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

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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 *Toll^{10b}* 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.**

Supplemental data available online

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 (Htl), 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-Gomez 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 (Kirre/Duf) (Ruiz-Gómez et al., 2000), Roughest/Irregular Chiasm (Rst/IrreC) (Strunkelnberg et al., 2001) and the intracellular adapter protein Rolling pebbles/Antisocial (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 *Toll^{10b}* mutant embryos. Cells of *Toll^{10b}* 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 blot analysis and in situ hybridization, providing validation for our approach. We investigated the phenotype of a selected set of newly identified genes and found that although some (e.g. *phyllopod*, *asteroid*) are crucial for the specification of particular muscles, others (e.g. *tartan*) are involved in the elaboration of specific muscle morphologies.

Materials and methods

Drosophila genetics

y w was used to reveal wild-type expression patterns. Fly stocks were: *phyl^{2}* (Dickson et al., 1995), *UAS-phyl* (Pi et al., 2001), *twist-Gal4* (Baylies and Bate, 1996), *Toll^{10b}* (Erdelyi and Szabad, 1989), *Dmef2-Gal4* (Carmena et al., 2002), *trn$^{25.4}$* (a gift from S. Cohen), *serpent-LacZ* and A490,2M3 (a gift from H. Skaer), *UAS-N^{intra}* (provided by T. Lieber) and *UAS-ras^{V12}* (provided by A. Michelson). *EfTuM^{L4569}* (CG6050), *Gs1^{1}* (CG2718), 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^{en11}lacZ]* 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, *EfTuM^{L4569}*, in the mitochondrial Elongation factor Tu gene (CG6050) was used. Embryos homozygous for *EfTuM^{L4569}* 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. *Gs1^{1}* is a lethal EMS-induced 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).

Histology techniques

Immunocytochemistry in embryos was performed as described by Rushton et al. (Rushton 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

a hot fixation 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-Trachealess (1:2000; HF; TSA), anti-Wg (1:200) and anti-Htl (1:2000; provided by A. Michelson). Antibodies against FasIII, 22C10, Trachealess 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-absorbed 1:900; TSA) (Chang et al., 1993) was achieved using streptavidin FITC. Anti-Kr (1:500; provided by J. Reinitz) was detected with the TSA Fluorescence System (PerkinElmer Life Sciences). Anti-Ase was used pre-absorbed 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'Neil and Bier, 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 ~12×30 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 amplification reagent. After ~4×15 minute washes, the embryos were incubated with a rabbit anti-β-Gal antibody at 4°C overnight to detect the *rp298* driven *lacZ* expression. This reaction was visualized using a biotinylated anti-rabbit secondary antibody and streptavidin-rhodamine (1:250; Jackson Laboratories).

Microarray methods

The microarrays used for the analysis contained over 8500 ESTs corresponding to 4949 unique genes plus a variety of controls. To generate microarray probes, 2.5 μg polyA$^+$ RNA was incubated with 4 μg of an equal ratio mixture of random hexamers and oligo dT, in a final volume of 15 μl at 70°C for 10 minutes. The reaction was then placed on ice and 6 μl 5× first strand buffer (Gibco BRL), 3 μl 0.1 M DTT, 3 μl Cy3 or Cy5-dUTP dye, 0.6 μl dNTPs [25 mM dATP, 25 mM dCTP, 25 mM dGTP and 10 mM dTTP] and 2 μl SuperScript II reverse transcriptase (Gibco BRL) was added. The reaction was placed at 42°C for 2 hours. RNA base hydrolysis was used to stop the reaction by adding 1.5 μl 1 M NaOH, 20 mM EDTA and placing at 65°C for 10 minutes. The pairs of Cy3 and Cy5 reactions were pooled together with 60 μl water and 15 μl NaAc (pH 5.2). Unincorporated dyes were removed by adding 500 μl Qiagen PB buffer and purifying using the Qiaquick purification system according to manufacturer's instructions. The reactions were eluted off the Qiaquick mini columns using two 30 μl EB buffer washes. The samples were concentrated using a Microcon-30 spin column. For array hybridization, the sample volume was adjusted to 10.4 μl and 3 μl 20×SSC, 1.2 μl 10 mg/ml polyA$^+$ RNA and 0.4 μl 10% SDS were added. The sample was heated to 100°C for 2 minutes, briefly spun and immediately added to the array. The arrays were hybridized in a chamber with 3×SSC at 65°C for 12-14 hours. Following hybridization, the array was successively 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.

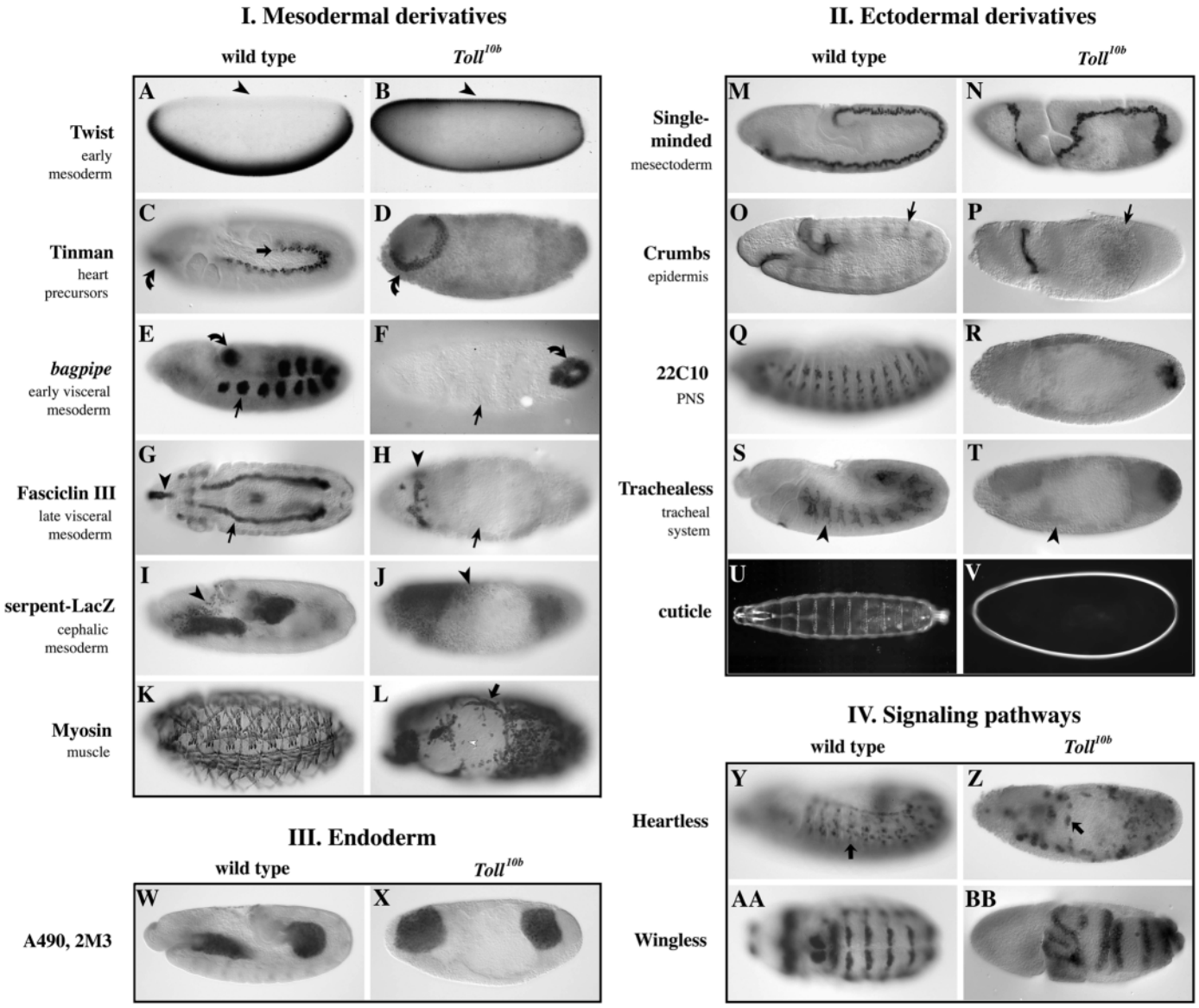
Results

Characteristics of *Toll^{10b}* 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 *Toll^{10b}*. 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 *Toll^{10b}* mutant embryos. Accordingly, several molecular probes were used to identify mesoderm, ectoderm and endoderm tissues in *Toll^{10b}* 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 *Toll^{10b}* 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 *Toll^{10b}* embryos, *serpent* expression indicated that blood cell progenitors were specified (Fig. 1I-J). Fused, myosin-stained syncytia were readily observed throughout *Toll^{10b}* embryos, indicating the presence of somatic muscle (Fig. 1K-L). *Toll^{10b}* embryos did not form epidermis, nervous system, trachea or cuticle (Fig. 1O-V), but did make mesectoderm cells and endoderm (Fig. 1M,N,W,X). Therefore, *Toll^{10b}* mutant embryos differentiate primarily as somatic 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 *Toll*^{10b} mutant embryos. *dpp* and *hh* transcripts were absent from *Toll*^{10b} 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). *Toll*^{10b} 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 *Toll*^{10b} 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 *Toll*^{10b} mutant embryos.

Toll^{10b} mutant myoblasts respond to Ras and Notch signaling like their wild-type counterparts

For *Toll*^{10b} 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 *Toll*^{10b} 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 *Toll*^{10b} 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 *Toll*^{10b} mutant, the number of FCs decreased (Fig. 2C,F) or increased (Fig. 2D,G), respectively, compared with *Toll*^{10b} 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. *Toll^{10b}* 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 *Toll^{10b}* 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 *Toll^{10b}* 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 *Toll^{10b}* mutant embryos (B). (C,D) Tinman was expressed in heart precursors (straight arrow, C), dorsal somatic muscle and foregut (bent arrow, C). Only some putative foregut expression remained in *Toll^{10b}* 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 *Toll^{10b}* 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). *Toll^{10b}* 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 *Toll^{10b}* mutant embryos (arrowhead, J). (K,L) Myosin heavy chain staining of wild-type (K) and *Toll^{10b}* mutant (L) embryos. The final muscle pattern was disrupted in *Toll^{10b}* mutant embryos but there was an abundance of Myosin-positive myoblasts and muscle fibers (arrow, L).
(M-V) Ectodermally derived tissues were missing from *Toll^{10b}* 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 early mesectoderm marker, was maintained in the *Toll^{10b}* mutant background but showed an aberrant pattern. However, further mesectoderm differentiation did not occur, as antibodies to neuronal markers such as HRP failed to detect differentiated neurons (not shown). (O,P) Crumbs, a marker for apical-basal polarization of epidermal cells, was expressed in ectodermal stripes (arrow) and in foregut ectoderm in wild-type embryos (O). All the ectodermal stripe expression was absent in *Toll^{10b}* mutant embryos (arrow) but foregut ectoderm expression remained (arrow, P). (Q,R) The anti-22C10 antibody labeled all neurons in the peripheral nervous system in wild-type embryos (Q) but was almost completely absent in *Toll^{10b}* mutant embryos (R). (S,T) Anti-Trachealess, a marker for tracheal cell fate, revealed invaginating ectodermal cells (arrowhead in S) in wild-type embryos but showed only marginal posterior expression in *Toll^{10b}* mutant embryos (arrowhead, T). (U,V) A terminal ectodermal specialization, the larval cuticle, was readily detected in wild-type larvae (U) but was completely absent in *Toll^{10b}* mutant larvae. Image shows vitelline membrane and absence of cuticle (V). (W,X) An endoderm-specific enhancer-trap line crossed into the *Toll^{10b}* mutant background revealed that the endodermal cell fate was readily specified in these mutant embryos. Panels show 5- to 9-hour AEL embryos. (Y-BB) Several paracrine signaling pathways remain active in *Toll^{10b}* mutant embryos. All embryos were 5- to 9-hour AEL.
(Y-Z) Htl expression in wild-type stage 12 embryos was restricted to clusters of myoblasts (arrow, Y). *Toll^{10b}* mutant embryos similarly aged also showed expression in clusters (arrow, Z). (AA,BB) Wg expression in *Toll^{10b}* mutant embryos was found in stripes reminiscent of wild-type expression.

Gene expression profile of *Toll^{10b}* embryos with Ras or Notch activated in the somatic mesoderm

RNA from 5- to 9-hour *Toll^{10b}* embryos expressing activated Ras (FC-enriched) or activated Notch (FCM-enriched) were collected and hybridized to cDNA arrays containing 4988 Drosophila genes [for details, see Materials and methods, and Furlong et al. (Furlong et al., 2001)]. The differences between expression levels for each gene under the two conditions were measured as the value of activated Ras divided by the value for activated Notch. We defined the Fold difference (F; Table 1) as the mean of the ratios from three independent hybridization experiments. Thus a fold difference of 1 indicates that the gene in question did not appear to be regulated differently by either Ras or Notch (hence was 'equally expressed' in both FCs and FCMs). Values greater than 1 result from genes with transcripts 'enriched in FCs', whereas values less than 1 indicate genes encoding transcripts 'enriched in FCMs'.

We applied stringent selection criteria: (1) a fold difference of 2 or more (Ras conditions) or 0.5 or less (Notch conditions) in three independent hybridization experiments; or (2) significance by SAM statistical analysis with a fold difference of 1.8 or above (Ras conditions) or 0.6 and below (Notch conditions). We identified 83 genes that were differentially expressed in FCs compared with FCMs. Transcript levels from 35 genes were higher under activated Ras (FC-enriched) conditions, and 48 transcripts were higher under activated Notch (FCM-enriched) conditions. The genes were divided into classes based on known biological roles, conserved domains or similarities to known proteins (Table 1). 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 *kirre*/*duf*,

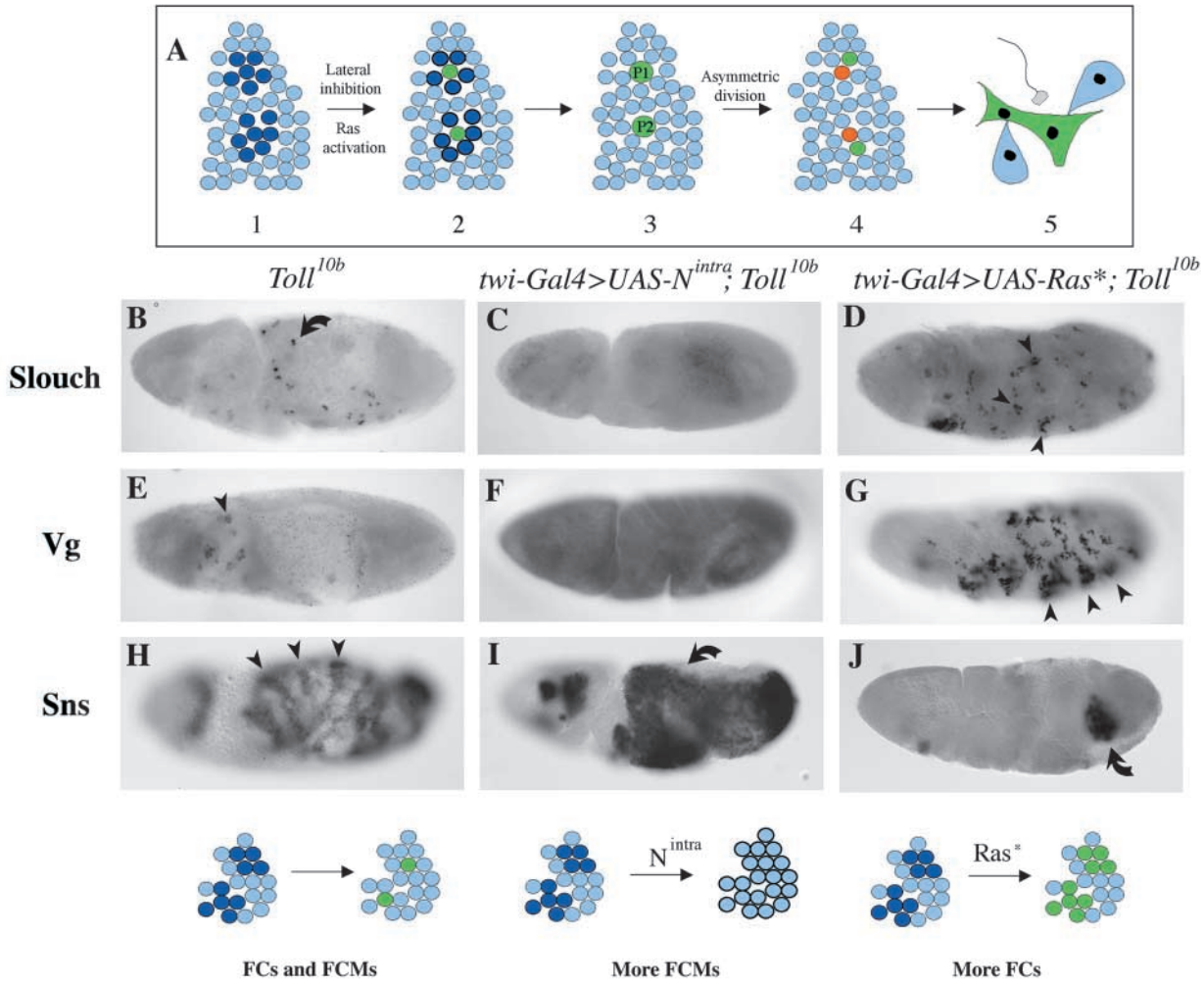


Fig. 2. *Toll*^{10b} mutant embryos specify the FC and FCM fate, and respond to alterations in the Ras and Notch signaling pathways. (A) Representation of the steps involved in FC specification (1, 2, 3, 4) and some aspects of terminal muscle differentiation (5). Dark blue indicates clusters of equipotent myoblasts in which the Ras signaling pathway is active, whereas an increase in the thickness of the outline of the cell represents activation of the Notch signaling pathway. Cells (in 1) are depicted in a transitional state when the coordinated activities of the Notch and Ras pathways (and Argos activity, not represented) single out the muscle progenitor cell (green in 2). In these clusters (1), all cells show some Notch activity, but a particular cell achieves a stronger Delta signaling ability, which leads to a strong activation of the Notch pathway in surrounding cells (dark blue, 2). Surrounding cells are therefore prevented from becoming muscle progenitors. Concomitantly with this process, a burst of Ras signaling activity in the progenitor cell (green in 2) leads to activation of progenitor cell markers, such as Eve, and feedback loops, such as Argos. Muscle progenitor cells (P1 and P2 in 3) undergo asymmetric division giving rise to two FCs represented as green and orange cells in 4. Finally, FCs fuse to surrounding FCMs (represented as blue cells with processes in 5) to form syncytial muscles, growth cones extend to innervate the muscles, and muscle precursors extend towards their normal epidermal attachment sites. (B-J) All panels show 5- to 9-hour AEL embryos with anterior to the left. Anti-Slouch (B-D) and anti-Vg (E-G) antibody staining of *Toll*^{10b} mutant embryos (B,E), and similar embryos expressing an activated form of Notch (*UAS-N*^{intra}; C,F) or Ras (*UAS-ras*^{V12}; D,G) under the control of the *twist-Gal4* driver at 29°C. Activation of the Notch pathway throughout the embryo led to a complete inhibition of the FC fate (note lack of staining in C,F), whereas activation of the Ras pathway enhanced FC fate as shown by an increased staining (D,G; arrowheads). Conversely, the FCM marker Sns was readily expressed in *Toll*^{10b} embryos (arrowheads, H), and showed more (bent arrow, I), or less (J, bent arrow indicates residual staining), expression upon Notch or Ras activation, respectively. Cartoons beneath the panels represent schematically the results of experimental manipulation. Color scheme is the same as in A.

rst/*irreC*, *slouch*, *Kr*, *vg* or *apterous*, were not present on the array whereas others, such as *sns*, *rols*/*ants* 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 *Toll*^{10b} 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 FCMs. 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 FCMs (Table 1). Three of these, *parcas*, *asteroid (ast)* and *goliath* (*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 FCM 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 (Pyd) 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. FCs may have their own endopeptidase system: *tequila*, a gene encoding a serine-type endopeptidase, was found to be enriched under Ras conditions and is therefore predicted to be in FCs (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 (Kumagai et al., 1999), are induced in FC-enriched embryos. Northern blot analysis confirmed that

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

A. Genes enriched under Ras conditions

Gene	Description	F*	S†	Biology‡
Transcription factors				
CG3258	*asense*	2.4	y	A,**, Fig. 4
CG10388	*Ultrabithorax*	2.3	y	FB, A, Fig. 4
Signaling-related molecules				
CG1225	*RhoGEF3*	2.0	y	A, SM
CG7223	heartless	5.3	y	FB. DD
CG10108	*phyllopod*	2.7	y	Fig. 6, A
CG17348	*derailed*	2.0		FB
CG31317	*stumps*	3.8	y	FB
CG31349	*polychaetoid*. Guanylate cyclase (EC:4.6.1.2)	1.8	y	SM
Extracellular matrix component				
CG12908	*nidogen*	14	y	N,DD,A,Fig. 4
Cell adhesion				
CG10275	Contains EGF-like repeat and laminin G domain	2.4	y	M, A
CG11280	*tartan*	3.3	y	N,FB,A,Figs 4, 5
Putative or known enzymatic activities				
CG7780	Deoxyribonuclease II (EC:3.1.22.1)	1.8	y	M,A
CG8147	Alkaline phosphatase	3.8	y	M, A,§§
CG9520	(EC:3.1.3.1)	1.9	y	A
CG17323	Galactosyltransferase? UDP-glucuronosyltransferase (EC:2.4.1.17)	4.0	y	M,A
Serine proteases				
CG4821	*tequila* (EC:3.4.21.-)	2.5	y	M,A
Chaperone				
CG14207	Hsp20/alpha crystallin family	1.9	y	M,¶, A
Molecular transporters				
CG4722	*big brain*. Gap junction component	2.1	y	FB,A,M
Novel genes				
CG2083	Contains NLS	3.5	y	A, SM
CG8588	Transmembrane protein	2.0	y	A
CG17492	Ankyrin repeats, zinc-binding domain and RING finger	2.2	y	N,M,A,SM
Role in muscle development uncertain				
gypsy	Transposable element	2.0	y	M
GH16741	repetitive sequence	2.0	y	M,§§
CG1394	No remarkable features	2.4		NE, NA,¶
CG1743	*Glutamine synthetase 2* (EC:6.3.1.2)	1.9	y	A, NE,¶
CG4128	nicotinic *Acetylcholine Receptor alpha 30D*	2.4	y	M, NE, NA
CG6024	LDL receptor domain class A and CUB domain	1.9		N, NA
CG6531	*wengen*. TNFR domain	2.2	y	M, A,¶
CG6980	Contains a tetratricopeptide repeat (TPR)	2.9	y	NA
CG7157	*Accessory gland peptide 36DE*	5.6	y	NA
CG10131	Contains a 3-hydroxyacyl-CoA dehydrogenase domain	2.0	y	M, NA
CG10433	Putative prenyl group binding	2.1		NE, NA
CG12071	site	2.8	y	NE, NA
CG14307	C2H2 zinc finger domains	3.9	y	M, NA
CG14989	*fruitless* Contains mitochondrial targeting peptide	2.4		NE, NA,§

B. Genes enriched under Notch conditions

Gene	Description	F*	S†	Biology‡
Transcription factors				
CG4136	Contains homeobox	0.52	y	N,A,¶
CG5441	*delilah*	0.53	y	N,DD,M,Fig. 3,A, SM M,A,SM
CG14548	*E(spl) region transcript m!*	0.46	y	
Signaling-related molecules				
CG1803	*regucalcin*. Calcium binding	0.48	y	M,A,¶
CG2679	*goliath*	0.33	y	FB,N,M,SM,Fig. 3
CG4426	*asteroid*	0.55	y	FB,M,A,Fig. 5
CG4889	*wingless*	0.45	y	FB
CG7761	*parcas*	0.33	y	FB, N,A,M
CG9085	SR Protein Kinase (SRPK)	0.06	y	M late,CK01209
CG11059	*calsyntenin-1*	0.39	y	M early,A,SM
Microtubule motor				
CG9279	Component of dynactin motor	0.46	y	A,SM
Myoblasts fusion and cell adhesion				
CG1363	*blown fuse*	0.56	y	FB,A,M
CG1915	*sallimus* (*kettin*/*D-titin*)	0.55	y	FB,M,A,CK00556
CG7449	*hibris*	0.28	y	FB,A,DD
CG8343	C-type mannose binding lectin	0.36	y	M,A
Cytochrome P450 superfamily				
CG1944	*Cyp4p2*	0.42	y	M,A
CG8733	*Cyp305a1*	0.52	y	M,A
Putative or known enzymatic activities				
CG2718	*Glutamine synthetase 1* (EC:6.3.1.2)	0.49	y	M,A
CG4123	*Mipp 1* (EC:3.1.3.-)	0.56	y	A
CG6339	*rad50*. *RAD50* ortholog	0.17	y	A
CG9674	Glutamate synthase NADPH (EC:1.4.1.13)	0.42	y	M,A
CG13348	*Phenylalanyl-tRNA synthetase* (EC:6.1.1.20)	0.46	y	FB,M,A
CG16758	Purine-nucleoside phosphorylase (EC:2.4.2.1)	0.25	y	M,A
CG32031	*Arginine kinase* (EC:2.7.3.3)	0.25	y	FB,M,SM
Serine proteases and inhibitors				
CG8342	*E(spl) region transcript m1*. Serine protease inhibitor	0.42	y	M,A
CG16749	Serine-type endopeptidase (EC:3.4.21.-)	0.49	y	M,A
CG33045	*Kaz1*. Kazal-type serine protease inhibitor domain	0.34	y	
Protein synthesis elongation factors				
CG6050	*EfTuM*	0.51	y	M,A
Molecular transporters				
CG3460	*Nmd3*. Ribosomal large subunit nucleus export	0.51	y	M,A,¶
CG7212	*cadmus*. Importin 13 homolog	0.51	y	A
Novel genes				
CG2668	*peb*. Contains Pro-rich region	0.42		
CG8503	MYND finger and SET domain	0.49	y	N,DD,M,CK0321 A
CG9336	No remarkable features	0.56	y	A
CG9889	*yellow-d*	0.46	y	M, A
CG12758	*serrano*	0.34	y	A
CG16791	No remarkable features	0.39	y	A
CG17210	*SCP-containing protein B*	0.49	y	M,NA,§§
CG31038	Immunoglobulin superfamily	0.41	y	M,SM
Role in muscle development uncertain				
CG1124	Contains a JHBP domain	0.50		A,††
CG3006	*Fmo-1*. Dimethylaniline monooxygenase. EC:1.14.13.8	0.37	y	M, NA
CG4276	*arouser*. *Eps8* ortholog	0.36	y	M,A,NE
CG7088	*bangles and beads*	0.47	y	A,§,¶
CG8329	Chymotrypsin (EC:3.4.21.1)	0.5	y	M, NE, NA
CG8910	RING finger domain?	0.53	y	M, NE, NA
CG9119	Mouse homologue exist	0.36	y	M, NE, NA
CG10842	*Cyp4p1* (EC: 1.14.14.1)	0.44	y	NA
CG10947	SAM^{i}-dependent methyltransferase domain	0.31	y	M, NE, NA
CG17800	*Dscam*	0.24	y	A,‡‡

*The fold difference (F) was defined as the mean of ratios of activated Ras/activated N from three independent hybridization experiments.

†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 hibridization in the somatic musculature in the BDGP expression pattern project (A or SM respectively; http://www.fruitfly.org/cgi-bin/ex/insitu.pl), a FC/FCM-specific expression pattern (Fig. 4). The relevance of some genes to muscle development is 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 mesectodermal expression as no mesodermal expression has been reported.

¶Maps outside regions of the X chromosome defined as containing genes whose products are involved in wild-type muscle development (Drysdale et al., 1993).

**A deficiency removing the *achaete-scute* complex, Df(1)sc19, yields a ventral muscle phenotype (Drysdale et al., 1993).

††Two ESTs from CG1124, CK00336 and RE70318 show expression in the ventral midline. Regulation could be due to midline expression.

‡‡There is no reported expression in the mesoderm for Dscam (Schmucker et al., 2000) (not shown).

§§Expressed in the somatic mesoderm (our results, data not shown).

Other abbreviations: DD, detected by Differential Display; M, predicted mesodermal expression by Furlong et al. (Furlong et al., 2001); NLS, Nuclear localization signal; SAM, S-adenosyl-L-methionine-(SAM^{i})-dependent methyltransferase; SET, (Su(var)3-9, Enhancer-of-zeste, Trithorax) domain; JHBP, Juvenile hormone binding protein domain in insects; EfTuM, Elongation factor Tu mitochondrial.

Origin of expression data: arouser (E.E.F., unpublished); CG1394, CG4128, CG8329, CG17210, CG9119, CG10433, CG10947, CG12071 and CG14989 (R.A., unpublished); CG8910 (E.E.F. and R.A., unpublished); CG31038, BDGP; CG2083, CG1225 and CG31349 (BDGP, our interpretation).

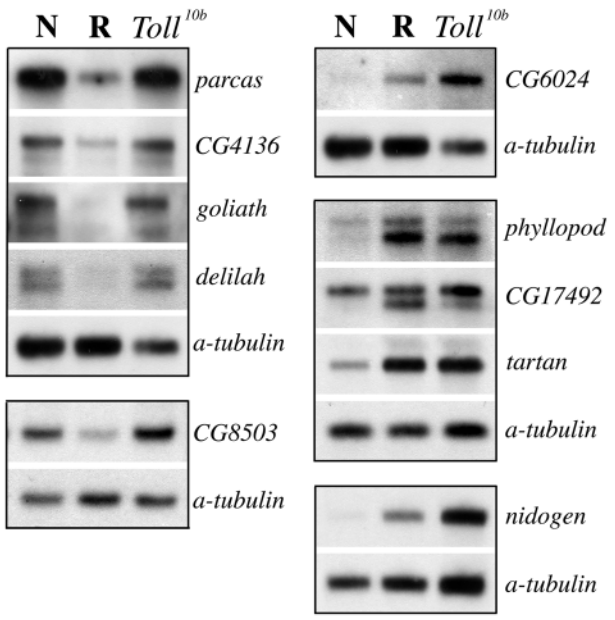


Fig. 3. Northern analysis confirmed the differential regulation of genes identified by microarray. Blots containing 1 μg of embryonic polyA$^+$-selected RNA from the cross *twist-Gal4*; *Toll^{10b}* X UAS-*N^{intra}* (N); *twist-Gal4*; *Toll^{10b}* X UAS-*ras^{V12}* (R) and *twist-Gal4*; *Toll^{10b}* X *yw* (*Toll^{10b}*) at 29°C were hybridized to the indicated genes, using the probes described in Material and methods. Equal loading was assessed using *α-tubulin*. The inclusion of the *Toll^{10b}* 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; *dei*, 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 *α-tubulin* 2 kb.

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).

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 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 *Toll^{10b}* background. A second group consists of genes that are normally expressed in tissues other than somatic mesoderm that are still present in *Toll^{10b}* 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 *Toll^{10b}* 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 promoters 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 hybridizaton. 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 *Egfr^{E1}* 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

Trn 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

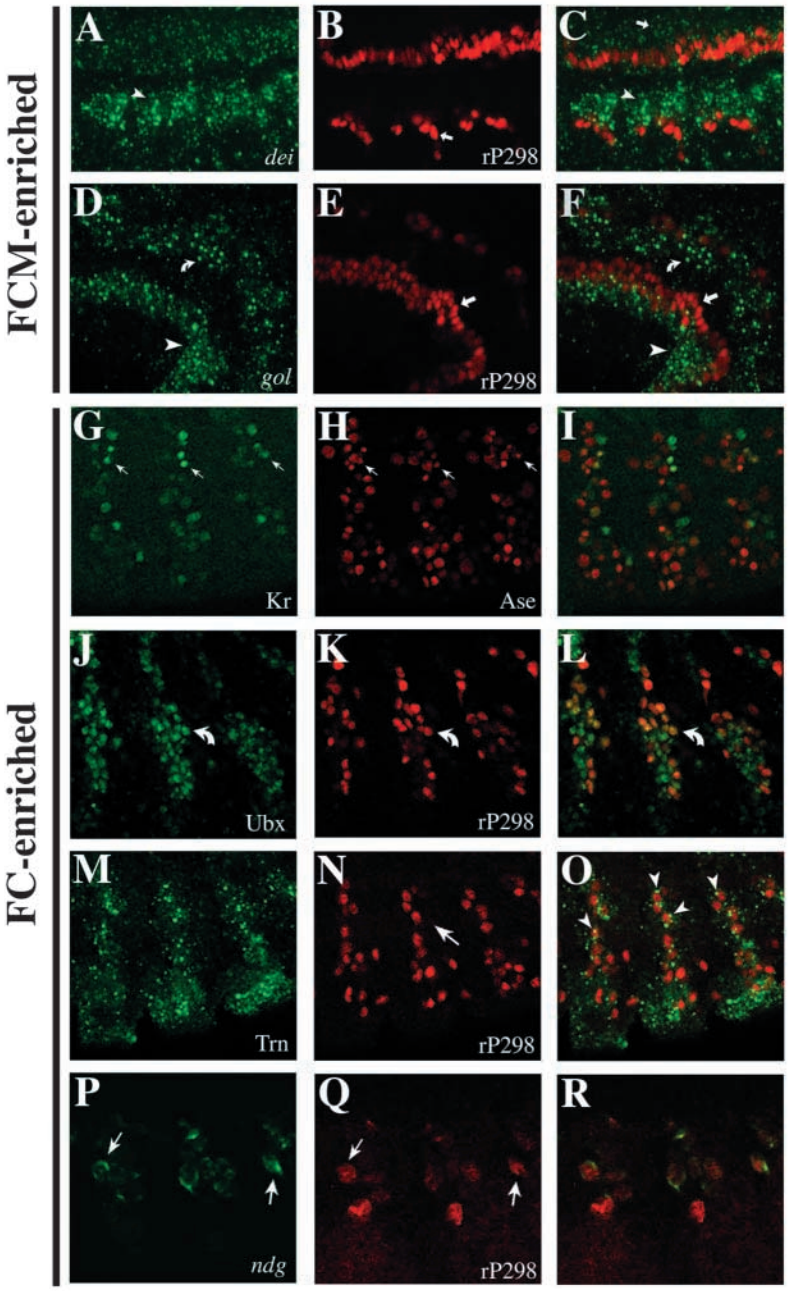


Fig. 4. FCM and FC specific expression of genes from array validates genetic and microarray strategy. (A-F) Simultaneous fluorescent detection of *dei* and *gol*, two genes predicted to be enriched in FCMs. *dei* (A,C) and *gol* (D,F) transcripts detected by in situ hybridization and anti-β-Gal staining (B,E) in stage 12 rP298 embryos. The enhancer trap insertion rP298 marks FCs in somatic (Ruiz-Gómez et al., 2000) and visceral mesoderm (Klapper et al., 2002; SanMartin et al., 2001). All panels are confocal images with C and F showing the red and green channels merged. *dei* transcripts in somatic (arrowhead, A,C) and visceral (arrow, C) mesoderm cells were detected in non β-Gal-expressing FCs (red nuclei; B,C), which were therefore putative FCMs. *gol* transcripts in somatic (bent arrow, D,F) and visceral (arrowhead, D,F) mesoderm did not overlap with β-Gal-expressing FCs (E,F). A similar situation was detected in visceral mesoderm: β-Gal-expressing nuclei (arrow, E) were not observed in *gol*-expressing cells (arrowhead, F). (G-I) Confocal images of wild-type embryos at mid-stage 12 immunostained for Kr (G) and Ase (H); the corresponding merged image is shown in I. Ase was detected transiently in Kr-positive FCs in close proximity to Kr, suggesting that Ase was expressed in FCs immediately after the progenitor cell had divided (arrows). (J-O) Confocal images of rP298 embryos at late stage 12, showing immunoreactivity to Ubx (J), Trn (M) and β-Gal (K,N). The corresponding merged images are shown in L,O. The yellow color (bent arrow, L) indicates expression of Ubx in FC. Trn was expressed in a punctate pattern in close proximity to nuclei expressing the rP298 reporter (arrowheads, O), suggesting that FCs express Trn. (P,R) *nidogen* transcripts detected by fluorescent in situ hybridization in a late stage 11 embryo (green, arrows in P) tightly surrounded β-Gal expressing FCs (arrows, Q), as shown in the merged image (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.

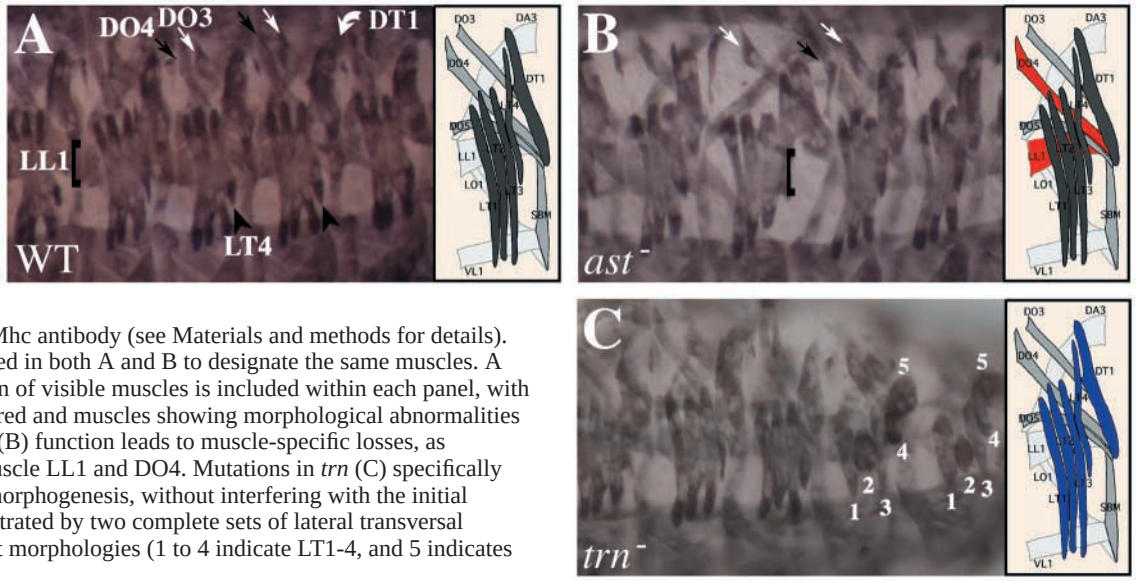


Fig. 5. Embryonic phenotype of a subset of genes from the screen reveal specific morphological defects. Lateral views (A-C) of late stage 16 embryos of the genotypes wild-type (A), *ast*^{1}*/Df(2R)ast4* (B) and *trn*$^{25.4}$*/In(3LR)C190* (C) stained with an anti-Mhc antibody (see Materials and methods for details). The same symbols are used in both A and B to designate the same muscles. A diagramatic representation of visible muscles is included within each panel, with missing muscles colored red and muscles showing morphological abnormalities colored blue. Loss of *ast* (B) function leads to muscle-specific losses, as exemplified by loss of muscle LL1 and DO4. Mutations in *trn* (C) specifically blocked normal muscle morphogenesis, without interfering with the initial specification. This is illustrated by two complete sets of lateral transversal muscles showing aberrant morphologies (1 to 4 indicate LT1-4, and 5 indicates DT1).

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 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:

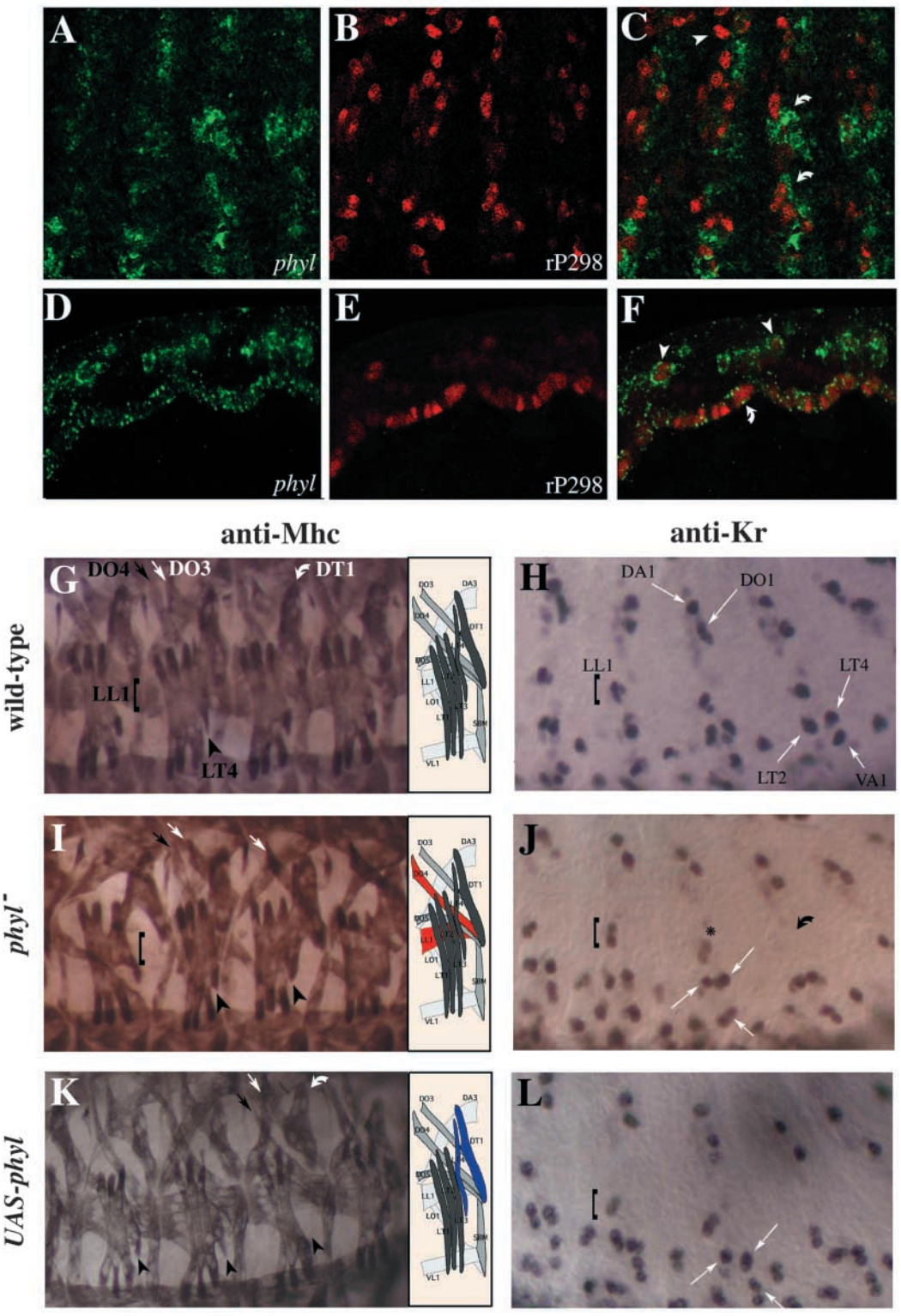


Fig. 6. *phyl* is expressed in FCs and both loss-of-function and overexpression cause specific muscle defects. (A-F) Confocal images of rP298 embryos in which *phyl* expression has been detected by fluorescent in situ hybridization (A,D; green), and FCs have been detected using the β-Gal reporter (B,E; 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 abnormalites 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.

EfTuM, *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 **(**Fig. 4 and data not shown**)**. By contrast, we have identified more complex patterns of gene expression in FCs, such as the transient transcription 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 segregation (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 *phyl* 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 ubiquitination for proper muscle patterning.

A role for ubiquitination 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, *hbs* 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 FC- 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.

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References

Arbeitman, M. N., Furlong, E. E., Imam, F., Johnson, E., Null, B. H., Baker, B. S., Krasnow, M. A., Scott, M. P., Davis, R. W. and White, K. P. (2002). Gene expression during the life cycle of *Drosophila melanogaster*. *Science* **297**, 2270-2275.

Armand, P., Knapp, A., Hirsch, A., Wieschaus, E. and Cole, M. (1994). A novel basic helix-loop-helix protein is expressed in muscle attachment sites of the Drosophila epidermis. *Mol. Cell Biol.* **14**, 4145-4154.

Artero, R., Castanon, I. and Baylies, M. (2001). The immunoglobulin-like protein Hibris functions as a dose-dependent regulator of myoblast fusion and is differentially controlled by Ras and Notch signaling. *Development* **128**, 4251-4264.

Bate, M. (1990). The embryonic development of larval muscles in *Drosophila*. *Development* **110**, 791-804.

Bate, M. (1993). The mesoderm and its derivatives. In *The development of* Drosophila melanogaster, Vol. II (ed. M. Bate and A. Martínez Arias), pp. 1013-1090. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Bate, M., Rushton, E. and Frasch, M. (1993). A dual requirement for neurogenic genes in *Drosophila* myogenesis. *Development Suppl.* 149-161.

Baylies, M. and Michelson, A. (2001). Invertebrate myogenesis: looking back to the future of muscle development. *Curr. Opin. Genet. Dev.* **11**, 431-439.

Baylies, M. K. and Bate, M. (1996). *twist*: a myogenic switch in *Drosophila*. *Science* **272**, 1481-1484.

Baylies, M. K., Martínez-Arias, A. and Bate, M. (1995). *wingless* is required for the formation of a subset of muscle founder cells during *Drosophila* embryogenesis. *Development* **121**, 3829-3837.

Baylies, M. K., Bate, M. and Ruiz-Gómez, M. (1998). Myogenesis: a view from *Drosophila*. *Cell* **93**, 921-927.

Beckett, K., Artero, R. and Baylies, M. (2002). Parcas, a novel regulator of RTK signaling is required in somatic myogenesis. *A. Dros. Res. Conf.* **43**, 658.

Borchers, A. G., Hufton, A., Eldridge, A., Jackson, P., Harland, R. and Baker, J. (2002). The E3 ubiquitin ligase GREUL1 anteriorizes ectoderm during Xenopus development. *Dev. Biol.* **251**, 395-408.

Bouchard, M. and Cote, S. (1993). The *Drosophila melanogaster* developmental gene g1 encodes a variant zinc-finger-motif protein. *Gene* **125**, 205-209.

Bour, B. A., Chakravarti, M., West, J. M. and Abmayr, S. M. (2000). *Drosophila* SNS, a member of the immunoglobulin superfamily that is essential for myoblast fusion. *Genes Dev.* **14**, 1498-1511.

Brand, A. and Perrimon, N. (1993). Targetted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Buff, E., Carmena, A., Gisselbrecht, S., Jimenez, F. and Michelson, A. M. (1998). Signalling by the *Drosophila* epidermal growth factor receptor is required for the specification and diversification of embryonic muscle progenitors. *Development* **125**, 2075-2086.

Campos-Ortega, J. A. and Hartenstein, V. (1985). *The embryonic development of* Drosophila melanogaster. Berlin: Springer Verlag.

Carmena, A., Bate, M. and Jiménez, F. (1995). *lethal of scute*, a proneural gene, participates in the specification of muscle progenitors during *Drosophila* embryogenesis. *Genes Dev.* **9**, 2373-2383.

Carmena, A., Gisselbrecht, S., Harrison, J., Jimenez, F. and Michelson, A. (1998a). Combinatorial signaling codes for the progressive determination of cell fates in the *Drosophila* embryonic mesoderm. *Genes Dev.* **15**, 3910-3922.

Carmena, A., Murugasu-Oei, B., Menon, D., Jimenez, F. and Chia, W. (1998b). *Inscuteable* and *numb* mediate asymetric muscle progenitor cell divisions during *Drosophila* myogenesis. *Genes Dev.* **12**, 304-315.

Carmena, A., Buff, E., Halfon, M., Gisselbrecht, S., Jimenez, F., Baylies, M. and Michelson, A. (2002). Reciprocal regulatory interactions between the Notch and Ras signaling pathways in the *Drosophila* embryonic mesoderm. *Dev. Biol.* **244**, 226-242.

Casal, J. and Leptin, M. (1996). Identification of novel genes in *Drosophila* reveals the complex regulation of early gene activity in the mesoderm. *Proc. Natl. Acad. Sci. USA* **93**, 10327-10332.

Chang, H. C., Solomon, N. M., Wassarman, D. A., Karim, F. D., Therrien, M., Rubin, G. M. and Wolff, T. (1995). phyllopod functions in the fate determination of a subset of photoreceptors in *Drosophila*. *Cell* **80**, 463-472.

Chang, Z., Price, B., Bockheim, S., Boedigheimer, M., Smith, R. and Laughon, A. (1993). Molecular and genetic characterization of the *Drosophila tartan* gene. *Dev. Biol.* **160**, 315-332.

Chen, C., Freedman, J., Bettler, D., Manning, S., Giep, S., Steiner, J. and Ellis, H. (1996). *polychaetoid* is required to restrict segregation of sensory organ precursors from proneural clusters in *Drosophila*. *Mech. Dev.* **57**, 215-227.

Chen, E. and Olson, E. (2001). Antisocial, an intracellular adaptor protein, is required for myoblast fusion in *Drosophila*. *Dev. Cell* **1**, 705-715.

Corbin, V., Michelson, A. M., Abmayr, S. M., Neel, V., Alcamo, E., Maniatis, T. and Young, M. W. (1991). A role for the *Drosophila* neurogenic genes in mesoderm differentiation. *Cell* **67**, 311-323.

Dickson, B. (1998). Photoreceptor development: breaking down the barriers. *Curr. Biol.* **8**, R90-R92.

Dickson, B. J., Dominguez, M., van der Straten, A. and Hafen, E. (1995). Control of Drosophila photoreceptor cell fates by Phyllopod, a novel nuclear protein acting downstream of the Raf kinase. *Cell* **80**, 453-462.

Dohrmann, C., Azpiazu, N. and Frasch, M. (1990). A new *Drosophila* homeo box gene is expressed in mesodermal precursor cells of distinct muscles during embryogenesis. *Genes Dev.* **4**, 2098-2111.

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

embryonic muscle development in *Drosophila melanogaster*: a survey of the X chromosome. *Roux's Arch. Dev. Biol.* **202**, 276-295.

Duan, H., Skeath, J. and Nguyen, H. (2001). *Drosophila* Lame duck, a novel member of the Gli superfamily, acts as a key regulator of myogenesis by controlling fusion-competent myoblast development. *Development* **128**, 4489-4500.

Dworak, H. and Sink, H. (2002). Myoblast fusion in *Drosophila*. *BioEssays* **24**, 591-601.

Dworak, H., Charles, M., Pellerano, L. and Sink, H. (2001). Characterization of *Drosophila hibris*, a gene related to human nephrin. *Development* **128**, 4265-4276.

Erdelyi, M. and Szabad, J. (1989). Isolation and characterization of dominant female sterile mutations of *Drosophila melanogaster*. *Genetics* **122**, 111-127.

Frasch, M. (1999). Controls in patterning and diversification of somatic muscles during *Drosophila* embryogenesis. *Curr. Opin. Genet. Dev.* **9**, 522-529.

Furlong, E., Andersen, E., Null, B., White, K. and Scott, M. (2001). Patterns of gene expression during *Drosophila* mesoderm development. *Science* **293**, 1629-1633.

Gabay, L., Scholz, H., Golembo, M., Klaes, A., Shilo, B. and Klämbt, C. (1996). EGF receptor signaling induces pointed P1 transcription and inactivates Yan protein in the *Drosophila* embryonic ventral ectoderm. *Development* **122**, 3355-3362.

Giebel, B. (1999). The Notch signaling pathway is required to specify muscle progenitor cells in *Drosophila*. *Mech. Dev.* **86**, 137-145.

Halfon, M. S., Carmena, A., Gisselbrecht, S., Sackerson, C. M., Jimenez, F., Baylies, M. K. and Michelson, A. M. (2000). Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. *Cell* **103**, 63-74.

Harrison, S. and Travers, A. (1990). The tramtrack gene encodes a *Drosophila* zinc finger protein that interacts with the *ftz* transcriptional regulatory region and shows a novel embryonic expression pattern. *EMBO J.* **9**, 207-216.

Hicks, M., O'Leary, V., Wilkin, M., Bee, S., Humphries, M. and Baron, M. (2001). DrhoGEF3 encodes a new *Drosophila* DH domain protein that exhibits a highly dynamic embryonic expression pattern. *Dev. Genes Evol.* **211**, 263-267.

Imam, F., Sutherland, D., Huang, W. and Krasnow, M. A. (1999). stumps, a *Drosophila* gene required for fibroblast growth factor (FGF)-directed migrations of tracheal and mesodermal cells. *Genetics* **152**, 307-318.

Joazeiro, C. A. and Weissman, A. M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell* **102**, 549-552.

Kiehart, D. P. and Feghali, R. (1986). Cytoplasmic myosin from *Drosophila melanogaster*. *J. Cell Biol.* **103**, 1517-1525.

Klapper, R., Stute, C., Schomaker, O., Strasser, T., Janning, W., Renkawitz-Pohl, R. and Holz, A. (2002). The formation of syncytia within the visceral musculature of the *Drosophila* midgut is dependent on *duf*, *sns* and *mbc*. *Mech. Dev.* **110**, 85-96.

Kotarski, M., Leonard, D., Bennett, S., Bishop, C., Wahn, S., Sedore, S. and Shrader, M. (1998). The *Drosophila* gene *asteroid* encodes a novel protein and displays dosage-sensitive interactions with *Star* and *Egfr*. *Genome* **41**, 295-302.

Kumagai, C., Fessler, L., Kramerov, A. and Baumgartner, S. (1999). NIDOGEN:Sequence, expression and isolation of an ECM protein during Drosophila development. *A. Dros. Res. Conf.* **40**, 64.

LeMosy, E., Hong, C. and Hashimoto, C. (1999). Signal transduction by a protease cascade. *Trends Cell Biol.* **9**, 102-106.

Leptin, M., Casal, J., Grunewald, B. and Reuter, R. (1992). Mechanisms of early *Drosophila* mesoderm formation. *Development Suppl.* 23-31.

Levin, H. (2002). Newly identified retrotransposons of the Ty3/gypsy class in Fungi, Plants, and Vertebrates. In *Mobile DNA II* (ed. Nancy L. Craig, Robert Craigie, Martin Gellert and Alan M. Lambowitz), pp. 681-701. ASM Press.

Li, S., Li, Y., Carthew, R. W. and Lai, Z. C. (1997). Photoreceptor cell differentiation requires regulated proteolysis of the transcriptional repressor Tramtrack. *Cell* **90**, 469-478.

Li, S., Xu, C. and Carthew, R. W. (2002). Phyllopod acts as an adaptor protein to link the Sina ubiquitin ligase to the substrate protein Tramtrack. *Mol. Cell. Biol.* **22**, 6854-6865.

Menon, S. and Chia, W. (2001). *Drosophila* Rolling pebbles: a multidomain protein required for myoblast fusion that recruits D-Titin in response to the myoblast atractant Dumbfounded. *Dev. Cell* **1**, 691-703.

Michelson, A. M. (1994). Muscle pattern diversification in *Drosophila* is determined by the autonomous function of homeotic genes in the embryonic mesoderm. *Development* **120**, 755-768.

Michelson, A. M., Gisselbrecht, S., Zhou, Y., Baek, K. and Buff, E. (1998a). Dual functions of the Heartless fibroblast growth factor receptor in development of the *Drosophila* embryonic mesoderm. *Dev. Genet.* **22**, 212-229.

Michelson, A. M., Gisselbrecht, S., Buff, E. and Skeath, J. B. (1998b). Heartbroken is a specific downstream mediator of FGF receptor signalling in *Drosophila*. *Development* **125**, 4379-4389.

Milan, M., Weihe, U., Perez, L. and Cohen, S. (2001). The LRR proteins Capricious and Tartan mediate cell interactions during DV boundary formation in the *Drosophila* wing. *Cell* **106**, 785-794.

O'Neil, J. W. and Bier, E. (1994). Double-label in situ hybridization using biotin, digoxigenin-tagged RNA probes. *BioTechniques* **17**, 870.

Patel, N., Snow, P. and Goodman, C. (1987). Characterization and cloning of Fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in Drosophila. *Cell* **48**, 975-988.

Pi, H., Wu, H. and Chien, C. (2001). A dual function of *phyllopod* in *Drosophila* external sensory organ development: cell fate specification of sensory organ precursor and its progeny. *Development* **128**, 2699-2710.

Rau, A., Buttgereit, D., Holz, A., Fetter, R., Doberstein, S., Paululat, A., Staudt, N., Skeath, J., Michelson, A. and Renkawitz-Pohl, R. (2001). *rolling pebbles* (*rols*) is required in *Drosophila* muscle precursors for recruitment of myoblasts for fusion. *Development* **128**, 5061-5073.

Ray, R., Arora, K., Nüsslein-Volhard, C. and Gelbart, W. (1991). The control of cell fate along the dorsal-ventral axis of the *Drosophila* embryo. *Development* **113**, 35-54.

Rothwell, W. F. and Sullivan, W. (2000). Fluorescent analysis of *Drosophila* embryos. In Drosophila *Protocols*, (ed. W. Sullivan M. Ashburner and R. S. Hawley), pp. 141-158. New York: Cold Spring Harbor Laboratory Press.

Ruiz-Gómez, M. and Bate, M. (1997). Segregation of myogenic lineages in *Drosophila* requires Numb. *Development* **124**, 4857-4866.

Ruiz-Gómez, M., Romani, S., Hartmann, C., Jäckle, H. and Bate, M. (1997). Specific muscle identities are regulated by *Krüppel* during *Drosophila* embryogenesis. *Development* **124**, 3407-3414.

Ruiz-Gómez, M., Coutts, N., Price, A., Taylor, M. V. and Bate, M. (2000). *Drosophila* dumbfounded: a myoblast attractant essential for fusion. *Cell* **102**, 189-198.

Ruiz-Gómez, M., Coutts, N., Suster, M., Landgraf, M. and Bate, M. (2002). *myoblasts incompetent* encodes a zinc finger transcription factor required to specify fusion-competent myoblasts in *Drosophila*. *Development* **129**, 133-141.

Rushton, E., Drysdale, R., Abmayr, S. M., Michelson, A. M. and Bate, M. (1995). Mutations in a novel gene, *myoblast city*, provide evidence in support of the founder cell hypothesis for *Drosophila* muscle development. *Development* **121**, 1979-1988.

SanMartin, B., Ruiz-Gómez, M., Landgraf, M. and Bate, M. (2001). A distinct set of founders and fusion-competent myoblasts make visceral muscles in the *Drosophila* embryo. *Development* **128**, 3331-3338.

Schmucker, D., Clemens, J., Shu, H., Worby, C., Xiao, J., Muda, M., Dixon, J. and Zipursky, S. (2000). *Drosophila* Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity. *Cell* **101**, 671-684.

Schneider, D. S., Hudson, K. L., Lin, T. Y. and Anderson, K. V. (1991). Dominant and recessive mutations define functional domains of Toll, a transmembrane protein required for dorsal-ventral polarity in the *Drosophila* embryo. *Genes Dev.* **5**, 797-807.

Schnorr, J. D., Holdcraft, R., Chevalier, B. and Berg, C. A. (2001). Ras1 interacts with multiple new signaling and cytoskeletal loci in *Drosophila* eggshell patterning and morphogenesis. *Genetics* **159**, 609-622.

Sinka, R., Jankovics, F., Somogyi, K., Szlanka, T., Lukacsovich, T. and Erdelyi, M. (2002). poirot, a new regulatory gene of *Drosophila* oskar acts at the level of the short Oskar protein isoform. *Development* **129**, 3469-3478.

Stojdl, D. and Bell, J. (1999). SR protein kinases: the splice of life. *Biochem. Cell Biol.* **77**, 293-298.

Strunkelnberg, M., Bonengel, B., Moda, L., Hertenstein, A., de Couet, H. G., Ramos, R. G. P. and Fischback, K. F. (2001). *rst* and its paralogue *kirre* act redundantly during embryonic muscle development in *Drosophila*. *Development* **128**, 4229-4239.

Takahashi, K., Matsuo, T., Katsube, T., Ueda, R. and Yamamoto, D. (1998). Direct binding between two PDZ domain proteins Canoe and ZO-1 and their roles in regulation of the Jun N-terminal kinase pathway in *Drosophila* morphogenesis. *Mech. Dev.* **78**, 97-111.

Tang, A. H., Neufeld, T. P., Kwan, E. and Rubin, G. M. (1997). PHYL acts to down-regulate TTK88, a transcriptional repressor of neuronal cell fates, by a SINA-dependent mechanism. *Cell* **90**, 459-467.

Taylor, M. (2002). Muscle differentiation: how two cells become one. *Curr. Biol.* **12**, R224-R228.

Tian, L., Nelson, D. and Stewart, D. (2000). Cdc42-interacting protein 4 mediates binding of the Wiskott-Aldrich syndrome protein to microtubules. *J. Biol. Chem.* **275**, 7854-7861.

Tusher, V. G., Tibshirani, R. and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. USA* **98**, 5116-5121.

Vincent, S., Wilson, R., Coelho, C., Affolter, M. and Leptin, M. (1998). The *Drosophila* protein Dof is specifically required for FGF signaling. *Mol. Cell* **2**, 515-525.

Yamadori, T., Baba, Y., Matsushita, M., Hashimoto, S., Kurosaki, M., Kurosaki, T., Kishimoto, T. and Tsukada, S. (1999). Bruton's tyrosine kinase activity is negatively regulated by Sab, the Btk-SH3 domain-binding protein. *Proc. Natl. Acad. Sci. USA* **96**, 6341-6346.

Zaffran, S., Kuchler, A., Lee, H. and Frasch, M. (2001). biniou (FoxF), a central component in a regulatory network controlling visceral mesoderm development and midgut morphogenesis in *Drosophila*. *Genes Dev.* **15**, 2900-2915.

Zhang, S. and Bernstein, S. (2001). Spatially and temporally regulated expression of myosin heavy chain alternative exons during Drosophila embryogenesis. *Mech. Dev.* **101**, 35-45.