Genetics of the Drosophila flight muscle myofibril: a window into the biology of complex systems

Jim O. Vigoreaux

Summary
This essay reviews the long tradition of experimental genetics of the Drosophila indirect flight muscles (IFM). It discusses how genetics can operate in tandem with multidisciplinary approaches to provide a description, in molecular terms, of the functional properties of the muscle myofibril. In particular, studies at the interface of genetics and proteomics address protein function at the cellular scale and offer an outstanding platform with which to elucidate how the myofibril works. Two generalizations can be enunciated from the studies reviewed. First, the study of mutant IFM proteomes provides insight into how proteins are functionally organized in the myofibril. Second, IFM mutants can give rise to structural and contractile defects that are unrelated, a reflection of the dual function that myofibrillar proteins play as fundamental components of the sarcomeric framework and biochemical “parts” of the contractile “engine”. BioEssays 23:1047–1063, 2001. © 2001 John Wiley & Sons, Inc.

Introduction
Functional genomics is making possible the description of biological processes from a broad, systems perspective. Genome sequence information reveals the identity of the principal molecular players that define life. As we embark into the “post-genome” era, great efforts are being expended in trying to elucidate when and where these molecular players are active and how their interactions determine the fate of the cell and the organism.

The genome era has unleashed a new data-driven, large-scale style of molecular science. Many biologists are facing the imminent task of consolidating their “bits” of information, derived from painstaking years of reductionist research, with “terabyte” datasets being generated daily by new, expansive technologies. The culmination of these efforts will be the grand realization of how genotype dictates phenotype. For this goal, model experimental systems that are amenable to interdisciplinary functional validation are highly desirable.

Research into muscle function has spanned from the molecular to the organismal, resulting in a broad body of knowledge about the properties of this remarkable tissue. It is often difficult to consolidate information gained from disparate disciplines, either because the research is conducted on different experimental specimens (organism or muscle type) or because we lack the conceptual tools to properly transpose the findings of one discipline to another.

This essay reviews the historical tradition in Drosophila IFM research and discusses how this experimental model can be exploited to provide an integrated view of muscle function. Genetics plays a pivotal role in affording an integrative approach and enabling the validation of functional assays across levels of biological organization. Genetics also provides the bridge between traditional, small-scale research and new, large-scale approaches. Examples of mutations in myofibrillar proteins are presented to illustrate the multiple faces of protein function and how genetics provides information about proteins as individuals and as pegs in the cellular proteome. An understanding of the broad properties and multifarious functions of myofibrillar proteins is seen as an important step towards a systems-wide understanding of muscle function.

Historical synopsis
It has been almost 100 years since Drosophila melanogaster entered genetics research. The fly has made outstanding contributions to our knowledge of genes and genomes, from the first genetic linkage map to the annotated sequence of the entire genome.(1) Fly genetics has played a primary role in the functional annotation of genes and in deciphering gene networks that control embryogenesis, development and differentiation.

For the past six decades, insect IFM has been a staple of research on muscle function. The pioneering work of Pringle and colleagues with Lethocerus IFM led to the discovery of stretch activation(2) (Fig. 1). Research on Lethocerus IFM also uncovered the ATP-dependent changes in crossbridge angle that contributed to the formulation of the “swinging crossbridge” model of muscle contraction.(3) The high structural order of insect IFM has lent itself well to X-ray diffraction studies, electron microscopy and three-dimensional reconstruction, the results of which continue to provide a privileged view of the fine details of actomyosin interaction.(3)

Drosophila IFM shares many of the structural features of its Lethocerus counterpart and, in addition, is amenable to...
genetic manipulation. The properties and features that make fly IFM an excellent experimental model are well documented. It is remarkable that the IFM has served to study biological phenomena ranging from the gene (transcriptional regulation) to the population (fitness traits), at practically every level of biological organization (Table 1). More significantly, genetics has afforded the integration of molecular structure and cellular function with muscle performance and animal locomotion. In addition to vertical integration across levels of biological organization, studies of *Drosophila* flight aerodynamics afford a horizontal integration across physiological systems that contribute to locomotion.

Genetics provides a useful experimental approach for uncovering protein function. As with a pickax, genetics can be utilized in two different ways. The pick end of the tool is used to “dig out” individual amino acids that dictate specific properties of the protein (herein referred to as molecular function). The broader, chisel-edge is used to “scrape away” the protein’s association with other proteins and its contribution to cellular processes (herein referred to as cellular function).

**Genetics and the molecular function of muscle proteins**

**Identification of myofibrillar protein genes**

Table 2 lists all known IFM myofibrillar proteins and summarizes how each gene or protein was first identified. Recent analysis of the complete *Drosophila* genome with gene prediction software has led to the identification of a potentially new myofibrillar gene, *stretchin-mlck*, that encodes several conceptual proteins, including a large 926 kDa titin-like kinase. Thus, an important application of genome data is to identify new myofibrillar genes. This is particularly important in light of the fact that additional genes whose mutations give muscle phenotypes or that are expressed in muscle (as determined by enhancer trap lines) remain to be identified. Some of these genes may encode novel myofibrillar proteins while others are likely to encode proteins involved in muscle development, muscle attachment, and/or neuromuscular interaction.

Molecular studies that identified myofibrillar protein genes were, in many instances, preceded by genetic studies of IFM function. The convergence of these two approaches has played a major role in the functional annotation of these genes.

**Early genetic studies**

A number of genetic loci involved in muscle development and contractile function were first identified through mutant screens designed to isolate flightless flies (Fig. 2A). These screens offered the advantage that flightless flies could be easily identified among their flighted cohorts so large numbers of flies were tested for autosomal dominant and/or sex-linked flightless mutations. Furthermore, several mutant strains recovered exhibited additional phenotypes such as abnormal wing position or indented thoracic cuticle indicative of a defective IFM. Later studies revealed that many of these loci encode the major myofibrillar proteins actin (*Act88F*), myosin heavy chain (*Mhc*), tropomyosin (*Tm*), tropinin I

---

**Figure 1.** The indirect flight muscles of *Drosophila* and the mechanics of insect flight. Side view of the thorax showing the IFM consists of two sets of antagonistic muscles that are oriented nearly perpendicular to each other, the dorsal longitudinal muscles (DLM, left to right) and the dorsal ventral muscles (DVM, top to bottom). Both sets of muscles are anchored at the cuticle by epithelial tendon cells and move the wings indirectly through deformation of the thoracic cuticle. Contraction of the DVM raise the wings and stretches the DLM; in turn, contraction of the DLM lower the wings and also stretches the DVM. The stretch (L, length change) results in a concomitant increase in force (F) that rapidly decays but rises again to a high level after a short delay. This “stretch activation” response underlies the ability of the IFM to perform oscillatory work and produce the high power necessary for flight. For clarity, other muscles of the thorax are not shown.
and troponin T (upheld/indentated (up/int)), among others. However, a handful of the mutant genes had more unexpected functions. For example, erect wing encodes a DNA-binding protein and flapwing encodes a protein phosphatase.\(^8\) Other studies revealed that mutations that affect muscle innervation can also result in dominant flightlessness.\(^4\) Results from these early genetic studies suggested that only a limited number of myofibrillar genes are haploinsufficient for flight and that the majority of mutations interfere with the expression of multiple proteins within the IFM.

**Saturation mutagenesis**

To establish if additional autosomal genes can be mutated to produce a dominant flightless phenotype, Cripps et al. screened over 40,000 flies and obtained 22 new mutants that mapped to three loci.\(^12\) Most of the mutants recovered were *Mhc* or *Act88F* alleles. However, a third dominant flightless and recessive lethal loci, named *lethal(3)Laker* was also uncovered. An and Mogami conducted a massive screen (~120,000 flies) on the third chromosome from which they recovered ten *Act88F* alleles and thirteen other dominant flightless mutations.\(^11\) The availability of genome sequence should speed up the identification of the gene or genes affected by these mutations, especially since their genetic position is already known.\(^11\) In summary, the saturation studies, together with those described in the previous section, demonstrate that sarcomeric protein genes can be identified from relatively simple, high throughput genetic screens. However, the number of loci identified from these screens appears to be limited to those encoding abundant sarcomeric proteins.

In addition to the discovery of *lethal(3)Laker*, the study of Cripps et al was significant for two other reasons. First, they identified two *Mhc* alleles, *Mhc*\(^13\) and *Mhc*\(^19\), whose phenotype differed from all other mutants recovered. These MHC mutations appear to have little or no effect on IFM development but resulted in severe and irreversible shortening of the muscle fibers in the adult. Later studies showed that these mutations harbor single amino acid changes within the coiled coil region and probably affect the interaction of flightin with thick filaments, an interaction that appears to be critical for muscle cell integrity.\(^13,14\) Second, their study concluded that “not all major muscle protein genes appear to mutate readily to give dominant flightlessness and other techniques must be applied in order to identify additional genes and mutations within them.” A subsequent study provided the first systematic screen for autosomal (second chromosome) recessive muscle mutations in the IFM (Fig. 2C).\(^15\) The study relied on direct examination of IFM fiber morphology using polarized light microscopy and resulted in the identification of eight new complementation groups that result in IFM defects. Five of the eight complementation groups were mapped genetically but none of the genes have been identified.

**Reverse genetics**

A second strategy for obtaining recessive flightless mutations relies on hemizygosity for a chromosomal deletion or deficiency. Once a cloned gene or cDNA is in hand, the cytological position of the gene can be identified by in situ

---

**Table 1.** Genetic and molecular genetic manipulations of *Drosophila* IFM afford an experimental model for broad ranging biological questions

<table>
<thead>
<tr>
<th>Level</th>
<th>Properties</th>
<th>Assays</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>Gene</td>
<td>Transcriptional regulation</td>
<td>Transgenic expression of marker gene</td>
</tr>
<tr>
<td>E</td>
<td>RNA</td>
<td>Alternative splicing</td>
<td>Transgenic expression of minigene/RT-PCR</td>
</tr>
<tr>
<td>N</td>
<td>Protein</td>
<td>Abundance, modification</td>
<td>2DE</td>
</tr>
<tr>
<td>E</td>
<td>Myofilament</td>
<td>Polymization</td>
<td>Stop flow kinetics, <em>in vitro</em> motility, optical trap</td>
</tr>
<tr>
<td>T</td>
<td>Myofilament</td>
<td>Assembly</td>
<td>Filament sedimentation, solid phase binding</td>
</tr>
<tr>
<td>I</td>
<td>Protein</td>
<td>Abundance, modification</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>C</td>
<td>Fiber</td>
<td>Biomechanics</td>
<td>Centrifugation</td>
</tr>
<tr>
<td>S</td>
<td>Fly</td>
<td>Assembly</td>
<td><em>In vivo</em> length measurements</td>
</tr>
<tr>
<td>S</td>
<td>Myofilament</td>
<td>Assembly</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>I</td>
<td>IFM</td>
<td>Morphology &amp; biomechanics</td>
<td>Force transducer rig</td>
</tr>
<tr>
<td>C</td>
<td>Fiber</td>
<td>Morphology</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>C</td>
<td>Fly</td>
<td>Aerodynamics (tethered)</td>
<td>Light microscope</td>
</tr>
<tr>
<td>S</td>
<td>Myofilament</td>
<td>Assembly</td>
<td>Flight box</td>
</tr>
<tr>
<td>S</td>
<td>Population</td>
<td>Myofilament spacing &amp; ordering</td>
<td>Flight arena (respirometry/kinematics chamber)</td>
</tr>
<tr>
<td>S</td>
<td>Fly</td>
<td>Biochemical &amp; biomechanics</td>
<td>X-ray diffraction</td>
</tr>
<tr>
<td>S</td>
<td>Population</td>
<td>Population fitness</td>
<td>Weld tunnel</td>
</tr>
<tr>
<td>S</td>
<td>Population</td>
<td>Free flight performance</td>
<td>Three-dimensional motion analysis</td>
</tr>
</tbody>
</table>
### Table 2. Myofibrillar proteins of *Drosophila* IFM

<table>
<thead>
<tr>
<th>Structure</th>
<th>Protein</th>
<th>Identification</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thick filaments</td>
<td>MHC (36B)</td>
<td>Cross hybridization</td>
<td>Alternative RNA splicing generates &gt; 14 isoforms. Embryonic isoform</td>
<td>4, 33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>competent for assembly but not function of IFM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMLC2 (99DE)</td>
<td>Abundantly expressed RNA during adult muscle development, <em>in vitro</em> translation</td>
<td>46 aa N-terminal extension; multiple phosphovariants</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>ELC1/3 (98B)</td>
<td>Abundantly expressed RNA during adult muscle development, <em>in vitro</em> translation</td>
<td>Alternative RNA splicing generates IFM-specific isoform which differs in C-terminal 12 aa</td>
<td>4</td>
</tr>
<tr>
<td>Flightin (76DE)</td>
<td>Antibody</td>
<td></td>
<td>IFM-specific protein; multiple phosphovariants</td>
<td>76</td>
</tr>
<tr>
<td>Parayosin (66D)</td>
<td>Antibody</td>
<td></td>
<td>Low levels in IFM, distributed throughout thick filament. Multiple phosphovariants</td>
<td>4, 77</td>
</tr>
<tr>
<td>Mini-parayosin (66D)</td>
<td>Cross hybridization</td>
<td></td>
<td>Unique N-term 114 residues not found in other paramyosins. Localized at the M line and both ends of thick filaments.</td>
<td>4, 77</td>
</tr>
<tr>
<td>Thin filaments</td>
<td>Actin (88F)</td>
<td>Cross hybridization</td>
<td>Act88F gene expressed in IFM only</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Arthrin (88F)</td>
<td>Mutation, antibody</td>
<td>IFM-specific ubiquinated Act88F actin</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Tropomyosin (88F)</td>
<td>Chromosome walking</td>
<td>C-term 27 residues encoded by IFM-specific exon; switching of this region with non-IFM sequence does not affect function</td>
<td>4</td>
</tr>
<tr>
<td>Troponin T (12A)</td>
<td>Antibody</td>
<td></td>
<td>Long (~136aa) C-terminal extension with polyglutamic tail; 17 aa insertion; phosphorylated, binds calcium</td>
<td>4, 78</td>
</tr>
<tr>
<td>Troponin I (16F)</td>
<td>Abundantly expressed RNA during IFM development, mapping to cytological region of known mutant</td>
<td>Alternative IFM-specific exon encodes N-terminal Ala-Pro rich 61aa sequence</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Troponin C (73F, 41C)</td>
<td>PCR/cross-hybridization</td>
<td></td>
<td>Encoded by three genes; the most distantly related gene is adult-specific; IFM expression has not been established</td>
<td>79</td>
</tr>
<tr>
<td>Troponin H (88F)</td>
<td>Antibody, chromosome walking</td>
<td>IFM-specific Tropomyosin fusion with ~200 aa hydrophobic, Pro-rich C-term extension; two spliced variants</td>
<td>80, 59</td>
<td></td>
</tr>
<tr>
<td>Z bands</td>
<td>GST-2 (53F)</td>
<td>Mass spectrometry</td>
<td>Not expressed in non-IFM muscles</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Actinin (2C)</td>
<td>Antibody</td>
<td>Muscle and non-muscle isoforms generated by alternative RNA splicing from same gene</td>
<td>65</td>
</tr>
<tr>
<td>Z(210) (—)</td>
<td>Antibody</td>
<td>Expression limited to IFM and large cells of TDT.</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Kettin (62C)</td>
<td>Antibody</td>
<td>Renamed zetalin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z(400/600) (—)</td>
<td>Antibody</td>
<td>Most likely Kettin</td>
<td></td>
<td>65</td>
</tr>
<tr>
<td>Projectin (102C/D)</td>
<td>Antibody, PCR</td>
<td>Mutation that deletes kinase domain affects crossbridge kinetics and stretch activation</td>
<td>65, 82, 43</td>
<td></td>
</tr>
<tr>
<td>Titin (62C)</td>
<td>Antibody, Mutation</td>
<td>Lacks characteristic domains found in A band and M line region of vertebrate Titin</td>
<td>83, 84, 85</td>
<td></td>
</tr>
<tr>
<td>Stretchin-MLCK (52DE?)</td>
<td>PCR, database mining</td>
<td>Seven proteins encoded by three transcription units. One unit encodes a standard MLCK. Tissue distribution not yet established but one form, A(225) identified in IFM A bands</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>ADP/ATP translocase (9EF)</td>
<td>Mass spectrometry</td>
<td>Abundant in IFM myofibrils; unclear whether it represents mitochondrial contamination or a novel isoform</td>
<td>67</td>
<td></td>
</tr>
</tbody>
</table>

1 cytological position of gene indicated in parenthesis; (—) no published reference.

2 Indicates how the *Drosophila* gene or protein was first identified.
hybridization to larval polytene chromosomes. Mutagenesis screens can then be designed to “target” the cytogenetic region of interest. For example, Warmke et al. used a synthetic deficiency of the myosin regulatory light chain (Dmlc2) gene to screen for recessive lethal mutations. The screen identified 44 mutations that mapped to 23 complementation groups but none corresponded to Dmlc2. However, a null allele of Dmlc2, E38 (renamed Dmlc2 E38), was obtained in a similar screen conducted independently by John Merriam. Interestingly, the screen by Warmke et al. generated four alleles of a noncomplementing group that maps outside the region uncovered by the deficiency and exhibits dominant lethal synergism with Dmlc2 E38. This group, named l(3)nc99Eb, was later shown to be within the Tm2 gene. This study epitomizes both the strength and weaknesses of the genetic approach. On one hand, they failed to obtain mutant alleles of Dmlc2 in two separate screens designed to recover dominant flightless and recessive lethal mutations, respectively, despite the fact that Dmlc2 E38 shows both characteristics. On the other hand, they uncovered an unexpected functional interaction.

Figure 2. Genetic strategies for isolation of mutations that affect IFM. A: The traditional approach for obtaining dominant IFM mutants is to subject male flies to a mutagen (irradiation or feeding a chemical, most commonly ethyl-methane sulfonate (EMS)), mate mutagenized males to normal females, and select flightless progeny that fall to and are captured at the bottom of a flight testing column. Recessive mutations on the X chromosome are obtained via a similar screen that selects only males, which are hemizygous for the sex chromosome. B: (top) Many flightless mutants also exhibit an abnormal wing position phenotype which can be selected for without the need for a flight test. (Bottom) Once a muscle mutant is available, intragenic or intergenic modifier mutations can be obtained by screening for revertants of the wing phenotype. C: Mutagenized flies can be scored directly for morphological muscle defects using polarized light to visualize the IFM after chemical clarification of the cuticle. D: Once the chromosomal position of a gene is established by in situ hybridization, it is possible to select for deletions or deficiencies of that region by screening for the absence of a visible marker (e.g., eye color) encoded by a transposable element inserted nearby. Southern blots of genomic DNA and in situ hybridization to polytene chromosomes are performed to ascertain the region of interest has been deleted in lines that fail to express the visible marker. The vast collection of single transposable element insertion lines makes this approach nearly universally applicable. The deficiency also serves to identify null mutations in recessive genes. De novo mutagenized males are mated to females carrying the deficiency chromosome and the progeny screened for the absence of the gene product. This approach permits the isolation of null mutations without the need to score for a specific muscle phenotype. E: Transgenic manipulation of Drosophila has enabled molecular genetic approaches to study protein function in vivo. Mutations engineered into cloned genes are introduced into the germline via P element mediated transformation. Expression of the transformed gene can be restricted to the IFM with the use of the tissue-specific Act88F promoter.
between Dmlc2 and Tm2 that may reveal important features of the muscle regulatory system.

Knowing the cytological location of a gene also allows the isolation of mutations without having to select for a particular functional phenotype. One example is the approach taken to isolate a null mutation for flightin that did not rely on a muscle phenotype. Instead, a protein dot–blot approach was used to detect the absence of the protein (Fig. 2E). This approach offers the advantage of high throughput, but it is useful only for identifying null mutations. More recently, another phenotype-independent, high throughput approach based on denaturing HPLC has been developed to identify missense mutations in any Drosophila gene.

Molecular genetics dissection of protein function

Molecular genetics has served to expand the repertoire of mutants obtained from the aforementioned studies. The availability of IFM-specific null mutants has enabled transgenic approaches to study protein structure–function in vivo without the preoccupation that mutations may affect viability (Fig. 2E). This has been the case for MHC (IIm(2)2, alias Mhc2), ACT88F(K368R), and Tm2 (IIm(3)Tm23). Null alleles and genetic deficiencies also permit the study of dosage requirements for IFM function and these have been shown to differ among contractile protein genes. Thus, while the expression of MHC, DMLC2, Tm2, and ACT88F actin have been shown to be sensitive to dosage effects, flightin, kettin, α-actinin, TnT and TnH are dosage insensitive. Interestingly, Mhc haploids are flightless but the presence of three genes appears to have little effect upon IFM function. If gene dosage is increased to four, however, defects in IFM structure appear and flight performance is compromised. In the case of Act88F, which is also haploinsufficient for flight, the presence of two extra gene copies has little effect upon flight performance.

The Act88F gene is not essential for viability of the fly and has allowed the only genetic study of a muscle actin gene. These genetic studies have provided information about actin polymerization and myofibril assembly, actomyosin kinetics and stretch activation, and torsional flexibility of cross-bridges. Missense alleles obtained from flightless screens point to obvious residues that are critical for proper actin function in the IFM. Nevertheless, this type of screen has the disadvantage that the selection is based on an extreme phenotype (lack of flight) and mutations that result in subtle but important functional changes remain undetected. Drummond et al. overcame this screening bias by performing random mutagenesis on a cloned C-terminal fragment of the Act88F gene and introducing the mutated genes back into flies to study their effect in vivo. All seven missense mutations generated in their study were antimorphic for flight but showed varying effects on IFM myofibrillar structure and flight performance. Generally, the alleles obtained from flightless screens tend to have more pronounced effects on muscle structure than the alleles obtained from random in vitro mutagenesis, no doubt a reflection of the strong selection bias used for the in vivo screens. Interestingly, only one mutant obtained from the random in vitro approach corresponds to a mutated residue (G347S) obtained from large-scale in vivo screens for dominant flightless mutants. The combination of in vivo and in vitro mutagenesis yields non-overlapping information about the role of specific amino acids in dictating actin function in vivo.

Actin mutant alleles that affect flight performance have been scrutinized to disclose the role of individual residues on protein function. Early studies of mutant alleles focused on in vivo effects on myofibrillar assembly, morphology, and accumulation of myofibrillar proteins. These studies revealed the large scale of changes that occur as a consequence of each mutation. Later studies examined more specific actin properties such as stability, conformation and polymerization.

In a wide-ranging study, Sparrow et al. documented the effects of Act88F(E93K) (an actin mutant in which glutamic acid 93 is replaced by lysine) on IFM ultrastructure, myofibrillar protein accumulation, actomyosin interaction, and mechanical properties of skinned fibers. Myofibrils from Act88F(E93K) mutant IFM are severely disorganized and have no discernable Z bands or sarcomeres. As a result, the fibers produce very little active tension and cannot be stretch activated. The lack of Z bands results from the absence of α-actinin, and perhaps reduced amounts of high molecular weight Z band proteins. Based on these results, the authors proposed that Act88F(E93K) mutation may affect the binding of actin to a protein, possibly α-actinin, critical for Z band assembly and/or stability.

The development of macroscale (from whole flies) and microscale (from dissected IFM) purification protocols for Act88F actin has permitted the application of biochemical assays to study the function of mutant actins in vitro. For example, Bing et al. conducted in vitro motility assays to determine that the charge reversal in Act88F(E93K) causes a shift in the position of tropomysin along the actin filament helix towards the “off” state. Further studies of Act88F(E93K) actin using stop-flow solution kinetics and single molecule mechanics (optical trap) showed that, while the mutation has no effect on force transmission, it does affect the ability of actin to bind rabbit skeletal S1 myosin. The reduced affinity for myosin suggests that actin residue 93 forms part of a secondary myosin-binding site. In summary, the combination of molecular assays has provided a detailed description of how a single amino acid change affects the biochemical properties of actin (Fig. 3, top).

It is important to correlate the functional properties of the mutant protein obtained from in vitro studies with measured fiber parameters in situ to gain insight on the in vivo molecular mechanism of function. Unfortunately, Act88F(E93K) affects the accumulation of several proteins in addition to actin and
causes major structural defects in IFM myofibrils. As a result, it is not possible to ascertain the direct effect of the mutant actin on IFM biomechanical properties. It is very likely, however, that the widespread disruption in sarcomere assembly caused by \textit{Act88F\textsuperscript{E93K}} is not a consequence of its affect on tropomyosin position or myosin binding (Fig. 3, middle). Instead, residue 93 may form part of a binding domain between Z band actin and another Z band protein, possibly \(\gamma\)-actinin. Thus the same amino acid appears to play distinct functional roles for actins that differ in their sarcomeric distribution.

The functional properties of MHC also have been investigated intensively by targeted mutagenesis.\textsuperscript{(33)} MHC is encoded by a single gene that undergoes alternative RNA splicing to generate isoforms whose expression is developmentally and/or spatially restricted.\textsuperscript{(4,33)} To understand how different MHC isoforms confer fiber-type-specific properties, Bernstein’s group has generated transgenic flies to express incorrect isoforms in the IFM. In one study, flies expressing an embryonic MHC containing five alternative exons not normally expressed in IFM, were able to assemble a normal IFM, which later became dystrophic.\textsuperscript{(33)} Transgenic studies also have shown that myosin molecules lacking the head region can assemble into thick filaments in vivo but the resulting sarcomeres and myofibrils have varying lengths and irregular dimensions.\textsuperscript{(34)}

In summary, in vivo and in vitro mutagenesis has been employed to study the function of contractile proteins. While the majority of the studies have examined the mutation effect upon in vivo muscle structure, more recent work has focused on the biochemical and biophysical properties of mutant proteins. However, this work has been limited largely to actin and MHC. Standardized purification protocols and in vitro

**Figure 3.** Multiple dimensions of protein function are revealed by the combination of genetics, biochemistry, and proteomics. (Top) Traditional genetics elucidate the role of particular amino acids in dictating the molecular function of a protein (e.g., enzymatic activity, binding properties). Each coordinate (e.g., \(m_1a_1\)) represents a "pixel" of functional information obtained from performing a biochemical assay on a mutant protein. Points along the y-axis (e.g., \(m_1a_1, m_2a_2\)) represent assays performed on the normal (non-mutant) protein. Points along x axis (e.g., \(m_1a_0, m_2a_0\)) represent different mutants. Molecular genetics provides additional pixels by allowing systematic dissection of the protein sequence. (Middle) Examination of each mutant effect on cell ultrastructure provides insight about the role of the protein in myofibril assembly and stability. In some cases in vitro biochemical studies reveal mutant characteristics that are incongruous with cellular phenotype (e.g., \(m_1\)) while in other cases in vitro and ultrastructural data are mutually supportive and portray a unified perspective on protein function (e.g., \(m_2\)). (Bottom) Proteomics provides an additional dimension to the study of muscle mutants. By enhancing the view of the mutant effect on the protein panorama of the cell, proteomics consolidates molecular and cellular function and enables interpretation of mutant effects within the context of the entire cell.
biochemical assays need to be developed for the other contractile proteins. Biochemical studies of low abundance contractile proteins and/or proteins that do not express well in recombinant systems remains a challenge.

**Molecular genetics and integrative approaches to protein function**

The aforementioned studies on Act88F E93K illustrate a problem common in studies of contractile protein function that many of these proteins serve essential roles during myofibril assembly. If the mutation in question interferes with normal assembly, its effect on muscle function cannot be ascertained. For this reason, alleles that have little or no effect on myofibrillar assembly are highly desirable. Three such mutations were engineered by Tohtong et al. who replaced two myosin light-chain kinase (MLCK)-phosphorylated serines in DMLC2 with alanines, singly and in combination. The double mutant gene, Dmlc2 S66A,S67A, is able to rescue the recessive lethality engendered by Dmlc2 E38 and has no discernible effect on IFM myofibril assembly ultrastructure. However, flight is impaired and mechanical studies on skinned fibers showed a dramatic decrease in oscillatory work production (i.e., stretch activation). Time-resolved X-ray diffraction showed a reduction in the number of power-generating cross-bridges is the most likely explanation for the drop in oscillatory work. Dickinson et al. performed an integrative functional analysis on all three Dmlc2 phosphorylation site mutants and were able to trace the power deficiencies from the molecular to the organismal level. Their study revealed how specific amino acid changes affect the in vivo function of the flight system. In the case of the double mutant Dmlc2 S66A,S67A, mechanical and metabolic power outputs were significantly decreased, as was wing beat frequency. Interestingly, double mutants were able to compensate for the drop in wing beat frequency by increasing stroke amplitude while hovering, presumably through the action of the synchronous steering muscles.

A multifaceted approach similar to that of Dickinson et al. was taken to study the role of the N-terminal 46 amino acid extension of DMLC2. Transgenic flies expressing a truncated DMLC2 (DMLC2 2–46) and no full-length DMLC2 are viable, are able to walk and jump, have normal IFM ultrastructure, and can fly, albeit with diminished performance. Under conditions that stimulated maximum flight performance in tethered flies, DMLC2 2–46 flies were capable of generating flight forces comparable to body weight, or \( \approx 35\% \) lower than those produced by wild-type flies. Skinned fiber mechanics experiments revealed that the elastic modulus (the in-phase component of the fiber’s dynamic stiffness) is significantly reduced. These studies suggest that the N-terminal extension of DMLC2 contributes to the elastic properties of the IFM, possibly by forming a parallel link between the thick filament and the thin filament, and that this structure is required for maximal mechanical efficiency of the IFM. In summary, genetic manipulations in Drosophila IFM afford an integrated approach to muscle protein function that spans from the molecular to the organismal level.

**Genetics and the cellular function of muscle protein**

The myofibril is a complex organelle where each protein is likely to interact with several other proteins. The interaction between two proteins may be influenced by other proteins that interact separately with either partner. Several hundred or more protein contacts underlie a functional myofibril; proper performance of the contractile machine is dependent upon correct assembly and stability of the myofibril. Only a small number of “functional interactions” between proteins have been identified from biochemical studies of partially reconstituted systems (e.g., actin-S1, troponin/tropomyosin). The difficulty in ascertaining the precise nature of protein contacts from biochemical studies is even more pronounced for proteins that interact transiently during assembly or during the contractile cycle. Genetics, and more recently Functional Genomics, offer complementary approaches to the study of protein function by revealing how a protein fits into the cellular web of interacting proteins, and how these interactions define biological processes and functions. Unlike conventional biochemical approaches, the study of protein function by genetics is not dependent on protein abundance but rather on functional relevance. Hence genetics can reveal functional features of protein complexes that are not easily accessible through biochemistry. Two genetic strategies have been undertaken to examine protein–protein interactions in IFM: (i) genetic interactions among extant mutants and (ii) genetic screens for dominant modifiers of extant mutants.

**Genetic interactions from pairwise combinations of known mutants**

Two fly strains carrying mutations in two separate genes can be mating to generate progeny that are heterozygous for the two mutations (double heterozygotes). A genetic interaction is evident if the phenotype of the progeny is unlike that of either parent (e.g., flightless progeny from two flighted parents) or if the progeny exhibits an otherwise recessive phenotype. There are several circumstances that can give rise to a genetic interaction. One common situation is when mutations in two genes affect binding between their encoded proteins. Genetic interactions also result even if the two mutant proteins are not in direct physical contact but function in the same or in parallel pathways, or are components of a large multiprotein complex. Such is the case in muscle cells, where proper assembly and structural stability of the myofibril is essential for function. Given the range of underlying molecular causes, interpretation of genetic interactions is not straightforward but is potentially
very important and informative. Fortunately, muscle research provides a panorama of ultrastructural, cellular, biochemical, and biomechanical information that enlightens the interpretation of genetic studies.

To date, the most exhaustive analysis of muscle mutant interactions was conducted by Homyk and Emerson in which they examined alleles for several myofibrillar protein genes including Act88F, Mhc, Tnl, TnT, Tm2 and a-actinin. The study uncovered two general types of interactions among the muscle mutants: combinations that resulted in synthetic lethality and combinations that resulted in flight impairment and/or wing posture defects. Lethality results from defects in the larval musculature while flight impairment and abnormal wing posture result mostly from defects in IFM. The inability of either muscle type to perform their functions could stem from abnormal development giving rise to a defective muscle lattice, or from a mechanical or biochemical defect which may prevent contractile force generation or propagation from a normally assembled muscle myofibril.

The most extensive set of interactions among non-Mhc genes was observed between hdp and up/int mutants. These loci were later shown to encode Tnl and TnT, respectively. The genetic interaction between Tnl and TnT mutants is not surprising given these two proteins function within the same complex and it is well established that they interact physically. One example is the interaction between int and hdp. Both mutant alleles are recessive for flight and show normal wing posture as heterozygotes, but the double heterozygote hdp+/+ int is flightless and shows abnormal wing posture. hdp is a missense mutation in Tnl that results in substitution of alanine 116 for valine. This mutation is located in a region that in vertebrate Tnl has been shown to interact with TnC. int is a TnT missense mutation that results in lysine substituting glutamate 100, a residue that is not part of the Tnl-binding site. The genetic interaction between hdp and int does not stem from improper binding between Tnl and TnT, assuming that the organization of the fly troponin complex is similar to the vertebrate troponin complex. This conclusion is also supported by the fact that the hdp interaction with up/int is not allele specific: several int alleles interact with several hdp alleles.

Since the mutations do not affect the contact sites between Tnl and TnT, what then is the basis for the genetic interaction and why are double heterozygous mutants flightless? One possible explanation is that each mutation has a destabilizing effect on the troponin complex, either affecting the regulatory function of troponin or stability of the whole thin filament. In homozygotes, int does not appear to affect myofibril assembly while hdp gives rise to minor sarcomeric irregularities. Nevertheless, in both mutant homozygotes myofibrils degenerate by eclosion, most likely as a result of stresses produced by contractile activity, and thin filaments are no longer visible in hdp adult IFM. Future experiments should address the underlying causes of muscle degeneration. For example, the effect of each mutation on Ca2+ regulation and IFM contractile properties can be ascertained from mechanical experiments of skinned fibers from very young int and hdp homozygote adults (i.e., before the onset of muscle breakdown), as well as from hdp+/+/int double heterozygotes. These studies should be complemented by studies in vitro that examine the properties of mutant troponin complexes (containing hdp Tnl, int TnT, or both) in regulating myosin ATPase activity. Finally, it is important also to examine muscle structure and function in the single mutant heterozygotes because early studies relied on flight tests as the sole criteria of IFM function. More recent studies have revealed that, while some mutant heterozygotes may perform well on the flight test, their IFM exhibit ultrastructural and/or contractile defects. In summary, an understanding of the changes in molecular properties caused by hdp and int will provide the foundation by which to interpret the genetic interaction; this, in turn, will serve to unravel the mechanistic and functional principles of the troponin complex.

Homyk and Emerson also uncovered genetic interactions that do not conform to current structural or biochemical models of muscle function. One example is the strong interaction between hdp and the recessive flightless a-actinin alleles fliA and fliA, fliA is a hypomorphic allele that results in greatly reduced expression of both nonmuscle and muscle isoforms. As a result, muscle insertions into the cuticle are defective but, surprisingly, sarcomere structure is only marginally affected. Like fliA, fliA heterozygotes can fly suggesting that one copy of the wild-type allele can compensate for the reduced production of the mutant allele. As mentioned above, however, the flight test is not a rigorous assay for muscle function and it is possible that underlying structural and contractile defects may have gone undetected in fliA and hdp heterozygotes. Regardless, double heterozygous fliA/hdp are flightless, and we must ask why? A direct interaction between a-actinin and Tnl has not been described in any system and therefore it is not considered a likely explanation for the phenotype. Furthermore, Tnl was not detected in a yeast two-hybrid screen for a-actinin-binding proteins. Nevertheless, both proteins physically interact with actin and the flightless phenotype most likely arises from separate, but additive effects of the two mutations on thin filament stability. Alternatively, the two mutations could act autonomously: hdp affect the ability of the myofibril to produce force and fliA affect the ability of the myofibril to sustain force. Thus, if hdp Tnl inhibits actomyosin interaction poorly or not at all, unregulated contractile force may reach levels that are unsustainable by a structurally compromised lattice.

In addition to TnT and a-actinin alleles, hdp also interacts with several Mhc alleles. This genetic promiscuity is a double-edge sword. On the one hand, hdp will serve to
uncover important features of myofibrillar architecture and mechanics; on the other hand, this mutant serves as a warning that more rigorous biochemical and biomechanical tests are needed before the nature of its multiple genetic interactions can be fully ascertained.

A less direct approach to identify genetic interactions that may shed new light on muscle function is the use of chromosomal "deficiency kits", collections of mutants in which each fly strain carries an unique deficiency. The kits provide contiguous coverage of most of the euchromatic portion of the fly genome. Crosses of deficiency strains to a mutant of interest helps to identify potential interacting genes. This approach offers the advantage that a large portion of the genome can be screened relatively quickly for interacting genes. Because of the possibility of non-specific and multiple locus interactions, this approach serves only as a first cue to identify potential candidate genes, which then must be subjected to further scrutiny. In addition, the large-scale *Drosophila* (P element) gene disruption project currently underway promises to deliver a mutant for every gene. Based on these results, the authors espoused the hypothesis that the suppressors may identify specific molecular interactions between MHC and TnI. This putative interaction could potentially couple kinetic and regulatory processes, perhaps as an added strategy for rapid activation/deactivation given the scarcity of sarcoplasmic reticulum in the IFM. However, it is unlikely that all four suppressors act by the same mechanism and physical interaction between the myosin residues affected in two suppressors, D41 and D45, and TnI appears unlikely. It is possible that D41 and D45 suppress TnI-induced degeneration indirectly, by curtailing the ability of the myosin motor to produce force. At any rate, the studies of Prado et al. and Kronert et al. have validated the in vivo approach of using mutations in one gene to identify interacting genes. The real challenge remains in elucidating the molecular basis of the interactions and distinguishing between specific protein interactions versus general effects. An understanding of how each suppressor works will require not only genetic tools but also biochemical, biomechanical and ultrastructural methods. The reward, a comprehensive appreciation of how molecular components of muscle work as a unit, is very much worth the effort.

**Unexpected genetic interactions**

*Drosophila* muscles are equipped with all the molecular components for dual thin filament and thick filament regulation of contraction. There is very little understanding of how the action of these separate regulatory systems is coordinated to achieve unified control, however, since very few studies have addressed this issue. Mutants discovered by Warmke et al. may be useful in the study of dual regulation of contraction. These authors uncovered a lethal interaction between *DmIco* and several *Tm2* alleles. Two of the alleles, J8 and L2, are essentially nulls (they encode very short Tm2 polypeptides) suggesting that lethality results from the additive haploinsufficiency of two essential larval muscle genes that act in tandem to regulate muscle contraction. The third allele, S2, encodes a single amino acid substitution (aspartic acid 121 to asparagine) and it is not clear why this mutant is lethal in heterozygous combination with *DmIco*. It is important to point out that Kreuz et al. were unable...
to reproduce the lethal effect between $Dmle2^{E38}$ and Tm2 mutants.\(^{(17)}\)

**Functional genomics**

Functional Genomics designates a variety of experimental and computational approaches that aim to assign function to each and every gene of an organism. The first step in this approach, gene annotation, involves finding the genes within the genome sequence and assigning function based on sequence similarities to known genes. An intense “annotation jamboree” was successful in identifying 13,601 *Drosophila* genes and assigning a putative function to $\approx 50\%$ of these genes.\(^{(49)}\) Validation of the assigned functions, and establishing the function of the remaining genes whose sequences do not match known sequences in the database, will require other experimental approaches.

A new method for assigning gene function, known as “guilt by association”, is based on microarray data from global gene expression experiments. An uncharacterized open reading frame can be assigned a tentative function if its expression pattern mimics that of a gene with known function.\(^{(50)}\) Other, more conventional techniques for identifying protein–protein interactions, such as the yeast two-hybrid system, have been customized for high throughput screening.\(^{(51)}\) Pooling data from high-throughput screens with that from published interactions has resulted in the generation of a global network of protein interactions in yeast.\(^{(52)}\) The function of newly discovered or uncharacterized proteins can be inferred from their interactions with proteins of known function. The combination of RNA and protein profiling, together with other functional genomics methods, is capable of providing a roaming view of cellular protein networks.\(^{(53)}\) In the post-genome era, traditional genetics will serve an increasingly important role in the validation of protein networks derived from functional genomics approaches.

**Proteomics**

An increasingly popular approach for assigning gene function is proteomics, whose initial aim is to provide a comprehensive catalogue of all proteins in a cell, tissue, or organism. Proteomics also endeavors to provide an in-depth description of protein function that includes knowledge of protein modifications, protein localization, protein interactions, and changes throughout development. The goal is to furnish an inclusive view of the cellular function of each protein. The panorama provided by proteomics would show how each protein fits naturally within the functional landscape of the cell and can serve to unify all aspects of protein function (Fig. 3, bottom).

The traditional platform in proteomics is to separate proteins by two dimensional gels (2DE) followed by mass spectrometry to identify proteins and their modifications (Fig. 4). While useful, a gel-based approach suffers from several handicaps, namely the exclusion of some proteins (e.g., hydrophobic proteins) and the inability to reliably detect low abundance proteins.\(^{(54)}\) One way to overcome the limitation in visualizing rare proteins is to pre-fractionate the sample before gel loading, thereby enriching for low abundance proteins. Pre-fractionation offers the additional advantage that it provides information about some property of the protein. Multidimensional chromatography (e.g., ion exchange and reverse phase)\(^{(55)}\) affords separation based on the protein’s intrinsic properties. A second approach is to fractionate proteins according to their subcellular distribution (Fig. 4).

**IFM proteomics in the pre-genomics era**

IFM proteomics dates back to the early 1980s when investigators began to assemble a 2DE database from normal and mutant flies.\(^{(9,56)}\) Coomassie blue-stained gels of whole thorax homogenates revealed 186 protein spots; of these $\sim 20$ spots were also detected in a myofibrillar fraction, and 12 of these protein spots appear to be IFM-specific.\(^{(56)}\) To date, only three of the 20 myofibrillar proteins (spots 98, 99, and 184) have not been identified. Furthermore, additional myofibrillar proteins have been identified (Table 2), including high mass proteins (e.g., titin, projectin, and kettin) and proteins that do not resolve well in standard 2DE protocols (e.g., mini-Pm, TnT).

The examination of mutants by 2DE was an attempt at establishing gene networks based on the presence or absence of a given protein in different mutant IFM. The premise of this approach is that, if two proteins function together, the absence of one protein would affect the accumulation of the other. Mogami and Hotta examined the IFM proteins of 57 newly isolated dominant flightless mutants and found that, in 26 lines, more than one protein spot was absent or reduced, as a result of the mutation.\(^{(9)}\) Analysis of the protein profile of the 26 lines revealed that the presence or accumulation of some protein spots is strictly dependent on the presence of other spots (Fig. 5). They proposed that this dependency reflects functional and/or architectural relationships among the proteins. In addition, they used this information to predict the primary site of the mutation.

The gene defects of several of the mutant strains generated by Mogami and Hotta are now known. A re-examination of the results from the early studies in light of the new information validates the gel comparison approach as a means of identifying functional protein relationships. Nevertheless the 2DE approach, limited by the resolution and range of the gel system and the sensitivity of the detection method, lacks the capacity to reveal the full spectrum of protein changes that may occur as a result of a mutation.

Figure 5 summarizes the interrelationships among several protein spots deduced from 2DE analysis of IFM mutants.\(^{(9)}\) Except for spot 184, the identity of all the other spots are known. Their results correctly predicted that spots 158 and
Figure 4. Building a functional map of IFM proteins through the study of mutant proteomes. 

A: Approach pioneered by Mogami and Hotta for examining mutant effect on IFM function. Myofibrillar proteins from wild-type IFM (left panel) and mutant IFM (right panel) are separated by 2DE and their profiles compared. Many IFM mutations affect the expression of multiple proteins and, in some cases, the presence of a protein is completely prevented (red circles). However, the range and resolution of the gel, and the sensitivity of the detection method (e.g., coomassie blue) limits the number of proteins that can be detected. 

B: A proteomics approach enhances the study of mutant effect on IFM function. A dissected IFM fiber is split and transferred sequentially through three different solutions that allow fractionation of proteins by their subcellular distribution: drop 1, soluble (cytosolic) proteins; drop 2, detergent-extractable (membrane and organelle) proteins; drop 3, insoluble (cytromatrix) proteins. The pre-fractionation reduces the complexity of the sample and enriches low abundance proteins, which are now detected by 2DE (blue squares). Individual protein spots (red circle) are cut out from the gel, digested with trypsin, and the resulting peptides analyzed by mass spectrometry. Alternatively, 2DE is bypassed altogether and proteins fractionated by HPLC or multidimensional chromatography before or after trypsin digestion. Proteins are identified by using peptide fingerprints (MS) and/or partial sequence of representative peptides (MS-MS) to scan a virtual translated Drosophila Genome. The protein profile of each mutant, along with relevant information from other studies (e.g., genetic interactions, in vitro assays) is used to assemble an IFM mutant database and the formulation of functional protein networks.
Two-dimensional gel analysis revealed that a reduction in actin (spot 184) is a common secondary manifestation of most mutant alleles. More than ten protein spots (DMLC2, ELC1/3, and flightin among them) were shown to be missing in Mhc7 IFM. All the missing proteins are presumed to be thick filament components since thin filaments and Z bands appear marginally affected in Mhc7.(57) More than ten protein spots (DMLC2, ELC1/3, and flightin among them) were shown to be missing in Mhc7 IFM. All the missing proteins are presumed to be thick filament components since thin filaments and Z bands appear marginally affected in Mhc7.(57)

Mogami and Hotta also examined several Act88F alleles, including Ifm(3)2 (renamed Act88F-R28C) and Ifm(3)7 (Act88FW356E-2), which affect the accumulation of known thin filament proteins, namely Tm2, arthrin, and TnH (Fig. 5). However, no changes were noted in other thin filament proteins such as GST-2 (spot 137), TnI or TnT. The latter two probably did not resolve well in their gel system, making their presence more difficult to ascertain. Interestingly, some TnT alleles also affect the accumulation of actin or other proteins.(42,58)

At least three important lessons are derived from the studies that analyzed muscle protein accumulation in IFM mutants. First, the total analysis of protein accumulation in mutants espoused by Mogami and Hotta is an extremely valuable approach for deciphering functional protein networks. The limitations encountered in their study are easily overcome using higher resolution 2DE systems and more sensitive stains, or replacing 2DE altogether with other means of protein separation (e.g., HPLC or multidimensional LC; Fig. 4).(65)

Second, the strict interrelationship between one isoform of TnH (spot 34) and spot 184 is intriguing and deserves further consideration. TnH co-purifies with the Troponin complex in Lethocerus(59) and binds GST-2 in Drosophila IFM,(60) but an interaction with protein 184 has not been uncovered by biochemical studies. A later study showed that the accumulation of spot 184 is affected by many Act88F alleles suggesting that this protein may be a thin filament component.(11) In addition, the interrelationship between TnH and spot 184 holds for 14 of 15 Act88F alleles.(11) Since both TnH and spot 184 are IFM-specific,(56) this putative interaction may play a defining role in stretch activation. So what is spot 184? One possibility is that this protein is another member of the troponin complex, perhaps TnC. In fact, if one queries the genome using the known properties of spot 184 (pl, molecular weight, cytoskeletal protein), the two most likely candidates that emerge are TnC and a calmodulin-related protein.

Third, some Act88F alleles affect accumulation of thick filament proteins, namely flightin and DMLC2. The effect is particularly pronounced in several antimorphic alleles in which DMLC2 is not detected at all.(11) These results are consistent with the proposal that DMLC2 interacts with actin, perhaps through its extended N terminus.(38) More importantly, however, these results indicate that the assembly of functional thin and thick myofilaments may not be as independent of each other as is often assumed.(61)

It is clear from the aforementioned studies that most mutant genes affect more than their encoded products, they also affect the expression of multiple other proteins some of which are likely to interact physically with the product of the mutant gene. These studies certainly represent “the tip of the iceberg” since they were based on visual inspection of 2DE gels of limited range and resolution. An enhanced view of the proteome would expose the full range of each mutation effect and should prove particularly beneficial in cases where the observed phenotype cannot be explained logically. A study of three single amino acid mutations in the MHC rod illustrates the validity of a proteomics-type approach, even if it is limited in scope. Three Mhc alleles (Mhc6, Mhc13, and Mhc19) were shown to have a pronounced effect on IFM structure and function and little or no effect on other muscles despite the fact that the mutations are located in an MHC constitutive exon.(13) Two-dimensional gel analysis revealed that a reduction in flightin accumulation was a common secondary manifestation of the three mutants. Furthermore, the severity of the muscle defect worsened in direct proportion to the reduction in flightin abundance, Mhc13 in which little or no flightin is present, has a more severe muscle defect than Mhc6, in which some unphosphorylated flightin is present.(13) More recently Reedy et al. described the isolation of fin7, a flightin null mutant whose

![Figure 5](Image)

**Figure 5.** Interpretation of functional gene networks deduced from 2DE analyses of IFM mutants (flow diagram from Mogami and Hotta).(9) Arrows point to spot numbers that are reduced or missing in all mutants where the spot from which the arrow originates is also reduced or missing. Numbers in boxes connected by doubleheaded arrows indicate spots that always decrease together. Note that reduction in actin (spot 101) always results in reduction of thin filament proteins (arthrin, Tm2, and TnH) and flightin, but do not affect DMLC2 or ELC 1/3. Flightin is the only myofibrillar protein whose accumulation is affected in all homozygous mutants that were studied. Furthermore, flightin expression is affected by practically all Act88F alleles that have been examined and by some TnT alleles.(9,11,58) Flightin is a thick filament associated protein(14) but these results suggest that flightin interacts with actin or some other protein of the thin filament. These results validate the mutant proteome comparative approach to identify functionally significant protein interactions.
phenotype is remarkably like that of Mhc\textsuperscript{13} despite the fact that MHC is unaffected in fln\textsuperscript{0}.\textsuperscript{(14)} The convergent phenotypes suggest that the mutations in two separate genes may affect a common aspect of muscle structure and/or function. Together, the studies of Kronert et al. and Reedy et al. provide persuasive evidence that the phenotypic manifestations of Mhc rod alleles result from their effect on flightin abundance, rather than to some intrinsic effect on the myosin molecule.

This situation is intriguingly similar to that described for vertebrate cardiac MHC and myosin-binding protein C (MyBP-C). On the one hand, mutations in the S2 region of \( \beta \) myosin that cause familial hypertrophic cardiomyopathy (FHC) have been shown to affect MyBP-C binding but have no effect on myosin coiled-coil structure or stability.\textsuperscript{(62)} On the other hand, more than 30 mutations in cardiac MyBP-C have been found in FHC patients and most of the mutations result in truncated MyBP-C that lack the myosin-binding domain.\textsuperscript{(63)} These examples in flies and humans illustrate how mutations in one gene (myosin) may engender muscle defects by interfering with the activity of a separate gene product (MyBP-C or flightin). These studies also highlight the need to look beyond the primary site of the genetic defect in order to obtain an accurate portrait of the molecular consequences and pathobiological manifestations of each mutation. In summary, the analysis of mutant IFM proteomes offers new perspectives on the cellular function of muscle proteins and provides fresh insight into the correlation of genotype and phenotype. This view may be particularly beneficial for the study of human genetic diseases, such as FHC, in which knowledge of the molecular defect has not translated into an understanding of disease pathogenesis.

The assignment of cellular function also requires knowledge of the protein’s subcellular distribution. Warmke et al. pioneered subcellular fractionation of IFM fibers in microliter volumes to obtain distinct cytosolic, organelle, and cytomatrix fractions (Fig. 4).\textsuperscript{(64)} This approach, as well as large-scale fractionation,\textsuperscript{(65)} permits the isolation of a nearly intact myofibril and allows myofibrillar protein composition to be directly correlated with mechanical performance.\textsuperscript{(64)} Furthermore, the myofibrillar fraction can be subsequently separated into thick filament, thin filament or Z disk subfractions.\textsuperscript{(34,65)} In a recent study, Reedy et al. combined genetics and subcellular fractionation to demonstrate that flightin remains associated with thick filaments composed of headless myosin, suggesting that flightin interacts primarily with the thick filament shaft.\textsuperscript{(14)}

**Summary and prospective**

The function of the myofibril, like the function of other complex subcellular machines, is predicated on the proper assembly and maintenance of a supramolecular structure. Innumerable protein contacts underlie a functional myofibril. The three cases highlighted here (Act\textsuperscript{88F E93K}, hdp\textsuperscript{2}, and Mhc\textsuperscript{13}/fln\textsuperscript{0}) emphasize the high degree of interdependency among myofibrillar proteins and demonstrate the multiple layers of function that are served by protein constituents of a mechanical ensemble. The fundamental complexity of the myofibril is evident in the varied functional expression of Act\textsuperscript{88F E93K}, the genetic promiscuity of hdp\textsuperscript{2}, and the congruent phenotypic manifestations of Mhc\textsuperscript{13} and fln\textsuperscript{0}. These studies serve as a humbling warning that no single experimental approach is a panacea for understanding muscle function. Fortunately, *Drosophila* IFM is well suited for the multifaceted experimental assault that will be needed to generate a comprehensive description of how muscle works. The long tradition in cross-disciplinary research, together with the adeptness of IFM to be queried from a top-down or bottom-up approach, provide a solid foundation from which to embark on a systems approach to function (Fig. 6). The knowledge gained will impact our understanding of insect flight and of muscle function in general.

![Figure 6. A systems view of the myofibril.](image-url)
We have made great strides towards our goal of understanding IFM function but many important challenges remain. Critical questions about the functional defects of many extant mutants have not been addressed, and the full potential of most of these mutants has not been unleashed. Large-scale interaction screens and modifier screens will provide much needed information about biologically significant protein interactions. The results of these studies will provide the impetus for the formulation of increasingly inclusive models of myofibril form and function. Existing methodologies, ranging from optical trapping of single molecules to X-ray diffraction of IFM in live flies, will provide ample and comprehensive means for validation of genetic data. The marriage of genetics and proteomics is enthralling and has tremendous potential for unveiling intimate details of contractile protein function, including unexpected relationships between proteins. Bioinformatics will play an increasingly important role in enhancing our ability to assimilate and integrate information from multidisciplinary investigations.

Years from now historians of science may view the current genomics revolution as the beginning of the end of reductionism. The ability of “Big science” to generate information has rapidly outpaced our ability to comprehend it. The situation is not unlike that faced by early 19th century western naturalists, who had gained little understanding about nature despite large compilations of geological and biological phenomena. The publication of The Origin of Species in 1859 provided the contextual framework by which a lot of the information began to make sense. Whether we will need a similar intellectual breakthrough in order to make sense of genome-wide data remains to be seen. What is evident is that time-tested genetic approaches will continue to reap important rewards in the post-genome era of large-scale biology.

Acknowledgments
I am grateful to David Maughan and Gretchen Ayer for critical comments on the manuscript, and to members of the UVM Drosophila group for many stimulating discussions. I would also like to acknowledge the reviewers for their insightful comments and suggestions. I want to thank Karen Moore for help with the graphics, to David Maughan for providing Figure 1, and the National Science Foundation for their support.

References


34. Cripps RM, Suggs JA, Bernstein SI. Assembly of thick filaments and myofilaments occurs in the absence of the myosin head. EMBO J 1999;18:1793–1804.


