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RNA interference screening in *Drosophila* primary cells for genes involved in muscle assembly and maintenance

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To facilitate the genetic analysis of muscle assembly and maintenance, we have developed a method for efficient RNA interference (RNAi) in *Drosophila* primary cells using double-stranded RNAs (dsRNAs). First, using molecular markers, we confirm and extend the observation that myogenesis in primary cultures derived from *Drosophila* embryonic cells follows the same developmental course as that seen in vivo. Second, we apply this approach to analyze 28 *Drosophila* homologs of human muscle disease genes and find that 19 of them, when disrupted, lead to abnormal muscle phenotypes in primary culture. Third, from an RNAi screen of 1140 genes chosen at random, we identify 49 involved in late muscle differentiation. We validate our approach with the in vivo analyses of three genes. We find that *Fermitin 1* and *Fermitin 2*, which are involved in integrin-containing adhesion structures, act in a partially redundant manner to maintain muscle integrity. In addition, we characterize *CG2165*, which encodes a plasma membrane Ca^{2+}-ATPase, and show that it plays an important role in maintaining muscle integrity. Finally, we discuss how *Drosophila* primary cells can be manipulated to develop cell-based assays to model human diseases for RNAi and small-molecule screens.

KEY WORDS: *Drosophila*, Myogenesis, RNAi, Primary cells, Muscle assembly, Human diseases

INTRODUCTION

Drosophila is an excellent model system in which to study muscle development. As in vertebrates, myogenesis occurs in two distinct phases (Nongthomba et al., 2004): (1) acquisition of myoblast cell fate and cell fusion that results in the formation of syncytial myotubes (Bate, 1990); and (2) assembly and maturation of myofibrils (Vigoreaux, 2001). Genetic analysis of naturally occurring and experimentally induced mutants has proven to be an excellent approach to study muscle development. In particular, studies in the *Drosophila* embryo have provided many insights into both the differentiation program of the myogenic pathway and myoblast fusion, illustrating the remarkable conservation of many aspects of myogenesis between flies and vertebrates (Baylies et al., 1998; Chen and Olson, 2004). However, genetic analyses of myofibril assembly have been limited because the functional disruption of genes involved in this process may not allow development to proceed to late larval stages, at which phenotypes are readily discernible (Bernstein et al., 1993). Furthermore, because muscles are multinucleate, screens based on the generation of mutant clones in late larval stages cannot be easily performed. Thus, genetic screens in *Drosophila* have been limited mostly to the identification of a few viable mutations in some major myofibrillar components, such as Indirect flight muscle (IFM) actin (Actin 88F) and Tropomyosin (Vigoreaux, 2001).

Owing to limitations in the use of traditional genetic screens to study muscle biology, we set out to establish a cell-based approach to identify genes involved in the regulation of myofibril assembly using RNA interference (RNAi). In principle, the use of an RNAi-based method could overcome the limitations discussed above, and would allow the examination of myofibril organization at a cellular level. As none of the existing *Drosophila* cell lines we examined is, or could be, transformed into myogenic cells capable of differentiating into mature muscles with organized myofibril structures (J.B., unpublished), we investigated whether muscle cells prepared from primary cells could replace cell lines.

Myogenesis in primary cultures has been used to study muscle biology in both normal and mutant animals (Donady and Seecof, 1972; Volk et al., 1990), and has contributed significantly to our understanding of muscle assembly and maintenance. As earlier studies were largely based on muscle-specific morphological features, such as multiple nuclei in primary myotubes, as well as on myofibril structures observed using light and/or electron microscopy (Bernstein et al., 1978; Echalier, 1997), we set out to confirm and extend previous analyses by following myogenesis in primary culture using muscle-specific molecular markers. We developed conditions for RNAi by culturing cells in the presence of double-stranded RNAs (dsRNAs), and used it to identify genes involved in muscle maintenance and integrity. We validated our approach with in vivo analyses of three genes. We find that *Fermitin 1* and *Fermitin 2*, which are involved in integrin-containing adhesion structures, act in a partially redundant manner to maintain muscle integrity. In addition, we characterized *CG2165*, which encodes a plasma membrane Ca^{2+}-ATPase (PMCA), and showed that it plays an important role in maintaining muscle integrity. Finally, we discuss how *Drosophila* primary cells can be manipulated to develop cell-based assays to model human diseases for RNAi and small-molecule screens.

MATERIALS AND METHODS

Drosophila genetics

Drosophila strains used in this study are *Dmef2-Gal4* (Ranganavakulu et al., 1996), *D42-Gal4* (Gustafson and Boulianne, 1996), *Hand-Gal4* (Arbrecht et al., 2006), *rp298-lacZ* (Ruiz-Gomez et al., 2000), *UAS-mitoGFP* (Cox and Spradling, 2003), *UAS-2EGFP* (Halfon et al., 2002), *G053* (SLS-GFP) (Morin et al., 2001), *MHC-τGFP* (Chen and Olson, 2001) and *5053A* (Mandal et al., 2004). w^{1118} was used as a wild-type strain.

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Embryonic primary cell cultures

Embryonic primary cell cultures were established as described previously (Bernstein et al., 1978). Briefly, eggs were collected on molasses plates streaked with killed yeast paste for 2 hours and incubated for an additional 4 hours at 25°C. Embryos were dechorionated in 50% bleach for 3 minutes, rinsed thoroughly with 70% ethanol and sterilized water, and dissociated into a cell suspension using Dounce homogenizers (VWR Scientific, Seattle, WA) (7 ml for smaller scale, 40 ml or 100 ml for larger scale preparations) in Shields and Sang M3 medium (Sigma). Cell suspensions were spun once at 40 ***g*** for 10 minutes to pellet tissue debris, large cell clumps and vitelline membranes. The supernatant was then transferred to a fresh tube and spun at 360 ***g*** for 10 minutes to pellet the cells. Cells were washed once and resuspended in primary cell medium [10% heat-inactivated fetal bovine serum (JRH Biosciences), 10 mU/ml bovine insulin (Sigma) in M3 medium]. Cells were seeded and grown in 384-well optically clear plastic plates (Costar) at 1.7-2.5×10^5 cells/cm^2 (no extra coating steps required).

Immunofluorescence microscopy and western blotting

Protocols for dissection of late embryos or first instar larvae, and for the staining of dissected tissues and primary cells, are described in detail elsewhere (Bai et al., 2007). Primary antibodies used were: rabbit anti-Dmef2 (Bour et al., 1995), rabbit anti-Lmd (Duan et al., 2001), mouse anti-Mhc and anti-α-Actinin (Actn) (from Dr J. Saide, Boston University, Boston, MA), and rat anti-Tropomyosin (The Babraham Institute, Cambridge, UK). Secondary antibodies were from Jackson Laboratories.

For western blotting, early first instar larvae (30 hours AEL at 25°C) were homogenized in sampling buffer, and whole-body protein extracts (equivalent to five larvae) were subjected to western blotting and probed with anti-*Drosophila* PMCA (Lnenicka et al., 2006) and mouse anti-α-tubulin (Sigma).

Primary cell RNAi and staining

Our protocol for a primary cell RNAi screen for muscle genes is outlined in Fig. 4A. Briefly, primary cells were isolated from post-gastrula embryos (4-6 hours AEL at 25°C), and seeded in 384-well plates containing different individual dsRNAs in each well using a MultiDrop (Thermo Scientific) liquid dispenser at ~3-4×10^4 cells (in 10 μl) per well. After 22 hours in serum-free M3 medium at 18°C, the MultiDrop was used to add to each well 30 μl of serum-containing medium to bring the final concentration of fetal calf serum to 10%. Primary cells were then cultured for an additional 10-11 days at 18°C before fixation with 4% formaldehyde. Cells were stained overnight at 4°C with phalloidin Alexa Fluor 568 (Molecular Probes; 1:2000) and DAPI (Sigma, 1:5000) in PBTB (PBS, 0.1% Triton X-100, 1% BSA), washed once in PBS and left in PBS containing 0.02% NaN_3.

RNAi screen and image annotation

dsRNAs were obtained from the *Drosophila* RNAi Screening Center (DRSC) at Harvard Medical School; details of dsRNA synthesis and the amplicons used in this study can be found at http://flyrnai.org/. Because primary myocytes were relatively large and were in general distributed sparsely and randomly in the well, rather than capturing the images using an automated microscope, we visually inspected the wells using an inverted microscope and then imaged those wells containing cells with abnormal muscle phenotypes. Phenotypes were classified into one of four categories (see Results), and the severity of the phenotypes was defined by the percentage of mutant muscles in the well: 'severe' describes cases in which over 80% of muscles showed a certain phenotype, whereas 'medium' describes cases in which ~50% showed a mutant phenotype.

To address the issue of off-targets associated with dsRNAs (Kulkarni et al., 2006; Ma et al., 2006), independent dsRNAs were used. For a list of those genes whose RNAi phenotypes were reproducibly observed with an independent second set of amplicons, see Table S2 in the supplementary material; for the IDs of the amplicons used for generating dsRNAs targeting these genes, see Table S3 in the supplementary material. For a list of all the genes screened in this study, see Table S4 in the supplementary material.

Embryo RNAi injection, in situ hybridization and confocal microscopy

Embryonic in situ hybridizations were performed as described (Hauptmann and Gerster, 2000). dsRNAs (prepared as described at http://flyrnai.org/) were injected at 2 μg/μl into *MHC-τGFP* embryos through their mid-ventral side according to a standard embryo injection protocol (Kennerdell and Carthew, 1998). Injected embryos were aged at 25°C for 20 hours and then analyzed with a Leica LSM NT confocal microscope.

Establishment of transgenic RNAi lines targeting *CG2165*

The snap-back hairpin construct targeting *CG2165* was made in the VALIUM (Vermilion-AttB-Loxp-Intron-UAS-MCS) vector (forward primer, 5′-GTCTAGAGACATGAGGGCACTTTGGAG-3′; reverse primer, 5′-AGAATTCCATTGCTATCACGAATACGCC-3′), and *UAS-CG2165 hp* transgenic flies were generated as described by Ni et al. (Ni et al., 2008).

Single-cell $[Ca^{2+}]_i$ imaging

Primary muscles used for single-cell $[Ca^{2+}]_i$ imaging were derived from cells dissociated from wild-type control embryos and those carrying *UAS-drc2/+; Dmef2-Gal4/UAS-CG2165 hp*, and were cultured in complete media in 8-well cover-glass chamber slides coated with human vitronectin (Chemicon) at 25°C for 3 days. Primary cells were washed twice with low-calcium Ringer solution (150 mM NaCl, 4 mM $MgCl_2$, 5 mM KCl, 0.5 mM $CaCl_2$, 10 mM HEPES, pH 7.2), loaded with Fura PE 3 [5 μM Fura PE 3-AM (Sigma F0918), 0.02% pluronic acid (Molecular Probes) in low-calcium Ringer solution at room temperature]. After a 90 minute incubation, cells were washed twice with Ringer solution, followed by a 30 minute incubation for further dye cleavage. The loaded cells were examined using a Nikon inverted epifluorescence microscope and a 100× oil-immersion lens. Only primary muscles with well-spread morphology were subjected to calcium ratio imaging analysis, with the excitation beams at 340 and 380 nm and the emission wavelength at greater than 510 nm. Images were acquired with Ratiotool software (Inovision, Raleigh, NC). $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. (Grynkiewicz et al., 1985): $[Ca^{2+}]_i = Kd \times [R - R\text{min}]/[R\text{max} - R]$, where Kd is the Fura PE 3 dissociation constant for calcium (251 nM) (Kermode et al., 1990), R is the ratio of intensities at 340 and 380 nm, and Rmin and Rmax are the R values at 0 and saturating levels of calcium (10 mM), respectively.

RESULTS

Myogenesis in primary cell cultures derived from *Drosophila* embryos

To characterize *Drosophila* myogenesis in vivo, primary cells were dissociated from gastrulating embryos following a mass homogenization (see Materials and methods). Embryos were collected at stage 10 or 11 [4-6 hours after egg laying (AEL) at 25°C] when myoblast cell fate is already specified but before fusion is initiated (Bate, 1993). To characterize the freshly dissociated cells, we fixed and stained them for a number of myoblast-specific markers (Fig. 1A-F). In these cultures, based on the number of cells expressing the myogenic transcription factor Dmef2 (*Drosophila* Mef2) (Bour et al., 1995), myoblasts represented 15±2% (±s.e.m., from five independent preparations) of the cell population. In addition, both founder cells and fusion-competent cells could be easily distinguished by the expression of specific markers, such as *rp298-lacZ* for founder cells (Ruiz-Gomez et al., 2000) (Fig. 1D-F), and Lame duck (Lmd) for fusion-competent cells (Duan et al., 2001) (Fig. 1F). Consistent with the observation that the expression of muscle Myosin begins at stage 13 when myoblast fusion has already occurred (Bate, 1993), we failed to detect any expression of muscle Myosin heavy chain (Mhc) in the newly isolated cells (data not shown).

Since fusion is a significant event in myogenic cell differentiation, we confirmed the observation that fusion occurs in culture. When cells originating from *Dmef2-Gal4* and *UAS-2EGFP* embryos,

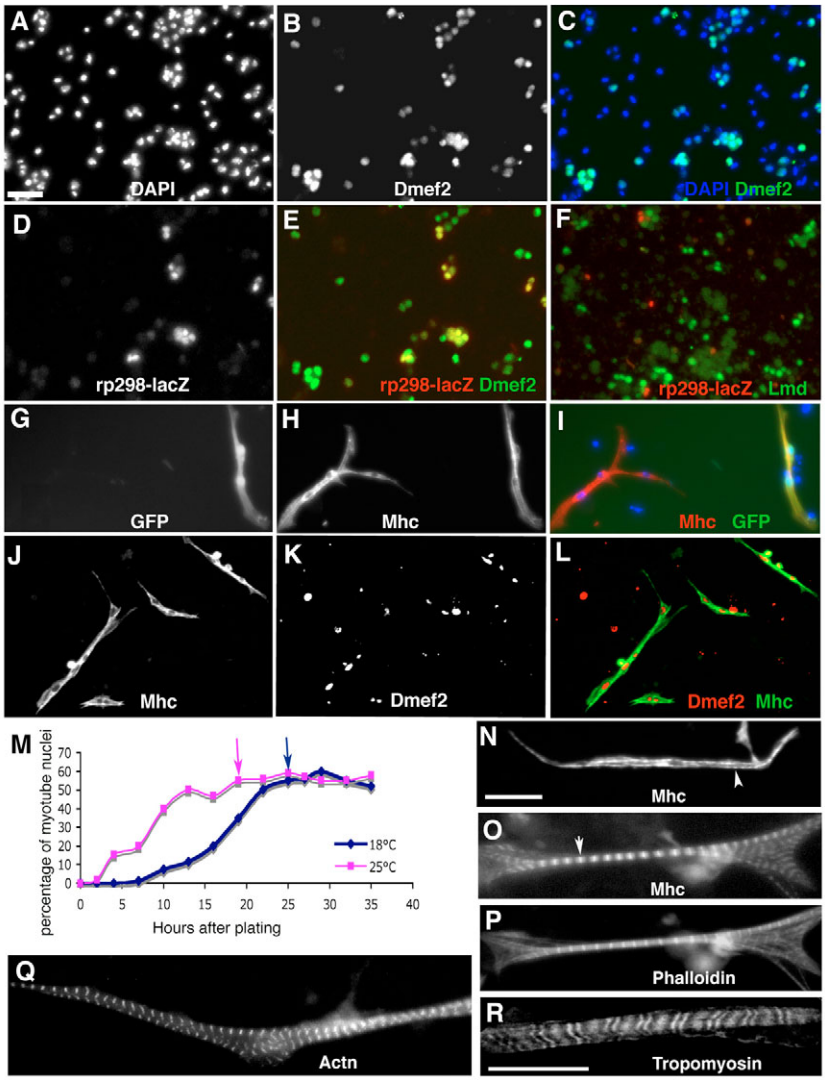

Fig. 1. Myogenesis in primary cultures derived from *Drosophila* embryos. (**A-F**) Fluorescence micrographs of freshly dissociated cells obtained from *Drosophila* gastrulating embryos carrying *rp298-lacZ* immediately following plating. Cells are stained using DAPI for nuclei (A, and blue in C), and antibodies targeting Dmef2 (B, and green in C,E), β-galactosidase (D, and red in E,F) and Lmd (green in F). (**G-I**) Primary cells derived from *Dmef2-Gal4* embryos were mixed with those from *UAS-2EGFP* and allowed to develop for 48 hours at 18°C in culture. The GFP-positive myotube (G, and green in I) resulted from fusion of cells supplied by two genetically different embryos, and the GFP-negative one is most likely derived from the fusion of cells from two genetically identical embryos. Both myotubes expressed Mhc (H, and red in I). (**J-L**) Multinucleated myotubes are identified by staining for Mhc (J, and green in L) and for Dmef2 (K, and red in L). Note that not all Dmef2-positive nuclei are found in myotubes. The percentage of myotube nuclei among the total number of Dmef2-positive nuclei was used as an indication of the amount of fusion. (**M**) Time-course of myoblast fusion at 18°C and 25°C. Primary cell cultures were fixed and stained for Dmef2, Mhc or Actin at the times indicated. The number of Dmef2-positive nuclei was counted using Autoscope and Metamorph software. The number of nuclei in the myotubes was determined manually. The percentage of myotube nuclei was estimated by the number of myotube nuclei among the total Dmef2-positive nuclei, and used as an indication of the extent of myoblast fusion. Each point represents the average results of two or three trials. Arrows point to the time when fusion is nearly complete (pink for 25°C and blue for 18°C). (**N**) Fluorescence micrograph of a primary myotube from a 2-day culture at 18°C stained for Mhc. The white arrowhead points to the immature myofibril that formed along the side of the myotube. (**O-R**) Primary myotubes from 11-day cultures at 18°C, stained for Mhc (O), Actin (as detected using phalloidin) (P), Actn (Q) and Tropomyosin (R). The short arrow in O indicates the bundled myofibrils. Scale bars: 20 μm, in A for A-L and in N for N-Q.

respectively, were mixed, we detected GFP expression in a fraction of multinucleated myotubes (Fig. 1G-I and see Movie 1 in the supplementary material). These GFP-expressing cells indicated the fusion of myoblasts supplied by two different classes of embryos, one expressing *Gal4* and one carrying *UAS-GFP*. We next followed the time-course of myoblast fusion in cultures at 25°C and 18°C (Fig. 1J-M). We found that fusion began ~2 hours after plating, and became rare after 16 hours at 25°C. However, fusion takes place at a much slower pace at 18°C (J.B., N.P., J. Lu and A. Michelson, unpublished), as it initiated at ~7 hours after plating and could last for another 18 hours. Cell density had no significant effect on the fusion rate, although it did affect subsequent muscle differentiation, which probably required myotubes to spread well in culture (data not shown). We further determined the number of nuclei in myotubes in 2-day-old cultures following plating, when fusion is essentially complete at both temperatures. In contrast to myotubes

in vivo that have an average of 10-11 nuclei by the completion of fusion (Bate, 1993), the number of nuclei per myotube in culture was 3.48±0.5 (range from 2 to 15), with 2-5 nuclei seen most commonly (scored in ~100 myotubes in three independent cultures); for representative examples, see Fig. 1L. As visceral muscles have fewer fusions in vivo than somatic muscles, we investigated whether the primary myotubes in our cultures might be primarily of visceral muscle origin by examining the cultures for several visceral muscle markers, including *Hand-Gal4*, *UAS-2EGFP* (circular visceral and cardiac muscles) (Arbrecht et al., 2006), and *5053A*, *UAS-2EGFP* (longitudinal visceral muscles) (Mandal et al., 2004). Only very few myotubes (~2%) were found to co-express GFP in primary cultures derived from embryos carrying *5053A*, *UAS-2EGFP* (data not shown) and ~20% were labeled with the *Hand-Gal4*, *UAS-2EGFP* combination (see Fig. S1 in the supplementary material), indicating that most primary myotubes in culture are derived from somatic muscle cells. Thus, we speculate that the fewer fusions observed in the primary culture might result from the dispersed distribution of myoblasts among other cell types. Relatively pure myoblast preparations may give rise to myotubes having more nuclei (Storti et al., 1978).

Next, we followed the assembly and maturation of myofibrils by staining cells in culture for Actin and Mhc at different time points. Thin and thick filaments were detected as regular patterns at 13 hours after plating at 25°C or when grown for 22 hours at 18°C (data not shown). Later, they aligned and began to organize into parallel bundles and striation became clearly visible at around 19 hours at 25°C or 29 hours at 18°C. These newly formed myofibrils were thin and often found along the lateral sides of myotubes (Fig. 1N). Within ~5-7 days at 25°C (or 10-13 days at 18°C), these strip-like myotubes became mature and stable, with much thicker and more bundled myofibrils (Fig. 1O-R), indicating that the maturation process is achieved by adding more myofibrils laterally. Approximately 52% (52±0.8%) of the myoblasts initially plated in the culture were able to survive to the later stage and developed a fiber-like morphology. About 75% of those with a fiber-like morphology had well-defined striated thick and thin filaments, as revealed by staining for muscle markers, such as Mhc, Actin, Tropomyosin and Actn (Fig. 1O-R). We estimated that ~48% of myoblasts initially plated died after being cultured for an extended period of time. The maximum length of sarcomeres in mature myofibrils was ~8 μm, comparable to that in late L3 body-wall muscles. The average length (6±0.73 μm) of the recognizable sarcomeres remained unchanged during in vivo maturation of the primary muscles. Importantly, some of these primary muscle cells were actively contracting in culture, indicating that they were fully functional (see Movies 1, 2 in the supplementary material). In addition, we found that primary muscles could be detected on the basis of their phalloidin staining alone, as other cell types such as neurons did not display strong phalloidin staining (Fig. 2A-D). Thus, by simply monitoring the strong Actin staining of muscle cells with phalloidin, we can follow myotube differentiation into organized branch-like shapes with a striated structure, and distinguish them from other cell types, including those known for their roles in regulating muscle function in vivo, such as neurons (Fig. 2E-H) and tendon cells (data not shown) (Tucker et al., 2004).

RNAi is an effective method to perturb gene activity in primary cells

Because the use of RNAi in *Drosophila* primary cells had not been previously reported when we started this work, we conducted experiments to establish whether the addition of dsRNAs to primary cells could elicit a robust gene interference response. As serum starvation can significantly facilitate effective cellular uptake of dsRNAs from the medium (Clemens et al., 2000), we

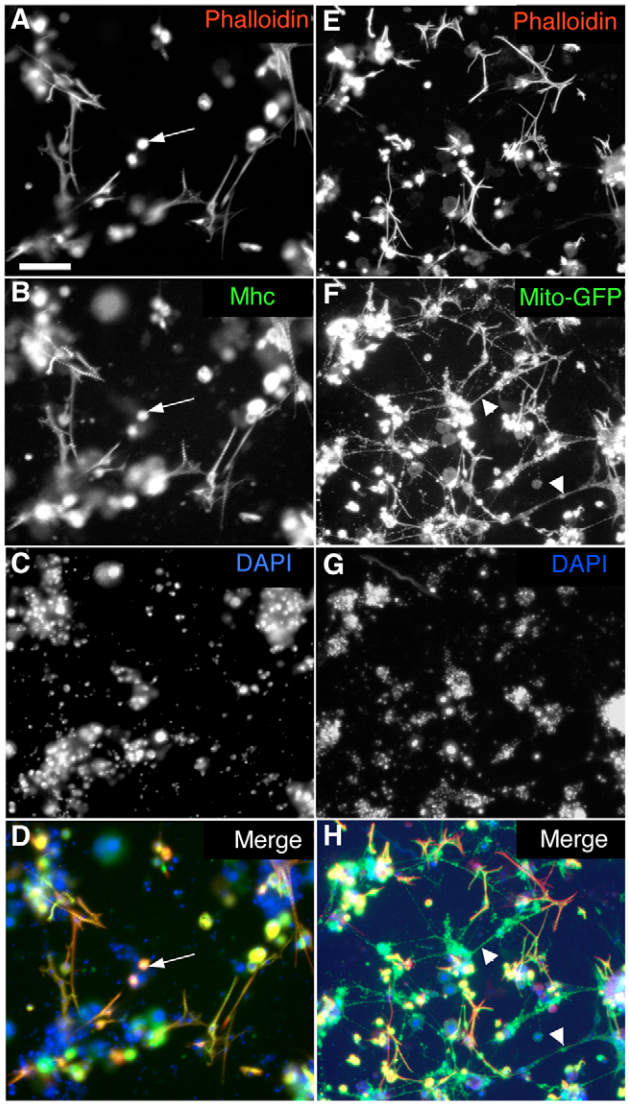

Fig. 2. Primary cultures derived from *Drosophila* embryonic cells contain a mixture of different cell populations that include muscles and neurons. (**A-D**) Primary myotubes strongly stained by phalloidin (A, red in the merged image in D) are all stained for Mhc (B, green in the merged image in D). Note that other cell types whose nuclei are revealed by DAPI (C) are faintly visible by phalloidin staining. As myotubes mature they become more contractile, some detach from the tissue culture surface, and are seen as round muscles (arrows in A,B,D). (**E-H**) Primary cells were isolated from *Dmef2-Gal4*, *D42-Gal4*, *UAS-mito-GFP* embryos in which *Dmef2-Gal4* and *D42-Gal4* drive expression of mito-GFP, a mitochondrial marker transgene that fuses the mitochondrial targeting signal to the N-terminus of EGFP, in muscles and motoneurons, respectively. Muscle structure is visualized by phalloidin staining of Actin (E, red in the merged image in H), and neurons can be seen in F (green in the merged image in H) as they stain strongly with mito-GFP but not phalloidin (triangles in F,H). In addition to neurons and muscles, other cells are present in the culture, as revealed by the staining with DAPI (G, blue in the merged image in H). In H, muscles are shown in red and yellow, neurons and their extensions in green only. Scale bar: 50 μm.

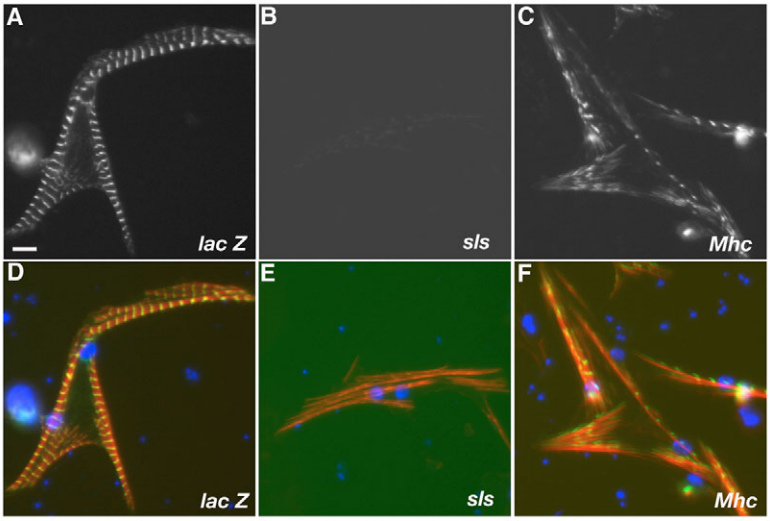

Fig. 3. Gene-specific RNAi effects in primary cells. Primary cells were isolated from *G053 Drosophila* embryos expressing the SLS-GFP fusion protein and were treated with dsRNAs targeting *lacZ* (**A**,**D**), *sls* (**B**,**E**) and *Mhc* (**C**,**F**). SLS-GFP expression was detected by GFP (A-C, green in D-F). Muscle structure was revealed by SLS-GFP in green, phalloidin staining of Actin in red, and DAPI staining of nuclei in blue (D,E,F). Scale bar: 20 μm.

first determined how it would affect myogenesis in culture. Although myoblast fusion did not require a serum supplement and could proceed to completion in its absence (see Fig. S2 in the supplementary material), myofibrils rarely formed myotubes without serum, probably owing to a lack of stimulatory factors required for their efficient assembly (Volk et al., 1990) (data not shown). Thus, we chose to starve cells for 22 hours at 18°C, when myofibril assembly initiates, and then added serum back to the cultures. This treatment did not perturb the time-course of myogenesis (see Fig. S2 in the supplementary material). Furthermore, we found that simple bathing of *Drosophila* primary cells in serum-free medium containing dsRNAs for 22 hours, followed by incubation in serum-containing medium at 18°C, was sufficient to elicit a robust and specific RNAi response. Primary cells were prepared from embyos of line *G053*, a homozygous viable enhancer-trap line carrying an in-frame insertion of *GFP* in the gene *sallimus* (*sls*), which encodes a sarcomeric protein related to vertebrate titin (Morin et al., 2001). Treatment of primary cells using control dsRNA targeting *lacZ* does not affect myofibril structure or change SLS-GFP expression (Fig. 3A,D). However, *sls* dsRNA abrogated the expression of the SLS-GFP and disrupted myofibril structure (Fig. 3B,E), whereas treatment with a dsRNA against *Mhc* interfered with its striated pattern (Fig. 3F) but did not affect SLS-GFP expression (Fig. 3C). In both cases, the RNAi knock-down was observed in 90% of existing muscle cells. In addition, we did not observe any difference in the RNAi effects within myotubes containing different numbers of nuclei, indicating that myotubes derived from more fusion events are as sensitive to RNAi treatment as those derived from fewer fusion events. Importantly, both the *sls* and *Mhc* RNAi phenotypes (Fig. 3E,F) faithfully mimicked those found in vivo in *sls* and *Mhc* mutant muscles, respectively (O'Donnell and Bernstein, 1988; Zhang et al., 2000). Furthermore, the use of SLS-GFP allowed us to follow the RNAi effect in live myotubes, which we could detect as early as 2 days after serum addition. At this time, the expression of SLS-GFP was hardly detectable in live myotubes in wells containing dsRNAs targeting *sls*, whereas live myotubes in control wells started to show organized SLS-GFP expression and myofibril structure (data not shown). The RNAi effect was more robust after 8-11 days (Fig. 3), when the expression of muscle proteins such as Actin, Myosin and SLS-GFP became stronger, and the myotubes more differentiated.

RNAi phenotypes of *Drosophila* genes that are homologous to human genes associated with muscle diseases

Using the approach outlined in Fig. 4A, we analyzed the RNAi phenotypes in primary muscle cells of *Drosophila* genes that are homologous to human genes associated with muscle diseases (see Table S1 in the supplementary material). We attempted to analyze the functional role of these *Drosophila* homologs of human disease genes in myofibril assembly and maintenance of muscle integrity. Among the 28 genes that we analyzed, 19 of them, when disrupted by RNAi, led to various muscle phenotypes (see Table S1 in the supplementary material) that fell into four distinct categories. In Class I, over 70% of myotubes failed to extend and usually rounded up, whereas neurons differentiated well in the same culture (Fig. 4C and see Fig. S3 in the supplementary material). Rounded-up muscles might indicate that the muscles failed to spread or maintain spreading on the surface of plates. Both *multiple edematous wings* (*mew*) and *myospheroid* (*mys*), which are the orthologs of the human integrin alpha- and beta-subunit genes, respectively, belong to this class (Fig. 4C and data not shown), indicating that muscle spreading on the plate requires integrin-mediated adhesion (Estrada et al., 2007; Volk et al., 1990). Phenotypes in Classes II and III consisted of disrupted sarcomeric structures with severely compromised or no striation, as detected by phalloidin staining (Fig. 4D,E). Actin filaments of Class II muscles usually had no discernible striations, whereas Myosin filaments still showed a striated pattern. Moreover, the myofibril length of Class II muscles was in general shorter than that of wild-type muscles. A representative example of this class is the *sls* gene (Fig. 3E, Fig. 4D). The ratio between the length and width of the myofibrils in *sls* RNAi muscles was ~7.5 per nucleus, which was much less than that of wild-type muscles (~21 per nucleus). In Class III muscles, both Actin and Myosin filaments lacked striation, and Actin filaments appeared more spread out (Fig. 4E). Genes in this class have been implicated in the regulation of Myosin function [*Mhc* and *Myosin light chain 2* (*Mlc2*)], or serve to mediate interactions between thin and thick filaments [*wings up A* (*wup A*), *upheld* (*up*) and *bent* (*bt*) (Vigoreaux, 2001)]. Their RNAi phenotypes demonstrate that both thick filaments and the interactions between thin and thick filaments are essential for sarcomeric order and periodicity (Clark et al., 2002). Finally, in Class IV, muscles had normal myofibril structures, but were thinner or shorter than in the wild type (Fig.

4F). dsRNAs simultaneously targeting all Actin isoforms led to this phenotype (Fig. 4F), probably reflecting the temporal effect of knocking-down Actin by RNAi after myoblast fusion. Since Actin is essential for building myofibrils, this phenotype might result from an arrest in myofibril assembly owing to a lack of available Actin monomers.

Strikingly, dsRNAs targeting *Drosophila* homologs of human dystrophin complex genes did not cause any obvious muscle phenotypes (see Table S1 in the supplementary material, and data not shown). A potential explanation is that these genes, mutations in which are associated with various types of human muscular dystrophies, are required for maintaining muscle strength and integrity (Dalkilic and Kunkel, 2003) (see Table S1 in the supplementary material). This is in contrast to congenital myopathies and cardiomyopathies, which are usually caused by the disruption of genes encoding sarcomeric components (Bornemann and Goebel, 2001; Clarkson et al., 2004; Seidman and Seidman, 2001) (see Table S1 in the supplementary material). Therefore, it is possible that muscles in culture do not experience the same mechanical stress as they do in vivo. In summary, our study of *Drosophila* genes homologous to known human muscle disease genes demonstrates the potential of RNAi in *Drosophila* primary muscles to analyze the loss-of-function of these genes, which might provide clues to the further understanding of the mechanism underlying human muscle diseases.

A screen for new genes involved in muscle assembly and maintenance

To estimate the number of genes in the *Drosophila* genome that are involved in muscle assembly, we analyzed a random set of dsRNAs targeting 1140 genes. Among these, 49 genes were confirmed to be associated with distinctive and reproducible phenotypes (four belong to Class I, 28 to Class II, 5 to Class III and 12 to Class IV) (see Table S2 in the supplementary material). Interestingly, 22 of the 49 genes (45%) have not been previously reported to be involved in late muscle differentiation, and 27 out of 49 (55%) are either expressed or putatively enriched in the mesoderm (either myoblasts and/or muscle tissues) (see Table S2 in the supplementary material). Finally, as these 1140 genes represent ~8% of the *Drosophila* genome (~14,000 genes covered with dsRNAs available in DRSC), we estimate that the total number of candidate genes implicated in muscle differentiation and maintenance (as defined by the morphological criteria used in this study) in a genome-wide screen would be around 580 (~4% of the genome).

In vivo validation by injection of dsRNAs into embryos or transgenic RNAi

We selected three Class I genes for in vivo validation, as the rounded-up muscle phenotype can be easily detected. We chose *Fermitin 1* (*Fit1*) and *Fermitin 2* (*Fit2*) because their function in *Drosophila* muscles had not been previously recognized, and in *C*.

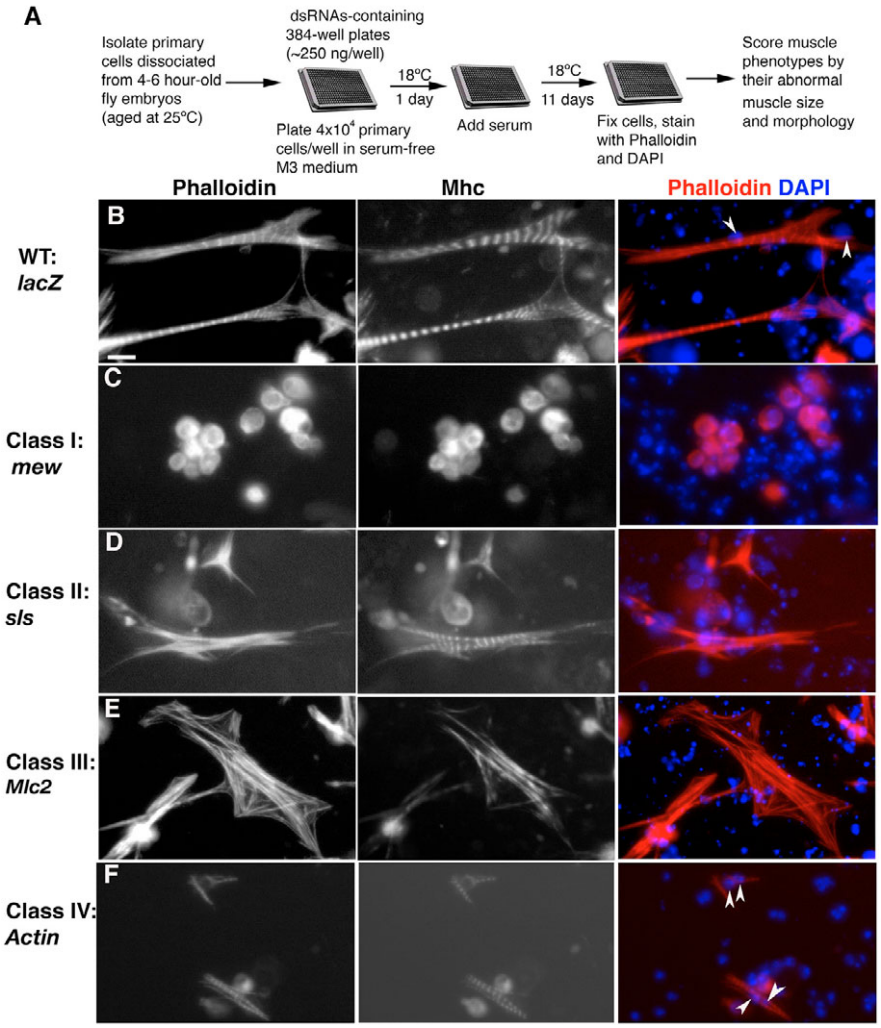

Fig. 4. Phenotypic classes identified from the RNAi screen. (**A**) Protocol for RNAi screening in primary cultures. (**B-F**) Four distinct classes of muscle phenotype were distinguished based on the staining of Actin using phalloidin (left panels, and red in right panels), Mhc (middle panels) and of nuclei with DAPI (blue in right panels). (B) Wild-type control myotubes treated with dsRNAs targeting *lacZ*. (C) Class I (treated with *mew* dsRNAs). (D) Class II (treated with *sls* dsRNAs). (E) Class III (treated with *Mlc2* dsRNAs). (F) Class IV (treated with *Actin* dsRNAs simultaneously targeting all Actin isoforms, including *Act42A*, *Act57B*, *Act5C*, *Act79B*, *Act87E* and *Act88F*). Note that the four phenotypic classes did not result from fewer fusions, as muscles contained the same number of nuclei as controls. Arrowheads point to the nuclei. Scale bar: 15 μm.

elegans the orthologous protein, UNC-112, had been shown to be involved in the assembly of integrin-containing adhesion structures (Rogalski et al., 2000). In our screen, knock-down of *Fit1* and *Fit2* individually by their corresponding dsRNAs only caused partial rounded-up muscle phenotypes, i.e. some muscles rounded up but some with branch-like morphology were still present (see Fig. S3A-C in the supplementary material). However, knock-down of these two genes together led to a complete rounded-up muscle phenotype (see Fig. S3D-F in the supplementary material, compared with wild type in Fig. 2), suggesting that their functions are partially redundant.

To validate this observation in vivo, we first examined *Fit1* and *Fit2* expression during embryogenesis by in situ hybridization. Strikingly, both genes are expressed in the musculature (Fig. 5A-E), in a pattern reminiscent of *mys* (MacKrell et al., 1988). Next, we interfered with the function of these genes by directly injecting dsRNAs against *Fit1* and *Fit2* into embryos. To ensure specificity, we used both negative (*lacZ*) and positive (*mys*) control dsRNAs in our injection. Embryos injected with *lacZ* dsRNAs did not show any discernible phenotype (Fig. 5F), whereas embryos injected with *mys* dsRNAs displayed the expected germ band retraction and round muscle phenotypes (Fig. 5G) (MacKrell et al., 1988). When *Fit1* or *Fit2* dsRNAs were injected alone, we observed that some muscles consistently rounded up (Fig. 5H,I, short arrows), although some did not seem to be affected (Fig. 5H,I, long arrows). However, the vast majority of muscles showed a rounded-up phenotype when embryos were co-injected with both *Fit1* and *Fit2* dsRNAs (Fig. 5J). These results suggest that *Fit1* and *Fit2* have overlapping roles in vivo, a conclusion that has been independently confirmed by a genetic analysis of *Fit1* and *Fit2* mutations (D. Devenport and N. Brown, personal communication).

We also validated *CG2165*, another Class I gene, using a transgenic line carrying a snap-back hairpin construct targeting this gene (see Materials and methods). Although dsRNAs targeting *CG2165* (referred to as *CG2165* RNAi) caused complete rounded-up muscle phenotypes after primary cells were cultured for 11 days at 18°C (Fig. 6D), time-course examination of 4-day and 8-day cultures showed that the majority of *CG2165* RNAi primary muscles were well spread on day 4 of culture (Fig. 6C), but few were found to have elongated morphology on day 8 of culture (Fig. 6E). These results indicated that *CG2165* may not be required for the initial spreading of primary muscles in culture, but is required for maintaining muscle morphology.

CG2165 is located at 102B5-102B5 on the fourth chromosome, and currently there are no available mutations in this gene. *CG2165* is the only gene in the *Drosophila* genome that encodes a plasma membrane Ca^{2+}-ATPase (PMCA), the putative function of which is to extrude calcium from cells, thereby maintaining a low cytosolic calcium concentration ($[Ca^{2+}]_i$) (Gwack et al., 2006). The gene is expressed ubiquitously in all tissues, including muscle (Lnenicka et al., 2006; Roos et al., 2005) (data not shown). The function of PMCA in muscle cells has not been described previously in *Drosophila* or vertebrates. To investigate the function of *Drosophila* PMCA in muscle cells in vivo, we used the *Gal4-UAS* binary system (Brand and Perrimon, 1993) to drive expression of the hairpin construct in muscles using *Dmef2-Gal4* along with the overexpression of Dicer-2 (Dcr-2) [*UAS-Dcr-2/+; Dmef2-Gal4/UAS-CG2165 hp* (referred to as muscle-specific *CG2165* RNAi)]. Dcr-2 was used to increase the RNAi effect (Dietzl et al., 2007), as we have observed the same muscle phenotype with and without Dcr-2 (*Dmef2-Gal4/UAS-CG2165 hp*), although the phenotype is less penetrant without Dcr-2 (data not shown). Moreover, larvae with overexpression of Dcr-2 alone (*UAS-Dcr-2; Dmef2-Gal4*) showed wild-type muscle morphology (Fig. 6H and data not shown). Muscle-specific *CG2165* RNAi significantly reduced the expression of its corresponding protein PMCA (Fig. 6F). Disruption of *CG2165* function did not appear to affect muscle development, as the majority of larvae expressing muscle-specific *CG2165* RNAi hatched (141/200 versus 130/161 observed in *UAS-Dcr-2; Dmef2-Gal4* controls), although all larvae died during early first instar. While still alive, these larvae were sluggish and generally were shorter and appeared hypercontracted

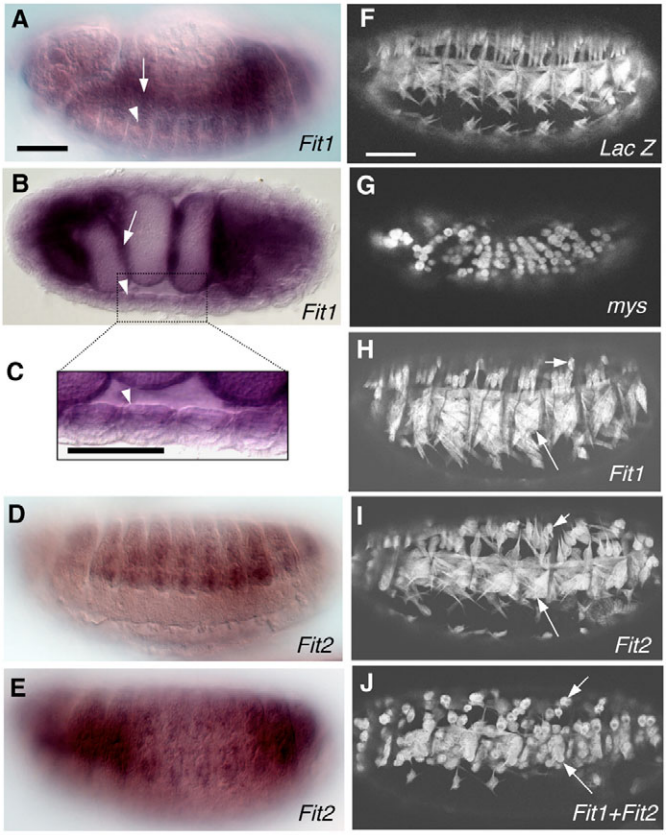

Fig. 5. In vivo validation of *Fit1* and *Fit2* using dsRNA injection. (**A-E**) Micrographs of whole-mount in situ hybridizations of *Drosophila* embryos with Dig-labeled antisense probes specifically targeting *Fit1* (A-C) and *Fit2* (D,E), oriented anterior to the left. (A,D) Lateral view of stage 14 embryos. (B) Dorsal view of a stage 16 embryo focusing on visceral muscle and somatic body wall muscles. (C) High-magnification image showing *Fit1* somatic body wall muscle expression. Arrowhead, somatic body wall muscles; arrow, visceral gut muscles. (E) Lateral view of a stage 16 embryo. (**F-J**) Fluorescence micrographs of stage 17 embryos carrying MHC-τGFP. dsRNAs targeting (F) *lacZ* (2 μg/μl), (G) *mys* (2 μg/μl), (H) *Fit1* (2 μg/μl), (I) *Fit2* (2 μg/μl) and (J) *Fit1* (1 μg/μl) + *Fit2* (1 μg/μl) were injected into MHC-τGFP embryos. MHC-τGFP allows visualization of all somatic muscles, as shown in F, where the embryos were injected with a negative control dsRNA targeting *lacZ* (*n*=67, none showed muscle phenotypes). Note that severely rounded muscles are present in the embryos injected with dsRNAs targeting *mys* (G) (100% penetrance, *n*=87, where *n* is the number of embryos injected) and *Fit1* + *Fit2* (J) (96% penetrance, *n*=150), whereas dsRNAs targeting either *Fit1* (H) or *Fit2* (I) alone only caused some muscles to round up (short arrows in H,I). Long arrows in H-J point to the ventral acute muscles that are still present as fibers in H and I, but round up in J. Scale bars: 50 μm in A for A-E, in F for F-J.

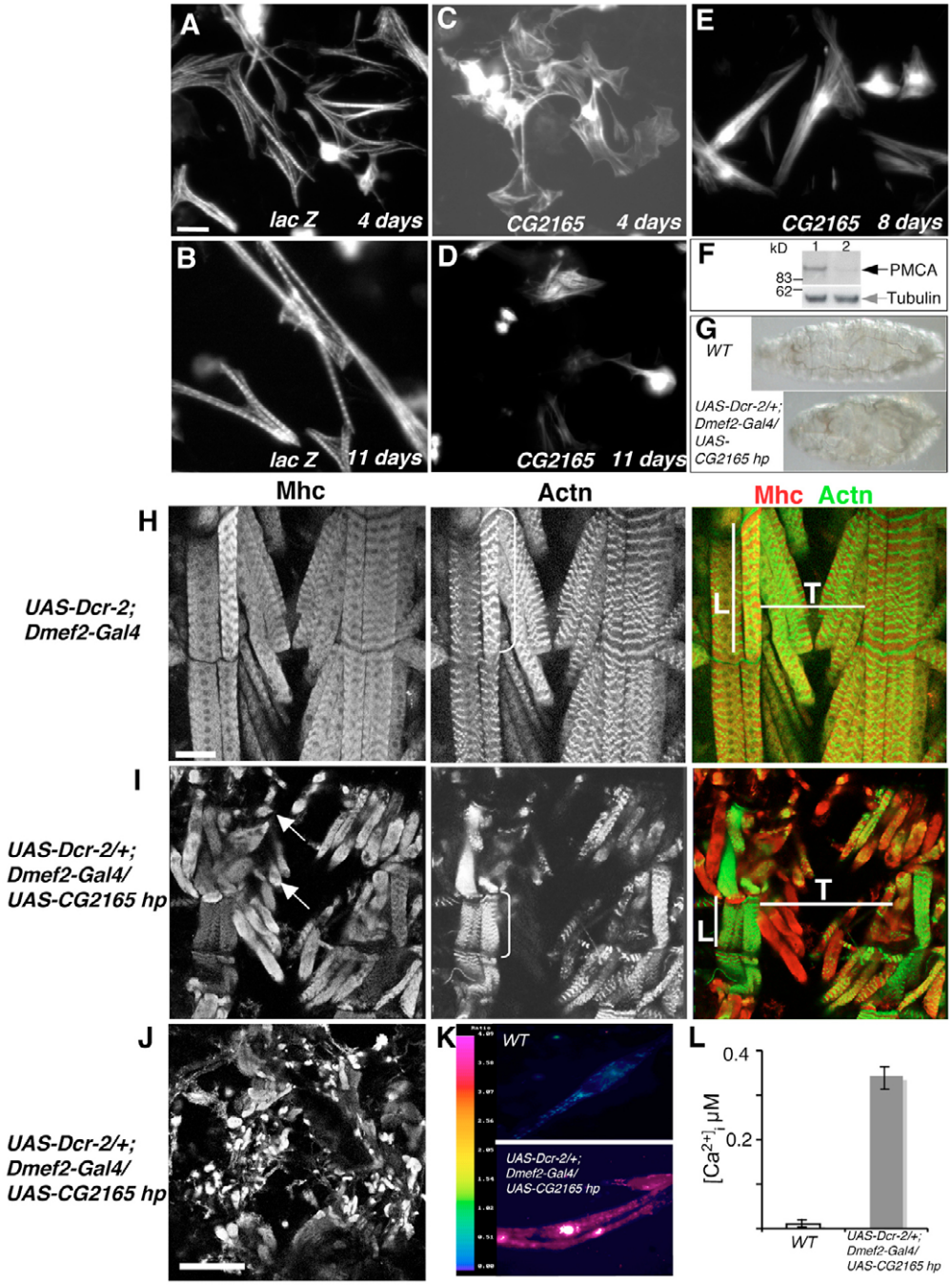

Fig. 6. In vivo validation of *CG2165* using transgenic RNAi. (**A-E**) Wild-type (A,B) and *CG2165* RNAi (C-E) primary muscle phenotypes at 18°C cultured for 4 days (A,C), 8 days (E) and 11 days (B,D), revealed by phalloidin staining for Actin. (**F**) Western blots probed with rabbit anti-*Drosophila* PMCA (top) and mouse anti-tubulin (bottom, as loading controls). Note that the expression of PMCA was significantly reduced in muscle-specific CG2165 RNAi larvae (lane 2) compared with wild type (lane 1). (**G**) Comparison of the body size in wild-type (top) and muscle-specific CG2165 RNAi (bottom) first instar larvae of the same age (30 hours AEL at 25°C). Note the short size and hypercontracted appearance of muscle-specific CG2165 RNAi larvae. (**H**,**I**) Confocal fluorescent micrographs showing the ventral internal muscles of first instar larvae of *UAS-Dcr-2; Dmef2-Gal4* (H) and muscle-specific CG2165 RNAi (I) stained for Mhc and Actn. Arrows in I point to the rounded-up muscles. Note that although both control and muscle-specific CG2165 RNAi VL4 muscles (brackets) contained a comparable number of sarcomeres longitudinally, the length of CG2165 RNAi VL4 muscles is only half that of the wild type (lines labeled L), and thus the sarcomere size was only ~50% of that in wild type. Also note that the transverse distance (T) between two VL4 muscles in the same segment in muscle-specific CG2165 RNAi larvae is much greater than that in the wild type (lines labeled T). (**J**) Fluorescent micrograph showing a larva with almost complete rounded-up muscles as revealed by staining for Mhc. (**K**) Fura PE 3 ratiometric calcium imaging micrographs of primary muscles derived from embryos of wild type (top) and *UAS-Dcr-2/+; Dmef2-Gal4/UAS-CG2165 hp* (bottom) and cultured at 25°C for 3 days. The color indicates the ratio between the emission intensities excited at 340 nm and 380 nm, and reflects a measurement of calcium concentration. (**L**) Bar chart showing $[Ca^{2+}]_i$ as average±s.e.m. for wild-type control cells (0.344±0.0162 μM; *n*=35 muscle cells in two representative experiments, white bar), and for muscle-specific CG2165 RNAi (0.0105±0.0012 μM; *n*=71 in three representative experiments, gray bar). Scale bars: 50 μm in A for A-E; 20 μm in H for H,I; 75 μm in J.

compared with control larvae of the same age (Fig. 6G). These phenotypes indicated that muscle contraction was not affected because defects in contraction would have been expected to lead to an elongated body. We further examined the muscle morphology of muscle-specific *CG2165* RNAi larvae by fluorescent confocal microscopy (Fig. 6H-J). The stainings for Mhc and Actn revealed that some larvae showed almost completely rounded-up muscles (Fig. 6J), whereas others still contained muscles with recognizable striated morphology (Fig. 6I). This is in contrast to control larvae of the same age, which always had nicely patterned muscles (Fig. 6H). Moreover, those muscles that still had the striated myofibril structure also exhibited a hypercontracted morphology, as indicated by their dramatically shortened sarcomere sizes and muscle lengths (Fig. 6I). We further investigated whether disruption of *CG2165* would lead to increased $[Ca^{2+}]_i$ in muscles. We conducted single-cell calcium imaging using Fura PE 3 on primary muscle cells derived from muscle-specific *CG2165* RNAi embryos (Fig. 6K,L) and found that the $[Ca^{2+}]_i$ in these primary muscles was over 30 times higher than that in wild-type control muscles (Fig. 6L). This confirmed that the phenotypes observed in the muscle-specific *CG2165* RNAi larvae were associated with an abnormal increase in $[Ca^{2+}]_i$ in muscle cells. Altogether, our findings suggest that *Drosophila* PMCA plays an important role in maintaining muscle integrity.

DISCUSSION

Drosophila primary cultures have been used to study muscle biology in normal and mutant animals (Donady and Seecof, 1972; Volk et al., 1990). A significant advantage of this approach is that it obviates the difficulty associated with the dissection of early first instar larvae and allows visualization of myofibril organization at a cellular level using conventional microscopy. Here, we have established a robust method for RNAi screening in *Drosophila* primary cells and found that simple bathing of these cells in dsRNA-containing medium is sufficient for an effective and specific RNAi effect. This technology allows the analysis of late-stage differentiation processes such as muscle assembly and maturation, which is difficult to tackle with classical *Drosophila* genetics.

Drosophila primary cultures have distinct advantages over vertebrate culture systems for systematically analyzing gene functions involved in muscle assembly and maintenance. Myotube cultures derived from primary myoblasts in vertebrates can have a high degree of sarcomeric maturity, and thus are often used for studies on myofibril assembly. However, preparations of primary myoblasts from freshly harvested tissues can be technically demanding, time consuming and costly (Cooper et al., 2004), in contrast to the ease with which large numbers of *Drosophila* primary cells can be isolated from embryos. Established vertebrate clonal muscle cell lines such as C2C12 have overcome the requirement for repeated myoblast isolation from fresh tissue (Cooper et al., 2004). Despite extensive fusion and myotube formation during early stages of differentiation, it has been difficult to derive C2C12 myotubes with a mature sarcomeric structure using traditional culture methods, although co-culturing cells on a primary fibroblast substratum has been reported to be more successful (Cooper et al., 2004). *Drosophila* primary cultures, however, consist of mixed cell populations, whereby non-muscle cells may facilitate muscle differentiation (J.B., unpublished). The method described in this study, however, might not be very useful for identifying genes involved in myoblast fusion, as newly isolated myoblasts have already adopted their cell-intrinsic developmental programs and have expressed those proteins required for fusion (Fig. 1) (Estrada et al., 2006). In addition, fusion takes place 2 hours after plating at 25°C and 7 hours at 18°C, too short a time to allow efficient RNAi (J.B., J. Lu, A. Michelson and N.P., unpublished).

In this study, we have described four distinct muscle phenotypes associated with knock-down of *Drosophila* homologs of human genes involved in muscle diseases. Both congenital myopathies and cardiomyopathies are also called 'sarcomere diseases' (Bornemann and Goebel, 2001; Clarkson et al., 2004; Seidman and Seidman, 2001). Indeed, the primary muscle phenotypes caused by RNAi on the *Drosophila* homologs of these human disease genes indicate that they are involved in different aspects of sarcomeric organization and muscle maintenance. Furthermore, we used this approach to conduct a screen to identify genes involved in muscle assembly and maintenance. In addition to the genes already discussed, we found that several of the proteins encoded by Class II genes are components of various cellular machineries. For example, four proteins are related to the ubiquitin/proteosome system (UPS), whereas four others function in metabolic pathways, and five are involved in basic transcription or translation. This indicates that development and maintenance of striated muscles rely on the turnover of regulatory and structural components as well as the maintenance of metabolic homeostasis in muscles (Hass et al., 2007; Lecker et al., 2006). In addition, three genes encoding ribosomal protein components were identified as Class IV genes that regulate muscle myofibril size. Of note, 22 genes identified from the screen have not been previously reported to be involved in late muscle differentiation (see Table S2 in the supplementary material).

Furthermore, we have demonstrated that the in vivo functions in muscle of genes identified from this approach can be validated and further characterized by injecting dsRNAs into embryos (Kennerdell and Carthew, 1998), by expressing snap-back hairpin constructs (Dietzl et al., 2007; Ni et al., 2008), or by using genetic mutations that disrupt gene function (Bai et al., 2007). Here, we have confirmed in vivo the primary muscle RNAi phenotypes of *Fit1*, *Fit2* and *CG2165*. In particular, we have analyzed the effects of *CG2165*, a previously uncharacterized gene identified from this screen, on the maintenance of muscle cell integrity in primary cell culture as well as in vivo. Our results demonstrate that disruption of *Drosophila* PMCA does not affect muscle development or contraction, but rather the excitation-contraction coupling process. Importantly, single-cell calcium imaging in primary muscles derived from muscle-specific *CG2165* RNAi embryos reveals that the increased $[Ca^{2+}]_i$ could be the primary cause of the rounded-up muscle phenotypes. Although we expect that the majority of the genes identified from this screen act autonomously in muscles, some genes expressed in tendons or neurons, such as *mew* (Estrada et al., 2007) and *Mgat2* (Tsitilou and Grammenoudi, 2003), may affect muscle morphology in a non-cell-autonomous manner. Further in vivo verification will be needed to address the tissue specificity of these genes by knocking down their function in a tissue-specific manner.

Our demonstration that RNAi works effectively in primary cells broadens considerably the types of studies that can be undertaken with *Drosophila* primary cultures. The major advantage of using primary cells for functional genomics is that they better model their in vivo counterparts than do immortalized mammalian cells. As the different cell types can be tracked in primary cultures using a tissue-specific GFP, antibodies or other markers, primary-cell-based RNAi screens may be used to identify genes required in other differentiated cells as well [e.g. primary neurons (K. Sepp and N.P., unpublished) (Sharma and Nirenberg, 2007)]. Importantly, RNAi screens in *Drosophila* primary cultures can be carried out by a simple bathing

method for dsRNA uptake. This is in contrast to the difficulties that have been reported with RNAi in mammalian cell lines and primary cells, which requires delivery of siRNAs into cells by chemical transfection or electroporation (Ovcharenko et al., 2005; Sharma and Nirenberg, 2007).

We anticipate that RNAi in primary cells will contribute to the understanding of human muscle biology in a number of ways. First, further deciphering the molecular relationships among genes whose RNAi phenotypes belong to the same phenotypic class will help frame the molecular mechanisms underlying muscle assembly, both in normal development and in pathological conditions. This approach might reveal candidate molecules for myopathies whose genetic lesions have not yet been identified. Second, using the Gal4/UAS system (Brand and Perrimon, 1993), expression of wild-type or mutant proteins relevant to human diseases in primary cells will lead to the development of cell-based assays to model human diseases that can then be used for RNAi and small-molecule screens. For example, RNAi in primary cells from *Drosophila* embryos overexpressing Actins with dominant mutations that cause human nemaline myopathy can be used to dissect the molecular mechanisms underlying the formation of nemaline rods under pathological conditions. Our study provides a paradigm for the use of *Drosophila* primary cells in designing cell-based assays for functional genomics using such screens.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/8/1439/DC1

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