Notch and Ras signaling pathway effector genes expressed in fusion competent and founder cells during Drosophila myogenesis

Ruben Artero¹,*,‡, Eileen E. Furlong²,†,‡, Karen Beckett¹, Matthew P. Scott² and Mary Baylies¹,§

¹Developmental Biology Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA
²Departments of Developmental Biology and Genetics, Howard Hughes Medical Institute, Beckman Center, B300, 279 Campus Drive, Stanford University School of Medicine, Stanford, California 94305-5329, USA
*Present address: Laboratory of Developmental Genetics, Department of Genetics, University of Valencia, Dr Moliner 50, 46100 Burjasot, Spain
†Present address: Developmental Biology Programme, European Molecular Biology Laboratory, Heidelberg, Germany
‡These authors contributed equally to this work
§Author for correspondence (e-mail: m-baylies@ski.mskcc.org)

Accepted 21 August 2003

Diversity in muscle identity begins in Drosophila with the specification of two types of myoblasts: founder cells (FCs) and fusion-competent myoblasts (FCMs). To better understand muscle diversity and morphogenesis, we performed a large-scale gene expression analysis to identify genes differentially expressed in FCs and FCMs. We employed embryos derived from Toll10b mutants to obtain primarily muscle-forming mesoderm, and expressed activated forms of Ras or Notch to induce FC or FCM fate, respectively. The transcripts present in embryos of each genotype were compared by hybridization to cDNA microarrays. Among the 83 genes differentially expressed, we found genes known to be enriched in FCs or FCMs, such as heartless or hibris, previously characterized genes with unknown roles in muscle development, and predicted genes of unknown function. Our studies of newly identified genes revealed new patterns of gene expression restricted to one of the two types of myoblasts, and also striking muscle phenotypes. Whereas genes such as phyllopod play a crucial role during specification of particular muscles, others such as tartan are necessary for normal muscle morphogenesis.

Summary

Drosophila muscles originate from the fusion of two types of myoblasts, founder cells (FCs) and fusion-competent myoblasts (FCMs). To better understand muscle diversity and morphogenesis, we performed a large-scale gene expression analysis to identify genes differentially expressed in FCs and FCMs. We employed embryos derived from Toll10b mutants to obtain primarily muscle-forming mesoderm, and expressed activated forms of Ras or Notch to induce FC or FCM fate, respectively. The transcripts present in embryos of each genotype were compared by hybridization to cDNA microarrays. Among the 83 genes differentially expressed, we found genes known to be enriched in FCs or FCMs, such as heartless or hibris, previously characterized genes with unknown roles in muscle development, and predicted genes of unknown function. Our studies of newly identified genes revealed new patterns of gene expression restricted to one of the two types of myoblasts, and also striking muscle phenotypes. Whereas genes such as phyllopod play a crucial role during specification of particular muscles, others such as tartan are necessary for normal muscle morphogenesis.

Key words: Myogenesis, Founder cells, Myoblast fusion, phyllopod, Toll, Microarrays

Introduction

The myogenic program displays remarkable versatility, giving rise to muscle types as specialized as cardiac and skeletal muscle, and to muscle morphologies as diverse as small ocular muscles and large thigh muscles. Studies of the fly Drosophila melanogaster have provided insights into several aspects of the myogenic program. These experiments have shown how interactions among signaling pathways lead to the differentiation of particular muscle tissues and how a gene hierarchy controls the myoblast fusion process (reviewed by Baylies et al., 1998; Dworak and Sink, 2002; Taylor, 2002). In this paper, we explore the basis of muscle diversity and morphogenesis in Drosophila by identifying genes that define the unique identity of a muscle. Specific muscle fibers are distinguished by size, shape, innervation and attachment to the epidermis of each muscle, all properties with clear counterparts in vertebrate development.

Diversity in muscle identity begins in Drosophila with the specification of two types of myoblasts: founder cells (FCs), which contain information required for morphogenesis of a given muscle; and fusion-competent myoblasts (FCMs), which fuse to a FC and become entrained to a particular muscle program (Bate, 1990; Dohrmann et al., 1990). Production of the two myoblast types requires a combination of signals from the ectoderm and mesoderm (reviewed by Baylies and Michelson, 2001; Frasch, 1999). In the dorsal mesoderm, two secreted signals, Wingless (Wg) and Decapentaplegic (Dpp), converge to define a region competent to respond to inductive signals mediated by Ras signaling. Localized Ras activation in dorsal cells instructs clusters of myoblasts to adopt the FC fate (Fig. 2A) (Buff et al., 1998; Carmena et al., 1998a). The FC fate is restricted to one cell within each cluster, the muscle progenitor, by a process of lateral inhibition mediated by Notch / Delta signaling (Bate et al., 1993; Carmena et al., 1995; Corbin et al., 1991; Giebel, 1999) and the activity of Argos, a diffusible inhibitor of the Drosophila Epidermal Growth Factor Receptor (Egfr, previously known as DER) (Carmena et al., 2002). Cells receiving the Delta signal from a neighboring muscle progenitor are determined to become FCMs. Thus, during specification of muscle progenitors from clusters of equivalent myoblasts, the Ras pathway induces the muscle progenitor fate, whereas the Notch pathway promotes the FCM fate.

Several points of cross-talk between the Ras and Notch signaling pathways guarantee a proper response during muscle progenitor specification. For example, Ras signaling cooperates with Notch signaling by inducing expression of Delta and Argos in FCs, and the activity of these proteins leads to the inhibition of FC fate in surrounding mesodermal cells. By contrast, Notch signaling competes with Ras signaling by
blocking production of Fgf/Egf pathway components, such as Heartless (Ht), Stumps and Rhomboid (Carmena et al., 2002). Notch signaling is required again when progenitors divide asymmetrically to form FCs and adult muscle progenitors (Carmena et al., 1998b; Ruiz-Gómez and Bate, 1997).

After FCs and FCMs are specified, fusion ensues under the control of cell-cell interaction regulators. Cell surface recognition proteins are produced specifically in the founder myoblast, which mediates fusion to FCMs. The recognition proteins include Kin of Irre/Dumbfounded (Kire/Duf) (Ruiz-Gómez et al., 2000), Rough/Reg/Regular Chiasm (Rst/Irrc) (Strunkelnberg et al., 2001) and the intracellular adapter protein Rolling pebbles/Antioscial (Rols/Ants) (Chen and Olson, 2001; Menon and Chia, 2001; Rau et al., 2001). FCMs, which are produced in response to the activation of Notch, appear to have their own distinct differentiation program. FCMs produce specific proteins required for fusion to FCs, such as Sticks and Stones (Sns) (Bour et al., 2000), Hibris (Hbs) (Artero et al., 2001; Dworak et al., 2001) and the transcriptional regulator Lame Duck (Lmd) (Duan et al., 2001; Furlong et al., 2001; Ruiz-Gómez et al., 2002). Although all these proteins have specific functions within either FCs or FCMs, a comprehensive understanding of the fusion process is clearly lacking.

The specific combination of inputs that a given FC receives results in the production of the unique set of molecular determinants that gives each muscle fiber its shape, size and connection pattern. Transcription factors, such as Krüppel (Kr) or Even-skipped (Eve), are produced in specific FCs in response to the signals. These transcription factors, through as yet unknown mechanisms, regulate the attributes of each muscle (Carmena et al., 1998a; Halfon et al., 2000; Ruiz-Gómez et al., 1997). The number of known molecular determinants is substantially less than the number of muscle fiber variants, which suggests that more molecular determinants remain to be discovered.

Here, we report the identification of additional genes that regulate the properties and functions of FCs and FCMs, using a genetic strategy coupled to a cDNA microarray approach. We exploited the response of the somatic mesoderm to Ras and Notch signaling to specifically enrich embryos in FCs, which are Ras-dependent, or in FCMs, which are Notch-dependent. FCs and FCMs are low-abundance cell types, so the overexpression experiments were carried out in Toll10b mutant embryos. Cells of Toll10b embryos differentiate primarily as somatic mesoderm, and the embryos are relatively enriched for FCs and FCMs (Casal and Leptin, 1996; Leptin et al., 1992; Ray et al., 1991) (this work). Newly identified genes predicted to be enriched in FCs or FCMs were confirmed using northern blots with the corresponding genes in parenthesis were: GH09755 (parcas), SD10254 (CG4136), GH20973 (gol), CK00397 (dei), CK00321 (CG8503), GH20549 (CG6024), pPhyl/BS (phyl) (Chang et al., 1995; Dickson et al., 1995), LD36757 (CG17492), GH10871 (trn) and CK00552 (nidogen). RNA was sequenced at the Cornell University DNA sequencing facility (Ithaca, NY).

Histology techniques

Immunocytochemistry in embryos was performed as described by Rushon et al. (Rushon et al., 1995), with the following modifications. Antibodies were pre-absorbed against fixed wild-type embryos, and in combination with the TSA system (PerkinElmer Life Sciences) or

Materials and methods

**Drosophila genetics**

yw was used to reveal wild-type expression patterns. Fly stocks were: phyl1 (Dickson et al., 1995), UAS-phyll (Pi et al., 2001), twist-Gal4 (Baylies and Bate, 1996), Toll10b (Erdelyi and Szabad, 1989), Dmef2-Gal4 (Carmena et al., 2002), trn114 (a gift from S. Cohen), serpent-LacZ and A490,2M3 (a gift from H. Skara), UAS-Ntr (provided by T. Lieber) and UAS-rasV12 (provided by A. Michelson). Ettum4450 (CG6050), Gs11 (CG2718), c deficiencies that uncover the aforementioned genes [Df(2R)CX1 and Df(2L)PM1, respectively] and other stocks were obtained from the Bloomington Stock Center. Combinations of mutations and stocks required for overexpression experiments (Brand and Perrimon, 1993) were generated by standard genetic crosses. For phenotypic studies, we generated embryos carrying a given mutation over a deficiency from the region (with relevant stocks balanced over CyO P[w+ wg n11lacZ] or TM3 Sb Ubx-lacZ). In this way we further reduced gene function in cases of hypomorphic alleles, and at the same time complemented second site mutations.

**Fly stocks for newly identified genes**

A P element insertion, Ettum4450, in the mitochondrial Elongation factor Tu gene (CG6050) was used. Embryos homozygous for Ettum4450 are lethal, whereas embryos that are transheterozygous for the P insertion and a deficiency from the same region [Df(2R)CX1] reveal a partial fusion block and a gut phenotype. Gs11 is a lethal EMS mutation in glutamine synthetase 1 (CG2718), which, in trans with a deficiency from the region (Df(2L)PM1), reveals several subtle morphological defects. For example, the dorsal muscles were sometimes split in two, the DO2 muscle was thinner than usual and the dorsal muscles show aberrant attachment to the apodemes. Because of our characterization of the nearby gene hibris, we initially had available deficiency combinations that removed parcas and two additional predicted genes that are not expressed in the embryo. In these mutant embryos, we detected a muscle phenotype, including muscle losses and attachment defects. A mutation in parcas (Sinka, et al., 2002), in trans with our deficiencies, has a similar muscle phenotype. The complete analysis of parcas function during muscle development will be published elsewhere. In the case of CG7212, the gene trap insertion BG02608 is a P insertion 27 bp downstream of the start site and is predicted to be a null allele. Although the initial specification of the muscles appeared normal by FC marker analysis, the LT1-4 and DT1 muscles, for example, were frequently shorter (data not shown). Muscles in CG7212 mutants also had defects in their growth toward the epidermal attachment sites and displayed abnormal shapes (e.g. LT4 muscles). We propose the name cadmus (cdm; after the mythological figure that changed his shape into a snake) for the predicted gene CG7212. The muscle phenotype detected in BG02608 homozygous mutant embryos was the same as this allele over a deficiency from the relevant region. The percentage of mutant hemisegments was calculated by counting between 65 (ast) to 120 (trn, phyl) myosin-stained hemisegments.

**Molecular biology**

RNA was extracted from appropriate samples using Tri-Reagent (Sigma), and polyA+ RNA was purified with Oligotex mRNA spin-columns (Qiagen). Northern blots were processed following manufacturer recommendations for digoxigenin-labeled probes (Roche Molecular Biochemicals). Probes employed in the northern blots with the corresponding genes in parenthesis were: GH09755 (parcas), SD10254 (CG4136), GH20973 (gol), CK00397 (dei), CK00321 (CG8503), GH20549 (CG6024), pPhyl/BS (phyl) (Chang et al., 1995; Dickson et al., 1995), LD36757 (CG17492), GH10871 (trn) and CK00552 (nidogen). DNA was sequenced at the Cornell University DNA sequencing facility (Ithaca, NY).
a hot fixmation protocol (Rothwell and Sullivan, 2000), as indicated by the acronym TSA or HF, respectively. Antibody dilutions were: anti-Mhc (1:1000) (Kiehart and Feghali, 1986), anti-β-Gal (1:2000; Promega), anti-Slouch (1:200), anti-Vg (1:100), anti-Sns (1:1000; HF; TSA) (Bour et al., 2000), anti-FasIII (1:100) (Patel et al., 1987), anti-Twist (1:5000; provided by S. Roth), anti-Tinman (1:2500; TSA), anti-Crumbs (1:50), anti-Single-minded (1:1000; HF; TSA; provided by S. Crews), anti-22C10 (1:20), anti-Tracheless (1:2000; HF; TSA), anti-Wg (1:200) and anti-Ht (1:2000; provided by A. Michelson).

Antibodies against FasIII, 22C10, Tracheless and Wg were obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Fluorescent immunohistochemistry with anti-Ubx (1:300; TSA) and anti-Trn- (pre-adsorbed 1:900; TSA) (Chang et al., 1993) was achieved using streptavidin FITC. Anti-Kr (1:50; provided by J. Reinitz) was detected with the TSA Fluorescence System (PerkinElmer Life Sciences). Anti-Ase was used pre-adsorbed at a 1:2500 dilution and detected with Cy3-conjugated streptavidin. Biotinylated secondary antibodies were used in combination with Vector Elite ABC kit (Vector Laboratories, CA). Specimens were embedded in Araldite and images captured using an Axiocam camera (Zeiss) using Adobe Photoshop software. RNA localization was detected using digoxigenin-labeled RNA probes as described (O’Neill et al., 1994).

Fluorescent in situ hybridizations to detect dei, gol, nidogen and phyl transcripts were performed using digoxigenin-labeled RNA probes under standard conditions (50% formamide at 65°C overnight). Following repeated washing at 65°C, the embryos were rehydrated in step wise fashion and incubated in blocking solution [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% blocking reagent; NEN] for 1 hr at 22°C. The embryos were then incubated at 4°C overnight in 250 µl blocking solution with a 1:2000 final concentration of anti-digoxigenin-POD (Roche), pre-adsorbed against fixed embryos. Following ~12x30 minute washes, the embryos were developed with the TSA-Plus Fluorescence Palette system (NEN), using a 3 minute incubation with a 1:50 dilution of fluorescein isothiocyanate (IBA). The embryos were then incubated 15 minutes at 65°C and 30 µl 20 M NaOH, 20 mM EDTA and placed at 37°C for 2 hours. The samples were washed in three solutions: (1) 1×SSC, 0.03% SDS; (2) 0.2×SSC and (3) 0.05×SSC. Following drying by centrifugation at 129 g for 5 minutes, the slides were immediately scanned using an Axon Scanner. For each of the two experimental conditions, three independent embryo collections (aged 5-9 hours AEL) and hybridizations were used.

Statistical analysis was performed as described (Tusher et al., 2001). The fold difference was defined as the mean of ratios of activated Ras/activated Notch conditions from three independent hybridization experiments. Genes were considered to be differentially expressed whenever one of the following conditions were met: (1) a fold difference of 2 or above (Ras conditions) or 0.5 and below (Notch conditions) in three independent hybridization experiments; or (2) significance by SAM statistical analysis (Tusher et al., 2001) with a fold difference of 1.8 or above (Ras conditions) and 0.6 and below (Notch conditions), when data from at least two independent hybridization experiments were available. Curated information from Drosophila genes was obtained from http://www.flybase.org/ and expression patterns from the BDGP expression pattern project at http://www.fruitfly.org/cgi-bin/ex/insitu.pl/. Published array databases for mesoderm (Furlong et al., 2001) or developmental stages (Arbeitman et al., 2002) (http://www.fruitfly.org/cgi-bin/ex/basic.pl) were also analyzed to confirm mesodermal and/or embryonic expression.

Results

Characteristics of Toll10b mutant embryos

To generate embryos enriched in either FCs or FCMs, we overexpressed activated Ras or Notch in the presence of the gain-of-function mutation Toll10b. The Toll receptor is essential for determining dorsoventral polarity in the embryo (reviewed by LeMosy et al., 1999). Constitutive activation of Toll causes ectopic transcription of twist and other ventral genes, and repression of dorsal genes (Leptin et al., 1992; Schneider et al., 1991). The induction of ventral genes, especially twist (Fig. 1A,B), results in most cells of the embryo adopting a mesoderm cell fate (Baylies and Bate, 1996; Casal and Leptin, 1996; Leptin et al., 1992; Ray et al., 1991). No publication has reported the cell fates or combinations of tissue types present when FC and FCM differentiation takes place in Toll10b mutant embryos. Accordingly, several molecular probes were used to identify mesoderm, ectoderm and endoderm tissues in Toll10b mutant embryos (Fig. 1).

Specific markers for progenitors of heart and midgut visceral mesoderm (Fig. 1C-H) showed that these cell types were absent from Toll10b embryos. Tinman, which is normally found in the precursors of heart, dorsal somatic muscles and foregut, was detected only in foregut precursors in the mutant embryos. Likewise, transcripts of bagpipe, which are normally present in eleven groups of visceral mesoderm progenitors, as well as the primordial cells of the foregut and hindgut (Zaffran et al., 2001), were found only in the hindgut primordia. In the cephalic region of Toll10b embryos, serpent expression indicated that blood cell progenitors were specified (Fig. 1I-J). Fused, myosin-stained syncyia were readily observed throughout Toll10b embryos, indicating the presence of somatic muscle (Fig. 1K-L). Toll10b embryos did not form epidermis, nervous system, trachea or cuticle (Fig. 1O-V), but did make muscle precursors, with blood cell progenitors, mesectoderm and endoderm.

Signaling pathways required for mesoderm differentiation include Wg, Hedgehog (Hh), Dpp, Notch and Ras, all of which
we evaluated in Toll10b mutant embryos. dpp and hh transcripts were absent from Toll10b embryos (not shown), with the exception of transient, early dpp expression at the anterior and posterior ends of syncytial blastoderm embryos (Ray et al., 1991) (data not shown). Toll10b mutant embryos stained with an anti-Htl antibody had clusters of Htl-positive cells in the mesoderm, reminiscent of wild-type controls (Fig. 1Y-Z) (Michelson et al., 1998a). Anti-Wg staining revealed stripes of expression (Fig. 1AA-BB), in contrast to the wild-type situation in which Wg is normally undetectable in the mesoderm at this time (Baylies et al., 1995). Successful Ras and Notch signaling was inferred from biological responses in Toll10b mutant embryos expressing activated Ras or Notch (Fig. 2; see below). Hence, Wg, Ras and Notch signaling pathways that are crucial to FC specification are active in Toll10b mutant embryos.

**Toll10b mutant myoblasts respond to Ras and Notch signaling like their wild-type counterparts**

For Toll10b embryos to be useful for our screen, FCs and FCMs must be specified and responsive to Ras and Notch signaling despite the altered tissue composition. Two genes expressed in FCs, slouch (Dohrmann et al., 1990) and vestigial (vg) (Bate et al., 1993), marked groups of myoblasts in Toll10b mutant embryos, indicating that FC specification occurs (Fig. 2B,E). FC genes that are normally expressed in dorsal cells (e.g. eve or runt) were not detected, presumably because of the absence of ectoderm-derived dpp (Halfon et al., 2000) (data not shown). Large numbers of FCMs developed in Toll10b mutants, as shown by the presence of the FCM-specific protein Sns (Fig. 2H).

When activated forms of Notch or Ras were expressed using the twist-Gal4 driver in a Toll10b mutant, the number of FCs decreased (Fig. 2C,F) or increased (Fig. 2D,G), respectively, compared with Toll10b embryos. Moreover, the FCM marker sns showed increased expression when FC markers decreased: activated Notch increased sns expression and Ras activation reduced sns expression (Fig. 2I,J). The dramatic changes in FC and FCM numbers when Ras or Notch were activated in mesoderm enriched embryos made it possible to analyze changes in transcript levels in normally scarce muscle progenitor cells.
Fig. 1. *Toll10b* mutant embryos differentiate largely as somatic mesoderm. Anterior is to the left and dorsal up unless otherwise noted. All stages are according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985). All views are lateral except for those shown in G,H,U,V,AA,BB, which are ventral. Wild-type and *Toll10b* mutant embryos were stained with the indicated antibodies, subjected to in situ hybridization (E,F) or processed to reveal the larval cuticle (U,V), as described in Materials and methods. (A-L) The somatic mesoderm is the chief mesodermal tissue present in *Toll10b* mutant embryos. All embryos were 5- to 9-hour AEL except for those shown in A,B and K,L, which were stage 5 and stage 16, respectively. (A,B) Arrowhead denotes lack of dorsal Twist staining in a wild-type embryo (A) and ubiquitous expression of Twist in *Toll10b* mutant embryos (B). (C,D) Tinnman was expressed in heart precursors (straight arrow, C), dorsal somatic muscle and foregut (bent arrow, C). Only some putative foregut expression remained in *Toll10b* mutant embryos (bent arrow, D). (E,F) *bagpipe* transcripts were expressed in circular visceral mesoderm precursors (straight arrow, E), foregut and hindgut precursors (bent arrow, E). Only the expression in putative hindgut precursors was maintained in *Toll10b* mutant embryos (bent arrow, F). Straight arrow in F denotes lack of the circular visceral mesoderm marker *bagpipe* expression in the trunk. (G,H) Fasciclin III, a marker for differentiated visceral mesoderm, labeled the muscle sheet surrounding midgut (arrow, G) and pharyngeal muscles (arrowhead, G). *Toll10b* embryos showed expression in the putative remains of the pharyngeal musculature (arrowhead, H). Arrow in H denotes the absence of Fasciclin III expression in the trunk where the visceral mesoderm would have developed. (I,J) A serpent-lacZ reporter revealed that pro-hematocytes migrating from the head mesoderm (arrowhead, I) were also present in *Toll10b* mutant embryos (arrowhead, J). (K,L) Myosin heavy chain staining of wild-type (K) and *Toll10b* mutant (L) embryos. The final muscle pattern was disrupted in *Toll10b* mutant embryos but there was an abundance of Myosin-positive myoblasts and muscle fibers (arrow, L). (M-V) Ectodermally derived tissues were missing from *Toll10b* mutant embryos. All embryos were 5- to 9-hour AEL, except for those shown in Q,R, which were 7.5- to 10.5-hour AEL, and those shown in U,V, which were first instar larvae. (M,N) Single-minded expression, an in genes identified in the screen (Figs 5, 6). A summary of the genes that are differentially expressed in FCs and FCMs, noting known or predicted subcellular locations of the encoded proteins, is given in the supplemental figure available online (see Fig. S1 at http://dev.biologists.org/supplemental/). Several approaches were used to validate the screen, including analysis of known muscle genes, FlyBase and BDGP database searches, northern blot analysis (Fig. 3), confocal microscopy (Fig. 4) and analysis of muscle development in embryos carrying mutations in genes identified in the screen (Figs 5, 6).

**Expression profiles of known muscle genes**

Two groups of genes functioned as controls for the screen. The first group consisted of known muscle genes that are uniformly expressed in both FCs and FCMs, including *Drosophila* Mef2, *daughterless*, *myoblast city*, *Myosin heavy chain* (*Mhc*) and *muscleblind*. As expected, none of these genes were identified as being differentially expressed by using microarrays (see Table S1 online at http://dev.biologists.org/supplemental/).

The second control group contains muscle identity and fusion genes already known to be expressed only in one type of myoblast. Three genes previously shown to be necessary for FC specification, *stumps*, *htl* and *big brain*, were identified (Table 1) (Corbin et al., 1991; Imam et al., 1999; Michelson et al., 1998b; Vincent et al., 1998). Both *htl* and *stumps* are specifically expressed in FCs, validating our approach. Genes required for myoblast fusion, including *hbs* and *sallimus/D-Titin* (*sal*), had increased transcript abundance under activated Notch conditions. *hbs* is expressed in FCMs (Artero et al., 2001; Dworak et al., 2001). Expression of *sal* occurs in both types of myoblasts, yet transcripts are more abundant in FCMs than in FCs (Menon and Chia, 2001). Several known genes that were expected to be differentially expressed, such as *kirrel/duf*,...
rst/irreC, slouch, Kr, vg or apertuous, were not present on the array whereas others, such as sns, rolslants and connectin, showed altered expression as expected but did not meet our stringent statistical requirements (see Table S1 at http://dev.biologists.org/supplemental/). Other known genes, such as eve, were not expected to be differentially expressed because their expression requires Dpp signaling, which is absent from Toll10b embryos.

Taken together, the controls indicated that the screen was able to identify genes differentially expressed between FCs and FCMs. Below we highlight several classes of genes involved in different facets of muscle morphogenesis. In addition to our stringent statistical requirements, the genes discussed have the expected biological properties, such as expression in the proper cells and/or relevant mutant phenotypes (Table 1; Figs 3-6).
Transcription factors expressed specifically in FCs or FCMs

The discovery of transcription factors expressed specifically in subsets of FCs (Dohrmann et al., 1990) and in FCMs (Duan et al., 2001; Furlong et al., 2001; Ruiz-Gómez et al., 2002) suggests that individual FCs and FCMs have different potentials. We have identified additional transcription factors that are enriched in FCs and FCMs. delilah (dei), which encodes a basic helix-loop-helix (bHLH)-containing protein (Armand et al., 1994), is expressed within FCs. The differential transcription of dei was confirmed by using northern blot analysis (Fig. 3) and in situ hybridization (Bouchard and Cote, 1993) (Fig. 4A-C). dei transcript levels decline in the somatic mesoderm as fusion proceeds. dei expression did not recur in developing muscles, suggesting that it is required to control some aspect of FCM identity before fusion. Two other FCM-enriched transcription factors were identified in the screen, the bHLH containing protein mβ of the E(spl) complex and the homeobox-containing gene CG4136 (see blot in Fig. 3). The involvement of the E(spl) complex in muscle development has been reported previously (Bate et al., 1993; Corbin et al., 1991), but the particular member of the complex that is involved remained unknown. Our data suggest that the mβ transcript is one of the members of the E(spl) complex required for muscle development.

Two transcription regulators, asense (ase) and Ultrabithorax (Ubx), were induced under activated Ras conditions and predicted to be active in FCs. Ase, an achaete-scute (ac-sc) complex member, is a bHLH transcription factor that functions as a proneural gene in bristle development. During muscle development in mid-stage 12 embryos, Ase was transiently expressed in Kr-positive FCs (Fig. 4G-I). The homeotic gene Ubx was predicted to be expressed in FCs based on previous reports that showed that Ubx is involved in muscle pattern diversification (Michelson, 1994). Confocal analysis confirmed that Ubx was expressed in FCs (Fig. 4J-L).

Signaling-related molecules enriched in FCMs or FCs

Seven genes that encode proteins predicted to be involved in cell signaling were enriched under activated Notch conditions and therefore predicted to be active in FCs (Table 1). Three of these, parcas, asteroid (ast) and golliath (gol), encode novel molecules. parcas is the Drosophila homolog of the human SAB gene. SAB protein inhibits the auto- and transphosphorylation of BTK, a tyrosine kinase crucial for B cell development (Yamadori et al., 1999). Regulation of parcas under our experimental conditions was confirmed by northern blot (Fig. 3). parcas is transcribed in embryos during somatic muscle development. Embryos in which parcas function has been removed have muscle phenotypes (Beckett et al., 2002) (see Materials and methods). Ast is a novel 815 amino acid protein. Expression and phenotypic analyses of ast are presented in detail below. A third FC signaling gene, gol, encodes a RING-finger protein. Its differential expression was confirmed by northern analysis (Fig. 3) and its FCM expression was confirmed by in situ hybridization (Fig. 4D-F). The Xenopus Gol homolog GREULI functions as an E3 ubiquitin ligase. It has been proposed that GREULI modulates signal transduction pathways required for neuralization of ectoderm (Borchers et al., 2002).

Three putative signaling proteins that were previously characterized in other tissues had transcripts enriched in FCs, suggesting roles in muscle development: RhoGEF3, polychaetoid and phyllopod (phyl). RhoGEF3 encodes the Drosophila ortholog of the human protein PEM2, a GEF regulator of Rho activity that is transcribed in muscle cells during morphogenesis (Hicks et al., 2001). Polychaetoid (Pdy) is a cytoplasmic PDZ and SH3 domain-containing protein required for dorsal closure (Takahashi et al., 1998), and for proper segregation of sensory organ precursors from proneural clusters (Chen et al., 1996). Phyllopod (Phyl) is discussed in more detail below. These findings highlight possible mechanisms through which signals are conveyed to the cytoskeleton to elaborate muscle morphology.

Myoblast fusion and cell adhesion

Myoblast fusion between FCs and FCMs occurs through the interaction of proteins that are produced in one or the other muscle cell type, for example Kirre/Duf in FCs and Sns in FCMs (Bour et al., 2000; Ruiz-Gómez et al., 2000). Our data identified three genes known to be required for myoblast fusion that are enriched in FCMs: hibris, blown fuse and sallimus/D-Titin. A novel gene induced by Ras signaling, CG17492 (Table 1), encodes a protein predicted to contain domains involved in protein-protein interactions also found in the adaptor protein Ants/Rols. Our in situ hybridization data indicate that it is strongly expressed in the somatic mesoderm (data not shown).

Work by Chen and Olson (Chen and Olson, 2001) has shown that two forms of Kirre/Duf, a cleaved and an uncleaved version, can be detected when Kirre/Duf is expressed in S2 cells. They hypothesize that the cleaved form functions as the attractive signal, which emanates from FCs. Because of its redundancy in structure and function, Rst/IrreC has been proposed to behave similarly (Dworak and Sink, 2002). Although the enzyme that cleaves Kirre/Duf is unknown, we have found a serine-type endopeptidase and two serine protease inhibitors that were induced under activated Notch conditions and are therefore predicted to be expressed in FCMs. FCMs may have their own endopeptidase system: tequila, a gene encoding a serine-type endopeptidase, was found to be expressed under Ras conditions and is therefore predicted to be expressed in FCMs (Table 1). It is tempting to suggest that these protease activities and inhibitors are involved in regulating production of the signaling-competent forms of Kirre/Duf and Rst/IrreC, and/or its diffusion among FCMs.

Two genes putatively involved in cell adhesion, tartan (trn) and the predicted gene CG10275, were enriched in activated Ras conditions. Trn, a Leucine-Rich Repeat (LRR)-containing transmembrane protein, contributes to the formation of the dorsoventral boundary in the developing wing (Gabay et al., 1996; Milan et al., 2001). The expression of tartan in FCs and the muscle phenotype of tartan mutants are described below.

In parallel with cell adhesion, substrate adhesion may also guide muscle morphogenesis. We found that transcripts of Drosophila nidogen, which encode an extracellular matrix protein that co-localizes with laminin in the basement membranes of muscles (Kumagi et al., 1999), are induced in FC-enriched embryos. Northern blot analysis confirmed that
A. Genes enriched under Ras conditions

**Table 1. N and Ras regulated genes from the microarray analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>F*</th>
<th>S*</th>
<th>Biology†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcription factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG1238</td>
<td>averse</td>
<td>2.4</td>
<td>y</td>
<td>A, **Fig. 4</td>
</tr>
<tr>
<td>CG10568</td>
<td>Ultraspirax</td>
<td>2.3</td>
<td>y</td>
<td>FB, A, Fig. 4</td>
</tr>
<tr>
<td><strong>Signaling-related molecules</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG1122</td>
<td>RhoGEF3</td>
<td>2.0</td>
<td>y</td>
<td>A, SM</td>
</tr>
<tr>
<td>CG7233</td>
<td>heartless</td>
<td>5.3</td>
<td>y</td>
<td>FB, DD</td>
</tr>
<tr>
<td>CG10108</td>
<td>phipilie</td>
<td>2.7</td>
<td>y</td>
<td>Fig. A, 6</td>
</tr>
<tr>
<td>CG17348</td>
<td>detailed</td>
<td>2.0</td>
<td>y</td>
<td>FB</td>
</tr>
<tr>
<td>CG13137</td>
<td>stumps</td>
<td>3.8</td>
<td>y</td>
<td>SB</td>
</tr>
<tr>
<td>CG13149</td>
<td>phosducin, Guanylate cyclase (EC:4.6.1.2)</td>
<td>1.8</td>
<td>y</td>
<td>SM</td>
</tr>
<tr>
<td><strong>Extracellular matrix component</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG12969</td>
<td>nidogen</td>
<td>14</td>
<td>y</td>
<td>N,DD,A,Fig. 4</td>
</tr>
<tr>
<td><strong>Cell adhesion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG10273</td>
<td>Contains EGF-like repeat and laminin G domain</td>
<td>2.4</td>
<td>y</td>
<td>M, A</td>
</tr>
<tr>
<td>CG11280</td>
<td>turtan</td>
<td>3.3</td>
<td>y</td>
<td>N,FB,A,Figs 4, 5</td>
</tr>
<tr>
<td><strong>Putative or known enzymatic activities</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG7780</td>
<td>Deoxyribonuclease II (EC:3.1.21.2)</td>
<td>1.8</td>
<td>y</td>
<td>MA</td>
</tr>
<tr>
<td>CG9147</td>
<td>Alkaline phosphatase (EC:3.1.3.1)</td>
<td>3.8</td>
<td>y</td>
<td>M, A, §§</td>
</tr>
<tr>
<td>CG9520</td>
<td>Galactosyltransferase? UDP-glucuronosyltransferase (EC:2.4.1.17)</td>
<td>4.0</td>
<td>y</td>
<td>MA</td>
</tr>
<tr>
<td><strong>Serine proteases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG14207</td>
<td>tequila (EC:3.4.21.1)</td>
<td>2.5</td>
<td>y</td>
<td>M, A</td>
</tr>
<tr>
<td><strong>Chaperone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG14207</td>
<td>Hsp20alpha crystallin family</td>
<td>1.9</td>
<td>y</td>
<td>M, A</td>
</tr>
<tr>
<td><strong>Molecular transporters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG4772</td>
<td>big brain. Gap junction component</td>
<td>2.1</td>
<td>y</td>
<td>FB,A,M</td>
</tr>
<tr>
<td><strong>Novel genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG2083</td>
<td>Contains NLS</td>
<td>3.5</td>
<td>y</td>
<td>A, SM</td>
</tr>
<tr>
<td>CG3588</td>
<td>Transmembrane protein</td>
<td>2.0</td>
<td>y</td>
<td>A, SM</td>
</tr>
<tr>
<td>CG17492</td>
<td>Aukin repeat, zinc-binding domain and RING finger</td>
<td>2.2</td>
<td>y</td>
<td>N,MA,SM</td>
</tr>
<tr>
<td><strong>Role in muscle development uncertain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eegyn</td>
<td>Transposable element</td>
<td>2.0</td>
<td>y</td>
<td>M</td>
</tr>
<tr>
<td>GH16741</td>
<td>repetitive sequence</td>
<td>2.0</td>
<td>y</td>
<td>M, §§</td>
</tr>
<tr>
<td>CG1743</td>
<td>No remarkable features</td>
<td>2.4</td>
<td>y</td>
<td>NE, NA, A</td>
</tr>
<tr>
<td>CG4128</td>
<td>nicotinic Acetylcholine Receptor alpha 30D</td>
<td>2.4</td>
<td>y</td>
<td>M, NE, NA</td>
</tr>
<tr>
<td>CG9024</td>
<td>LDL receptor domain A and CUB domain</td>
<td>1.9</td>
<td>y</td>
<td>N, NA</td>
</tr>
<tr>
<td>CG6531</td>
<td>wengen. TNFR domain</td>
<td>2.2</td>
<td>y</td>
<td>M, A</td>
</tr>
<tr>
<td>CG6980</td>
<td>Contains a tetracaine-CoA repeat</td>
<td>2.9</td>
<td>y</td>
<td>NE, NA, A</td>
</tr>
<tr>
<td>CG7157</td>
<td>Accessory gland peptide 36E</td>
<td>5.6</td>
<td>y</td>
<td>NA</td>
</tr>
<tr>
<td>CG10153</td>
<td>Contains a 3-hydroxy-CoA dehydrogenase domain</td>
<td>2.0</td>
<td>y</td>
<td>M, NA</td>
</tr>
<tr>
<td>CG10433</td>
<td>Putative prion gene</td>
<td>2.1</td>
<td>y</td>
<td>NE, NA</td>
</tr>
<tr>
<td>CG12071</td>
<td>site</td>
<td>2.8</td>
<td>y</td>
<td>NE, NA</td>
</tr>
<tr>
<td>CG14307</td>
<td>C2H2 zinc finger domains</td>
<td>3.9</td>
<td>y</td>
<td>M, NA</td>
</tr>
<tr>
<td>CG14898</td>
<td>frataxin</td>
<td>2.4</td>
<td>y</td>
<td>NE, NA, A</td>
</tr>
</tbody>
</table>

*The fold difference (F) was defined as the mean of ratios of activated Ras/activated N.
†The result of a SAM analysis (S column) is given as a ‘y’ if significant. Classes are given according to molecular functions (e.g. transcription factors) or biological processes (e.g. myoblast fusion process).
‡Observations that provide support for observed differential expression are given in the ‘Biology’ column: differential expression under our two experimental conditions by northern blot ('N’, see Fig. 3), description of an embryonic muscle phenotype (our data, Figs 5 and 6, or others, ‘FB’ – FlyBase), expression detected in a developmental time course microarray (5-9 hours AEL developmental window) or by in situ hybridization in the somatic musculature in the BDGP expression pattern project (A or SM respectively; http://www.fruitfly.org/cgi-bin/ex/exinsitu.pl), a FCP/PCM specific expression pattern (Fig. 4). The relevance of some genes to muscle development was unclear because they were not expressed in the somatic mesoderm as detected by standard in situ hybridization (NE, our not shown data) or were not detected in a developmental time course microarray (5-9 hours AEL developmental window) in the BDGP expression pattern project (NA). When the expression pattern was available from the CK EST expression project at BDGP (http://weasel.lbl.gov/cgi-bin/EST/community_query/cloneReport.pl), the name of the CK clone was included in the Biology column.
§Regulation observed may come from mesodermal expression as no mesodermal expression has been reported.
¶Maps outside regions of the X chromosome retained as containing genes whose products are involved in containing wild-type muscle development (Drysdale et al., 1993).
classified the genes with no clear mesoderm function into subgroups. For example, wingless, CG6024 and CG4136 form a group because they are not normally expressed in wild-type somatic mesoderm but their differential expression has been confirmed by northern blot (Fig. 3). This group of genes may normally be repressed in the mesoderm by a regulator that is absent in the Toll10b background. A second group consists of genes that are normally expressed in tissues other than somatic mesoderm that are still present in Toll10b embryos. For example, bangles and beads, CG1124 and CG14989 are expressed in the mesectoderm during the 5-9 hour developmental window used in our experiments (BDGP expression data, and data not shown). Similarly, other genes expressed in the endoderm, blood progenitors or other tissues present in Toll10b embryos, may be subject to Notch and/or Ras regulation. A third group is comprised of two repetitive retrotransposons: gypsy and 412. Retrotransposable elements are flanked by Long Terminal Repeats with promoter activity (Levin, 2002). For some copies of the transposons, these promotors might act as reporters for nearby differentially expressed genes, and thus appear specifically enriched in one of the conditions. The analyses to date, therefore, lead us to expect that the true false-positives emerging from the screen will be a small minority of the total.

**Phenotypes associated with new FC/FCM regulatory genes**

The differential expression of a number of genes identified in the screen were confirmed by northern blot and/or immunostaining/fluorescent in situ hybridization. We assayed a subset of genes from the screen to determine whether these genes have an essential role in normal muscle development. We obtained available loss-of-function mutants in ast (a FCM-enriched signaling-related protein), trn (a FC-enriched cell adhesion molecule) and phyl (a FC-enriched signaling-related molecule) (see Materials and methods). In each case, mutant embryos showed defects in the somatic musculature, ranging from aberrant muscle morphologies and partial fusion blocks (trn), to missing/duplicated muscles (phyl, trn and ast). We discuss the phenotypes of these three genes in more detail below.

**Asteroid, a FCM signaling-related protein**

Mutations in ast dominantly enhance Star mutations, and also enhance the Ellipse mutation EgfK1 in the Drosophila eye. ast transcripts have been detected in the somatic mesoderm up to stage 12 (Kotarski et al., 1998). Although we have been unable to detect ast expression in embryos by in situ hybridization, embryos carrying mutations in ast have defects in the development of a subset of the somatic muscles, particularly LL1 and DO4 (Fig. 5B). DO4 is affected in approximately 88% of the hemisegments, whereas LL1 is affected in 10% of the hemisegments. Founder cell specification, as judged by the FC marker Kr, which is expressed in the founder cell for LL1, appears normal in ast mutant embryos (data not shown).

**Tartan, a FC-enriched cell adhesion protein**

Tm is an Egf signaling transcriptional target that organizes the specific affinities of cells in the dorsal compartment of the wing disc (Gabay et al., 1996; Milan et al., 2001). We have confirmed that, in the embryonic mesoderm, Trn is expressed

---

**Fig. 3.** Northern analysis confirmed the differential regulation of genes identified by microarray. Blots containing 1 µg of embryonic polyA+ selected RNA from the cross twist-Gal4; Toll10b X UAS-N motif (N); twist-Gal4; Toll10b X UAS-rasV12 (R) and twist-Gal4; Toll10b X yw (Toll10b) at 29°C were hybridized to the indicated genes, using the probes described in Method and methods. Equal loading was assessed using a-tubulin. The inclusion of the Toll10b lane allows an assessment of why a gene is enriched in a particular condition. For genes enriched in the microarray under activated Notch signaling conditions, the major contribution to the differential expression of the genes tested came from Ras signaling acting as an inhibitory signal. By contrast, the behavior of genes enriched under activated Ras conditions revealed greater complexity. Whereas phyl and trn displayed strong repression by Notch signaling and little activation by Ras signaling, CG17492 showed two transcripts, one that did not change, and a smaller transcript that showed a strong regulation by Ras and Notch signaling. Finally, CG6024 and nidogen exhibited repression by both signaling pathways; however, the repression by Notch was stronger than that by Ras, which led to the observed differential expression. Approximate sizes are: parcas, 2.8 kb; CG4136, 5 kb; gol, 3.5 kb; de, 2.6 and 2.8 kb; CG8503, 1.9 kb; CG6024, 5.5 kb; phyl, 3.7 and 4.2 kb; CG17492, 3.8 and 4.2 kb; trn, 3.7 kb; nidogen, 5.3 kb; and a-tubulin 2 kb.

**Genes with uncertain roles during muscle development**

Although all 83 genes that met our stringent statistical criteria are shown in Table 1, some of the genes are not yet clearly linked to a role in wild-type muscle development by expression or phenotype. We therefore listed these genes as having ‘specific role in muscle development uncertain’ (Table 1). However, we emphasize that these genes may have been correctly identified as responsive to the genetic conditions used in our screen, which may influence genes in addition to muscle genes.

During the screen validation process we have further

**nidogen** was expressed at higher levels under activated Ras conditions (Fig. 3), and fluorescent in situ hybridization showed that the transcript is specifically localized in a subset of FCs (Fig. 4P-R).
only in FCs (Fig. 4M-O). Homozygous trn mutant embryos had abnormal somatic muscle morphology, with 60% of the hemisegments showing some obvious muscle defects. Muscles such as LT1-4 and DT1 had aberrant shapes and attachments (Chang et al., 1993) (Fig. 5C). Although anomalous in shape, these muscles contained several nuclei suggesting that the fusion process was not impaired in trn mutant embryos. Muscle losses and gains were also detected for LT1-4, despite wild-type expression of the FC marker Kr (data not shown). Although we show a lateral view of a stage 16 embryo in Fig. 5, all three muscle groups – dorsal, lateral and ventral – are affected in trn mutant embryos.

Phyllopod, a FC-enriched signaling related protein phyl transcripts were enriched under activated Ras conditions, predicting FC-specific expression for phyl. Expression of phyl was confirmed in a subset of FCs in the somatic mesoderm (Fig. 6A-C), and in the visceral mesoderm (Fig. 6D-F). Phyl, a novel adaptor protein, was already known to be required for determination of photoreceptor and external sensory cell fates (Li et al., 1997; Pi et al., 2001). Together with Seven-in-absentia (Sina), Phyl promotes photoreceptor differentiation by targeting the transcriptional repressor protein Tramtrack for degradation (Dickson, 1998; Tang et al., 1997), by acting as part of an E3 ubiquitin protein ligase complex (Li et al., 2002).

We found that phyl is a crucial regulator of somatic muscle differentiation. Loss of phyl caused reproducible loss of a subset of the somatic muscles, including muscles LL1 and DO4 (Fig. 6I). LL1 was lost in approximately 52% of the hemisegments analyzed. To address whether phyl is required for specification of FCs, we examined Kr expression in phyl null embryos. Kr expression was often missing or at lower levels in approximately 26% of the LL1 FC, whereas expression of Kr and other FC markers in other FCs was normal (Fig. 6J). These data suggest that although Phyl is required for the specification of a subset of FCs, perhaps through the maintenance of FC determinants such as Kr, other FC identity genes, which work in combination with Kr, may also be targets.
Ectopic expression of *phyl* with the GAL4/UAS system resulted in alterations in muscle identities. For example, LL1 was occasionally affected by *phyl* overexpression, and DT1 and LT4 consistently had altered morphology. DT1 failed to attach normally, resulting in a ‘ball of muscle’, like that seen in *myospheroid* mutant embryos. LT4 failed to grow dorsally, which resulted in a smaller muscle (Fig. 6K). The specification of the Kr-positive LT4 FC appeared normal in these embryos (Fig. 6L), in keeping with detection of LT4, albeit in abnormal form. In conclusion, these data indicate a previously unknown role for Phyl during muscle specification and morphogenesis.

**Discussion**

We find that the *Toll*10b mutation gives rise to embryos composed primarily of somatic mesoderm. In these embryos, FCs and FCMs were readily detected, and they responded to the Ras and Notch signaling pathways in the same way as their wild-type counterparts. We took advantage of this fact to enrich *Toll*10b mutant embryos for FCs or FCMs, which allowed us to concentrate on the transcription in these two specific cell types within the context of the entire embryo (Table 1). Genes known to be expressed and regulated in FCs or FCMs emerged from the screen in the proper categories. Not all known FC/FCM genes were detected in our screen for several reasons: the high stringency set for interpretation of the array data; the presence of only about one-third of the genome on the arrays; the loss of stringency set for interpretation of the array data; the presence of only about one-third of the genome on the arrays; the loss of Dpp in the *Toll*10b background; and the specific window of myogenesis (5- to 9-hours) that we focused on (Supplemental Table 1). However, a plethora of potential new muscle regulators were uncovered, including known genes with no previously recognized function in the mesoderm (such as *phyl* and *ast*), and genes predicted from the *Drosophila* genome sequence but not previously analyzed.

Various tests were applied to ascertain the validity of our results. Available databases (see Materials and methods) were analyzed to find evidence that the known and predicted genes in Table 1 were expressed at the correct time and place (Table 1, Biology). In addition, northern analysis with eleven genes tested the reliability of our microarray detection and selection criteria (Fig. 3); the results from all genes tested agreed with the array data.

A *Toll*10b sample on the northern blots allowed us to ascertain why a gene is enriched in a particular condition. For example, in the case of FC enriched genes, the signal in the Ras and Notch lanes can be compared with *Toll*10b alone to determine whether the Ras/Notch ratio for a gene is due to activation by Ras or repression by Notch. Those genes in Table 1 that are ‘enriched under Notch conditions’, for example, could reflect a variety of transcription mechanisms that would result in a ratio of less than 0.6. By northern analysis, we find many of the ‘Notch-regulated’ genes, and hence the predicted FCM genes, are repressed by Ras signaling and slightly activated by Notch. As a case in point, we showed that *hibris* was induced by Notch (2-fold) and repressed by Ras (10-fold), both by northern analysis and by in situ hybridization in embryos (Artero et al., 2001).

We used a combination of in situ hybridization, immunostaining and confocal microscopy to verify that the differential expression changes that we observed in these overexpression embryos reflected true differential expression in the wild-type situation. We analyzed the expression of nine genes from different functional categories in Table 1 (Figs 4, 6, and data not shown). For seven of these, we detected expression in the predicted type of myoblast. For two, *ast* and *cadmus* (see Materials and methods), we were not able to detect any specific staining in embryos by in situ hybridization. For those genes that fell into the category of ‘specific role in muscle development uncertain’, in situ hybridization of several (28%) showed expression in tissues other than somatic mesoderm that are present in the *Toll*10b background. These genes changed their expression levels in response to Ras or Notch, and may be Ras and Notch targets in non-mesodermal tissues.

We applied the most stringent test, mutational analysis, to a set of genes for which mutations are available. In addition to the three described in this paper, we have carried out preliminary analyses of another four FCM-enriched genes.
**Fig. 6.** *phyl* is expressed in FCs and both loss-of-function and overexpression cause specific muscle defects. (A-F) Confocal images of rP298 embryos in which *phyl* expression has been detected by fluorescent in situ hybridization (A,D; green), and FCs have been detected using the β-Gal reporter (B,E; red). Both channels are shown merged in C and F. *phyl* expression in the somatic (arrowheads; C,F) and visceral (bent arrow; F) mesoderm closely followed the β-Gal expression (C,F), indicating that both signals originated from FCs. Based on this level of analysis, *phyl* is expressed only in a subset of FCs.

Phenotypic analysis of *phyl* mutant embryos and embryos overexpressing *phyl* revealed specific morphological defects in the muscle pattern. Lateral views of anti-Mhc-stained late stage 16 embryos (G,I,K) and ventrolateral views of anti-Kr-stained late stage 12 embryos (H,J,L) of the genotypes wild-type (G,H), *phyl*2/Df(2R)Trix (I,J) and *twi-Gal4; Dmef2-Gal4* driving UAS-*phyl* (K,L). The same symbols are used to designate the same muscles in G-L. A diagrammatic representation is included with muscle loss indicated in red and morphological abnormalities indicated in blue. Null mutations in *phyl* reiterated some defects found in *ast* mutant embryos, such as losses of the LL1 (bracket, I) and DO4 (black arrow, I) muscles. In addition to muscle losses, the final muscle morphology in these embryos was compromised.

Overexpression of *phyl* throughout the mesoderm lead to specific morphological problems (K). The LL1 and DO4 muscles were usually present in these embryos, but LT4 (arrowheads, K) frequently showed an abnormal shape. These muscles contained more than one nucleus, indicating that a fusion block was not responsible for the observed defect. Similar problems were found in the DT1 (bent arrow, K) and SBM (not shown) muscles. Loss of *phyl* function interfered with Kr expression in the LL1 muscle FC. The LL1 FC was absent in some hemisegments (black bent arrow, J) or showed reduced Kr expression in others (asterisk, J), whereas other FCs, such as LT2-4 and VA1, were present. These data suggest that the muscle losses detected stem from defects in initiation or maintenance of muscle FC determinants such as Kr. Conversely, *phyl* overexpression in the mesoderm (L) did not reproducibly affect Kr expression in LT4 FCs and, therefore, the morphological defects found in the final LT4 muscle must stem from a later interference of Phyl during the morphogenetic process.
Regulators of myoblast development

EffuM, Glutamine synthetase 1, cadmus and parcas. All four mutants have muscle defects, including muscle losses and aberrant muscle morphologies (see Materials and methods for details). Thus all the genes tested show some muscle defect, supporting the usefulness of our genetic and genomic approach.

Taken together, our data suggest that the majority of genes listed in Table 1 will play important roles in FCs or FCMs during muscle development. Some of these genes might not have been found in traditional forward genetic screens because of partial or complete genetic redundancy. Our data complement traditional forward genetic approaches for finding genes crucial for muscle morphogenesis.

Cell type-specific transcriptional regulators control FC and FCM differentiation

Each of the thirty FCs per abdominal hemisegment is hypothesized to produce its own unique combination of transcriptional regulators, though the evidence for this is limited. In turn the combination of regulators would control the morphology of the final muscle. Although several transcriptional regulators have been linked to FC identity, the molecular description is far from complete. Our screen contributed two more FC-specific genes. Previously known markers, such as slouch or eve, once induced in the muscle FC, are maintained throughout the remainder of development. Ubx, which emerged from our screen, is a similarly simple case, as its transcripts are steadily present in most FCs which emerged from our screen. It may underlie temporal transcriptional inactivation of ase in a subset of FCs. The subsequent transcriptional inactivation of ase may underlie temporal changes in cell properties.

Even less is known about transcriptional regulators controlling FCM differentiation. Only one gene, lame duck, has been shown to have a role in FCMs. Our screen has uncovered three more potential players: dei, E(spl)mβ and CG4136, confirming that FCMs follow their own, distinct, myogenic program. Discovering what aspects of FCM biology are controlled by these transcriptional regulators awaits analysis of the loss-of-function phenotypes.

FCs and FCMs each integrate Notch and Ras signaling pathways, but in different ways

Notch and Ras signaling pathways interact during muscle progenitor specification (Carmena et al., 2002). Our results suggest that phyl and polychaetoid (pyd) may be additional links between the two signaling pathways in FCs. phyl and pyd both interact genetically with Notch and Delta (Chen et al., 1996; Pi et al., 2001). The transcription of phyl, which promotes neural differentiation, is negatively regulated by Notch signaling during specification of SOPs and their progeny (Pi et al., 2001). Our study shows a similar regulation in muscle cells, where Notch signaling repressed pyd expression and Ras signaling increased phyl expression (Fig. 3). Likewise, in the nervous system, the segregation of SOPs requires pyd, a Ras target gene, to negatively regulate ac-sc complex expression. Similarly, Pyd may restrict the muscle progenitor fate to a single cell, perhaps by regulating lethal of scute transcription. Thus, Pyd would collaborate with Notch signaling to restrict muscle progenitor fate to one cell.

FCMs appear to integrate Ras and Notch signaling differently. Two genes whose transcripts were enriched under activated Notch conditions, parcas and ast, have been implicated in Ras signaling in other tissues, directly (ast) (Kotarski et al., 1998) or indirectly (parcas) (Yamadori et al., 1999; Schnorr et al., 2001). These data are suggestive of a role for Ras signaling in the FCMs, in addition to its role in FC specification. In addition, Notch signaling to FCMs may prime cells for subsequent Ras signaling during muscle morphogenesis, much as occurs in FCs where Ras signaling primes the cell for subsequent Notch signaling during asymmetric division of the muscle progenitor (Carmena et al., 2002).

Roles for ubiquination during muscle specification and morphogenesis

Embryos that lack or ectopically express phyl have morphological defects in specific muscles, for example, in LL1 and DO4 in response to diminished phyl function, and in DT1 and LT4 in response to increased phyl function. The morphological defects in the loss-of-function embryos appear to be due to a failure to specify particular FCs, a conclusion that is based upon missing or abnormal production of the FC marker Kr. In eye development and SOP specification, Phyl directs degradation of the transcriptional repressor Tramtrack (Dickson, 1998). In a subset of the primordial muscle cells, Phyl may work similarly, targeting Tramtrack for degradation (Harrison and Travers, 1990). The presence of Tramtrack would contribute to the specific identity program of the muscle. As Tramtrack is expressed in the mesoderm (Harrison and Travers, 1990), this possibility is likely. Alternatively, Phyl may be required for targeted degradation of some other protein in a subset of FCs. The molecular partner for Phyl during muscle differentiation is unknown, although preliminary data suggest that sina is also expressed in somatic mesoderm and thus may be its partner (K. Gonzalez and M.B., unpublished).

Our studies have identified a new role for Phyl in muscle progenitor specification and suggest the importance of targeted ubiquitation for proper muscle patterning.

A role for ubiquination in muscle differentiation is further reinforced by the identification of the RING finger-containing protein Gol, induced by activated Notch conditions, and CG17492, induced by activated Ras conditions. Several RING-containing proteins function as E3 ubiquitin ligases, with the ligase activity mapping to the RING motif itself (Joazeiro and Weissman, 2000). Ligase function has been experimentally confirmed for the Gol ortholog GREUL1 in Xenopus (Borchers et al., 2002). Thus, targeted protein degradation during muscle morphogenesis could serve a host of crucial functions, such as protein turnover, vesicle sorting, transcription factor activation and signal degradation.

Muscle morphogenesis is controlled by both FCs and FCMs

The simplest view of the ‘founder cell’ hypothesis is that each FC contains all the information for the development of a particular muscle. By contrast, FCMs have been seen as a naïve group of myoblasts, entrained to a particular muscle program upon fusion to the FC. Our work indicates that these two groups of myoblasts have distinct transcriptional profiles. These data raise the possibility of a greater role for FCMs in
determining the final morphology of the muscle and emphasize a need to characterize fully those FCM genes listed in Table 1. For example, our screen identified a protein kinase of the SR splice site selector factors (SRPK) (Stojdl and Bell, 1999) whose transcripts are enriched in FCMs, suggesting that regulation of the splicing machinery is important for muscle morphogenesis. The Mhc gene undergoes spatially and temporally regulated alternative splicing in body wall muscles conferring different physiological properties on these muscles (Zhang and Bernstein, 2001). This FCM-specific expression of SRPK may indicate that the production of a particular Mhc isoform is regulated by the FCMs that contribute to that muscle, rather than by the particular FC that seeds the muscle. In addition, a number of observations suggest that FCMs may be a diverse population of myoblasts, with different subsets having different potential to contribute to the final muscle pattern. For example, lfs expression suggests that only a subset of FCMs express the gene (Artero et al., 2001), and twist expression in lame duck mutant embryos persists in a subset of FCMs (Ruiz-Gomez et al., 2002). Our study provides additional genes for exploring whether FCMs are a heterogeneous population of myoblasts as well as determining the nature of FCM contribution to the final muscle.

The molecular events underlying complex morphological changes, such as migration, cell fusion, cell shape changes or changes in the physiology of a cell, require a rich and dynamic program of transcription changes. We have described approximately one-third of this transcriptional profile. The FCM- or FCM-specific transcription of seven genes, and the mutant phenotype of four selected genes, allowed the definition of new muscle mutations that specifically affect the morphological traits of a subset of muscles. We now have the exciting prospect of exploring the functions of the numerous genes identified in this screen, and finding the molecular interactions among them that build perfectly organized muscles.

We thank M. Bate, A. Martinez-Arias, K. Anderson, Rosa de Frutos and members of both laboratories for critical reading of the manuscript, and thank S. Guzman for technical assistance. This work was supported in part by a Muscular Dystrophy Association Research grant, the New York Academy of Medicine – Speaker’s Fund for Biomedical Research Toward the Science of Patient Care, and the National Institutes of Health (GM 56989) to M.B. E.E.F. was supported by a grant from DARPA.

## References

- **Drysdale, R., Rushton, E. and Bate, M.** (1993). Genes required for


Regulators of myoblast development 6271


