig. 1. Scheme for making the *f hdp^2*; 3A stocks. (Stock for *f hdp^2*; 3D was made in the same way.)

Jumping test: The jumping ability of the flies with different copy numbers of *3A*, *3D* and *hdp^2* showed variable results. Significant differences were found between most of the genotypes compared to the wild type flies when tested with ANOVA. Flies with one or two copies of *3A* both in males and females all showed significant or highly significant difference compared to the wild type genotype. Highly significant difference was also found between males with one and two copies of *3A*. Variation within days and genotypes into days was mostly found non-significant among different genotypes with one or two copies of *3A*. In the case of genotypes with two copies of *3D* allele, no significant difference was found with the wild type. Apart from that the rest of the result was similar to *3A*. The summary of the statistical analysis of the jumping data obtained from different genotypes is shown in Tables 1 and 2. Note that the *hdp^2* flies showed almost no jumping ability therefore analysis was not done by the comparison of different genotypes with them. However, all the genotypes were able to jump better than the *hdp^2*. The mean distance jumped by the flies with different genotypes is shown in Fig. 2.

Walking test: The walking abilities of various genotypes with *3A* and *3D* were found to be different. Most showed statistically significant differences compared to wild type flies when tested with ANOVA. All the genotypes showed significant difference compared to the *hdp^2* flies without the suppressor alleles. The summary of the statistical analysis of the walking data obtained from different genotypes is shown in the Tables 3 and 4. The mean time taken by 50% of the flies of different genotypes to cross the 10.5 cms distance is shown in the Fig. 3.

Table 1. Jumping ability of flies with 3A allele compared to wild type. Key:
Sig. * = Significant at P< 0.05.

Genotype	Source of variation between	Comparison to wt F value	P< 0.01
hdp^2 / Y ; 3A / 3A	Genotypes	29.05	highly Sig.
	Days	0.902	non Sig.
	Genotypes X days	0.59	non Sig.
hdp^2 / *hdp*^{2} ; 3A / 3A	Genotypes	45.26	highly Sig.
	Days	2.09	non Sig.
	Genotypes X days	0.94	non Sig.
hdp^2 / *Y* ; 3A / +	Genotypes	2.59	Sig. *
	Days	3.32	Sig. *
	Genotypes X days	0.35	non Sig.
hdp^2 / *hdp*^{2} ; 3A / +	Genotypes	41.49	highly Sig.
	Days	0.61	non Sig.
	Genotypes X days	0.28	non Sig.
+/ *Y* ; 3A / +	Genotypes	9.96	highly Sig.
	Days	1.01	non Sig.
	Genotypes X days	0.81	non Sig.
hdp^2 / + ; 3A / +	Genotypes	6.79	Sig. *
	Days	1.87	non Sig.
	Genotypes X days	1.71	non Sig.

Genotype	Source of variation between	F value	P< 0.01
hdp^2 / Y ; 3A / 3A	Genotypes	13.97	highly Sig.
Vs	Days	1.43	non Sig.
hdp^2 / Y ; 3A / +	Genotypes X days	1.92	non Sig.

Table 2. Jumping ability of flies with 3D allele compared to wild type.
Key: Sig. * = Significant at P< 0.05.

Genotype	Source of variation between	Comparison to wt F value	P< 0.01
hdp^2 / Y ; 3D / 3D	Genotypes	0.25	non Sig.
	Days	0.41	non Sig.
	Genotypes X days	0.41	non Sig.
hdp^2 / *hdp*^{2} ; 3D/ 3D	Genotypes	0.05	non Sig.
	Days	0.75	non Sig.
	Genotypes X days	0.7	non Sig.
hdp^2 / *Y* ; 3D / +	Genotypes	8.92	highly Sig.
	Days	0.33	non Sig.
	Genotypes X days	1.20	non Sig.
hdp^2 / *hdp*^{2} ; 3D / +	Genotypes	17.16	highly Sig.
	Days	0.53	non Sig.
	Genotypes X days	0.15	non Sig.
+/ *Y* ; 3D / +	Genotypes	23.33	highly Sig.
	Days	1.14	non Sig.
	Genotypes X days	0.29	non Sig.
hdp^2 / + ; 3D / +	Genotypes	3.03	Sig. *
	Days	4.31	highly Sig.
	Genotypes X days	3.69	highly Sig.

Genotype	Source of variation between	F value	P< 0.01
hdp^2 / Y ; 3D / 3D	Genotypes	2.92	Sig. *
Vs	Days	0.14	non Sig.
hdp^2 / Y ; 3D / +	Genotypes X days	0.46	non Sig.

Table 3. Walking ability of flies with 3A allele compared to wild type and hdp^2 flies.
Key: Sig. * = Significant at P< 0.05.

Genotype	Source of variation between	Comparison to wt F value	P< 0.01
hdp^2 / Y ; 3A / 3A	Genotypes	118.60	highly Sig.
	Days	5.62	highly Sig.
	Genotypes X days	1.59	non Sig.
hdp^2 / hdp^2 ; 3A / 3A	Genotypes	8.819	highly Sig.
	Days	0.361	non Sig.
	Genotypes X days	2.035	non Sig.
hdp^2 / *Y* ; 3A / +	Genotypes	24.35	highly Sig.
	Days	17.11	highly Sig.
	Genotypes X days	6.493	highly Sig.
hdp^2 / hdp^2 ; 3A / +	Genotypes	0.38	non Sig.
	Days	1.625	non Sig.
	Genotypes X days	1.955	non Sig.
+/ *Y* ; 3A / +	Genotypes	5.17	Sig. *
	Days	5.15	highly Sig.
	Genotypes X days	1.475	non Sig.
hdp^2 / + ; 3A / +	Genotypes	64.77	highly Sig.
	Days	0.808	non Sig.
	Genotypes X days	2.048	non Sig.

Genotype	Source of variation between	F value	P< 0.01
hdp^2 / Y ; 3A / 3A	Genotypes	74.07	highly Sig.
Vs	Days	8.88	highly Sig.
hdp^2 / Y ; 3A / +	Genotypes X days	3.31	Sig. *
hdp^2 / Y ; 3A / 3A	Genotypes	2251.2	highly Sig.
Vs	Days	193.01	highly Sig.
hdp^2 / Y ; +/ +	Genotypes X days	165.79	highly Sig.

Table 4. Walking ability of flies with 3D allele compared to wild type and hdp^2 flies.
Key: Sig. * = Significant at P< 0.05.

Genotype	Source of variation between	Comparison to wt F value	P< 0.01
hdp^2 / Y ; 3D / 3D	Genotypes	15.195	highly Sig.
	Days	1.77	non Sig.
	Genotypes X days	4.135	Sig. *
hdp^2 / hdp^2 ; 3D/ 3D	Genotypes	12.166	highly Sig.
	Days	1.810	non Sig.
	Genotypes X days	3.201	Sig. *
hdp^2 / *Y* ; 3D / +	Genotypes	6.242	Sig. *
	Days	23.12	highly Sig.
	Genotypes X days	8.266	highly Sig.
hdp^2 / hdp^2 ; 3D / +	Genotypes	115.48	highly Sig.
	Days	2.906	non Sig.
	Genotypes X days	0.872	non Sig.
+/ *Y* ; 3D / +	Genotypes	1.839	non Sig.
	Days	1.252	non Sig.
	Genotypes X days	1.322	non Sig.
hdp^2 / + ; 3D / +	Genotypes	5.92	non Sig.
	Days	2.66	Sig. *
	Genotypes X days	1.233	non Sig.

Genotype	Source of variation between	F value	P< 0.01
hdp^2 / Y ; 3D / 3D	Genotypes	3.618	non Sig.
Vs	Days	10.99	highly Sig.
hdp^2 / Y ; 3D / +	Genotypes X days	18.67	highly Sig.
hdp^2 / Y ; 3D / 3D	Genotypes	3135.82	highly Sig.
Vs	Days	235.08	highly Sig.
hdp^2 / Y ; +/ +	Genotypes X days	246.83	highly Sig.

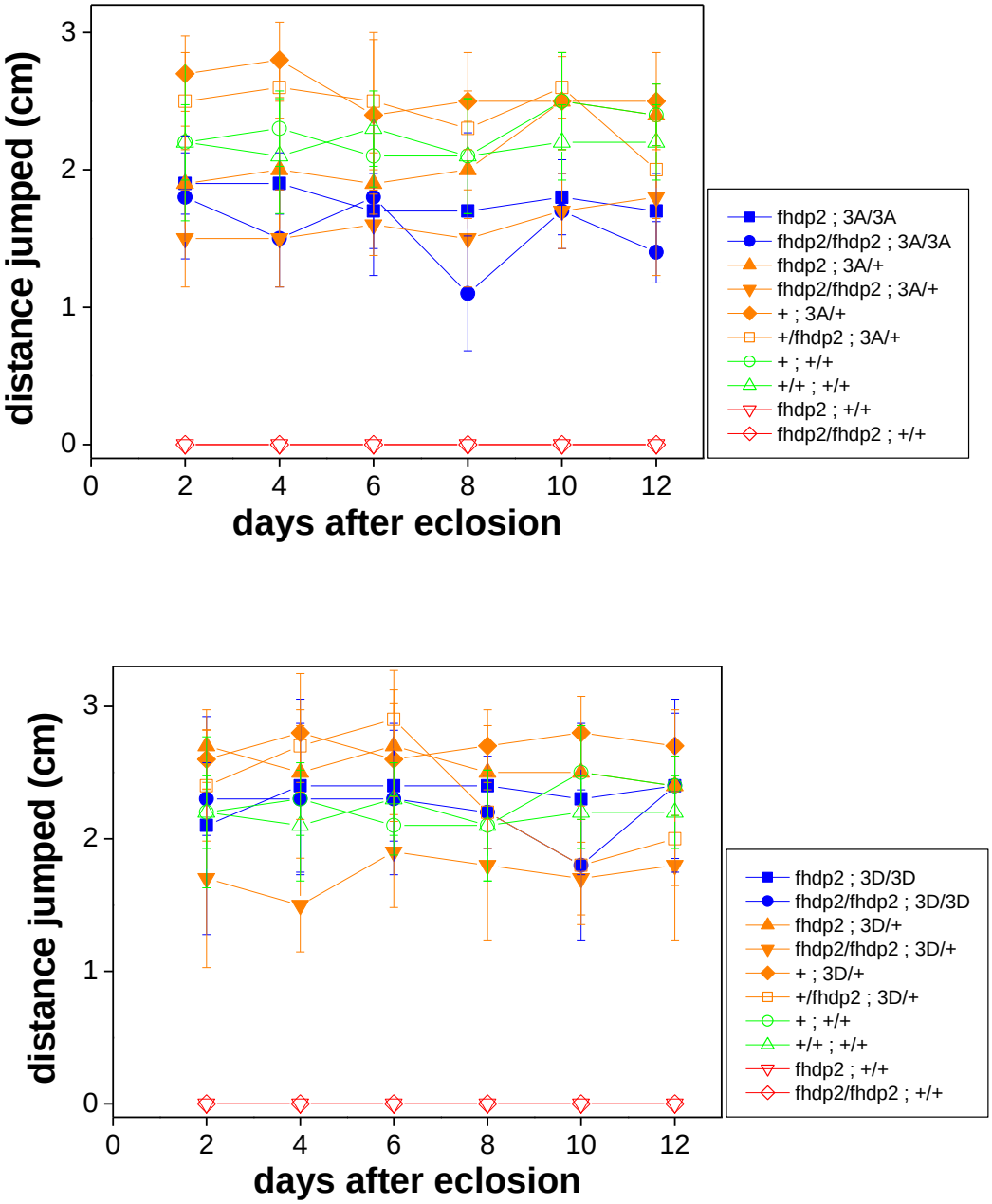

Fig. 2. Graph showing the mean distance jumped by flies with different copies of 3A and 3D alleles. The bars represent the standard deviations.

Flight testing: All *hdp*^{2} flies were flightless therefore no test was performed on them. Hemi-, hetero- and homozygous *hdp*^{2} flies with one or two copies of *3A* or *3D* allele were flight tested as described in the materials and methods. Also wild type flies with and without *3A* and *3D* alleles were tested. All the *hdp*^{2} flies regardless of the presence of one or two copies of *3A* or *3D* were found to fly downwards. Wild type flies with or without *3A* or *3D* all flew up. This suggested that *3A* and *3D* although cannot restore the flight in the *hdp*^{2} flies to the normal level but significantly improves it and also they on their own do not have any effect on the flight ability as seen in the case of wild type.

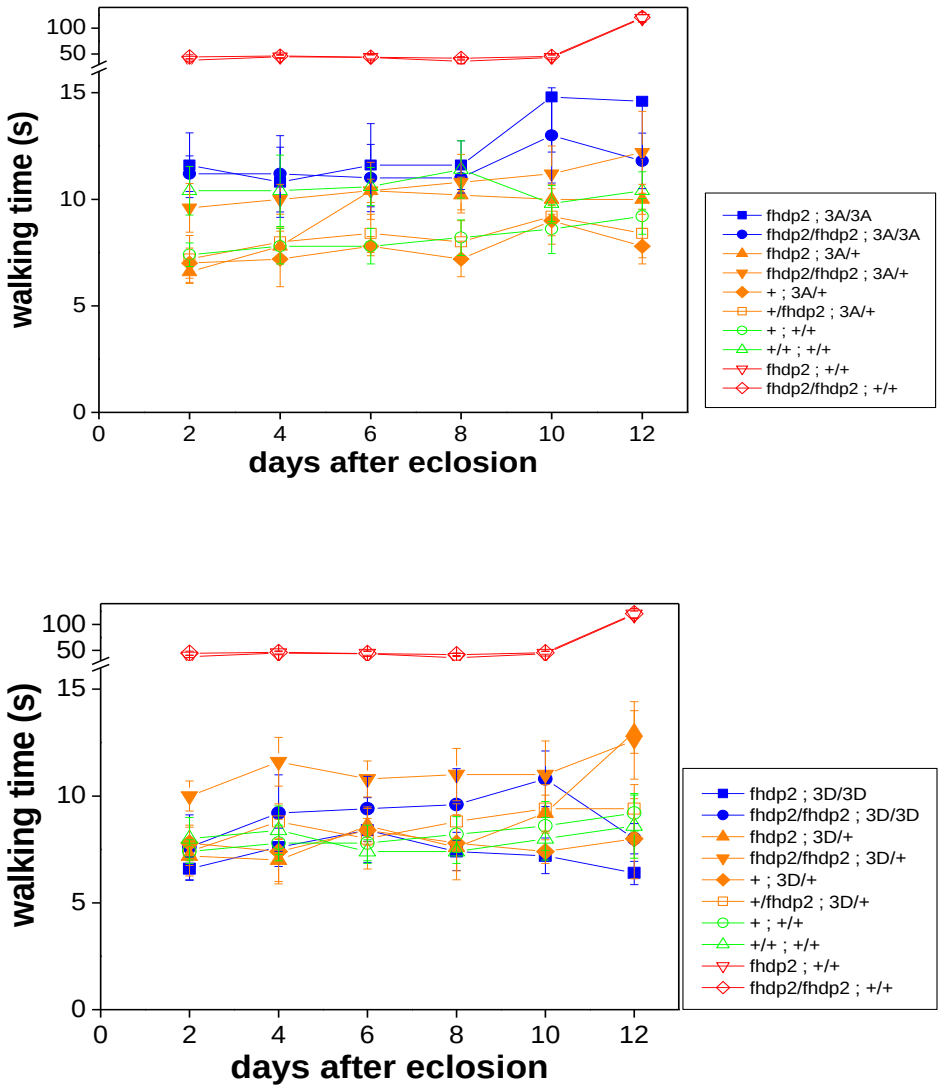

Fig. 3. Graph showing the mean time taken by flies with different copies of 3A and 3D allele to cross the 10.5 cms distance. The bars represent the standard deviations.

Polarized light microscopy of the IFMs: The polarized microscopy of the *hdp*^{2} homozygous, hemizygous and heterozygous flies all showed normal IFM phenotypes with both *3A* and *3D*. The gene dosage showed no effect on the IFM phenotype as seen in the Fig 4. The presence of *3A* or *3D* in the wild type does not show any change in the IFM suggesting that they both do not have the phenotypic effect of their own.

Mapping of the suppressors *3A* and *3D*: It was noted that whenever flies having the three markers *cu sr e*^{s} present together were crossed to the *hdp*^{2} females none of the progeny was flighted. This indicated that these flies were unable to pick the *hdp*^{2} suppressor alleles *3A* or *3D* due to no recombination in this area. Thus *3A* and *3D* were thought to be in the area between 50 to 70.7 on the third chromosome.

Sequence analysis: No change was noted in the DNA sequence of the actin and tropomyosin 2 gene compared to that of the wild type and Flybase data sequences (http://fly.ebi.ac.uk:7081). The sequencing of the *Tm1* cDNA revealed that in both 3A and 3D, a single base change of G to A had occurred at position 732 (Fig. 5) that corresponds to codon 244. This will change the arginine at this position in constitutive exon 13 to histidine (R244H). The *hdp*^{2} *Tm1* sequence showed no change as to the wild type sequence at this position.

Fig. 4. Polarized microscopy of flies with *hdp*^{2} suppressors 3A and 3D alleles.

All *hdp*^{2} flies showed normal IFM with one or two copies of the suppressors. Wild type flies also showed normal IFM with the copy of suppressors indicating that suppressors do not have the affect of their own on the IFM.

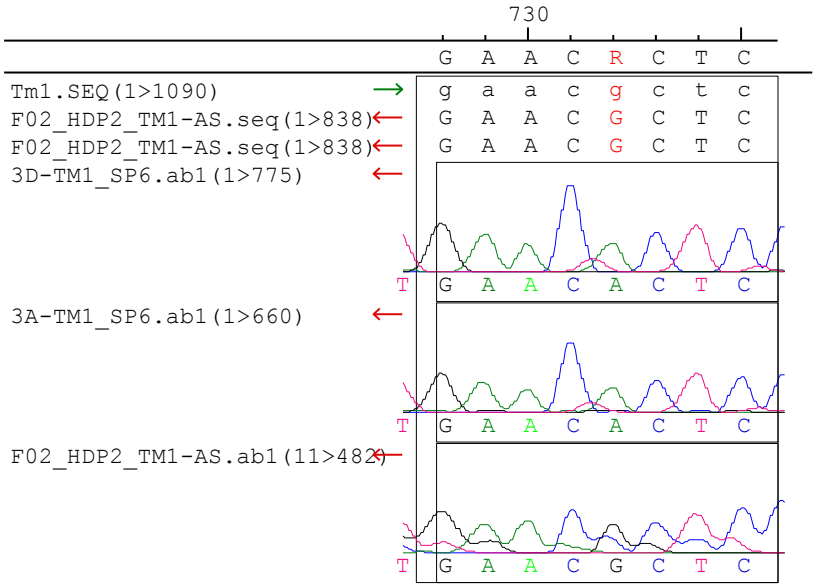

Fig. 5. DNA sequence of the *Tm1* from *3A*, *3D* and *hdp*^{2} IFM.

Note that the G of *hdp*^{2} is changed to A in 3A and 3D at base position 732 of the coding sequence. This changes the amino acid 244 of the protein from arginine to histidine. The numbers in the brackets indicates the total length of the sequence and the number on the top indicates the number of bases from translation start of mRNA.

DISCUSSION

Mutating a protein to disturb the function of muscles and than restoring the function by another suppressor mutation is a very powerful approach to clarify the interactions relevant to muscle function in vivo (Kronert *et al*., 1999). Both the hdp^2 suppressors *3A* and *3D* were found to be due to the same mutation causing the change from arginine to histidine in exon 13 of the *Tm1* gene. The arginine at this position is highly conserved in many species, including the humans, as seen in the Fig. 6. To our knowledge this is the first report of a viable *Tm1* point mutant that suppresses the effects of another gene, although previously Naimi *et al.* (2001), have reported a *Tm2* mutant that suppressed the affects of TnI mutation hdp^2 and TnT mutation up^{101}.

The *3A* and *3D* suppressors were isolated in the lab in an EMS screen for suppression of hdp^2, which caused the wings to be held in the normal, rather than the "wings-up" position. Although both the mutants 3A and *3D* were found to be the same with respect to the mutation in the *Tm1* gene, in the case of the *3D* suppressor another gene also seems to be involved therefore the results obtained with the two mutants were a bit different. We therefore treated the two suppressors as different genotypes.

The IFM were found normal with one or two copies of the *3A* and the walking speed was also found to be better although not as good as wild type statistically. The comparison of the jumping ability with the wild type flies shows again that both *3A* and *3D* are very good in suppressing the affects of hdp^2 on the jumping abilities of the flies. The hdp^2 flies were found to have almost no jumping ability but the presence of one or two copies of either *3A* or *3D* was able to restore the jumping ability. The performance of *3A* in either one or two copies was found to be statistically different significantly from the wild type but the presence of two copies of *3D* in hdp^2 flies showed no significant difference on the walking ability compared to wild type. The flightnessness caused by the hdp^2 mutation is however not restored completely as all the hdp^2 flies with one or two copies of *3A* or *3D* were able to fly only downwards. Interestingly both *3A* and *3D* on their own do not seem to have any effect as wild type flies with a copy of *3A* or *3D* were as flighted as the one without any *3A* or *3D* allele.

The hdp^2 mutation of alanine residue at position 116 to valine corresponds to the conserved alanine position 25 in the vertebrate skeletal muscle TnI sequence and is present in the N-terminal α-helix of the molecule that makes hydrophobic contacts with TnC (Farah *et al*., 1994; Tripet *et al*., 1997) and more precisely to the residues 98 (cysteine), 101 (isoleucine) and 102 (phenyl alanine) of the TnC 'E helix' (Tanaka *et al*., 2003). The *Drosophila* thin filament proteins show substantial homology to their vertebrate counterparts (Naimi *et al*., 2001) although there are some extensions in the C or N-terminal of the *Drosophila* proteins (Barbas *et al*., 1991; Beall and Fyrberg, 1991; Fyrberg *et al*., 1990; Karlik *et al*., 1984 and Hanke and Storti, 1988). The role of the mutated amino acid of hdp^2 can be inferred from the functional and structural studies of the vertebrate TnI. The model of TnI/TnC interface in the rabbit skeletal muscle proposed by Vassylyev *et al.* (1998), shows clear hydrophobic interactions between TnI and TnC. Although the TnI-TnC interaction is quite stable (Farah *et al*., 1994), the binding of Ca^{++} to TnC releases the NH_2-terminal of TnI that allows the binding of the inhibitory region of TnI to TnC (Tripet *et al*., 1997; Vassylyev *et al*., 1998). Lehrer and Geeves (1998) have proposed a model of thin filament regulation in which Tn-Tm can exists in three different states on the F-actin. In the absence of Ca^{++}, the binding of TnI to actin holds the Tn-Tm complex in the 'blocked state' so that the myosin-binding site on F-actin is covered and myosin cannot bind to actin. Neural stimulation causes the release of Ca^{++} into the sarcoplasm which binds to TnC causing a conformational change which in turn results in the release of TnI binding from actin and a small shift of the Tn-Tm complex on the actin surface and to a state called the 'closed state'. The small movement of the Tn-Tm complex allows small number of myosin heads to bind to F-actin, leading to the further displacement of the Tn-Tm complex. This is called the 'open state'. In this state the myosin heads can bind to any available targets on actin and muscle activation is achieved.

Models were proposed for the hdp^2 defects in the past on the basis of the identification of various suppressors in the regulatory proteins. Kronert *et al.* (1999) suggested that the hdp^2 mutation hastens the release of the α-helix of TnI at lower Ca^{++} concentrations, resulting in the more ready binding of TnI inhibitory domain to TnC resulting in the un-regulatory actin-myosin interaction. Naimi *et al.* (2001) proposed that the substitution A116V in hdp^2 increases the residue size which affects TnC-TnI binding by changing TnI α-helix and TnC 'E' helix interactions. This could result in the lowering the threshold for Ca^{++} activation or affect the ability of the Tn-Tm complex to return to the relaxed state. Recently Cammarato *et al*., (2004) have shown that Ca^{++} had no significant effect on tropomyosin position in the thin filament of the hdp^2 mutant and tropomyosin was in the Ca^{++} induced position on the inner domain of the actin regardless of whether Ca^{++} was present or absent. Thus the steric regulation was not functional in hdp^2 filaments and myosin-binding sites on actin are exposed at all times.

Suppression could result from altered stoichiometry or from specific amino acid alterations affecting protein interactions (Naimi *et al*., 2001). Gene dosage should produce the former type of interaction whereas the latter should be allele-specific (Naimi *et al*., 2001). The suppression of the hdp^2 mutation by the suppressor *3A* and *3D*

could be through the same mechanism as proposed for the suppression by *Tm2* mutant suppressor *D53* (Naimi *et al*., 2001) which is S185F. The *3A* and *3D* change from arginine to histidine may alter the Tn-Tm complex movement across the F-actin surface or by changing the Tn complex orientation on the Tm through the Ca^{++} sensitive Tn-Tm binding site.

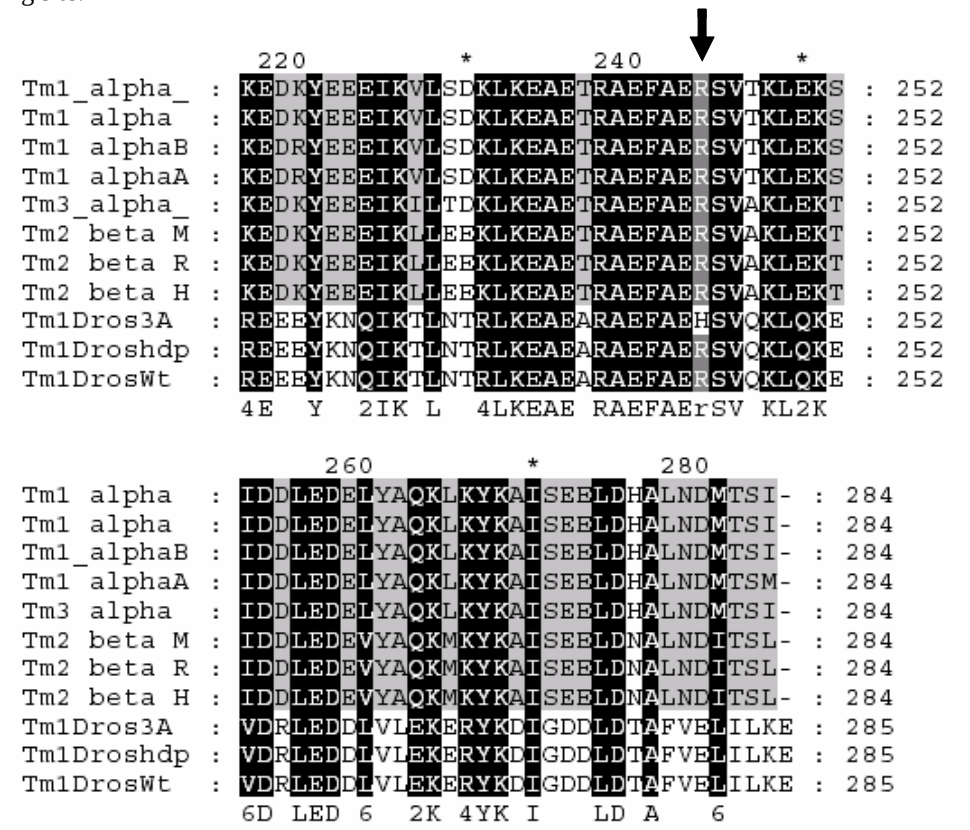

Fig. 6. Alignment of amino acid sequences showing the conserved regions in the C-terminal region between various tropomyosin.

The black shaded area indicates complete homology. The arrow on the top indicates the amino acid position 244, which is highly conserved among species but is mutated in 3A and 3D from R to H. The accession numbers of these sequences are given below in the order of alignment.
Tm1(alpha)Mouse P58771; Tm1(alpha)Rabbit P58772 ; Tm1(alphaB)Human P09493;
Tm1(alphaA)Human NP_000357; Tm3(alpha)Human P06753; Tm2(beta)Mouse P58774
Tm2(beta)Rabbit P58776; Tm2(beta)Human P07951; Tm1DrosWt SWP.P06754

Significant difference was found in the effect of presence of one or two copies of the suppressors *3A* and *3D* when analysed statistically (except in the case of *3D* on the walking ability) still both *3A* and *3D* were able to suppress the hdp^2 effects to a great degree regardless of the copy number. The IFM were found to be unaffected with the copy number whether there are one or two *3A* or *3D* copies. This suggested that the affect of the 3A suppression of hdp^2 is not structural mechanism but is regulatory. A copy of *3A* can also suppress the effects of the TnT mutation up^{101} (data not shown). The up^{101}; *3A* flies showed normal IFM with normal wing position. However flight was not restored. This further confirmed that the suppressor *3A* is affecting the regulatory mechanism, as up^{101} also appears to be due to the mis-regulation of muscle contraction (Nongthomba *et al*., 2003). Both hdp^2 and up^{101} seem to be acting in similar ways as earlier it was shown that the *Tm2* suppressor *D53* was able to partially suppress both the mutants. It is possible that the *3A* mutation in the *Tm1* gene is close to the TnI/TnC complex which allows it to overcome the small hindrance that might have been caused in the movement of Tm in the case of the hdp^2 mutation. The suppression could be due to the slight change in the shape of the molecule due to the change of the arginine to histidine.

The *3D* suppressor behaves like *3A* in all aspects of its suppression except that it seemed that there is another mutation present in some protein other than *Tm1, Tm2* and actin as the sequence of these three showed no other

changes except that of arginine to histidine in *Tm1*. Support for this idea comes from the fact that the results of *3D* were found to be different from the *3A*. Firstly during the mapping the recombinant genotype *sr* (stripe) *e* (ebony) with *3A* gave flighted progeny when crossed to hdp^2 females whereas the progeny of *sr e* with *3D* were all flightless. Secondly *3A* was able to suppress the IFM hypercontraction phenotypes in all the up^{101} flies, whereas the *3D* suppressed only about 50% of the flies suggesting that whenever there is a presence of the unknown mutation even with the suppressor *3D*, IFM hypercontraction due to up^{101} could not be suppressed. Thirdly, quite a few differences were noted in the jumping and walking behaviours of the flies with different combinations of *3A* and *3D*. This unknown mutation seems to be affecting up^{101} more than the hdp^2 suggesting that also has an effect like up^{101} itself. The question arises why approximately 50% of the up^{101} flies showed the hypercontraction with the new mutation? The reason could be that the unknown mutation was present in the heterozygous state and thus only half of the F1 flies can carry the gene (the mutation may be lethal in homozygous state that is why found always in the heterozygous condition). This proposal needs to be proven, as there was no reduction in the progeny number noted during the study.

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