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Signalling by the *Drosophila* epidermal growth factor receptor is required for the specification and diversification of embryonic muscle progenitors

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SUMMARY

Muscle development initiates in the *Drosophila* embryo with the segregation of single progenitor cells, from which a complete set of myofibres arises. Each progenitor is assigned a unique fate, characterized by the expression of particular identity genes. We now demonstrate that the *Drosophila* epidermal growth factor receptor provides an inductive signal for the specification of a large subset of muscle progenitors. In the absence of the receptor or its ligand, SPITZ, specific progenitors fail to segregate. The resulting unspecified mesodermal cells undergo programmed cell death. In contrast, receptor hyperactivation generates supernumerary progenitors, as well as the duplication of at least one SPITZ-dependent myofibre. The development of individual muscles is differentially sensitive to variations in the level of signalling by the epidermal growth factor receptor. Such graded myogenic effects can be influenced by alterations in the functions of *Star* and *rhomboid*. In addition, muscle patterning is dependent on the generation of a spatially restricted, activating SPITZ signal, a process that may rely on the localized mesodermal expression of RHOMBOID. Thus, the epidermal growth factor receptor contributes both to muscle progenitor specification and to the diversification of muscle identities.

Key words: myogenesis, *Drosophila*, epidermal growth factor receptor, progenitor cell

INTRODUCTION

The somatic muscles of the *Drosophila* embryo are arranged in a stereotyped pattern, with each myofibre characterized by its unique size, shape, position, orientation, epidermal attachments and innervation (Bate, 1990, 1993). How this pattern is generated poses a number of questions regarding the mechanisms of cell fate specification, differentiation and diversification that are relevant not only to the musculature but also to the development of many other tissues in a wide variety of organisms.

One of the earliest steps in *Drosophila* muscle development is the segregation of progenitor cells from a region of the mesoderm that expresses the *twist* (*twi*) gene at high levels (Carmena et al., 1995; Dunin Borkowski et al., 1995). Muscle progenitors are singled out from small clusters of mesodermal cells that express the proneural gene, *lethal of scute* (*l'sc*), a process that involves lateral inhibition mediated by the neurogenic genes (Bate et al., 1993; Carmena et al., 1995; Corbin et al., 1991). Progenitors then divide to yield two founder cells, each of which seeds the formation of an individual myofibre or another mesodermal derivative (Rushton et al., 1995). Particular cell fates are determined at the progenitor stage, as manifested by the expression of genes such as *Krüppel* (*Kr*), *nautilus* (*nau*), *vestigial* (*vg*), *even skipped* (*eve*), *apterous* (*ap*) and *S59* (Bourgouin et al., 1992; Dohrmann et al., 1990; Frasch et al., 1987; Gaul et al., 1987; Michelson et al., 1990; Paterson et al., 1991; Williams et al., 1991). The products of these genes confer specific myogenic identities (Bourgouin et al., 1992; Keller et al., 1997; Ruiz Gomez et al., 1997). The expression of a particular identity determinant persists in only one of the sibling cells derived from the division of a progenitor. Such asymmetric divisions, which are dependent on the functions of *numb* and *inscuteable*, assure the segregation of all of the myogenic fates characteristic of the complete muscle pattern (Carmena et al., 1998; Ruiz Gomez and Bate, 1997).

Once formed, founder cells fuse with adjacent nonfounder myoblasts to generate multinucleated muscle precursors. These cells develop into mature myofibres by undergoing additional fusions and by expressing terminal differentiation markers (Bate, 1990, 1993). The constellation of genes expressed by each founder determines the unique properties of the muscle fibre to which it gives rise (Bourgouin et al., 1992; Keller et al., 1997; Ruiz Gomez et al., 1997; Rushton et al., 1995). Furthermore, since the diversity of muscle cell types is first revealed in the unique patterns of gene expression that accompany progenitor segregation, the specification of these cells is a pivotal early step

in myogenesis. In addition to requiring high levels of TWI (Baylies and Bate, 1996; Dunin Borkowski et al., 1995), muscle progenitor specification is influenced by an extrinsic mechanism involving intercellular signalling molecules such as those encoded by the *wingless* (*wg*) and *decapentaplegic* (*dpp*) genes (Bate and Baylies, 1996; Baylies et al., 1995; Frasch, 1995; Lawrence et al., 1995; Park et al., 1996; Ranganayakulu et al., 1996; Staehling-Hampton et al., 1994). If these diffusible signals are not present, specific subsets of progenitors and founders do not segregate, and the corresponding muscle fibres fail to form.

Another relevant signalling pathway is the Ras-MAPK cascade, which is activated by a number of receptor tyrosine kinases (RTKs; Fantl et al., 1993; van der Geer et al., 1994). Among the many known RTKs in *Drosophila*, a fibroblast growth factor receptor encoded by *heartless* (*htl*) plays a central role in the directional migration of early mesodermal cells (Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998; Shishido et al., 1997). After migration is complete, HTL has a second mesodermal function in specifying certain muscle and cardiac cell fates (Michelson et al., 1998). Another RTK, the *Drosophila* epidermal growth factor receptor (DER), participates in a later stage of myogenesis that involves an inductive interaction between growing muscles and the adjacent ectoderm (Yarnitzky et al., 1997). In this case, DER acts within epidermal tendon cells to influence their differentiation, and is stimulated by a neuregulin-like ligand, VEIN, secreted by the apposed myotubes. Of note, earlier aspects of mesodermal patterning are unperturbed in *vein* mutants (Yarnitzky et al., 1997).

A second DER ligand, a transforming growth factor α (TGFα) homolog, is encoded by the *spitz* (*spi*) gene (Rutledge et al., 1992). *spi* is one member of a group of functionally related genes that also includes *rhomboid* (*rho*), *Star* (*S*) and *pointed* (*pnt*; Mayer and Nüsslein-Volhard, 1988). The primary SPI translation product is a membrane-bound precursor that undergoes processing to generate the biologically active, secreted form of the ligand (Schweitzer et al., 1995b). STAR and RHO may paticipate in the production of secreted SPI, although their precise mechanism of action has not been determined (Perrimon and Perkins, 1997; Schweitzer et al., 1995b; Schweitzer and Shilo, 1997). *pnt* codes for two related ETS domain transcription factors that function as nuclear transducers of DER signalling (Gabay et al., 1996; Klambt, 1993). Of interest, mutations in *spi*, *rho* and *pnt* are associated with similar muscle pattern abnormalities, which are distinct from those of *vein* (Bier et al., 1990; Klambt, 1993; Rutledge et al., 1992; Yarnitzky et al., 1997). The mesodermal phenotype of *S* has not been reported but is likely to be equivalent to that of *spi*, based on other similarities between these genes. Since the final arrangement of the somatic muscles is strongly influenced by the pattern of their epidermal attachments, it has been suggested that the abnormal muscles seen in embryos of the *spi* group could be secondary to the known ectodermal defects of these mutants (Bier et al., 1990; Klambt, 1993; Rutledge et al., 1992). However, alternative possibilities, such as a requirement for SPI-mediated DER signalling for muscle progenitor and founder cell specification, have not been investigated.

We now report that DER has a function in muscle development that is entirely independent of its role in epidermal tendon cell differentiation. Like HTL, DER is required for the initial segregation of specific muscle progenitor and founder cells. Furthermore, our data suggest that individual muscles may form at different threshold levels of DER activity, and that limiting amounts of STAR may influence such graded RTK signalling in the mesoderm. In addition, we show that the early myogenic DER signal must be activated in a spatially restricted manner and, as in other developmental contexts (Bier et al., 1990; Golembo et al., 1996a; Sturtevant et al., 1993), that this may be achieved through the localized expression of RHO.

MATERIALS AND METHODS

Drosophila strains and genetics

The following mutant *Drosophila* strains were used in this study: spi^{IIA}, S^{IIN}, rho^{7M} and flb^{1F26} (a temperature-sensitive allele of *DER*, referred to herein as flb^{ts}). All mutant stocks have been described (Clifford and Schüpbach, 1992; Mayer and Nüsslein-Volhard, 1988; Raz and Shilo, 1992). *Oregon R* was the reference wild-type strain. Targeted ectopic expression of transgenes was accomplished using the GAL4/UAS system (Brand and Perrimon, 1993). Mesodermal GAL4 drivers included *twi-GAL4* or *24B-GAL4* in combination with *twi-GAL4* (Brand and Perrimon, 1993; Greig and Akam, 1993). *UAS-rho*, *UAS-LacZ*, *UAS-activated DER* and *UAS-secreted spi* were provided by E. Bier, A. Brand, T. Schüpbach and B. Shilo, respectively (Brand and Perrimon, 1993; Queenan et al., 1997; Schweitzer et al., 1995b). *UAS-DER*, *UAS-DNDER* and *UAS-Star* were generated, as described below. Combinations of mutations and transgene insertions were obtained by standard genetic crosses. Balancer chromosomes containing *ftz-LacZ* transgenes were used for identification of homozygous mutant embryos.

Temperature shift experiments

Embryos were collected from flies of the flb^{ts} strain for 0.5-2 hours at the permissive temperature of 18°C, and aged at 18°C for various times prior to shifting to the restrictive temperature of 29°C. Embryos were maintained at 29°C until they had developed to stages 13-14, at which point they were fixed and stained, as described below. Reciprocal shifts were done in a similar manner except that embryo collections were of 0.5-1 hour duration at 29°C. The times expressed in Fig. 3 were corrected for equivalent age at 25°C using the following conversion factors: 1 hour at 25°C equals 1.8 hours at 18°C or 0.8 hours at 29°C (Wieschaus and Nüsslein-Volhard, 1986).

Immunohistochemistry and in situ hybridization

Embryos were fixed and subjected to immunohistochemical staining or in situ hybridization using standard protocols (Carmena et al., 1995; Michelson, 1994; O'Neill and Bier, 1994; Tautz and Pfeifle, 1989). Modified conditions for use of anti-RHO antibody were employed (Sturtevant et al., 1996). Confocal and light microscopy, as well as digital image processing, were performed as previously described (Carmena et al., 1995; Gisselbrecht et al., 1996).

Apoptosis assays

TUNEL was carried out according to published procedures (White et al., 1994) using commercially available reagents (Boehringer-Mannheim). The protocol for Acridine Orange staining of *Drosophila* embryos has been described (Abrams et al., 1993). Phagocytosis of apoptotic myoblasts was assessed by double-labeling with anti-DMEF2 and anti-peroxidasin antibodies (Abrams et al., 1993; Lilly et al., 1995; Nelson et al., 1994).

Quantitation of muscle defects

Stage 13-15 embryos of various genotypes were stained with either anti-EVE or anti-KR antibodies. The numbers of hemisegments containing EVE-positive DA1 or KR-positive LL1 and VA2 muscle precursors were counted under a dissecting microscope. 18 hemisegments per embryo (T2-3 and A1-7 on both sides of each

embryo) were assessed for the presence or absence of DA1, and 14 hemisegments per embryo (A1-7 on both sides of each embryo) were assessed for the presence or absence of LL1 or VA2. At least 80 embryos of each genotype were examined. The data in Figs 3, 5 and 6 are expressed as the percentage of all scored hemisegments that contain the indicated muscle.

Plasmid constructions

A dominant negative form of DER (referred to hereafter as DNDER) was generated by introducing a stop codon 20 amino acids C-terminal to the transmembrane domain of the wild-type receptor. This deletes the entire intracellular tyrosine kinase domain and yields a receptor that is capable of dimerization but not signal transduction. A similar construct has been generated by others (Freeman, 1996; Szüts et al., 1997). The cDNAs encoding both full-length and truncated forms of DER were introduced into the pUAST vector (Brand and Perrimon, 1993).

A full-length *Star* cDNA was obtained by screening a 9-12 hour embryonic cDNA library with probes directed against the published 5′- and 3′-untranslated regions of *Star* (Kolodkin et al., 1994). The resulting *Star* cDNA was subcloned into the *Eco*RI site of pUAST (Brand and Perrimon, 1993).

Germline transformation

Embryos derived from a *yw* strain of *Drosophila* were injected with pUAST constructs containing *Star*, full-length *DER* or *DNDER* cDNAs along with an appropriate helper plasmid (Spradling, 1986). w^+ germline transformants were isolated, and transgene insertions were mapped by standard genetic crosses.

RESULTS

DER signalling is essential for the development of a large number of embryonic muscles

In a screen for lethal mutations that disrupt the normal embryonic muscle pattern, we identified multiple alleles of two of the *Drosophila spi* group genes, *S* and *spi* (L. Mezitt, E. Shin, C. Tuchinda and A. M. M., unpublished results). The muscle phenotype of a strong *spi* mutant, as revealed by expression of myosin heavy chain (MHC), is illustrated in Fig. 1B. Approximately half of the normal myofibres are missing, while those that do develop have morphologies, positions and orientations that allow them to be assigned wild-type identities (compare the *spi* mutant in B with the wild-type embryo in A). For example, all of the lateral transverse muscles form normally in the absence of *spi* function, whereas gaps are present in the set of ventral longitudinal muscles. In addition, only one of the normal three ventral oblique muscles is present in a *spi* mutant. Of note, muscle defects are not more severe in ventral regions where the *spi* group genes are known to be required for ectodermal patterning (Golembo et al., 1996a; Mayer and Nüsslein-Volhard, 1988).

Since *spi* encodes a ligand for DER, the *Drosophila* epidermal growth factor receptor (Rutledge et al., 1992; Schweitzer et al., 1995b), we examined the DER loss-of-function phenotype. A temperature-sensitive allele was used to examine the mutant DER muscle phenotype in an attempt to bypass the early pleiotropic requirements for DER signalling in embryonic development (Clifford and Schüpbach, 1992; Raz and Shilo, 1992). Many of the *spi*-dependent muscles were also found to require DER function, but this analysis was limited by the finding that the temperature-sensitive period for DER involvement in myogenesis overlaps with that of other developmental roles of this receptor (E. B. and A. M. M., unpublished results). To circumvent this problem, we studied the myogenic function of DER in isolation by targeting the expression of a dominant negative form (DNDER) to the mesoderm using the GAL4/UAS expression system (Brand and Perrimon, 1993). DNDER was constructed by deleting the intracellular domain of the protein, a strategy that has proved effective in other systems for inhibiting full-length RTKs (Amaya et al., 1991).

The *spi* mutant muscle pattern was phenocopied by mesodermal expression of DNDER (Fig. 1C). The severity of this phenotype was dependent on the copy number of the *UAS-*

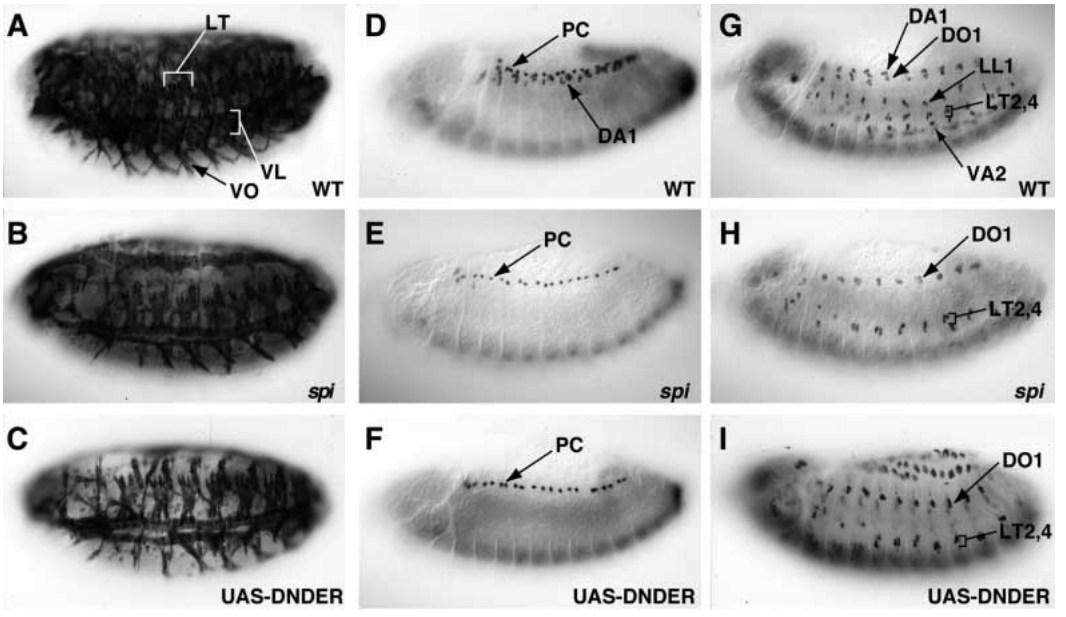

Fig. 1. Muscle phenotypes produced by the absence of SPI/DER signalling. OreR (A,D,G), spi^{IIA} (B,E,H), and embryos expressing two copies of *UAS-DNDER* under the control of *twi-GAL4*; *24B-GAL4* (C,F) or *twi-GAL4* (I) mesodermal GAL4 drivers are shown. (A-C) Stage-16 embryos stained with a myosin heavy chain antibody. Many myofibres are missing from all muscle groups in *spi* and *DNDER* embryos. Stage-13 embryos stained with anti-EVE (D-F) or anti-KR (G-I) show that SPI and DER are required for the formation of muscle precursors, including DA1, LL1 and VA2. Additional ventral muscle precursors expressing KR (Ruiz-Gomez et al., 1997) also are dependent on SPI and DER for their formation but are out of the plane of focus in G-I. PC, pericardial cell precursors. Muscle nomenclature follows the system of Bate (1993). DA, dorsal acute; DO, dorsal oblique; LL, lateral longitudinal; LT, lateral transverse; VA, ventral acute. In this and all subsequent figures, lateral views of embryos are shown with anterior to the left and dorsal up.

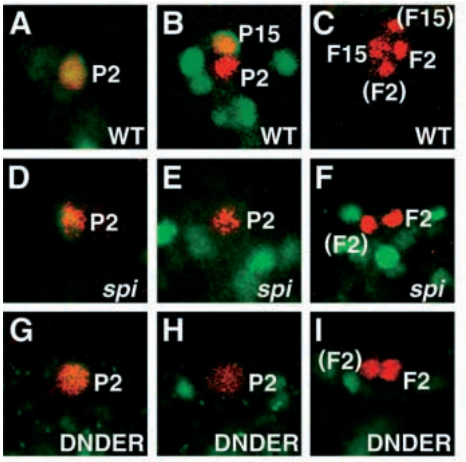

Fig. 2. SPI/DER signalling is essential for the specification of muscle progenitors and founders. Confocal micrographs of the dorsal regions of individual segments from embryos double-stained with anti-L'SC (green) and anti-EVE (red) antibodies are shown. (A-C) Wild type. (D-F) spi^{IIA} mutant. (G-I) Two copies of *UAS-DNDER* expressed under control of *twi-GAL4*. Consecutive panels of each genotype illustrate comparable embryos of increasing age between early and late stage 11. In wild type, P2, the progenitor of the EVE pericardial cells, forms first (A). The specification of P2 is not affected by loss of SPI/DER signalling (D,G). P15, the muscle DA1 progenitor, forms slightly later in wild type (B), but not in either *spi* or *DNDER* embryos (E,H). P2 and P15 each divide to yield two EVE-expressing founder cells, F2 and F15, only one of which retains EVE expression in each case (C and data not shown). Both F15 (but not F2) sibling cells are missing from *spi* and *DNDER* embryos (F,I).

DNDER transgene, and the specificity of the response to the truncated receptor was demonstrated by the ability of wild-type DER to reverse its effect (data not shown; see below). This strongly suggests that SPI signalling through DER is essential for normal myogenesis. Furthermore, the targeted ectopic expression of DNDER establishes that the receptor functions autonomously in mesodermal cells, as opposed to the known ectodermal abnormalities associated with loss of DER function having an indirect influence on mesoderm development. As a control for the latter possibility, cuticle preparations from embryos expressing DNDER in the mesoderm were analyzed. While very mild cuticle abnormalities were seen in some experimental embryos (largely confined to the ventral midline and attributable to the effects of mesectodermal expression of the *twi-Gal4* driver), these were of insufficient severity and frequency to account for the strong and highly penetrant muscle defects (data not shown). This, combined with the finding that the *spi*/*DER* muscle phenotype includes regions of the embryo where these genes are not involved in ectodermal patterning, argues strongly that DER acts directly in mesodermal cells to influence myogenesis.

spi and *DER* are required for the specification of a subset of somatic muscle progenitors

To determine at what stage of muscle development *spi* and *DER* are required, we examined the effect of loss-of-function of these genes on the expression of several early myogenic markers. The mature myofibres seen in stage-16 embryos differentiate from muscle precursors that are formed by myoblast fusion starting at stage 12 and continuing through stage 15 (Bate, 1990, 1993). Additional dorsal mesodermal cells segregate as heart precursors during this time. The segmentation genes, *eve* and *Kr*, are expressed in distinct but partially overlapping subsets of mesodermal precursors (Frasch et al., 1987; Gaul et al., 1987; Gisselbrecht et al., 1996; Ruiz Gomez et al., 1997; Fig. 1D,G). The precursor of muscle DA1, which expresses both EVE and KR, is missing in the absence of *spi* and *DER* functions (Fig. 1E,F,H,I). Additional KR-positive muscle precursors, including LL1, VA2 and several other internal ventral precursors, are also *spi*/*DER*-dependent (Fig. 1H,I; data not shown). However, the EVE-expressing pericardial cell precursors, as well as certain dorsal and lateral KR-expressing muscle precursors (DO1, LT2 and LT4), form normally in these genetic backgrounds (Fig. 1E,F,H,I). Moreover, the presence or absence of these precursors correlates completely with the mature myofibre pattern of *spi* and *DER* mutant embryos. These results demonstrate that *spi* and *DER* are required for the formation of some but not all muscle precursors.

At an even earlier stage of mesoderm development,

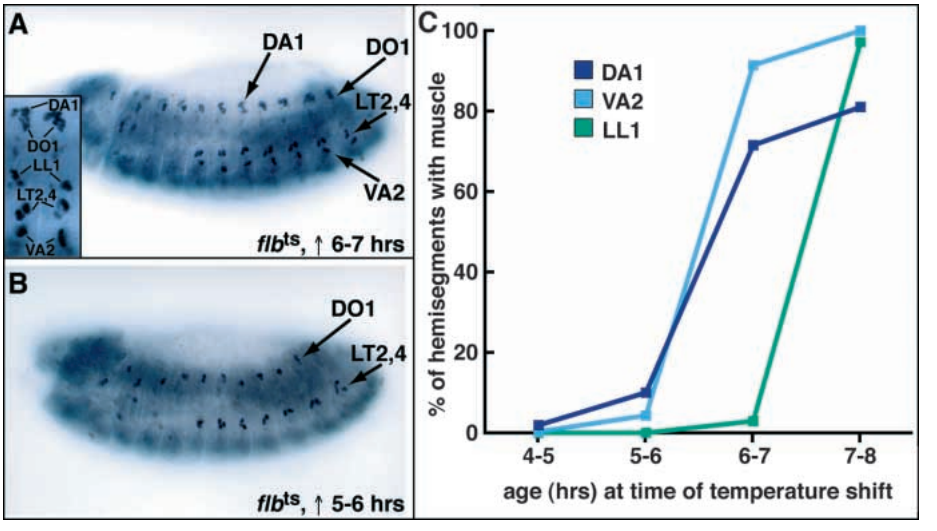

Fig. 3. SPI/DER signalling is required at different times for the development of specific muscles. (A,B) Stage 13 flb^{ts} embryos stained with anti-KR antibody show the different muscle precursors formed when embryos are shifted from 18°C to 29°C at 6-7 hours or 5-6 hours of development. The inset in A shows two segments of a wild-type embryo for comparison. (C) Quantitation of muscle precursor formation confirms that DER is required earlier for the development of muscles DA1 and VA2 than for LL1. Muscle precursor abbreviations are the same as in Fig. 1. Additional KR-positive ventral muscle precursors are out of the plane of focus in A and B.

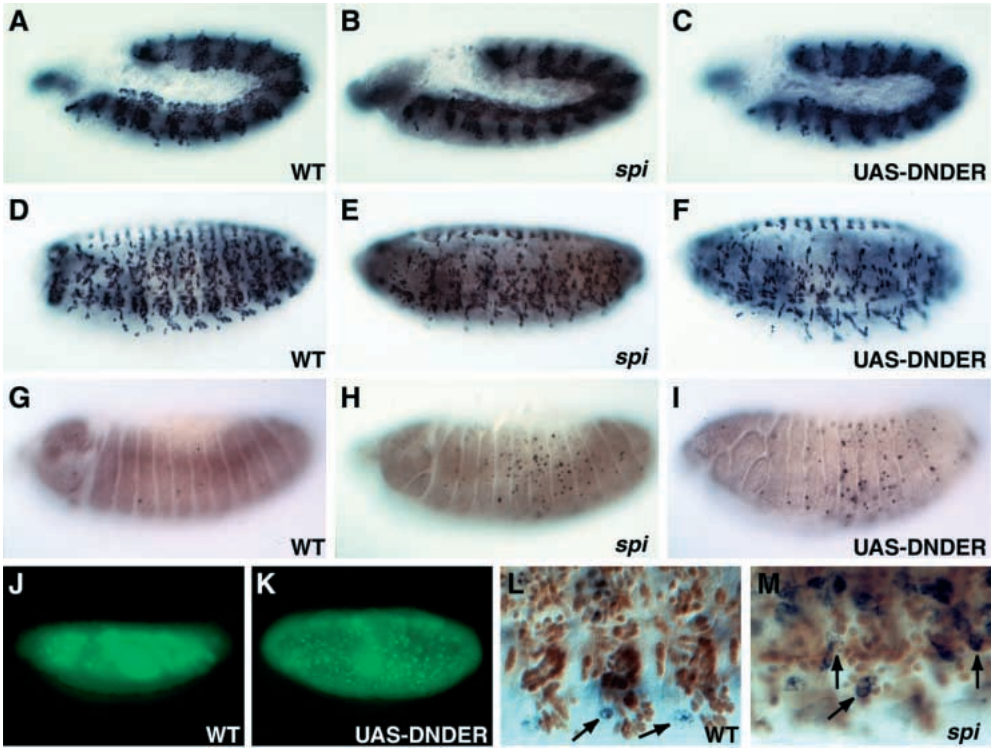

Fig. 4. Mesodermal cells not specified by SPI/DER signalling undergo programmed cell death. OreR (A,D,G,J,L), *spi*^{IIA} (B,E,H,M) and embryos expressing two copies of *UAS-DNDER* under the control of *twi-GAL4;24B-GAL4* (C,F,I,K) are shown. At stage 11, the expression of DMEF-2 in *spi* (B) and *DNDER* (C) embryos is essentially identical to wild type (A). By stage 16, *spi* (E) and *DNDER* (F) embryos have fewer DMEF2-expressing cells (D). TUNEL labeling reveals increased mesodermal apoptosis in stage 13 *spi* (H) and *DNDER* (I) embryos compared to wild type (G). Acridine Orange staining also indicates more apoptotic cells in *DNDER* (K) than in wild-type (J) embryos. Stage-15 embryos double-stained with anti-DMEF2 (brown) and anti-peroxidasin (black) show DMEF2-positive myoblasts engulfed by macrophages (arrows) in a *spi* embryo (M), a phenomenon not seen in wild type (L). Arrows in L indicate macrophages adjacent to wild-type ventral myofibres.

mononucleated progenitor cells segregate and divide to generate sibling founder cells, each seeding the formation of one muscle precursor (Carmena et al., 1995). Progenitors initially express the proneural gene, *lethal of scute* (*l'sc*), as well as muscle identity genes such as *S59*, *eve* and *Kr* (Carmena et al., 1995, 1998; Ruiz Gomez and Bate, 1997; Ruiz Gomez et al., 1997). Expression of identity genes persists while that of *l'sc* fades prior to progenitor division. This developmental sequence is illustrated for the two EVE progenitors, P2 and P15, in Fig. 2. P2 forms first and initially expresses both L'SC and EVE (Fig. 2A). By the time L'SC disappears from P2, P15 forms and co-expresses L'SC and EVE (Fig. 2B). Both progenitors then divide, each giving rise to two EVE-positive founder cells (F2 and F15 in Fig. 2C). EVE is retained in only one founder cell of each pair. The F2 founder in which EVE persists divides again, giving rise to a pair of pericardial cells in each hemisegment, while the EVE-expressing F15 contributes to muscle DA1; the subsequent fates of the F2 and F15 founders that lose EVE expression remain unknown (data not shown). With loss of either *spi* or *DER* function, P15 does not develop, whereas P2 and its founders segregate normally (Fig. 2D-I). This is consistent with the prior finding that DA1 muscle precursors, but not the EVE pericardial cells, are dependent on *spi* and *DER*. Additional L'SC-expressing muscle progenitors also are missing from *spi* mutant embryos (A. C. and F. J., unpublished results). Thus, SPI/DER signalling is involved in the earliest step of somatic myogenesis, the specification of muscle progenitors.

SPI/DER signalling specifies particular muscles at different developmental times

Individual muscle progenitors are specified at different times during stages 10 and 11 of embryogenesis (Carmena et al., 1995). We were interested in determining if the temporal requirement for the spi/DER signal correlates with the stages when particular muscles develop. Investigating this using a temperature-sensitive allele of *DER* (*flb*^{ts}) was limited because of the severe dysmorphology of embryos obtained from later temperature down-shift experiments (Clifford and Schüpbach, 1992; Raz and Shilo, 1992; data not shown). However, monitoring the formation of specific muscles in a series of temperature up-shift experiments proved to be informative (Fig. 3).

When *flb*^{ts} embryos at 6-7 hours of development were shifted to 29°C and then stained for expression of KR, only the LL1 muscle precursor failed to develop (Fig. 3A). In contrast, a similar up-shift of *flb*^{ts} embryos at 5-6 hours of development resulted in the absence of not only LL1, but also DA1 and VA2 precursors (Fig. 3B). A quantitative developmental assessment of these cells in additional up-shift experiments confirmed that DER activity is required earlier for the formation of DA1 and VA2 than for LL1 (Fig. 3C).

Unspecified mesodermal cells undergo programmed cell death in the absence of SPI/DER signalling

Although approximately half of the normal myofibres fail to develop in the absence of SPI/DER signalling, there are few myosin-expressing unfused myoblasts at later stages (Fig. 1B,C). Moreover, DMEF2 expresssion, while initially normal in *spi* and DNDER embryos (Fig. 4A-C), shows a progressive loss of mesodermal cells by stage 16 (Fig. 4D-F). Either unspecified myoblasts are present but lack myosin and DMEF2 expression, or these cells die when unable to differentiate.

Apoptosis was examined by three independent methods. First, TUNEL labeling (White et al., 1994) revealed a

significant increase in apoptotic death in the mesoderm of *spi* and *DNDER* embryos compared to wild type (Fig. 4G-I). Second, Acridine Orange, which specifically labels apoptotic cells in live *Drosophila* embryos (Abrams et al., 1993), stained more mesodermal cells in *DNDER* than in wild-type embryos (Fig. 4J,K). Finally, DMEF2-expressing myoblasts frequently were found to be engulfed by macrophages in *spi* but not in wild-type embryos (Fig. 4L,M). Taken together, these results indicate that mesodermal cells that remain uncommitted in the absence of SPI/DER signalling undergo programmed cell death. The magnitude of this cell loss suggests that both unspecified progenitors and the myoblasts with which they fuse must die when DER signalling does not occur.

Star modifies myogenic signalling by DER

As determined in our original screen, *Star* mutants also have a *spi*-like muscle phenotype (Fig. 5A-C). This is similar to findings for these two genes in other developmental contexts (Freeman, 1994; Heberlein et al., 1993; Kolodkin et al., 1994; Mayer and Nüsslein-Volhard, 1988; Tio et al., 1994). Since *Star* is known to interact with *DER* (Heberlein et al., 1993; Kolodkin et al., 1994), we next examined the ability of *Star* to modify the myogenic function of this RTK. Ectopic mesodermal expression of DNDER yielded a sensitized background in which to quantitate genetic interactions with *Star*. One copy of *UAS-DNDER* caused a partial reduction in the development of DA1 and VA2 (Fig. 5D). This effect was suppressed by co-expression of full-length DER or STAR, whereas another *UAS* transgene, *UAS-LACZ*, had no such influence. Ectopic expression of STAR or full-length DER in a wild-type genetic background had no effect on muscle development (data not shown). The ability of wild-type DER to reverse the effects of DNDER demonstrate the specificity of the dominant negative receptor, while the neutrality of *UAS-LACZ* indicates that there is no competition among the multiple *UAS* sequences for binding to GAL4. The *UAS-Star* results also indicate that STAR is required autonomously for DER function in the mesoderm. Furthermore, *Star* dominantly enhanced the effect of DNDER on muscles DA1 and VA2 (Fig. 5D), suggesting that STAR is normally limiting for muscle development.

Different muscles develop at distinct threshold levels of DER activity

In the experiments demonstrating a genetic interaction between *Star* and *DER*, the development of muscles DA1 and VA2 responded in a qualitatively similar manner to each of the genetic manipulations. However, each muscle exhibited a different quantitative sensitivity to loss of DER function. This was even true of the respective responses observed with two copies of *UAS-DNDER* (Fig. 5D). In all cases, the development of VA2 was resistant to a particular reduction of DER signalling relative to that of DA1.

To further examine the effect of altered DER activity on muscle development, we used a temperature-sensitive *DER* allele to vary the level of DER function more precisely. For example, conditions were identified where muscle LL1 could be included in this analysis. This was not possible with our *UAS-DNDER* insertions since these almost completely blocked LL1 formation (data not shown), implying that this muscle is particularly sensitive to loss of DER activity. This impression was confirmed by quantitating the formation of all three muscles in flb^{ts} mutant embryos under different conditions (Fig. 6). At 18°C, development of only DA1 was significantly diminished. This phenotype was enhanced by a 50% reduction of *Star* but not of *spi*. Of note, formation of muscle LL1 was precipitously affected by dominant enhancement of flb^{ts} by *Star* at 18°C, whereas VA2 remained essentially unaffected and DA1 showed an intermediate decline under this same condition. These responses are consistent with STAR being limiting for myogenesis (see above). An effect of heterozygous *spi* on development of all three muscles was revealed in combination with heterozygous *Star* and homozygosity for flb^{ts} at 18°C. Under these conditions, formation of muscle VA2 also is markedly reduced. Finally, when DER activity was decreased further by maintaining flb^{ts} embryos at 25°C, development of all three muscles was almost completely eliminated (Fig. 6).

In summary, the formation of each of three muscles exhibits a unique dose response to DER activity. Although DA1 is most sensitive to a small decrease of receptor function (flb^{ts} at 18°C), it demonstrates a gradual decline as DER is reduced further. In contrast, VA2 and LL1 development have comparatively sharp DER activity thresholds, with VA2 requiring relatively less DER signal than LL1 for its formation.

RHOMBOID is required for muscle DA1 formation and is expressed in the mesoderm in proximity to the DA1 progenitor

rho is another member of the *spi* group genes that is associated with DER function and is involved by an unknown mechanism in myofiber patterning (Bier et al., 1990). As we found for *spi*, *Star* and *DER*, *rho* is also required for development of the Eve-expressing muscle DA1 precursor but not for formation of the adjacent pericardial cells (Fig. 7A). Because RHO is a positive regulator of DER and its expression is frequently localized to sites where DER signalling is active (Bier et al., 1990; Ruohola-Baker et al., 1993; Sturtevant et al., 1993), we examined the expression of *rho* in the vicinity of DA1 during the course of its development.

rho transcripts are found in segmentally repeated dorsal mesodermal cells in stage-11 embryos (Fig. 7B). These cells are located at the peaks of the mesodermal crests that lie between the tracheal pits, precisely where the EVE-expressing P2 and P15 progenitors and their founders arise (Dunin Borkowski et al., 1995; Fig. 8A). Moreover, by double-labeling with RHO and EVE antibodies, we found that RHO is co-expressed with EVE in P2 (Fig. 7C). This is a particularly intriguing finding since the specification of P2 (the pericardial progenitor) precedes that of P15 (the muscle DA1 progenitor), these two cells segregate in very close proximity to each other (Fig. 2A,B), and only P15 is DER-dependent (Fig. 2H). Even under conditions where muscle DA1 forms in the absence of EVE pericardial cells, such as with partial inhibition of HTL activity (Michelson et al., 1998), RHO is expressed in a mesodermal cell that resembles a normal P2 but lacks EVE (A. C. and F. J., unpublished results). Given the known effects of RHO in modifying DER activity in other developmental contexts (Lage et al., 1997; Noll et al., 1994; Okabe and Okano, 1997; Ruohola-Baker et al., 1993; Sturtevant et al., 1993), the temporal and spatial expression of RHO in the

dorsal mesoderm is consistent with a functional role for RHO in the DER signalling responsible for P15 induction (see Discussion).

Hyperactivation of DER generates supernumerary muscle founders and the duplication of a DER-dependent muscle

Since increased DER signalling often is associated with an expansion of DER-dependent cell fates (Lage et al., 1997; Noll et al., 1994; Okabe and Okano, 1997; Queenan et al., 1997; Ruohola-Baker et al., 1993; Schweitzer et al., 1995b; Sturtevant et al., 1993), we next investigated the effect of DER hyperactivation on muscle development. DER was hyperactivated in the mesoderm in three different ways. First, wild-type RHO was targeted throughout the mesoderm, a manipulation that is associated with increased DER activity in other tissues (Noll et al., 1994; Ruohola-Baker et al., 1993; Sturtevant et al., 1993). Second, a ligand-independent, constitutively activated form of DER was expressed in all mesodermal cells (Queenan et al., 1997). Third, the biologically active, secreted form of the SPI ligand was induced throughout the mesoderm (Schweitzer et al., 1995b). In all three cases, there was a marked overproduction of EVE-positive (Fig. 8B-D) and KR-positive (data not shown) founder cells. This agrees with our prior demonstration that constitutively activated Ras is capable of generating extra EVE founder cells (Gisselbrecht et al., 1996; Michelson et al., 1998). Overexpression of the unprocessed form of the SPI precursor, in contrast, had no such effect (data not shown). The excess EVE cells included both pericardial and muscle founders even though only the latter are normally DER-dependent. This is due to nonspecific overstimulation of the general RTK signalling cassette, which also affects HTL-dependent pericardial progenitor specification (Michelson et al., 1998).

Having demonstrated that excess founder cells segregate in response to DER hyperactivation, we next were interested in the consequences of this for later stages of muscle development. However, it was not possible to meaningfully assess this question using the very strong conditions of DER activation described above since the subsequent muscle pattern was grossly deranged (data not shown). This probably reflects the known requirement for RTK signalling in later aspects of muscle development (Callahan et al., 1996; Yarnitzky et al., 1997). We were able to circumvent this problem by generating an intermediate level of DER hyperactivity through partial suppression of the very strong effect of ectopic RHO by simultaneous expression of DNDER. This produced a greater than wild-type number of EVE founders but fewer than that generated by ectopic RHO alone (Fig. 8E). Under these conditions, a second EVE-expressing dorsal muscle developed, which had the same morphology and position as a normal muscle DA1 (Fig. 8F). This could represent either a true duplication of DA1 or a transformation of the adjacent muscle DA2 into a fate characterized by EVE expression. To distinguish between these two possibilities, we stained similar embryos with antibodies directed against VESTIGIAL (VG) and KR proteins. Both VG and KR are expressed in DA1, while KR alone is expressed in DO1 and VG alone is expressed in DA2. This analysis revealed that embryos with ectopic RHO and DNDER contain an extra muscle DA1 and a normal complement of DO1 and DA2 (Fig. 8G). Thus, increased activity of DER is associated not only with the segregation of supernumerary EVE founder cells, but also with the duplication of a DER-dependent muscle derived from these founders.

DISCUSSION

Considerable progress has been made recently in our understanding of how a general myogenic fate is established in a number of developmental systems (Baylies and Bate, 1996; Cossu et al., 1996; Molkentin and Olson, 1996; Rawls and Olson, 1997; Tajbakhsh and Cossu, 1997). In *Drosophila*, where muscle progenitors and founders can be followed at single cell resolution, information also is available for how specific muscle identities are acquired. For example, genes such as *Kr*, *nau* and *ap* are expressed in distinct subsets of muscle founders, and their products determine the properties of the corresponding myofibres (Bourgouin et al., 1992; Keller et al., 1997; Ruiz Gomez et al., 1997). In addition, homeotic genes control intersegmental differences in muscle patterning (Greig and Akam, 1993; Michelson, 1994). What remains an important, but as yet poorly understood, problem is how the specification of muscle progenitors is initiated. We have shown that epidermal growth factor receptor signalling induces the formation of determined myogenic cells in *Drosophila*, and that differential activity of this pathway may contribute to progenitor diversification.

DER provides an inductive signal for the specification of muscle progenitors

In the absence of *spi* or *DER* function, a large subset of myofibres and their progenitors fail to form, and the resulting unspecified cells undergo apoptosis. By providing an inductive signal for the determination of muscle progenitors, this RTK acts at the earliest stage at which specific myogenic identities are established.

This function of DER differs in a number of respects from its other myogenic role in tendon cell differentiation (Yarnitzky et al., 1997). First, DER acts autonomously in mesodermal cells to promote the commitment of muscle progenitors; DER functions in the epidermis to influence the development of muscle attachments. Second, SPI is the ligand for DER in its muscle specification function; VEIN activates DER in tendon cells. Third, progenitor segregation occurs at an early stage of myogenesis; the formation of muscle attachments is a much later event. These dual functions of DER signalling in myogenesis underscore the highly pleiotropic effects of this RTK in *Drosophila* development (Freeman, 1997; Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997).

Whereas decreased DER function causes a loss of myogenic cells, receptor hyperactivation generates the reciprocal phenotype of progenitor overproduction. Since constitutively active DER also induces a duplication of muscle DA1, the extra progenitors must possess all of the information required to form a complete myofibre. Although a three- to fourfold increase in the number of EVE founders is induced by hyperactivated DER, only one extra DA1 muscle forms under these conditions. This may reflect the availability of a limited

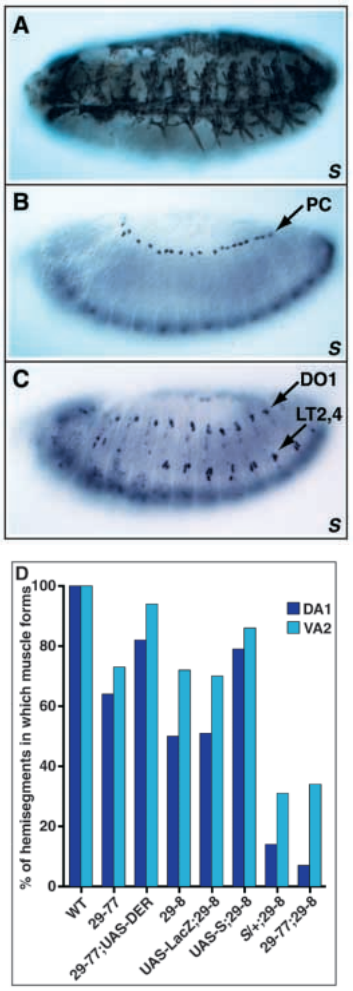

Fig. 5. STAR is required autonomously for DER function in the mesoderm. (A,B,C) *Star* mutant embryos stained for expression of myosin heavy chain (A), EVE (B) or KR (C). The same myofibres and muscle precursors are missing from *Star* and *spi* embryos (compare with Fig.1B,E,H). (D) Quantitative assessments of DA1 and VA2 muscle precursor formation in wild-type (WT) embryos and embryos expressing the indicated transgenes under the control of *twi-GAL4;24B-GAL4*. 29-77: a second chromosome *UAS-DNDER* insertion; 29-8: a third chromosome *UAS-DNDER* insertion. The inhibitory myogenic effect of DNDER is dominantly enhanced by *Star* and suppressed by simultaneous overexpression of full-length DER or STAR. Expression of LACZ from a *UAS* construct has no suppressing effect on *UAS-DNDER*, demonstrating that the suppression by *UAS-DER* and *UAS-Star* is specific and not due to titration of GAL4 protein by additional *UAS* target sites.

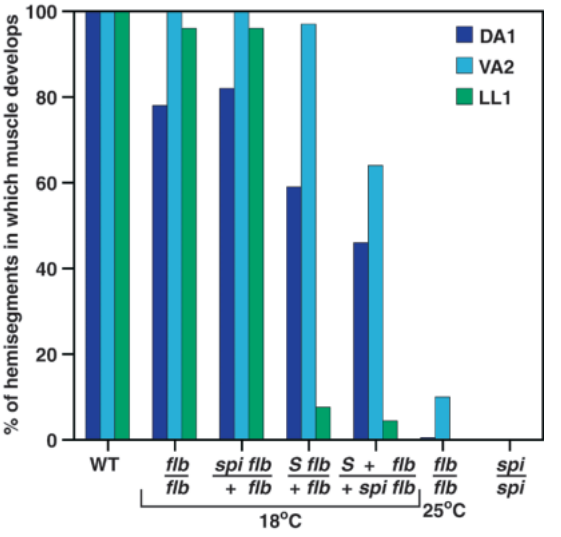

Fig. 6. Distinct thresholds of SPI/DER signalling are associated with the formation of different muscles. The bar graph shows the percentage of hemisegments in which DA1, VA2 and LL1 muscle precursors form in different genetic backgrounds. flb^{ts} has a mild phenotype at 18°C and a much more severe one at 25°C. *Star* dominantly enhances the weak flb^{ts} muscle phenotype. Heterozygous *spi* alone has little effect on flb^{ts}, but shows additional enhancement together with heterozygous *Star*. Each muscle responds differently to progressive reductions in DER activity, suggesting that distinct DER thresholds exist for the formation of individual muscles.

number of uncommitted neighboring myoblasts capable of fusing with the additional EVE founders.

Restriction and modulation of the myogenic DER signal

One of the earliest signs of mesodermal differentiation is the modulation of TWI expression levels, a process essential for the subsequent development of muscle founders (Dunin Borkowski et al., 1995; Baylies and Bate, 1996). Thus, DER functions within a prepatterned mesoderm to activate muscle identity gene expression and to stimulate the segregation of muscle progenitors. Additional factors that might influence this prepattern include WG and DPP, which are required at early stages for development of at least some DER-dependent muscles (Baylies et al., 1995; Frasch, 1995; Lawrence et al., 1995; Park et al., 1996; Ranganayakulu et al., 1996; Staehling-Hampton et al., 1994). Furthermore, TINMAN (TIN), a TWI target gene (Lee et al., 1997; Yin et al., 1997), acts in parallel to and is required for the effects of RTK signalling in the dorsal somatic mesoderm (Michelson et al., 1998). Although DER is a critical component of the muscle patterning machinery, its effects are superimposed on this pre-existing regulatory framework, and its function must be considered in this broader context.

Increased DER activity generates supernumerary muscle progenitors, but these cells are neither uniformly nor randomly distributed. For example, the additional EVE-positive cells induced by secreted SPI, ectopic RHO or activated DER are confined to clusters having positions similar to those of their wild-type counterparts. Localized factors such as WG, DPP and TIN likely contribute to this pattern by restricting and refining the output of mesodermal DER signalling, even when the receptor is highly activated throughout the mesoderm. An analogous prepattern has been identified for DER function in follicle cell development (Queenan et al., 1997).

Only a single progenitor of a given type is normally specified by DER, but additional cells having the same identity form when DER is upregulated. This implies that the normal mesodermal DER signal is spatially localized. Since both the receptor and its ligand precursor are ubiquitously expressed (Rutledge et al., 1992; Zak et al., 1990), the requisite regulation must be achieved by other means, possibly by local production

of the secreted form of SPI (Golembo et al., 1996a; Schweitzer et al., 1995b). RHO has been implicated in this process in other *Drosophila* tissues, and its expression correlates with sites of DER activity (Bier et al., 1990; Ruohola-Baker et al., 1993; Sturtevant et al., 1993). Moreover, RHO is known to have both cell autonomous and nonautonomous effects on development (Garcia-Bellido, 1977; Golembo et al., 1996a; Ruohola-Baker et al., 1993; Sturtevant et al., 1993). Thus, it is intriguing that RHO is expressed in the P2 progenitor, which develops before and in close proximity to the DER-dependent P15 progenitor. RHO might generate a short-range signal, possibly secreted SPI, that emanates from P2 and induces P15 in its immediate vicinity. The muscle phenotype of *rho* mutants, as well as the effects of ectopic mesodermal expression of RHO and secreted SPI, are consistent with this possibility. A similar mechanism has been suggested for the recruitment of chordotonal organ precursors in the *Drosophila* embryo (Lage et al., 1997; Okabe and Okano, 1997).

STAR also is capable of modifying the myogenic activity of DER. Given the known relationships among the *spi* group genes, this effect of STAR may derive from a functional interaction with RHO (Golembo et al., 1996a; Schweitzer et al., 1995b). In addition, modulation of DER activity can be achieved by the negative regulator, ARGOS (Golembo et al., 1996b; Schweitzer et al., 1995a), and we have also found that this mechanism is relevant to the mesodermal functions of DER (E. B., A. C., F. J. and A. M. M., unpublished results).

Both *DER* gain-of-function and neurogenic gene loss-of-function are associated with an overproduction of muscle progenitor and founder cells; similarly, these cells are lost in the absence of DER or the presence of constitutively active NOTCH (present work and data not shown; Baker and Schubiger, 1996; Bate et al., 1993; Corbin et al., 1991). These findings suggest that DER and NOTCH signals antagonize each other in myogenic specification. Since sufficiently strong activation of either pathway is capable of overcoming the effect of the other, the two signals must be balanced precisely to insure the proper segregation of muscle progenitors.

DER function in muscle diversification

DER signalling is required for the formation of approximately half of the muscles in an embryonic hemisegment. Since this

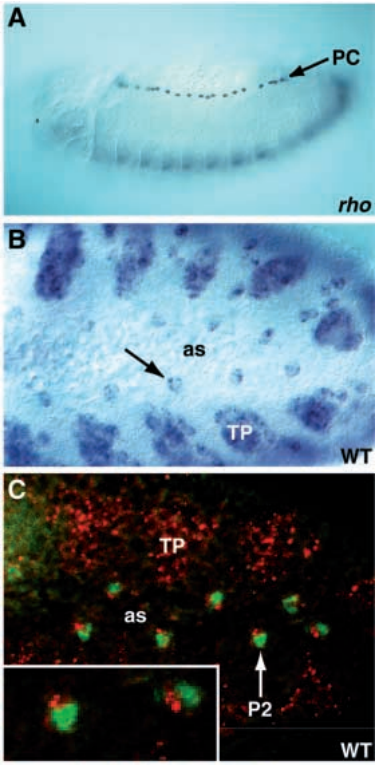

Fig. 7. RHO is expressed in progenitor P2 and is required for muscle DA1 formation. (A) A stage-13 *rho* mutant embryo stained with anti-EVE shows absence of muscle DA1 but not pericardial cell precursors. (B) In situ hybridization of a *rho* riboprobe to stage 11 wild-type embryos. *rho* is expressed in dorsal mesodermal cells (arrow) that are located between the tracheal pits (TP). (C) Confocal micrographs of embryos double-stained with anti-RHO (red) and anti-EVE (green) antibodies demonstrate the co-localization of RHO and EVE in P2, the EVE pericardial cell progenitor. The inset shows a magnification of two segments from the same embryo, illustrating the typical punctate pattern of RHO expression (Sturtevant et al., 1996).

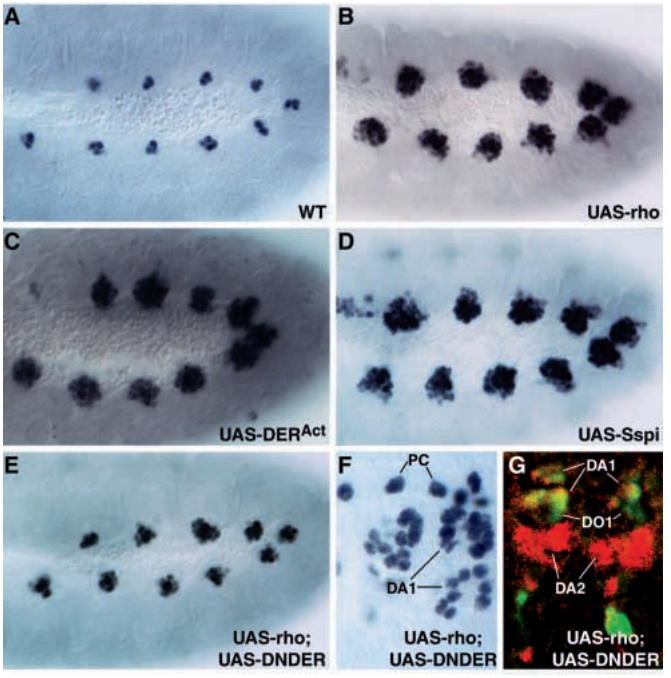

Fig. 8. Hyperactivation of DER generates supernumerary mesodermal founders and a duplication of muscle DA1. (A-E) Stage-11 embryos stained with anti-EVE antibody. Ectopic expression of RHO (B), constitutively activated DER (C), or secreted SPI (D) induced by *twi-GAL4* all generate a marked overproduction of EVE-positive mesodermal founder cells as compared to wild type (A). Co-expression of RHO and DNDER (E) results in an intermediate number of EVE-positive cells, reflecting an intermediate level of DER activity. (F) A stage-14 embryo ectopically expressing both RHO and DNDER contains an extra EVE-positive DA1 muscle in some segments. (G) Confocal micrograph of a stage-14 embryo with ectopic RHO plus DNDER double-stained with anti-KR (green) and anti-VG (red) antibodies. Although an extra muscle DA1 forms under the influence of increased DER activity, a normal complement of other dorsal muscles is present. This suggests that the additional DA1 is formed by duplication and not by transformation of another muscle precursor.

represents a considerable diversity of myofibre types, DER must be capable of contributing to multiple muscle progenitor identities. How can one signalling pathway be responsible for such a large number of different specificities?

One way of modifying the myogenic specificity of DER could be through interactions with regionally restricted factors. For example, specification of P15, the muscle DA1 progenitor, is dependent not only on DER but also on WG and DPP (S. G., A. C., F. J. and A. M. M., unpublished results). Since the domains of WG and DPP expression are highly localized and intersect each other precisely where P15 develops, the integration of WG, DPP and DER signals might provide the requisite specificity for this progenitor. Consistent with this possibility, we found that DER hyperactivation is capable of duplicating muscle DA1 adjacent to its normal counterpart. The different temporal requirements of specific muscles for DER activity could also influence the nature of such interacting factors.

Differences in the strength or duration of an RTK signal can influence its effects (Marshall, 1995). This could be relevant to the myogenic determination functions of DER via the modifying influences of STAR, RHO and SPI. The output of DER also may be augmented by a second RTK in the formation of at least one muscle, DA1, whose specification is dependent on both DER and the HTL FGF receptor (Michelson et al., 1998). The combined action of two RTKs could affect the magnitude and/or the persistence of the inductive signal, thereby generating a unique response for a particular muscle. A similar requirement for dual RTK input has been described for *Drosophila* photoreceptor development (Freeman, 1996; Tio and Moses, 1997).

The importance of muscle-specific DER signalling is highlighted by the different myogenic responses to altered levels of DER activity. Thus, the formation of each muscle may be triggered at a unique DER activity threshold. This mechanism would enable DER to contribute to multple myogenic identities. Graded RTK signalling has significant developmental consequences in other systems, as well (Golembo et al., 1996a; Greenwood and Struhl, 1997; Katz et al., 1995; Lillien, 1995; Queenan et al., 1997).

The postulated role of DER in determining muscle identity is distinct from the influence of homeotic genes in this process. Homeotic factors generate intersegmental differences in muscle pattern by mediating a choice between alternative identities (Greig and Akam, 1993; Michelson, 1994). Unlike local inductive signals such as DER, homeotic products are not responsible for initiating progenitor specification. This distinction is underscored by our finding that mesodermal cells lacking the normal inductive DER signal undergo apoptosis, not, as occurs in homeotic mutants, transformation into an alternative fate. Considered another way, homeotic function is superimposed on and modulates a basic pattern that is initially generated by DER signalling. Similarly, the muscle diversification role of asymmetric mesodermal cell divisions follows a progenitor pattern that is first established by DER (Carmena et al., 1998; Ruiz Gomez and Bate, 1997).

In summary, SPI activates DER to induce a large subset of early muscle cell fates in the *Drosophila* embryo. Modulation of DER activity may be important for specifying different myogenic identities, and such graded signalling could be generated by a variety of interacting factors. Some muscles require the combined inputs of multiple intercellular signals. An important challenge for future work is to understand the relationships among these disparate pathways and the mechanisms by which their inputs are integrated by the responding cells.

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