CF2 Represses Actin 88F Gene Expression and Maintains Filament Balance during Indirect Flight Muscle Development in Drosophila

Kathleen M. Gajewski1, Robert A. Schulz2

1 Department of Systems Biology, University of Texas M. D. Anderson Cancer Center, Houston, Texas, United States of America, 2Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana, United States of America

Abstract

The zinc finger protein CF2 is a characterized activator of muscle structural genes in the body wall muscles of the Drosophila larva. To investigate the function of CF2 in the indirect flight muscle (IFM), we examined the phenotypes of flies bearing five homozygous viable mutations. The gross structure of the IFM was not affected, but the stronger hypomorphic alleles caused an increase of up to 1.5X in the diameter of the myofibrils. This size increase did not cause any disruption of the hexameric arrangement of thick and thin filaments. RT-PCR analysis revealed an increase in the transcription of several structural genes. Ectopic overexpression of CF2 in the developing IFM disrupts muscle formation. While our results indicate a role for CF2 as a direct negative regulator of the thin filament protein gene Actin 88F (Act88F), effects on levels of transcripts of myosin heavy chain (mhc) appear to be indirect. This role is in direct contrast to that described in the larval muscles, where CF2 activates structural gene expression. The variation in myofibril phenotypes of CF2 mutants suggest the CF2 may have separate functions in fine-tuning expression of structural genes to insure proper filament stoichiometry, and monitoring and/or controlling the final myofibril size.

Introduction

The indirect flight muscles (IFM) of Drosophila are exquisitely adapted to provide the maximum power to the wings. They contain unique isoforms of structural proteins such as actin, the troponins, and myosin heavy chain (mhc). Thick and thin filaments are arranged in a distinctive hexagonal pattern (with each thick filament surrounded by six thin filaments) that allows for maximum contact between actin and myosin molecules. A number of mutations in structural genes are known that allow relatively normal development and functioning of other muscle types, but severely disrupt IFM development and function. Some of these mutations affect splicing of an IFM specific exon (for example the help[3] mutation of troponin I (Tn I) [1]) or coding regions of genes unique to the IFM (such as actin 88F (Act88F)), but some are null or strong hypomorphic mutants of genes that are expressed in all muscle types. For example, flies homozygous for the Mhc[1] null allele cannot fly, although heterozygous larvae have no discernable locomotion phenotype and heterozygous adults can walk [2]. Likewise, flies lacking a copy of Act88F are flightless. Interestingly, flies that are doubly homozygous for null mutations in mhc and Act 88F (therefore having a 1:1 mhc: actin ratio) can fly, although not as well as wild type (with a 2:2 ratio) [3]. Therefore, it is not just the amounts of these proteins, but also their stoichiometry, that influences flight muscle structure and function.

Sarcomeres assemble from the center outwards in the pupal myotubes. The first thick and thin filaments can be detected at about 42 hours after pupa formation (APF). Additional filaments are added at the periphery until the myofibril reaches its final size of approximately 35 thick filaments across (~1.5 μm in diameter) in the late pupal stages [4]. Observations of myofibril assembly in Act88F null mutants [3] or mhc null mutants [2] show that thin or thick filaments can assemble in the absence of the other. Z-discs can still form without the presence of thick filaments, and M-lines can be observed if thin filaments are missing. But the presence of both is required for normal sarcomere size, order, periodicity, and consequentially function [3]. When filament stoichiometry is altered, the peripheral regions of the myofibril tend to be the most severely affected. When there is only one functional copy of mhc, the thick filaments at the edges of the myofibril are surrounded by 9–10 thin filaments, instead of the normal six (which is maintained in the central portions) [2]. Extra doses of mhc (the equivalent of four copies) result in excess thick filaments at the periphery [3]. Since filament stoichiometry is so crucial to IFM development, it should be very tightly controlled, but little is known about the mechanisms that sense and adjust thick: thin filament ratios.

One point of control is the transcription of muscle structural genes, and the actions of various transcription factors, particularity in the embryonic stages, have been characterized. The MADS box protein MeF2 is a major player in muscle differentiation. Binding
of MeF2 is essential for expression of structural genes such as mhc, myosin alkali light chain, and myosin light chain 2 in the embryonic dorsal vessel [6], paramyosin/miniparamyosin in various muscles in larvae and adults [7], Tn I in adult and embryonic muscle [8], and troponin I and 57B actin in embryonic muscles [9,10]. Chorion factor 2 (CF2) encodes a Zn finger transcription factor, which was first characterized as a repressor of dorsal fate in the follicle cells of the ovary [11]. It is also expressed in the nuclei of all muscle types of the embryo in a pattern similar to MeF2, being detectable at around stage 12, after the induction of MeF2 expression [12]. CF2 was the first collaborating factor for MeF2 to be characterized [13]. The combination of MeF2 and CF2 has a synergistic effect on the expression of 57B actin, Tn I, and mhc in embryonic muscles [13], and there are clusters of MeF2 and CF2 binding sites upstream of troponin T, troponin 1 and 2, and paramyosin [14]. But the role of CF2 in development of the IFM remains undefined. Previous work [14] reported impaired flight in two hypomorphic CF2 mutants, but there have been no studies investigating what type of role CF2 plays in IFM development.

In this paper we investigate the regulatory role of the Zn finger transcription factor CF2 in the development of the IFM. We show that the specific isoforms expressed in the IFM changes during the process of development, with pupal IFM expressing a different set of CF2 isoforms than adult IFM. In contrast to its reported role in embryonic and larval muscles, our data point to CF2 as a repressor, rather than an enhancer, of at least one IFM structural gene, Act88F. A reduction in CF2 function increases transcription of several structural genes and in more severe cases increases myofibril size. Gain of CF2 function has a deleterious effect on IFM development, resulting in greatly reduced or almost completely ablated muscles. Similar to its role in embryonic muscle, CF2 in the IFM may be needed for the fine-tuning of structural gene expression. Our data also suggests a role for CF2 in insuring correct myofibril size.

Results

CF2 is expressed in adult and developing pupal flight muscle

In the Drosophila embryo the CF2 protein is expressed in the nuclei of all three muscle types (somatic, visceral, and dorsal vessel) [12]. To determine whether the protein is also expressed in adultflight muscle, we immunostained dissected IFM with a polyclonal CF2 antibody (that detects all isoforms). As in the embryo, the stain is distinctly nuclear (Fig. 1A), and its specificity confirmed by a control with 2° antibody, but no 1° antibody (Fig. 1B). This nuclear expression pattern is consistent with the conclusion that CF2 is active in the IFM.

To examine the expression of CF2 transcript during development of the flight muscles, we used RT-PCR on cDNA from the dissected IFM of adults, 40 hr APF pupae (beginning of myofibrillogenesis), and 60 hr APF pupae (elongation of IFM complete). The primers used were designed to amplify all three reported CF2 isoforms (Fig. 2A), which are created by alternative splicing of exon 3 [15,16]. In the adult sample (Fig. 2B), only isoforms I and II are detected. However, in both pupal lanes, isoform III replaced isoform I. This result is confirmed by Western blots (Fig. 2D-E). In the pupal samples the predominant bands run at 53.3 kD and 56.3 kD, the expected sizes for isoforms II and III. This suggests a previously unknown role for isoform III, which had been reported to only be expressed in testes [15]. In the wild type adult sample, a band the size of isoform I (∼56.7 kD) is observed instead of isoform III, consistent with the PCR results.

Figure 1. CF2 exhibits nuclear localization in adult indirect flight musculature. A) Flight muscles of a wild type adult, stained with anti-CF2 antibody. B) Control staining without primary antibody. Arrowheads indicate a representative nucleus in each panel.

doi:10.1371/journal.pone.0010713.g001

We obtained four P-insertion mutant alleles of CF2 (diagrammed in Fig. 2C) (CF2[KG05342], CF2[KG08941], CF2[KG04624], and CF2[KG04624]), in fly lines referred to hereafter as 05342, 08941, 04624, and 01640, 05342, 08941, and 04624 have P-insertions 5’ to the ATG start site in exon 2. The P-element in the 01640 line is in exon 3, within sequences unique to isoform I. We generated a fifth mutant (CF2[KG08941-R3]), referred to hereafter as R3, by excision of the P-element from line 08941. The R3 mutant has a small deletion (∼2.7 kb) that removes the first exon of CF2, but leaves the second exon (with the ATG start site) intact. All mutants are homozygous viable, and exhibit no visible signs of IFM defects, such as abnormal wing position. Western blots of mutant adult thoraces reveal that four of the five mutations are hypomorphic, ranging from the least severe, 05342, to the most severe, R3, which has a greatly reduced level of all forms of CF2 as compared to wild type (Fig. 2D–E).

The fifth mutant, 01640, has no detectable levels of isoform I. There is one very strong band, but it does not match the sizes for any of the other CF2 isoforms found in the other lines. It runs higher than isoform II, but lower than isoforms I and III. 01640 is unique among the set of mutants in that it is the only one to have a P-element insertion within coding sequences, in the region of exon 3 that is found only in isoform I. To test the possibility that the 01640 mutation produced an aberrant protein, we did RT-PCR with several sets of primers designed to test the splicing of exon 3, and all CF2 isoforms, or the presence of P-element sequences (Fig. 2G). With primers sets specific for isoform I or II (and 5’ to the P insertion site), the expected PCR products (386 bp and 361 bp) are not observed in the mutant cDNA lanes, in contrast with the wild type cDNA lanes. Primers from exons 2 and 3 upstream of the P-site produce identical results (a 228 bp band) with both genotypes, confirming that CF2 transcription is initiated in 01640 mutants. As expected, when primers for CF2 exon2 and the P Bac{PB} P-element vector [17] are used, a 328 bp PCR product is found in the 01640 lane, but not the wild type lane. These results confirm that exon 3 is not properly spliced in 01640 mutants, the mRNA contains P-element sequences, and this likely produces a truncated form of the CF2 protein.
they tended to be slightly less sharp than wild type or

Figure 2. Expression patterns of CF2 isoforms in the IFM. A) Map of the CF2 gene, showing the location of the primers used for RT-PCR (red and green arrows). These primers will amplify all three CF2 isoforms. Red regions denote coding sequences that are common to all three isoforms, purple common to isoforms I and II, blue unique to isoform I, and green unique to isoform III. The lack of exon 3 in isoform III causes a frame shift in exon 4, so that the green colored region, while present in all three isoforms, is in frame only for isoform 3. B) Gel of RT-PCR reactions with IFM cDNA from adults, ~40 hr pupae, and ~60 hr pupae. The predicted exon content of each band is indicated by the colored boxes on the left. All PCR products were sequenced to confirm their exon content. C) Diagram of the CF2 gene and the sites of P-element insertions or genomic DNA deletion. The hatched bar denotes the region that contains the R3 deletion. The exact breakpoints are unknown, but PCR mapping confirms that it is upstream of the ATG site. D) Western blot of IFM protein from wild-type pupae and whole thorax protein from wild type and mutant adults, using a CF2 antibody. The red arrow head points to the altered form produced by the 01640 mutant. E) Longer exposure of D), to show the fainter bands. F) Loading control Western using an actin

Loss of CF2 function causes subtle defects in IFM structure

The IFM of CF2 mutant adult flies appear normal on the gross structural level, with no abnormalities in muscle number, size, or patterning observed (data not shown). However, at the ultrastructural level, differences in myofibrils are seen between the wild type and mutant flies (Fig. 3). In 0342, 08941, and 01640 myofibrils (Figs. 3B, C, and F), the filament pattern often appeared diffuse. The round shape of the myofibrils indicates a proper cross section cut (as opposed to a section at an angle, which would produce an oval shape to the myofibril), and filament patterns of simulta-

3.5x, mhc elevations of 18x, 7x, and 1.3x for Act88F, respectively, a statistically significant increase in myofibril size. Therefore a certain threshold of CF2 function appears to be required to insure the proper myofibril size.

CF2 loss of function increases the levels of mRNAs encoding structural proteins

To determine the mechanism for the observed ultrastructural phenotypes in some CF2 mutants, we examined expression of several IFM structural genes via RT-PCR, using whole thoraces (minus wings and legs). These results indicated elevations in mRNA levels for most of the CF2 mutants (data not shown). To verify and quantitate these results, we next performed qPCR on cDNA from the five CF2 mutants and a wild type control, using primers for Act88F, and the flight muscle specific isoforms of mhc and Tn I. The results of the most consistent qPCR run were diagrammed in a graph in Fig. 4A. The 08941 mutant showed the most dramatic effects, averaging (over 2 separate runs) a 71-fold increase in Act88F mRNA levels, a 50-fold increase for mhc, and a 8-fold increase for Tn I. The 0342 and 04624 mutants were the next strongest by this measure. 0342 had average transcript elevations of 18x, 7x, and 1.3x for Act88F, mhc, and Tn I; for 04624 the same RNA levels were up 7x, 20x, and 1.5x. The 01640 mutant had a weaker RNA phenotype, with Act88F up an average ~3.5x, mhc up 14x, and Tn I down 1.6x. The R3 transcription phenotype was the mildest, with only a 1.7-fold increase in Act88F,
a 3.7x increase for mhc, and a 3.6-fold decrease in Tn I. These results were subjected to student’s t-test to verify statistical significance, and with the exception of one of the R3 act88F assays, all P values were 0.05 or lower. Quantitative PCR testing of CF2 transcript levels in these mutants showed no significant changes between the mutants and wild type, (data not shown).

To further verify that these effects are due to loss of CF2 function, we repeated this experiment with two CF2-RNAi lines, expressed via an 88F-GAL4 driver (Figure 4B). CF2 specific primers (which detect all isoforms) show that the RNAi construct does indeed lower CF2 transcript levels, by over 50% 11924-R2 line, compared to the driver only control. The 11924-R3 line increased Act88F transcript levels almost 2.5x, mhc over 1.5x, and Tn I by almost 5 fold. These differences are not as great as those observed between the stronger CF2 mutants and wild type, but the 88F-GAL4 has its own effects of muscle structural genes. Compared to wild type, the driver alone caused elevations in mRNA levels of the three structural genes tested, in addition to CF2 itself (data not shown). Addition of a UAS-CF2-RNAi construct reduced the levels of CF2 mRNA, but caused further increases in Act88F, mhc, and Tn I transcripts, consistent with a reduction of CF2 function increasing levels of structural gene mRNAs. Taken together, analysis of six fly lines with altered CF2 function demonstrates that CF2 represses expression of these muscle genes in the IFM.

**CF2 overexpression disrupts IFM development**

If CF2 has a role as a repressor in the IFM, then an increase in function should cause a decrease in transcript levels of genes encoding structural proteins. To test this hypothesis we overexpressed wild type and mutant (T40A) forms of CF2 using an IFM specific GAL4 driver containing 1.3 kb of DNA upstream of Act88F. This driver mimics the IFM expression of Act88F, with expression first detected at around 40 hours APF. Ectopic CF2 expression caused major disruption of the IFM. Fig. 5A shows a cross section through a wild type thorax, with its characteristic indirect flight muscle pattern. When wild type CF2 is overexpressed (Fig. 5B), only a few shreds of muscle tissue can be found at the dorsal part of the thorax; the majority of the space is empty of any muscle tissue. The T40A mutant form (UAS-CF2[A40]), which removes a phosphorylation site crucial to control of the subcellular localization of CF2 (via nuclear export) [18] has an even more severe effect (data not shown).

The use of two different GFP reporters produced two strikingly different results. When an Act 88FGFP reporter was introduced into these genetic backgrounds, there is no expression of GFP in the CF2 gain of function, even in the remnants of muscle tissue (Fig. 5D, wild type control Fig. 5C). In contrast, a mhc-GFP marker is not affected. GFP expression is strong in the muscle remnants (Fig. 5F, wild type control Fig. 5E), which are concentrated in the dorsal and anterior most portion of the thorax. Examination of developing IFM in pupa with overexpressed CF2[A40] and a mhc-GFP marker revealed that much of the muscle tissue fails to develop (Fig. 5G). The animal had reached to stage where its eyes were pigmented, which makes it further along in its development than the animals shown in the bottom panels of Figs. 5B and C, yet those younger animals have a full set of IFM close to their final size. We also tested a mhc-GAL4 driver (constructed with a 580 bp IFM specific enhancer region upstream of mhc, see Fig. 6A) with the UAS-CF2 constructs, and looked at the IFM of pharate adults. The wild type CF2 produced a milder phenotype with this driver (Fig 5H), with the IFM thinner than wild type (Fig. 5I), but intact from anterior to posterior. Sections of these mutant thoraces (Fig. 5L) confirmed that the IFM were considerably thinner than
driver only control IFM (Fig. 5K). The A40 mutant construct produced IFM disruption as equally severe as seen with the 88F-GAL4 driver (Fig. 5J), with only a few shreds of IFM expressing RFP at the anterior of the thorax. These results demonstrate that an excess of the CF2 transcription factor has a detrimental effect on the development of IFM. The most likely cause is repression of the transcription of structural genes. This is not currently directly testable due to: 1) a reduction in muscle mass if excess CF2 is expressed during muscle development, and 2) the lack of an inducible system that does not have its own effect on structural gene mRNAs. For example, when the GAL80.ts system was tried in order to induce excess CF2 after IFM development was complete, the higher temperatures required caused a decrease in IFM structural genes of control flies, making such an experiment impossible to interpret (data not shown).

**CF2 may not directly affect mhc transcription**

Despite the effects of loss or gain of CF2 function on mhc transcripts, expression of a mhc-GFP reporter is not affected by overexpression of CF2. Since we did not know the precise location of the sequences driving that reporter, we used a series of upstream mhc reporters we had generated to examine more precisely the effects of CF2 on mhc transcription in the IFM. We had previously searched the 9 kb region upstream of mhc, using a series of overlapping constructs for muscle (in particular IFM) enhancer elements. Fig. 6A diagrams the location of regions that drive muscle expression. Our analysis revealed a great diversity in both spatial and temporal control of mhc expression. We found multiple elements for a number of different muscle types (listed in Table 1), and of most interest to us, three different types of IFM enhancers. The first type of element (denoted as Class 1, diagrammed in red in Fig. 6A) is expressed at the onset of myofibrilogenesis, in a pattern reminiscent of the Act 88F GFP reporter (Fig. 6B compares the two expression patterns). Expression in the IFM remains strong throughout the adult life of the animal. A Class 2 element (diagrammed in blue in Fig. 6A) also expresses at the beginning of myofibrilogenesis, but the expression is weaker, and it diminishes to undetectable levels within a few days after eclosion. Class 3 elements (diagrammed in green in Fig. 6A), are not expressed until after the IFMs have finished elongating (Fig. 6C compares this pattern with the Act 88F pattern).

We crossed a representative of each of the 3 types of mhc IFM enhancer reporter lines into a CF2 gain of function background. The Class 3 element we chose, F4-678, is also expressed in larval somatic muscle, and has a putative CF2-II binding site (∼381 to ∼372) in close proximity to a putative MEF2 site (∼412 to ∼404). It should be noted that constructs F4-728 and F4-678 are contained within a region that has been previously tested for mhc muscle enhancers [14,19]. All three types of enhancers continued to drive GFP expression even when an excess of CF2 protein is present (Fig. 6D). The muscle mass is clearly reduced, and bunched at the anterior of the thorax, but still strongly expresses GFP. Given the reduction of native mhc transcripts seen with ectopic expression of CF2 (Fig. 5H), if direct repression by CF2 were the cause, we would expect to see an effect on at least one of the mhc reporters. Our results suggest that a reduction in mhc mRNA could be caused by a mechanism other than direct transcriptional repression by CF2.

**Discussion**

In this paper we investigate the expression pattern and function of the Zn finger transcription factor CF2 in adult flight muscle...
development. We find that the isoform expression pattern in the IFM changes over time, with isoforms II and III present during the building of the muscles, and isoforms I and II expressed in the adult muscle. We have also characterized the IFM phenotypes of CF2 mutants. In the loss of function mutants, transcripts of three muscle structural genes (\textit{Act 88F}, \textit{mhc}, and \textit{Tn I}) are increased, and the stronger mutations cause an \textasciitilde 50\% increase in myofibril size. However the hexagonal arrangement of thick and thin filaments is not perturbed. CF2 gain of function severely impairs the development of the IFM, most likely by the downregulation of structural genes. Lastly, our data point to CF2 as a probable direct negative regulator of \textit{Act 88F}, but possibly indirect regulator of \textit{mhc}. The exact nature (direct or indirect) of the effects of CF2 on \textit{Tn I} remains to be resolved.

Dynamic isoform expression pattern of CF2 in the IFM

During the early to mid stages of pupal development, when the sarcomeres are still assembling, strong expression of isoforms II and III at both the mRNA and protein level is observed in the IFM, while isoform I replaces isoform III in the adult stage. This represents a novel expression pattern for isoform III, which was previously reported to be exclusive to the testes [15]. Our methods (RT-PCR and Western blotting) are more sensitive than the combination of PCR and Southern Blots used in this previous study [15], and in addition we used material from dissected IFM thoraces rather than whole animals. Isoform III shares the first three N-terminal zinc fingers found in isoforms I and II, but the lack of exon 3 causes a frame shift that eliminates the C-terminal zinc fingers. As zinc fingers 4, 5, 5', and 6 have been shown to be
is essential for DNA binding [16], isoform III would be unlikely to bind DNA. However, the common three N-terminal zinc fingers could facilitate protein-protein interactions. A possible function for isoform III is to modulate the activity of isoform II, by sequestering co-factors that bind the N-terminal region. Isoform III could be needed to prevent isoform II from being too active (and thus

![Figure 6](image)

**Figure 6.** CF2 overexpression does not directly inhibit mhc transcription in the IFM. A) Map of the upstream region of mhc, showing regions that drive muscle expression. Green bars (late IFM elements) denote elements that express later in IFM development (after ~70 hrs APF, class 3), red indicates an element (early IFM element) that is expressed at the onset of myofibrillogenesis (~40 hrs APF at 22°C, class I), and blue an early IFM element that ceases expression in early adulthood (class 2). The black bars denote enhancer elements that drive expression in muscle types other than IFM. B) Expression patterns of GFP and RFP in animals carrying both the mhcF3-580-GFP early enhancer and an 88F-RFP reporter. C) Expression patterns of GFP and RFP in animals carrying both the mhcF4-631-GFP late enhancer and an 88F-RFP reporter. D) Expression of the three different classes of mhc reporters in a CF2 gain of function background. doi:10.1371/journal.pone.0010713.g006

**Table 1.** Muscle expression patterns of upstream mhc enhancers.

| Element   | Location | Muscle Expression Pattern                                      |
|-----------|----------|---------------------------------------------------------------|
| mhcF2-479 | -7600 to -7111 | larval somatic muscle, jump muscle (mid pupa-adult), IFM (mid pupa-adult) |
| mhcF2-510 | -7201 to -6692 | larval somatic muscle, jump muscle (mid pupa-adult), IFM (mid pupa-adult) |
| mhcF2-491 | -6768 to -6278 | subset of larval somatic muscles, leg muscle (pupa-adult) |
| mhcF3-580 | -5165 to -4586 | IFM (40 hrs APF-adult), dorsal vessel (midpupa), subset of abdominal muscles and upperleg muscles (pharate adult), proboscis muscles (late pupa) |
| mhcF3-548 | -3851 to -3303 | jump muscle, dorsal vessel, leg muscle (pupa) |
| mhcF3-522 | -3360 to -2838 | larval somatic muscle, proboscis muscles, leg, jump muscle (adult) |
| mhcF4-631 | -2158 to -1528 | IFM (mid pupa-adult), dorsal vessel (midpupa) |
| mhcF4-728 | -6857 to -913 | larval somatic muscle, IFM (40 hrs APF-early adult) |
| mhcF4-678 | -1048 to -371 | larval somatic muscle, jump muscle (mid pupa-adult), IFM (mid pupa-adult), leg muscles, abdominal muscles |
| mhcF4-453 | -485 to -33 | larval somatic muscle, jump muscle (mid pupa-adult), IFM (mid pupa-adult), leg muscles, abdominal muscles |

Location refers to the position of the cloned sequences relative to the mhc transcription start site, which is considered to be position 0. Animals were examined at the larval, pupal, and adult stages for GFP expression. All elements are diagrammed in Fig. 6A.

doi:10.1371/journal.pone.0010713.t001
repressing the Act 88F level too much) during myofibrillogenesis. In the adult IFM, isoform III is replaced by isoform I. This isoform has an extra DNA binding Zn finger. It preferentially binds a 12 bp consensus site [16], but it too may compete with isoform II, for cofactors if not also for binding sites. Isoform I may be functioning in a “maintenance mode” after the IFM myofibrils have been built to their appropriate size. Earlier studies [14] reported that the 05342 and 08941 mutant lines are flight impaired, and this effect worsened with age. This phenotype could be due to the lowered levels of isoform I in the IFM of these CF2 mutants.

Reconciling protein/transcript levels and ultrastructural changes in CF2 mutant myofibrils

At first glance there looks to be a number of contradictions between the Western, qPCR, and myofibrillar phenotypes of the five different CF2 mutants. The Western blot data indicate a decreasing hypomorphic series (based on amounts of CF2 protein) with R3 > 04624 > 08941 = 05342 = 01640. This, which produces an altered protein, would fall outside this grouping. However, when effects on the amounts of Act88F and mhc mRNAs are considered, the order of severity is changed, with 08941 > 05342 = 04624 > 01640 > R3. The results from measurement of Tn I transcripts are more complicated. The 08941, 05342, and 04624 mutants all show increased levels of Tn I transcripts. The opposite is observed with 01640 and R3, which cause a decrease in Tn I transcript levels. Expression of CF2-RNAi in the IFM causes an increase in Tn I mRNA, lending support to the hypothesis that a reduction in CF2 function caused increased transcription of Tn I (in addition to Act88F and mhc). As mentioned above, 01640 is most likely not a simple hypomorphic mutation. Given the presence of P-element sequences in the CF2 mRNA of this mutant (Fig. 2G), and a band on the Western blot (Figs. 2D and E), that does not correspond to any of the three CF2 isoforms, it is most probable that the 01640 mutation produces a truncated CF2 protein. The 01640 P insertion is within the portion of exon 3 that is uniquely spliced into isoform I. The coding sequences upstream, which include Zn fingers 1–4 (and half of Zn finger 5) could reasonably be expected to be present in this new CF2 protein. Such a protein could have changed functions, which might account for the differences in effects on muscle gene expression from those seen with the hypomorphic 08941, 05342, and 04624 alleles.

Why does R3 appear to be the strongest CF2 mutant by protein levels, but is the weakest when the effects of muscle gene transcription are tested? The answer may be that it is not just a CF2 hypomorph. R3 was produced by excision of the 08941 P-element. Preliminary mapping via PCR analysis has indicated that while the ATG site within exon 2 is intact, exon 1 is missing. Furthermore, at least 2.7 kb of DNA upstream of exon 2 is also missing. The nearest gene upstream of CF2, GC3008, is only 304 bp upstream of the CF2 transcription start site. Therefore the R3 deletion will also affect this gene. GC3008 is predicted to be a kinase (http://flybase.org/reports/FBgn0031643.html), but there is no current data on its adult expression pattern or phenotypes of any mutants. There are also several uncharacterized genes not far downstream of GC3008 that could also be potentially affected by the R3 deletion. There is no doubt that R3 affects CF2, given the greatly reduced levels of CF2 protein and the enlarged myofibril phenotype, but changes in any of these neighboring genes could have their own effects on transcription in the IFM. These effects could mitigate some of the effects of reduced CF2 in the R3 allele. Further mapping of the R3 deletion and examination of these neighboring genes should clarify this matter. In summary, 08941, 05342, and 04624, which produce increases in muscle gene transcript levels that are in agreement with those produced by RNAi knockdown, are hypomorphic mutations. 01640 is most likely a neomorph, and R3, while a CF2 hypomorph, is also mutant for at least one additional gene.

When considering the ultrastructural changes in CF2 mutants, one correlation is unambiguous: the three lines with the greatest reductions in CF2 protein (08941, 04624, and R3) are the ones that display an enlarged myofibril phenotype. With the R3 and 04624 myofibrils, it is possible to get an accurate measure of the increased size by counting the numbers of thick filaments. 08941 myofibrils appear to be comparable in size, but the lack of any focus on distinct filament structures makes counting thick filaments impossible. There is a weaker correlation here between the lack of sharp filament structures and the increases in structural gene transcription. 08941, which has the greatest increases in mRNA levels, also has the “fuzziest” myofibrils. The myofibrils of 05342 mutants, which have strong mRNA increases, also lack a clear focus on filament structure. Instead of the hexameric pattern, vague diamond or lines patterns are commonly observed instead. Such patterns can be seen in wild type myofibrils cut at an angle, as opposed to a 90° cross section. However such a cut would create oval, as opposed to round, myofibril slices. Since the slices in our samples are round, indicating a proper cutting angle, we speculate that the filaments in these mutants may not be oriented properly, so that they get cut at an angle. As mentioned, sarcomeric structures are observed in longitudinal section, so obviously thick and thin filaments are present. However there may be subtle defects in how the filaments line up, caused by excess amounts of protein, which could interfere with proper filament alignment. 04624 has an intermediate phenotype; the increases in transcript levels are comparable to 05342, but the filament structure begins to come into focus, although it is not as sharply defined as it is in R3 myofibrils. 01640 does not fit in neatly here, as the transcription phenotype is weaker than 04624, but the myofibril filament structure is diffuse and does not come into focus.

These differing phenotypes argue against a simple model where an increase in the amount of structural proteins is the direct cause of an increase in myofibril diameter. This suggests an additional function for CF2 that involves sensing/control of myofibril size, in addition to insuring proper filament ratios. Our data show a small but significant increase in myofibril diameter in several of the CF2 mutants over wild type. Even in the case of a mutant such as R3, where the filament balance appears to be restored, and the hexameric filament pattern is intact and properly aligned, the increase in myofibril size could be suboptimal for flight, as it could mean less room for mitochondria. Such mutant flies could be capable of short bursts of flight, but lack endurance for longer flights. There is one other report in the literature of a mutant that increases myofibril diameter, flt H [20]. These flies are flightless, but there were other defects observed in addition to increased myofibril diameter, such as disorganized filaments and defects in Z-bands, which are not observed in R3 or 04624 mutants. The gene corresponding to the flt H mutation has not been identified, so nothing is known at the molecular level about its role in flight muscle development.

CF2 functions in adult muscles are not the same as in embryonic muscles

In the embryonic and larval somatic muscles, CF2 plays a role as a transcriptional activator. In cooperation with Me2, it activates transcription of a number of different structural genes. However, in the developing IFM, our data point to an opposite role. It should be noted that muscle genes that are activated synergistically by Me2 and CF2 tend to be expressed in both the
larval and adult stages [14]. But *Act 88F* is a gene uniquely expressed in the IFM, distinct from the 57B actin gene expressed in larval muscle. There are putative CF2 binding sites in the first intron of *Act88F*, but no putative Me2 sites nearby. We see an increase in *Act 88F* mRNAs with CF2 loss of function (heteromorphic mutant background or 88F>Cf2-RNAi). We also observe repression of the *Act 88F* reporter. These data point to a likely role for CF2 as a direct transcriptional repressor of *Act 88F*. The use of an 88F-GAL4 driver raises a question because it shares most if not all of its sequences with the 88F-GFP reporter; would not the ectopic CF2 negatively feedback and repress its own expression? We suspect that it most likely does, but not before such a massive overexpression of CF2 (as the 88F enhancer is very strong, [21]) does its damage and disrupts IFM development.

Because of the potential problems with negative feedback, we also tried a *mhc-GAL4* driver (made from the F3-580 upstream fragment diagrammed in Fig 6A) to drive ectopic CF2 expression, a reporter that is not quenched by ectopic CF2. The wild type CF2 construct had a milder effect with this driver, resulting in IFM noticeably thinner than wild type, but intact from anterior to posterior, in contrast to the effects of the 88F-GAL4 driver. CF2[A40] behaved the same with both drivers, causing an almost total ablation of muscle tissue. The severity is most likely due to the removal of the phosphorylation site, resulting in a protein that cannot be controlled by shutting it out of the nucleus [18].

*Tn I* transcript levels are also altered by hypomorphic CF2 mutations, although we cannot say with our present data whether this effect is direct or indirect. Previous studies [22] demonstrated that the overexpression of *Tn I* caused downregulation of the transcription of other thin filament genes, which implies a system of coordinate regulation of thin filament genes. CF2 could be acting directly on this gene or the effect could be indirect as a response to alterations in level of *Act 88F* transcripts. It has been reported that the 05342 and 08941 mutant lines exhibit a decrease in *Tn I* transcript levels in embryos, pupae, and adults [14], which appears to be in contradiction with our results. The conflicting results may be explained by the differences in how the experiments were performed. We used a primer set from *Tn I* exons 3 and 4 for our qPCR, which would detect only the IFM-specific isoform of *Tn I*. The earlier studies [14] used primers from *Tn I* exons 7 and 8, which would amplify all *Tn I* transcripts. It is possible that a strong downregulation in the non-IFM muscles of the CF2 mutants could mask an increase in *Tn I* transcript in the IFM.

Despite the same kind of effects observed on *mhc* mRNAs as seen with *Act 88F* and *Tn I*, we have no evidence for a direct role for CF2 in the regulation of this gene in the IFM. We tested four different *mhc* reporters (three with known *mhc* enhancer regions) in CF2 gain of function backgrounds, and found no negative effects on GFP expression such as we observed with the *Act88F-GFP* reporter. Intronic *mhc* enhancers have been reported [19] and we have not tested any of these. But the enhancers we did test were strong drivers of GFP expression, and one of them (F4-676) even had a close grouping of Me2 and CF2 sites, which likely accounted for strong larval somatic muscle expression. It is unlikely that CF2 could be causing changes in *mhc* levels by affecting an untested enhancer while having no effects on the enhancers we found, but we cannot completely rule out the possibility that we may have separated IFM enhancers away from more distant regulatory elements.

**CF2 may “fine tune” filament stoichiometry**

In *mhc* or *Act88F* heterozygotes, there is no upregulation of the remaining wild type copy sufficient to correct the imbalance in filament stoichiometry. This could mean that there is no system for such upregulation, or alternatively, any existing system is not strong enough to reverse that great an imbalance. It is interesting to note that in *Mhc(1)/+ IFM*, the myofilibrils are ~30% smaller than wild type [2], which could represent a not quite successful attempt by such a putative system to regain proper filament ratios. In contrast, two of the strongest CF2 mutants (as measured by CF2 protein level) have wild type-like hexagonal arrangements of filaments, in myofilibrils that are up to 50% to 60% larger than wild type. While we have examined only one thick filament gene and two thin filament genes, it is likely that expression of other structural genes is also modulated, directly or indirectly. It has been noted that CF2 is not absolutely required for initiation of expression of embryonic muscle genes, but rather it functions to modulate levels of gene expression [13,14]. CF2 may play a similar role in the control of *Act88F* expression, except as a repressor in the IFM context. As a modulator, a reduction in its function would have a milder effect, at least in the cases of alleles such as *04624* and *R3*, an effect possibly weak enough to allow correction within the capabilities of a potential stoichiometry sensing/maintenance system. The phenotypes of these two mutants could present an opportunity to screen for genes involved in sensing and/or maintaining myofilibril size or filament stoichiometry, by providing a sensitized background.

**Materials and Methods**

**Fly stocks and genetics**

All fly stocks were raised on standard media at 22°C unless otherwise specified. *yw[67c23]* served as the wild-type control, CF2 mutant stocks *CF2[KG05342]* and *CF2[KG08941]* were obtained from the Bloomington Stock Center (University of Indiana, Bloomington IN), *CF2[c04624]* and *CF2[c01640]* from the Harvard Exelixis collection (Harvard Medical School, Boston MA), and UAS-CF2-RNAi (11924-R2) from the National Institute of Genetics (Mishima, Shizuoka, Japan). The *CF2[KG08941-R3]* mutant was generated by P-element excision of the *CF2[KG08941]* stock. The *mhc-GFP* [23] and *UAS-CF2-II[wt]* and *UAS-CF2-II[A40]*/strains [18] have been described previously.

**Construction of IFM GAL4 driver lines**

A 1.3 kb fragment of *Act88F* upstream region was amplified via PCR using the primers 5′-ggtacctaaataaaacgtctttggaagtgc-3′ and 5′-ggatccctgacattgaggtcgcactc-3′. The fragment was cloned into a GAL4 vector described previously [24] and used to make transgenic strains by the standard methodology. Transformant lines were tested for IFM expression by crossing to UAS-GFP. GFP expression is first observed in the IFM at around 40 hours APF (after pupa formation), and in the upper legs of pharate adults, a pattern reported previously [25]. A chromosome II line, 81B, was recombined with *Act88F-GFP* (fly line from S. Bernstein) to produce line 81B-13. A chromosome III line, 69, was recombined with a *mhc-GFP* (from E. Chen) to produce line 69-5. The mhc-GAL4 driver was made with a 580 bp upstream fragment that first shows IFM expression at about 40 hours APF (see Figs. 6A and B). A *mhc-GAL4*, *mhc-RFP* recombinant line was made via standard techniques.

**Immunostaining of adult IFM**

Adults were fixed using the high-octane fixation protocol (R. Carthew). IFM were stained with anti-CF2 antibody (1:1000) and a biotinylated goat anti-rabbit 2′ antibody (1:1000, Vector, Burlingame, CA). Photographs were taken with a Zeiss Axioscope 2 microscope and digital camera using AXIOVISION V3.1 software.
qPCR
RNA from whole flies of the appropriate genotype was purified using Trizol (Invitrogen, Carlsbad, CA), and treated with RQ1 DNase (Promega, Madison, WI) to remove any residual genomic DNA. The Superscript II cDNA kit (Invitrogen) was used to make cDNA. All qPCR reactions (total volume of 10 µl) were done in triplicate with the Applied Biosystems (Foster City, CA) SYBR Green Master Mix, 400 ng of cDNA, 50 nM primers, in an Applied Biosystems 7500 Fast Real Time PCR System using the 7500 v2.0.1 software. The primers (exact sequences available upon request) were designed to include flight muscle specific exons when applicable (exon 3 for Tr I and exon 11e for mhc). Measurement of GAPDH1 RNA levels was used as the reference.

RT-PCR
RNA and cDNA from freeze-dried thoraces, dissected IFM, or whole flies was prepared as described previously [26]. The sequences of the primers used are available upon request.

Paraffin sections
Embedding and sectioning of adult thoraces was done as described previously [26]. Photographs were taken as described above with a GFP filter.

TEM of flight muscles
Indirect flight muscles were prepared from 1 to 2 day old adults for transmission electron microscopy and photographed as described previously [26]. Myofibril sizes were quantified by counting the thick filaments of 13 single myofibrils magnified at 60,000× for each genotype. Differences from wild type were tested for statistical significance with student’s t-test.

Construction of mhc and Act88F reporters
The β3Facin reporter was made by inserting the 1.3 kb fragment used for construction of the GALA driver into a Pelican vector with the GFP region replaced with an RFP construct (Ds-Red from BD Biosciences, San Jose, CA).

A 9-kb region upstream of the mhc gene was amplified via PCR to produce a series of overlapping fragments [primer sequences available upon request]. These fragments were cloned into the Green Pelican vector and used to create transgenic fly strains via standard techniques. Transgenic animals were monitored under fluorescent light during the larval and pupal stages to determine any muscle expression pattern. Live animals expressing GFP or RFP were photographed with a LeicaMZFLIII Stereomicroscope, using ImagePro 6.0.

Western blotting
Freeze dried IFM or whole thoraces of each genotype were pooled, ground in liquid nitrogen, and resuspended in 50 µl diH2O/50 µl Western sample buffer (125 mM Tris 6.8, 6% SDS, 20% glycerol, 0.025% bromphenol blue, 10% β-mercaptoethanol), and heated to 100°C for 10 minutes. Samples were resolved on 8% polyacrylamide gels and transferred to Immobilon-P (Millipore, Billerica, MA) using standard protocols. Membranes were incubated overnight at 4°C with CF2 antibody (a polyclonal that detects all three isoforms) at a dilution of 1:15,000 or actin antibody (MAB 1501, Millipore) at a dilution of 1:1000. Secondary antibody (anti-rabbit-HRP for CF2, anti-mouse-HRP for actin, Pierce, Rockford, IL) was used at a dilution of 1:4000. Protein bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Acknowledgments
We would like to thank T. Hsu for CF2 antibody and Kenn Dunner, Jr. for TEM work. We also thank G. Halder, Y. Furuta, and M. Barton for their support, W. Mattox for comments on the manuscript, L. McCord for help with figures, and R. Cripps for opinions on EM data. We are very grateful to K. Allton and W. Bossuyt for assistance with qPCR.

Author Contributions
Conceived and designed the experiments: KMG. Performed the experiments: KMG. Analyzed the data: KMG. Received the paper: KMG. Head of the lab-provided equipment and funding: RAS.

References
1. Barbas JA, Galceran J, Torroja L, Prado A, Ferrús A (1993) Abnormal muscle development in the heldp1 mutant of Drosophila melanogaster is caused by a splicing defect affecting selected tropoinin I isoforms. Mol Cell Biol 13: 1433–1439.
2. O’Donnell PT, Bernstein SI (1988) Molecular and ultrastructural defects in a Drosophila myosin heavy chain mutant: differential effects on muscle function produced by similar thick filament abnormalities. J Cell Biol 107: 2601–2612.
3. Beall CJ, Sepsanski MA, Fyrberg EA (1989) Genetic dissection of Drosophila myofibril formation: effects of actin and myosin heavy chain null alleles. Genes Dev 3: 131–140.
4. Reddy MG, Beall C (1995) Ultrastructure of developing flight muscle in Drosophila. I. Assembly of myofibrils. Dev Biol 160: 443–465.
5. Cripps RM, Becker KD, Mardahl M, Kronert WA, Hodges D, et al. (1993) Construction of mhc reporter with the GFP region replaced with an RFP construct (Ds-Red from BD Biosciences, San Jose, CA).
6. Cripps RM, Becker KD, Mardahl M, Kronert WA, Hodges D, et al. (1993) Abnormal muscle development in the heldp1 mutant of Drosophila melanogaster is caused by a splicing defect affecting selected tropoinin I isoforms. Mol Cell Biol 13: 1433–1439.
7. O’Donnell PT, Bernstein SI (1988) Molecular and ultrastructural defects in a Drosophila myosin heavy chain mutant: differential effects on muscle function produced by similar thick filament abnormalities. J Cell Biol 107: 2601–2612.
8. Beall CJ, Sepsanski MA, Fyrberg EA (1989) Genetic dissection of Drosophila myofibril formation: effects of actin and myosin heavy chain null alleles. Genes Dev 3: 131–140.
9. Reddy MG, Beall C (1995) Ultrastructure of developing flight muscle in Drosophila. I. Assembly of myofibrils. Dev Biol 160: 443–465.
10. Cripps RM, Becker KD, Mardahl M, Kronert WA, Hodges D, et al. (1993) Construction of mhc reporter with the GFP region replaced with an RFP construct (Ds-Red from BD Biosciences, San Jose, CA).
11. Hsu T, Bagni C, Sutherland JD, Kafatos FC (1996) The transcription factor CF2 is a myogenic marker downstream of MEF2 during muscle development. Dev Biol 177: 265–268.
12. Bagri C, Bray S, Gogos JA, Kafatos FC, Hsu T (2002) The Drosophila zinc finger transcription factor CF2 is a myogenic marker downstream of MEF2 during muscle development. Dev Biol 177: 265–268.
13. Tanaka KK, Bryantsev AL, Cripps RM (2008) Myocyte enhancer factor 2 and chorion factor 2 collaborate in activation of the myogenic program in Drosophila. Mol Cell Biol 28: 1616–1629.
14. Garcia-Zaragoza E, Mas JA, Vivar J, Arredondo JJ, Cervera M (2008) CF2 activity and enhancer integration are required for proper muscle gene expression in Drosophila. Mech Dev 125: 617–630.
15. Hsu T, Gogos JA, Kirsh SA, Kafatos FC (1992) Multiple zinc finger forms resulting from developmentally regulated alternative splicing of a transcription factor gene. Science 257: 1946–1950.
16. Gogos JA, Hsu T, Bolon J, Kafatos FC (1992) Sequence discrimination by alternatively spliced isoforms of a DNA binding zinc finger domain. Science 257: 1951–1955.
17. Thibault ST, Singer MA, Miyazaki WY, Milash B, Dompe NA, et al. (2004) A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. Nat Genet 36: 283–287.
18. Mantorova EV, Hsu T (1998) Down regulation of transcription factor CF2 by Drosophila RAS/MAP kinase signaling in oogenesis: cytoplasmic retention and degradation. Genes Dev 12: 1166–1175.
20. Koana T, Hotta Y (1978) Isolation and characterization of flightless mutants in *Drosophila melanogaster*. J Embryol Exp Morphol 45: 123–143.

21. Barthmaier P, Fyrberg E (1995) Monitoring development and pathology of *Drosophila* indirect flight muscles using green fluorescent protein. Dev Biol 169: 770–774.

22. Marco-Ferreres R, Arredondo JJ, Fraile B, Cervera M (2005) Overexpression of troponin T in *Drosophila* muscles causes a decrease in the levels of thin-filament proteins. Biochem J 386: 145–152.

23. Chen EH, Olson EN (2001) Antisocial, an intracellular adaptor protein, is required for myoblast fusion in *Drosophila*. Dev Cell 1: 705–715.

24. Gajewski K, Choi CY, Kim Y, Schulz RA (2000) Genetically distinct cardiac cells within the *Drosophila* heart. Genesis 28: 36–43.

25. Nongthomba U, Pasalodos-Sanchez S, Clark S, Clayton JD, Sparrow JC (2001) Expression and function of the *Drosophila* ACT88F actin isoform is not restricted to the indirect flight muscles. J Muscle Res Cell Motil 22: 111–119.

26. Gajewski KM, Wang J, Schulz RA (2006) Calcineurin function is required for myofilament formation and troponin I isoform transition in *Drosophila* indirect flight muscle. Dev Biol 291: 17–29.