Development 128, 5061-5073 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV7910

rolling pebbles (*rols*) is required in *Drosophila* muscle precursors for recruitment of myoblasts for fusion

Annette Rau[1,*], Detlev Buttgereit[1,*], Anne Holz[1,*], Richard Fetter[2], Stephen K. Doberstein[2,‡], Achim Paululat[1], Nicole Staudt[1,†], Jim Skeath[4], Alan M. Michelson[3] and Renate Renkawitz-Pohl[1,§]

[1]Developmental Biology, Philipps-Universität Marburg, 35032 Marburg, Germany
[2]Howard Hughes Medical Institute, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA
[3]Brigham & Womens Hospital/Howard Hughes Medical Institute, 20 Shattuck Street, Room 1021, Boston, MA 02115, USA
[4]Department of Genetics, Box 8232, Washington University School of Medicine, 4566 Scott Avenue, St. Louis, MO 63110, USA
*These authors contributed equally to this work
[†]Present address: MPI für Biophysikalische Chemie, Abt. Molekulare Entwicklungsbiologie, Am Fassberg, 37077 Göttingen, Germany
[‡]Present address: Exelixis, 170 Harbor Way, P.O. Box 511, South San Francisco, CA94083-0511, USA
[§]Author for correspondence (e-mail: renkawit@mailer.uni-marburg.de)

Accepted 27 September 2001

SUMMARY

Mutations in the *rolling pebbles* (*rols*) gene result in severe defects in myoblast fusion. Muscle precursor cells are correctly determined, but myogenesis does not progress significantly beyond this point because recognition and/or cell adhesion between muscle precursor cells and fusion-competent myoblasts is disturbed. Molecular analysis of the *rols* genomic region reveals two variant transcripts of *rols* due to different transcription initiation sites, *rols6* and *rols7*. *rols6* mRNA is detectable mainly in the endoderm during differentiation as well as in malpighian tubules and in the epidermis. By contrast, *rols7* expression is restricted to the mesoderm and later to progenitor descendants during somatic and pharyngeal muscle development. Transcription starts at the extended germ band stage when progenitor/founder cells are determined and persists until stage 13. The proteins encoded by the *rols* gene are 1670 (Rols6) and 1900 (Rols7) amino acids in length. Both forms contain an N-terminal RING-finger motif, nine ankyrin repeats and a TPR repeat eventually overlaid by a coiled-coil domain. The longer protein, Rols7, is characterized by 309 unique N-terminal amino acids, while Rols6 is distinguishable by 79 N-terminal amino acids. Expression of *rols7* in muscle founder cells indicates a function of Rols7 in these cells. Transplantation assays of *rols* mutant mesodermal cells into wild-type embryos show that Rols is required in muscle precursor cells and is essential to recruit fusion-competent myoblasts for myotube formation.

Key words: Myoblast fusion, Ankyrin repeats, TPR repeats, RING finger, Myogenesis, *Drosophila*

INTRODUCTION

In *Drosophila*, the establishment of muscle founder cells prefigures the larval pattern of body wall muscles (Bate and Martinez Arias, 1993). These cells are descendants of progenitor cells, which are determined by the Notch pathway via lateral inhibition (Carmena et al., 1995) and specified by signaling pathways such as the EGF receptor pathway (Buff et al., 1998). Each progenitor divides asymmetrically and gives rise to two founder cells, each of which expresses a distinct set of transcription factors and is determined to build a specific myofiber (Ruiz-Gomez, 1998; Frasch, 1999; Paululat et al., 1999a).

Founder cells first form bi- or trinucleated cells called precursor cells by fusion to the second class of mesodermal cells, the fusion-competent myoblasts (FCMs) and then enlarge by further fusion to form mature myotubes (Bate, 1990). Recently, Ruiz-Gomez et al. (Ruiz-Gomez et al., 2000) analyzed *dumbfounded* (*duf*), which encodes a member of the immunoglobulin family and presumably serves as an attractant in the founder cells. *duf* was the first gene shown to be essential in the founder cell to mediate already the first fusion to muscle precursor cells. These precursor cells then recruit additional FCMs from a common pool in the surrounding mesoderm to form myofibers. In *Drosophila*, myotube formation is finished within a few hours.

At the ultrastructural level, distinct steps in the fusion event are evident (Doberstein et al., 1997). First, cells recognize each other and align. At the area of fusion where the cell membranes will later be degraded, vesicles accumulate between the individual precursors and the FCMs. This prefusion complex dissolves and is replaced by electron-dense plaques of unknown composition; afterwards membrane breakdown occurs and the cells fuse. The electron microscopic studies of Doberstein et al. (Doberstein et al., 1997) were carried out with stage 13 and stage 14 embryos at a time when the majority of precursor cells have been established but abundant fusion to mature muscles still takes place.

Genetic approaches uncovered several genes essential for myoblast fusion (Paululat et al., 1999b; Frasch and Leptin, 2000; Taylor, 2000). The ultrastructural analysis of Doberstein et al. (Doberstein et al., 1997) revealed that mutations in individual fusion relevant genes interrupt myoblast fusion at distinct steps with regard to cell adhesion, prefusion complex and plaque formation. The gene *myoblast city* (*mbc*) is expressed in, besides other tissues, all myoblasts and is here required during an early step to mediate proper recognition and alignment between the founder cells and the FCMs (Rushton et al., 1995). Mbc shows homologies to the human DOCK180 protein and interacts with the *Drosophila* homolog of CRK, an adaptor protein (Erickson et al., 1997; Doberstein et al., 1997; Galletta et al., 1999). However, as it is present only in the cytoplasm, its precise function is still unclear. The same holds true also for Blown Fuse (Blow), a protein that is uniformly distributed in the cytoplasm of all myoblasts and essential to proceed from prefusion complex formation to plaque formation, all the more as it shows no homologies to proteins identified so far (Doberstein et al., 1997). In *sticks and stones* (*sns*) mutants, which correspond to the *43-49* fusion mutant originally found on the $rost^{P20}$ chromosome (Paululat et al., 1999b; Bour et al., 2000), myoblasts accumulate after plaque formation (Doberstein et al., 1997). SNS is expressed in FCMs, but not in founder cells, and encodes a 162 kDa nephrin-like protein that crosses the membrane and contains Ig repeats in the extracellular domain (Bour et al., 2000). Overexpression of a dominant negative form of the GTPase Drac1 leads to incomplete membrane breakdown (Luo et al., 1994). Interaction between Mbc and Drac1 may be required to rearrange the cytoskeleton during myoblast fusion as Mbc has been isolated independently as a suppressor of Drac1 (Nolan et al., 1998). Both proteins are involved in a number of developmental processes and are clearly essential, but not specific, for myoblast fusion.

Despite considerable insight into several steps of fusion, only a limited number of components and their interactions are known at the molecular level. An important question is whether the establishment of muscle precursor cells by initial fusion and further growth to muscles by recruiting further fusion competent cells is just a timewise difference, or whether distinct genes are required to proceed from the precursor to the mature muscle. We present data that suggest *rolling pebbles* (*rols*) might be a key component specifically required in the muscle precursor cell to recruit surrounding myoblasts for fusion. Alternatively the loss of Rols might reduce the efficiency of fusion so that only mini-muscles are formed.

MATERIALS AND METHODS

Drosophila stocks and genetic analysis

The *rols* relevant deficiency line *Df(3L)BK9* (which corresponds to Umea stock #54650) as well as the enhancer trap lines *P1027* and *P1729* (Spradling et al., 1995) were obtained from the Bloomington and Umea Stock Centers. *P1729* corresponds to stock numbers *11729* and *P{PZ}l(3)08232*, and carries a P-element at 68F01-03; *P1027* corresponds to stock number *11027* and *P{lArB}A490.2M3*, the P-element was placed in 68F. The alleles $rols^{AD328}$ *and* $rols^{XX117}$ were found in an EMS mutagenesis screen for the 3rd chromosome carried out by Skeath and Doe in which the FGF-receptor mutant *heartless* was found (Gisselbrecht et al., 1996).

$rols^{AD328}$, $rols^{XX117}$, $rols^{P1027}$ and $rols^{P1729}$ were all allelic, as determined by failure to complement each other and the deficiency *Df(3L)BK9*. *rols* alleles were kept over a marked balancer *TM3 Sb Dfd-lacZ* to distinguish homozygous mutants and heterozygous embryos at early stages. In the rP298 enhancer trap line, the P-element is localized on the X-chromosome (Nose et al., 1998) and the enhancer trap pattern corresponds to *dumbfounded* (Ruiz-Gomez et al., 2000). By appropriate crosses we established a fly stock carrying the *rols* deficiency on the 3rd chromosome over the marked balancer *TM3 Sb Dfd-lacZ* and the rP298 insertion on the X chromosome.

Remobilization of P-element insertions

The P-element in $rols^{P1729}$ and in $rols^{P1027}$ was remobilized by using the transposase source of the line $ry^{506}P[ry^{+}\Delta 2\text{-}3]/ry^{506}P[ry^{+}\Delta 2\text{-}3]$. The loss of the P-element (visible by loss of $rosy^{+}$ marker) was detected by crossing single jumpstarter males with females of the balancer line 120 (Bloomington Stock Center). Isogenic individual *rosy* lines were analyzed concerning lethality, phenotype and allelic situation to the deficiency *Df(3L)BK9*. As we noticed a second lethal hit on the *P1027* chromosome (outside the breakpoints of the deficiency), we crossed this strain five times (Davies et al., 1996) against the ry^{506} allele and named the corresponding strain with the P-element in the 68F locus after rebalancing $rols^{P1027ber}$. Although no second hit was observed in $rols^{P1729}$, the *P1729* line was crossed in the same way (and named $rols^{P1729ber}$).

Immunohistological staining of embryos

Immunostaining was performed as described previously (Paululat et al., 1995). We used antibodies against β3 tubulin (Leiss et al., 1988; Buttgereit et al., 1996) and against Mef2 (Bour et al., 1995) to stain mesodermal derivatives and developing muscles. A polyclonal anti β-galactosidase antibody (Biotrend, dilution of 1:5000) was used to visualize muscle precursors of the enhancer trap line rP298, the expression of the marked balancer, and the enhancer trap pattern in the *rolling pebbles* alleles $rols^{P1027}$ and $rols^{P1729}$. Anti-Eve (Frasch et al., 1987) was used to selectively stain dorsal acute muscle 1 (DA1) and pericardial cells. The Vectastain ABC Elite-kit (Vector Laboratories) was used as detection system. The embryos were embedded in glycerol or dehydrated and embedded in Epon and photos were taken under Normarski optics with a Zeiss Axiophot microscope, digitalized and processed in Adobe Photoshop.

Whole-mount in situ hybridization

Whole mount in situ hybridization was carried out essentially as described by Tautz and Pfeifle (Tautz and Pfeifle, 1989). We synthesized antisense DIG-labeled probes by single strand PCR using primers specific for the *rols6* transcript (st-Primer: 5′ ACTGCCA-GATCGACGACCGGTTG) or the *rols7* transcript (lt-primer: 5′ TCCAGCGAGGAGTTCATAGAT), respectively. In addition, for *sns* we used antisense probes from a 6 kb *sns* cDNA clone in pBSKII kindly provided by Susan Abmayr (Bour et al., 2000).

Electron microscopic analysis

The electron microscopic analysis was carried out as described by Doberstein et al. (Doberstein et al., 1997) using the $rols^{AD328}$ allele.

Plasmid rescue and isolation of the *rolling pebbles* genomic region, chromosomal walk and cDNA library screening

Plasmid rescue was carried out using standard molecular biology methods. Genomic DNA of $rols^{P1729}$ flies was digested with *Xba*I, ligated overnight and transformed into high efficiency ultra competent cells (XL2-Blue, Stratagene). Thus, we obtained a 5 kb genomic fragment flanking the P-element insertion. Accordingly, we obtained a 4 kb genomic fragment by digesting genomic DNA of $rols^{P1027}$ flies using *Sal*I.

A Canton-S genomic library (Stratagene, made from 0-12 hours

embryos) was screened using the 5 kb plasmid rescue fragment as an initial probe. Several overlapping clones were isolated, subsequently subcloned into the Bluescript plasmid vector (Stratagene) and used for additional screenings. A 5′ extension of 3 kb was obtained by the analysis of the P1 phage DS00075 (Kimmerly et al., 1996) so that the different phage clones finally spanned a genomic region of 60 kb.

We isolated three cDNAs from the embryonic LD-cDNA library from the BDGP EST Project (made by Ling Hong) by using a genomic 3 kb probe from the most 5′ region of the *rols* transcription unit. Two of these three cDNAs were identical (6.3 kb), whereas the third one, 5.8 kb length, differed in the 5′ region.

Sequence analysis, determination of ORFs and prediction of protein domains

To determine exon/intron boundaries we compared the sequences of the *rols* cDNAs to genomic sequences. For this purpose, we took advantage of the already published genomic sequences of *Drosophila* (Adams et al., 2000) and used the independent sequenced genomic fragments from our library screening. To search for protein domains, we applied the SMART-Tool of the EMBL (Schultz et al., 1998; Schultz et al., 2000).

Sequence data for *rols7* and *rols6* have been deposited with the EMBL/GenBank Data Libraries under Accession Numbers AF386647 (*rols7*) and AF386648 (*rols6*).

Determination of the developmental expression profile by RT-PCR

Primers were designed to amplify a common part of the *rols* mRNA leading to a *rols* PCR fragment of 580 bp from the mRNA and a genomic fragment of 830 bp (primer LDI-14, CCACCATCGC-TATGCGAGC; and LDI-20, CTTCAGGTT-GTGATTGCTCACG). A primer pair specific for *rols7* leads to a 705 bp fragment (primer LDA, 5′ GCCACATTGGATTATCAGTGA; primer LDR, 5′ CATGATGTCATTCCGATTGAAG flanking the *rols7* specific intron). A *rols6* specific combination leads to a 708 bp fragment (primer LDIIDO, 5′ TCGGCAAATCAT-CGCCGATAC; and primer LDIISP, 5′ACTGCCAGATCGACGAC-CGGTTG, flanking the small *rols6* specific intron). A primer pair (B1E1D, CCAAGCTGGTCAGTGC; and B1E2U, GAGCCTCGTT-GTCGATG) was used to amplify a 601 bp fragment of the β1-tubulin mRNA. All primers were chosen so that they flanked an intron in the pre-mRNA, to distinguish amplification from genomic DNA. As the β1-tubulin fragment (601 bp) is very similar in size to the *rols* PCR-product, β1-tubulin primers were omitted in the *rols* PCR reactions, but included as internal control in the *rols6* and *rols7* reactions. Per RT-PCR reaction with approx. 20 ng poly (A^+) RNA (isolated with the Qiagen One-step kit) amplifications were carried out at the appropriate annealing temperature with 30 to 35 cycles.

Transplantation procedures

As donor for transplantation, we used the offspring of *UAS-lacZ/UAS-lacZ; rols$^{Df(3L)BK9}$ /TM3 Sb Dfd-lacZ.* The *rols* allele *Df(3L)BK9* was marked with *UAS-lacZ* on the 2nd chromosome (P1776 from the Bloomington Stock Center, this *Drosophila* stock was additionally marked with *Ubx/Sb* on the 3rd chromosome) and balanced over *TM3 Sb Dfd-lacZ.* The strain *daughterless GAL4* (*daGAL4*) with ubiquitous *GAL4* expression (Wodarz et al., 1995) (supplied by R. Klapper) served as a recipient (Klapper, 2000). All transplantation experiments

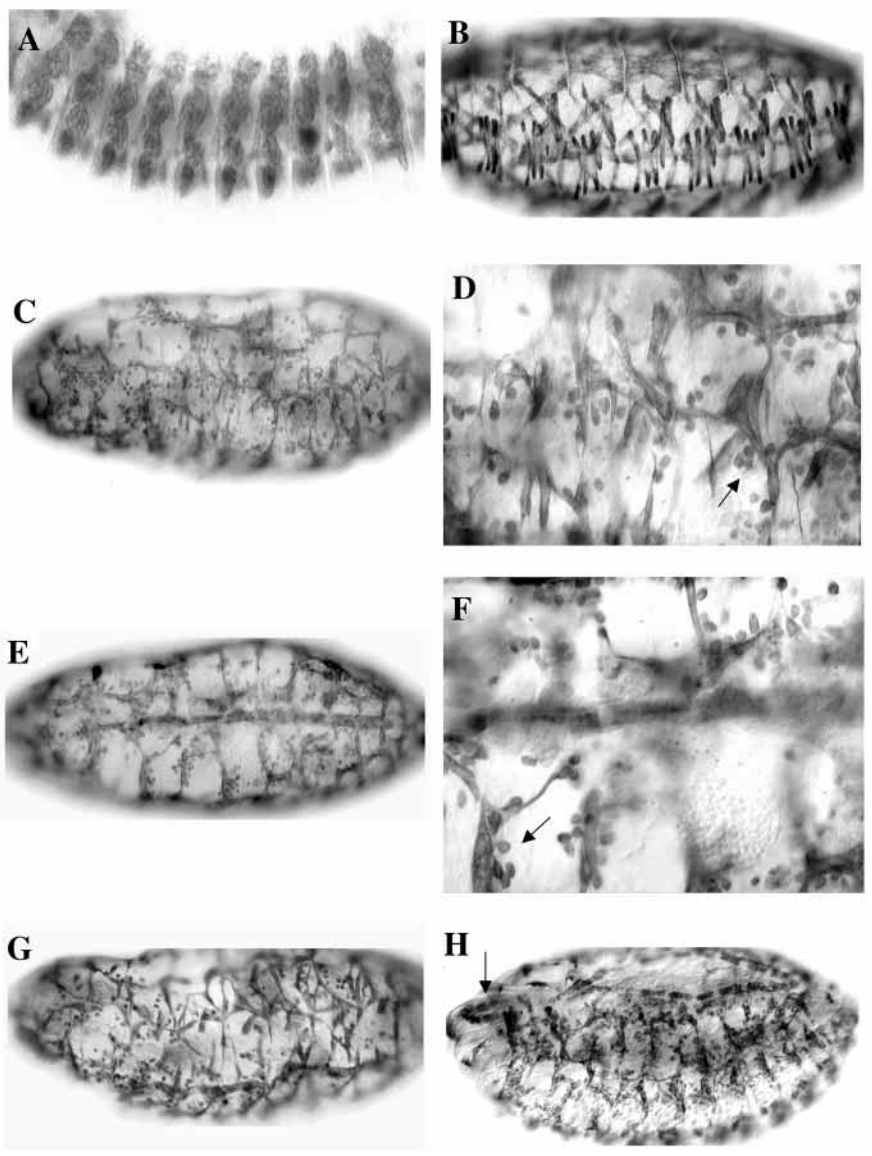

Fig. 1. Myoblast fusion is severely disturbed in *rols* mutants. The muscle phenotype of *rols* mutants (C-H) is compared with the wild-type (A,B) pattern of the body wall muscles. The mesoderm is visualized with antibodies against β3-tubulin. (C) *Df(3L)BK9* embryo, stage 16, lateral view; the number of myotubes as well as their orientation is altered significantly. (D) Same embryo as in C but at a higher magnification; the disarranged myotube pattern as well as single, unfused myoblasts (arrow) are shown. (E) *Df(3L)BK9* embryo, stage 16, dorsal view; the dorsal vessel is nearly undisturbed; most unfused myoblasts are arranged nearby formed myotubes. (F) Same embryo as in E, but at higher magnification; the alignment of unfused myoblasts (arrow) at individual muscles is evident. (G) *rols^{AD328}*, EMS-induced allele, late stage 16, lateral view; the pattern of the somatic muscles is also severely disturbed, however, owing to the later embryo stage, the number of unfused myoblasts is reduced in comparison with C. (H) *rols^{P1729ber}*, a P-element induced allele, stage 15, dorsolateral view. As in the other alleles, muscle number is reduced, and many unfused myoblasts are visible; also the pharynx (arrow) shows fusion defects. Again, the dorsal vessel is only weakly affected.

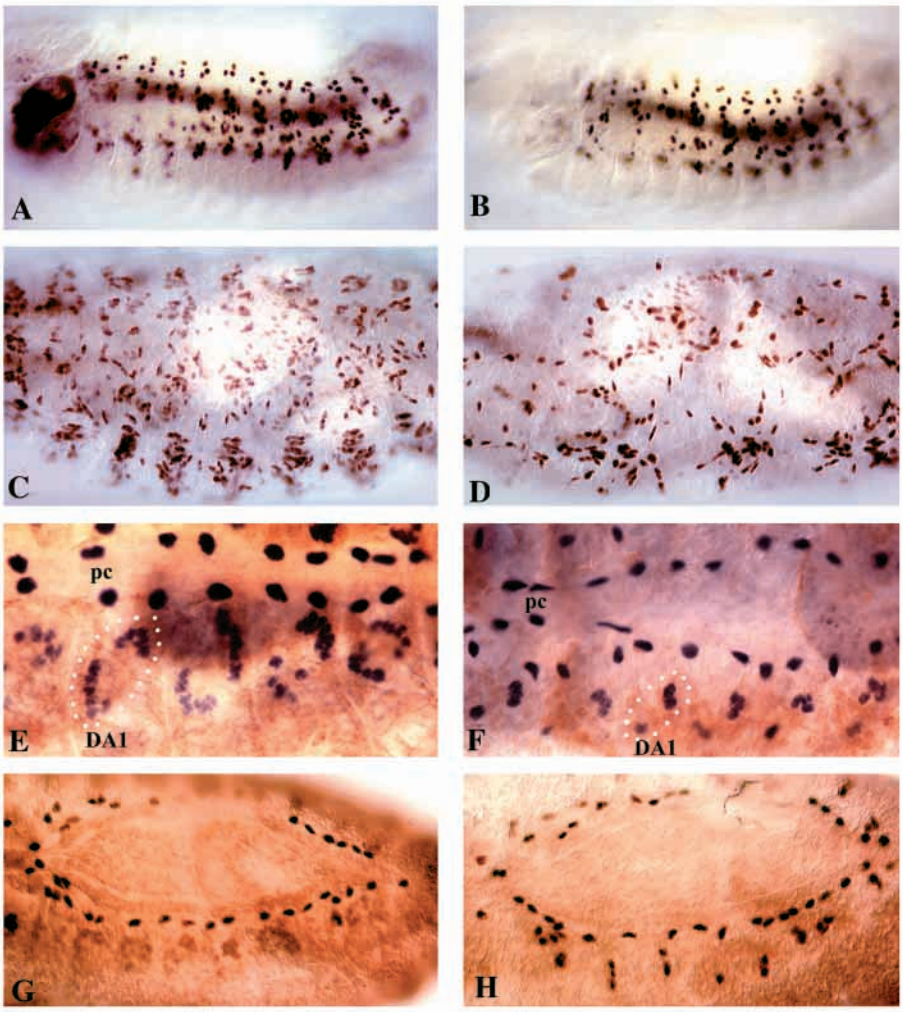

Fig. 2. Muscle founders/precursors are determined correctly in *rols* mutants. All nuclei of muscle founder cells are visualized at late stage 13 in the enhancer trap line rP298 by the anti-β-galactosidase antibody. The expression of rP298 was monitored in embryos homozygous for the deficiency *Df(3L)BK9* (B) and revealed essentially a wild-type pattern (A). However, at stage 15/16, the number of rP298 positive nuclei in *rols* mutant embryos (D) is significantly reduced compared with wild type (C). Eve expression in wild-type embryos marks the nuclei of dorsal muscle 1 (DA1, marked by a white circle in one segment) and pericardial cells (pc) (E). In *rols*^{P1729} mutants, Eve staining in DA1 is visible only in two to five nuclei (F, marked by a white circle in one segment). This is shown for a younger embryo homozygous for the deficiency (H) where two to three nuclei of dorsal muscle 1 are stained compared with a wild-type embryo with five to ten Eve-positive nuclei (G).

were carried out during blastoderm stage (Holz et al., 1996) and beginning gastrulation. Mosaics were generated by removing approximately 25 cells from the mesodermal anlage of donor embryos and transplanting three to five cells homotopically in up to six host embryos. Transplantations were carried out between 40-60% EL (egg length). To select hetero- and homozygous donor embryos, single developed donors were dissected and stained for β-galactosidase activity achieved by the *TM3 Sb Dfd-lacZ-* balancer. All host embryos with cells from an individual donor were raised together in culture and dissected as third instar larvae. Spreading of the musculature was achieved by briefly dipping the larvae into a 60°C water bath (Hooper, 1986). Histochemical demonstration of β-galactosidase expression was carried out as described by Meise and Janning (Meise and Janning, 1993). The detection of β-galactosidase expression was only possible in syncytial tissues where *UAS-lacZ* and *daughterless Gal4* are co-expressed. This method of syncytium detection was established by Klapper et al. (Klapper et al., 2001a).

RESULTS

The identification of the *rols* locus

We screened a collection of deletion mutants for defects in muscle formation to identify novel genes required for myoblast fusion (Bloomington and Umea stock collection, about 70% of the collection for 3rd chromosome). The mesoderm specific β3-tubulin isotype is expressed before myoblast fusion (Buttgereit et al., 1996). Thus, we used a β3-tubulin specific antibody to visualize mesodermal cells in mutants. This strategy has been successfully applied for P-element-induced mutations described previously (Burchard et al., 1995; Paululat et al., 1995). Most of the deletion mutants show severe distortions (e.g. in segmentation). However, embryos homozygous for a small deletion on the left arm of the third chromosome, *Df(3L)BK9* exhibited a clear muscle phenotype. This deletion maps to the cytological positions 68E2-3; 69A1. Mesoderm differentiation is severely disturbed compared with wild-type embryos (compare Fig. 1C-H with Fig. 1A,B); most reliably, we detect clusters of unfused myoblasts, which fail to form myofibers at late stages of embryogenesis (Fig. 1D,F). We named the transcription unit responsible for the fusion defects *rols*, owing to the appearance of the scattered distribution of the unfused myoblasts.

The *rols* gene is essential for myoblast fusion

The relative size of the deletion suggests that many genes are removed. To identify the gene responsible for the mutant phenotype, we analyzed lethal P-element inserts located within the deleted region for defects in muscle formation. We identified two P-element insertions, *P1027* and *P1729* from the Bloomington collection, which lead to embryonic lethality and show the same muscle fusion phenotype as the deficiency. The original *P1027* chromosome contains two mutations leading to lethality in the homozygous situation. These mutations were separated by meiotic recombination and the strain with a single P-element insertion at 68F was named *rols*^{P1027ber}. Embryos homozygous for *rols*^{P1027ber} and as well as for *rols*^{P1729ber} (a cleansed *rols*^{P1729} chromosome, see Material and Methods) show strong fusion defects (Fig. 1H). As *rols*^{P1027} fails to complement *rols*^{P1729}, we concluded that these two P-element

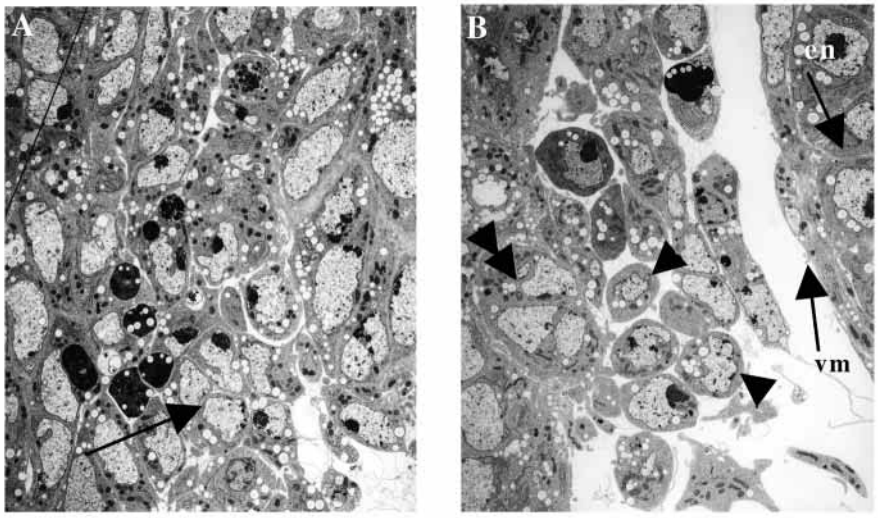

Fig. 3. Ultrastructural analysis of *rols* mutants show that Rols is required for alignment of muscle precursor cells with FCMs. Electron micrograph of an early stage 13 *rols*^{AD328} mutant embryo (A) reveals closely associated cells (arrow) but in contrast to the wild type, no fusion complexes are evident. At stage 14 (B) myoblasts in *rols* mutant embryos have lost contact with muscle precursor cells compared with wild-type where prefusion complexes (Doberstein et al., 1997) are evident. Arrowheads indicate myoblasts, whereas the double arrowhead shows a trinucleated precursor cell. Regions of the endoderm (en) and the visceral mesoderm (vm) are marked with arrows.

inserts were allelic. Additionally, remobilization of both P-elements in the cleansed P-element insertion lines restored viability, strong evidence that these P-elements disrupt the function of the *rols* gene (data not shown).

Furthermore, we identified two EMS-induced alleles from the collection of Skeath and Doe (Gisselbrecht et al., 1996), which failed to complement the deficiency as well as the P-alleles. As they exhibit an identical fusion phenotype, we named them *rols*^{AD328} and *rols*^{XX117}. Looking at the muscle morphology, fusion was severely disturbed as in all other *rols* alleles (Fig. 1G). Before dorsal closure, we often observed many unfused myoblasts per segment. In embryos at stage 16/17, only a small number of irregularly shaped myofibers were present, leading to a very rudimentary muscle pattern and a varying proportion of unfused myoblasts (see Fig. 1C-H). The disappearance of many myoblasts might be explained in part by cell death of unfused myoblasts which are cleared away by macrophages, as previously observed in another muscle fusion mutant (Rushton et al., 1995).

Embryos homozygous for the mutant *rols* alleles develop until shortly before hatching as dorsal closure is evident (shown for *Df(3L)*BK9, for example) (Fig. 1E). Many unfused myoblasts were present and we found some muscle-like fibers with only a few nuclei (Fig. 1C-H). We propose that these muscle-like fibers represent muscle precursor cells that stretched and tried to contact the epidermis, as originally observed for founder cells in *mbc* mutant embryos (Rushton et al., 1995). The persisting myoblasts often adhere to the myofiber-like cells (see Fig. 1D,F). Furthermore, we often observe that these myoblasts extend filopodia, which are

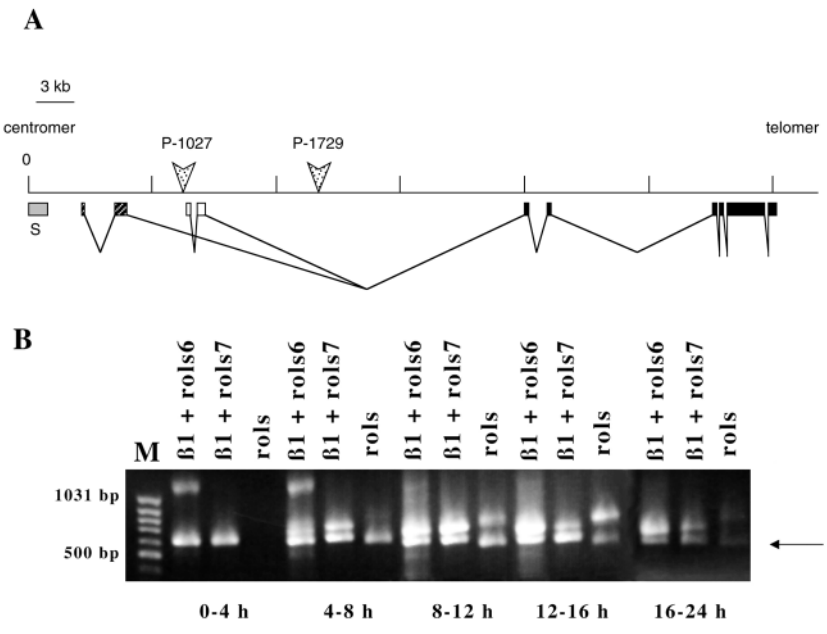

Fig. 4. (A) Genomic organization of the *rols* locus. The scheme summarizes the data of our genomic walk, the data from the *Drosophila* Genome Project and the sequence analysis of *rols* cDNAs, as well as the determination of the P-element insertion sites. The *rols* gene extends about 60 kb, as indicated by the scale and is flanked towards the centromer by an open reading frame coding for Semaphorin 5c (CG5661; S, gray box). The exons common to *rols7* and *rols6* are shown as black boxes, specific exons for *rols7* are shown as black and white hatched boxes, while the specific exons for *rols6* are shown as white boxes. The transcription initiation site of *rols6* is localized within the large second intron of *rols7*. The translation initiation codons for Rols7 and Rols6 are unique, they are localized in the second exon unique to each message. The P-element in the mutant *rols*^{P1027} is localized 70 bp upstream of the transcription initiation site of *rols6*, while the P-element *P1729* of the *rols*^{P1729} mutant is localized within the common part of the second intron. (B) Stage-specific mRNA levels detected by RT-PCR. Poly (A)$^+$ RNA was prepared from embryos collected for the times indicated, and RNA from 0-4 and 4-8 hours was purified twice in order to remove genomic DNA completely. For details of the RT-PCR see Material and Methods. Whereas the β1-tubulin mRNA (arrow) is present during all stages, *rols6* and *rols7* are first detected at 4-8 hours, corresponding to embryonic stages 8-11; *rols7* reaches maximum levels between 8-12 hours (stages 12-14), while *rols6* shows the highest levels at 12-16 hours (stages 15-16), whereas *rols7* already declines. During 16 hours until hatching, *rols6* is present at relative high levels compared with *rols7*.

ROLSSP 1670 AA

```
MFPLTNRSSS GNRTNNNPSN NNNNQTTSFI IGGTTNINNN NNQLGGTNSA KDKGNFENNW SCNKTTGRRS GSGNSCSFL  79
```

ROLSLP 1900 AA

```
MPSLQQIALA MEGDQSELQT PPLPINPPPK VMANGIVYGF RTRHLSCIEE NDSDSLHGSS IPHAYDMMQQ ADETFQKLNA  80

NEEQDESGSE AGGLENLDDT IAMIDESRLL EADYLDELVA EEQLQEQDED EEESSDLVDS GNVEEDFWQP SFNRNDIMMT  160

SVYGALNGSL TSEVSSTAAP GYSECATPRK EAEPVYATPE KRRASNRSFD STCASLTSSL YGGSMNSSLD LKYSSLGGGD  240

SVSGGISGTS TLRGTSVPPM DLSMISSDGV SALDWGSASV SCVLDSGDGN PAEELNEEMA AKCLSAAESD LQSIRRLLEH  320

                    RING-finger
DASGSVCPSC RISFDKGKRR KLIDTCGHER CYSCMFRNDQ CPMCMNSSLK DVDGANAQGY DTGIGGSTST IVSPLGSPQP  400

QLRSHAQRNA ALARYMQQHR QESQSPDYHH RYASNGKLPR AAALSTNGNG FTTGTTQTVT VDVHHQLGGG GGGAAAAAKQ  480

LMANGISGHS RSSSMSHNIH AAAYSELSAA PPAFATPPTR RRFFNHKNLR SALTGGSGGG SGVGGGVGGA GGVGGGHRRT  560

ASNGGCPIDT ASILSGDHTH QHHHRDNQPE GKESNALNTS TCSDSAVTRR RRKNVSNHNL KTSARHGASS ENRLNRLSLA  640

GTSVYAGHLS SLVFGKIKSL WSVNSSNSSE AGLNQLAGSD AIDHHSSFLN EKLQKDQLHA RLGLLLNDPG SNGNSSSSGS  720

GCEPISAHST TSTTSSSGVG AASTTTSGSS QNVSPEQTLA SGAGMLSGSQ LSVATSHGVK EDALSLCGKF KAGCSMLHVY  800

EALPSKSRKG NVRRSTRGQQ GSSSSASAAS AVGSRVTASS LAAVQLALKP LFFEVPLQEP DPPYVGRQWL VQQLSNILLG  880

     ATP/GTP binding site
TETRVVLING QPGTGKTAFC LQLVEYSCFG RRQMQDDPDG IYSQLQLGAH CERMRGLASH MVGYHFCQAD ANLTCQVPDF  960

VHSLAAQLCQ APQLTAYRDY LLSEPHLQDI LSVRECIADA ERVMKLAILE PLAHLHRAGK IPAKVAVIVV DALCEAEYHR  1040

PDHGHTIASF LAQLTPHFPA WLKLVATVRT QMLELVKAPS YTQLTLDSWA SSQALQQDML DYIGARLADS PEIRMNIGGG  1120

GGQNSQSGSQ PQTKFVSHLQ SLSRGSMLYA KLILDLIAXG QLVIKSSSYK VLPVSLAQIF LLHFNLRFPT ARSFEQAAPI  1200

LNICLAALYP LTLDEIYYSM EALSHGREAL SWPDFMQRFK LLDGFLIKRL DNTYMFFHSS LREWLMRRDE GESNKFLCDA  1280

RLGHAGIAFR LSRLQAPLSP QLTLELGHHM LKAHLYGGTS LTLLSPRDLQ SYWLAGAADN ISSSLGALRN VYSPNLKVSR  1360

                                    ANK1                ANK2
LVLLAGASPN HRTDYMGGAP ILCIAAHEGI LPMVSLLLEF GADVGLTNSQ GCTPLILAAM RGHCDVVRPL VAAGSSLGQL  1440

          ANK3
DITQRCALVH AARMGHLSVV KYLLACDWSP RPHSQDVTRS VALQQALIGA AAQAHCKILE DLLDLNETEF DLDVNGMEPS  1520

               ANK4                          ANK5                          ANK6
SGELALTAAA RHGCIDVVGI LLSRGAQIDA RNRQGYSALW LAVKEGHWSV VEHLLQRGAL LDEPLGQTRK TPLMIAAEEG  1600

                                    ANK7                ANK8
HLELVDLLLA RGAQREAQDH EGFTALSWAC LRGHLAAAKT LIEHGCNRHH EDHNGRTALD LAAYQGAASL VIYILEQGGN  1680

                    ANK9
LEHIDVHGMR PLDRAIACRN IQAVQVFLRK GAKLGPTTWS MAMGKPEILV ILLNKLLEDG NVLYRKNRFQ EAAHRYQYAL  1760

                                                        TPR-repeat
RKISGLEQLL ERNAIFAQLR TNLLLNLSRC KRKLNELDAS IDLATQAIAQ KPHSYEGYYA RAKARMELGA LNEALVDANE  1840

AMQQAAQSGV LCEVVEVLKR IQTELLTRIS ISSEDQTGRM SNIGGGASVA YEXHHEITDL  1900
```

Fig. 5. Deduced amino acid sequence of Rols7 and Rols6. From the analysis of the cDNA sequences, the possible open reading frames were deduced. According to conceptual translation, *rols6* encodes a protein of 1670 amino acids, 79 of which at the very N terminus are unique to Rols6. *rols7* encodes a protein of 1900 amino acids, 309 of which are unique to Rols7. The amino acid sequence of Rols7 is shown in total. The beginning of the common part of Rols7 and Rols6 is indicated by a black triangle, which corresponds to the first common intron-exon boundary of the mRNAs. Structural motifs were determined using the SMART-tool at the EMBL-server at Heidelberg (http:/smart.embl-heidelberg.de) (Schultz et al., 1998; Schultz et al., 2000). This analysis predicts four structural motifs within the common part of both proteins which are underlined and nominated in the sequence: a RING-finger motif in the N-terminal part, a predicted nucleotide binding motif, nine ankyrin motifs and a TPR-repeat (broken underline) close to the C terminus.

directed towards muscle-like fibers. However, the extent of the phenotype is variable mainly after stage 16; the number of unfused myoblasts, and the appearance and number of myofibers also differ significantly between embryos of the same stage. Cardioblast development is not obviously disturbed, as the typical repetitive pattern of four β3 tubulin stained cardioblasts and two unstained cardioblasts is evident (Fig. 1E,F,H). Analysis of gut morphogenesis often reveals incomplete formation in at least a quarter of the mutants when compared with the wild type (data not shown), which might be evidence for defects in the visceral muscles of the midgut. Recently, it was shown by Klapper et al. (Klapper et al., 2001b) and San Martin et al. (San Martin et al., 2001) that the visceral musculature also consists of small syncytia. Indeed, also the visceral mesoderm contains rP298 expressing cells, presumably founder cells, and *sns* expressing cells, presumably fusion competent cells. San Martin et al. (San Martin et al., 2001) and Klapper et al. (Klapper et al., 2001b) have shown that in *duf* mutants and in *sns* mutants, no fusion can be detected in the visceral mesoderm, implying that the founder cell hypothesis also holds true for the visceral musculature of the midgut.

Both P-elements in *P1027* and *P1729* contain a *lacZ* gene coupled to a basal promotor, which can be driven by genomic enhancer elements. The enhancer trap pattern in *rols*^{P1027} is mainly restricted to the endoderm (see Fig. 6A), while β-galactosidase is expressed in the muscle attachment sites (apodemes) in the epidermis of *rols*^{P1729} embryos (see Fig. 6B). Therefore we analyzed whether these tissues show morphologically detectable distortions in *rols* mutants. Apodeme integrity was checked using an antibody against Alien, which marks all muscle attachment sites (Goubeaud et al., 1996; Dressel et al., 1999). No major aberrations could be detected; therefore we conclude that at least apodeme

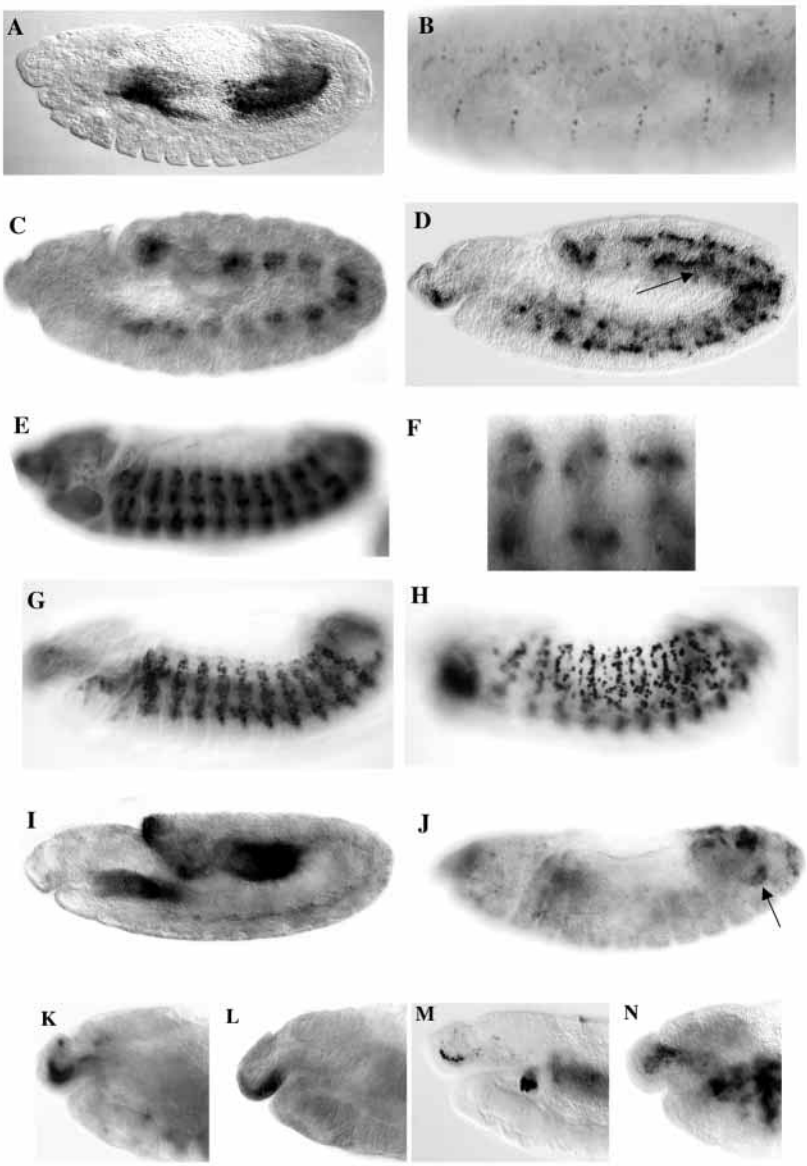

Fig. 6. Differential distribution of *rols*-transcripts during *Drosophila* embryogenesis. (A,B) Expression patterns from the enhancer-trap lines $rols^{P1027}$ and $rols^{P1729}$. (A) $rols^{P1027}$ embryo, stage 12, lateral view, focus on the interior. β-galactosidase expression is detectable in both midgut primordia. (B) $rols^{P1729}$, embryo stage 16, lateral view, focus on the epidermis. The nuclei correspond to the positions of the epidermal muscle attachment sites (apodemes). (C) *rols7* is first detected in the mesoderm at the extended germ band stage. (D) During germband retraction, the number of *rols7* expressing cells increases but remains restricted mainly to the somatic mesoderm; weak, transient expression is detected in the visceral mesoderm (arrow). (E) After stage 12, the mRNA remains restricted to a subset of myoblasts derived from the somatic mesoderm. (F) Higher magnification of muscle precursors of the embryo depicted in E reveals expression surrounding one of the nuclei in a precursor. (G) Anti Mef2 stains all nuclei of the somatic mesoderm. (H) rP298 stains a subset of myoblasts (the muscle founder/precursor cells in every segment), while the unfused fusion-competent cells are not stained. (I,J) *rols6* is mainly expressed during the invagination of the anterior and posterior gut primordium, and later on in the developing pharynx, the malpighian tubules (arrow) and in some ectodermal cells. (K) *rols7* is expressed in a small group of mesodermal cells of the clypeolabrum. (L) *rols6* is expressed in the ectoderm beneath the *rols7*-positive cells. (M) The rP298 enhancer trap pattern coincides with the *rols7* expression pattern in the clypeolabrum. (N) *sns* is transcribed in a group of mesodermal cells of the clypeolabrum flanking the *rols7*-positive cells on the dorsal side.

formation is normal in $rols^{P1729}$ mutant embryos (data not shown). As many genes have a function in myogenesis and neurogenesis, we checked in addition for the gross morphology of the nervous system by staining with Mab22C10 (Zipursky et al., 1984). Again, no major specific aberrations in the nervous system were detectable (data not shown).

Muscle founder cells are correctly determined in *rols* mutants and recruit myoblasts to form syncytial precursor cells

Myogenesis can be disturbed by disrupting muscle progenitor determination leading to a loss of founder cells, by interfering with muscle precursor formation or the recruitment and fusion of fusion-competent myoblasts. Therefore, we addressed the question of whether muscle founder cells and/or muscle precursor cells are correctly determined in *rols* mutants. We analyzed the formation and development of muscle founders and muscle precursors in the *rols* deficiency *Df(3L)BK9* using the enhancer trap line rP298. In a wild-type background, rP298 expresses β-galactosidase in the nuclei of all muscle founder cells at the extended germ band stage and in the nuclei of all muscle precursor cells at later stages of embryonic development (Fig. 2A) (Nose et al., 1998). We detect a similar pattern of β-galactosidase expression in embryos homozygous for the *Df(3L)BK9* deficiency. This result indicates that founder/precursor cell formation occurs normally in the absence of *rols* function (Fig. 2B). As we assume that the deficiency represents the null situation for *rols*, this shows that at least the majority of muscle founder/precursor cells are specified correctly in the absence of *rols* function.

As already mentioned, two steps may be distinguished during the formation of the myofibers at the morphological level (Bate, 1990). First, the founder cells recruit some myoblasts to form precursors with two to four nuclei. Second, these precursor cells attract further FCMs to form the multinucleated myotubes with up to 24 nuclei. When we analyze later embryonic stages according to the rP298 pattern, we observe from stage 15 onwards a reduced number of rP298-positive nuclei (compare Fig. 2C with 2D). To determine more precisely at which step myotube formation is blocked in *rols* mutants at least for one individual muscle, we analyzed the *even skipped* pattern, which marks dorsal muscle 1 and the pericardial cells (Fig. 2); as it was shown that Eve is expressed in fusion mutants (Paululat et al., 1999a). In embryos homozygous for the $rols^{P1729}$ allele, Eve expression in pericardial cells appears normal; however, the DA1 muscle expresses Eve in two to five nuclei, as opposed to up to 14

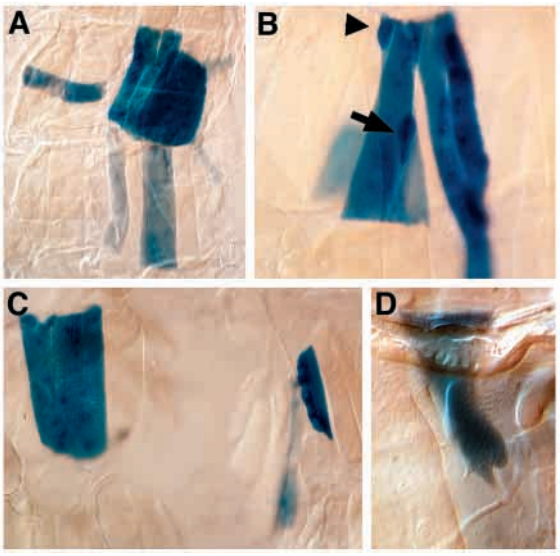

Fig. 7. *rols* mutant cells are able to fuse with wild-type myoblasts as fusion-competent cells and exhibit fusion defects as muscle precursor cells. Descendants of the transplanted cells are visualized by histochemical β-galactosidase staining only in syncytial tissues that co-express *GAL4* and *UAS-lacZ*. Clones in the ventral and lateral larval muscles after transplantation of homozygous *rols* null mutant cells are shown. (A) Ventrolateral larval muscle clone spanning two segments derived from a transplantation of three to five homozygous mutant cells. (B) Most descendants of *rols* mutant cells behave normally in wild-type background. This ventral muscle clone shows two additional structures, one spindle-like (arrow) and one more compact (arrowhead) with two to three nuclei each. (C) Ventral bilateral clone in two larval muscles, a normal syncytium and a small elongated striated syncytium with four nuclei, probably representing a muscle precursor. (D) Part of a ventral muscle clone with a very shortened mini-muscle attached correctly to the epidermis.

nuclei in wild-type embryos. As the P-element-induced mutation might not be a null mutation for *rols*, we analyzed Eve expression in the deficiency *Df(3L)BK9*. In this *rols* null mutant, development for dorsal muscle 1 proceeds beyond the founder cell stage, as we again observe two to three nuclei (Fig. 2H). This is in contrast to the situation in *mbc* mutants, where the Eve-positive cells are clearly mononucleated (Carmena et al., 1998). These data suggest that in the absence of *rols*, muscle founder cells fuse with a restricted number of myoblasts to become muscle precursor cells, but that muscle development becomes stalled at this stage.

Ultrastructural analysis of *rolling pebbles* mutant embryos

For the steps that follow muscle precursor formation, light microscopy is not sufficient to resolve at which step myogenesis is interrupted during myofiber formation. We therefore analyzed the *rols* phenotype of the strong EMS allele $rols^{AD328}$ at the electron microscopic level. At early stage 13, myoblasts are closely apposed to each other (Fig. 3A) and morphologically indistinguishable from wild-type myoblasts. Syncytial cells with three nuclei are present, which presumably represent muscle precursor cells. However, by stage 14, precursor cells and FCMs have lost their close alignment and have scattered throughout the mesoderm (Fig. 3B). In wild-type embryos, a prefusion complex forms between precursor cells and FCMs after cell adhesion (Doberstein et al., 1997). In $rols^{AD328}$ mutants, the prefusion complex and later steps of fusion are absent. Whether this failure of cell alignment and further progression in myogenesis is due to defects in the precursor cells, in the FCMs or both remains to be clarified. To address this and other issues, we cloned the *rols* gene.

rols is localized at 68F and spans approximately 60 kb

The *rols* allele $rols^{P1729}$ provided an entry point into the genomic region and facilitated the identification (for details see Materials and Methods) of a 5 kb fragment of genomic DNA that flanks the P-insert. We used the 5 kb genomic fragment to isolate several genomic clones from phage libraries. We used the phage clones to screen a cDNA library and we sequenced all positive cDNA clones. These data gave access to the corresponding sequence of the *Drosophila* genome in the database (Adams et al., 2000). From the analysis of the isolated cDNAs, we identified two putative *rols* transcripts (5.8 and 6.3 kb) that differ at the 5′ end. These transcripts are named *rols7* (long transcript) and *rols6* (short transcript) (Fig. 4A). The encoded proteins are named Rols7 and Rols6, accordingly. Sequence comparison between the cDNAs and the genomic region revealed a complex intron-exon structure as well as common and selective exons (Fig. 4A). The six most 3′ localized exons are common to both transcripts and include a single polyadenylation signal, as well as the majority of the open reading frame (Fig. 4A). *rols6* contains two unique 5′ exons with the translation initiation codon localized in the second exon. The longer transcript contains two specific exons as well as the translation initiation codon in the second exon unique for *rols7*. At the 5′ site, *rols* is flanked by another gene previously identified as coding for Semaphorin 5c (Khare et al., 2000). The positions of the P-element integrations in $rols^{P1027}$ and $rols^{P1729}$ were determined by cloning and analyzing flanking sequences. This places the P-element in *P1729* in intron sequences shared by the *rols7* and the *rols6* transcription units (Fig. 4A). In *P1027*, the P-element is inserted approximately 70 bp upstream of the transcription initiation site of *rols6*, which has been determined by primer extension analysis (data not shown). This P-element is localized in the second intron of *rols7*. Both P-elements are inserted into the same gene, and excision of both P-elements can revert the *rols* phenotype. This provides strong evidence that this gene is *rols*.

rols encodes a protein with an N-terminal RING-finger, nine ankyrin repeats and a TPR-repeat

Sequence analysis of *rols6* and *rols7* predicts two distinct large proteins to be encoded by *rols* (Fig. 5). Conceptual translation indicates that *Rols7* encodes a 1900 amino acid protein, whereas *rols6* reveals an open reading frame of 1670 amino acids. Rols6 harbors 79 specific amino acids at the N terminus with a high level of acetic amino acids, while Rols7 is characterized by 309 specific amino acids at the N terminus (Fig. 5).

In order to gain insight into the possible function of Rols we analyzed the predicted domains of the protein using the SMART-tool of the EMBL (Schultz et al., 1998; Schultz et al., 2000). Two striking structural features are depicted in the

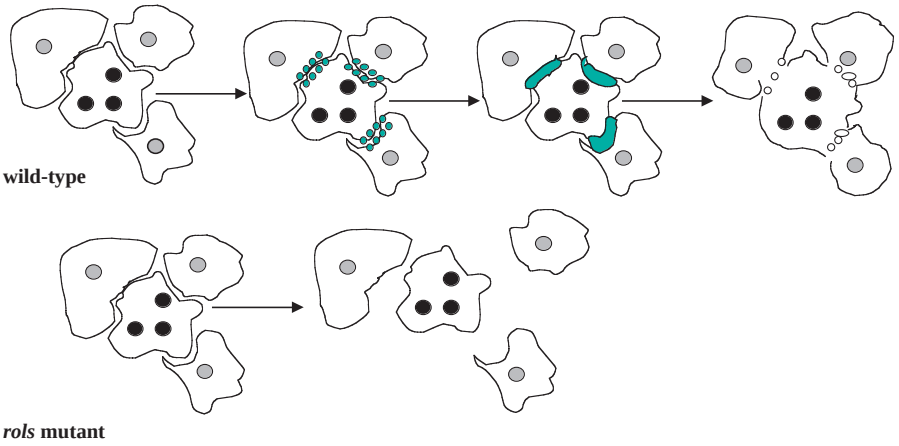

Fig. 8. Rolling pebbles signaling between precursor cells and FCMs is necessary to stabilize cell contacts and to initiate progression in fusion such as prefusion complex formation. In wild-type myoblasts fusion proceeds after adhesion of FCMs to precursor cells by forming the prefusion complex of paired vesicles followed by plaque formation of the opposing membranes and subsequent membrane breakdown. In *rols* mutants the initial adhesion of precursor cells with FCMs is observed but cells scatter instead of proceeding to prefusion complex formation, showing its first function before *sns* and *blow* but after the initial Duf function.

common part of Rols6 and Rols7 (Fig. 5). Both proteins contain a RING-finger motif that has been shown to participate in protein-protein interactions (Freemont, 2000). In some cases, specialized RING-fingers are essential parts of proteins involved in the ubiquitination pathway having E3-ligase activity (Freemont, 2000). Furthermore, nine ankyrin repeats are predicted which represent a classical structure for protein-protein interactions. Close to the C terminus, an additional protein-protein interaction motif is found, a so-called TPR motif eventually overlaid by a coiled-coil region (Blatch and Lassle, 1999). Apart from these structural domains, the database research revealed no homologous proteins in other organisms. The predicted structure of Rols implies multiple protein-protein interactions during myoblast fusion.

Developmental expression profile of *rols7* and *rols6* during embryogenesis

We determined the temporal expression profiles for *rols6* and *rols7* by RT-PCR using primers specific for each transcript. Poly $(A)^+$ RNA from embryos collected at 4 hours intervals was isolated and purified. Using this approach, we first detected the characteristic fragment for *rols7* between 4 and 8 hours of development. *rols7* reaches a maximum level of expression at 8-12 hours and then declines to low levels in 12- to 16- and 16- to 24-hour-old embryos (Fig. 4B). We first detect the *rols6* transcript between 4-8 hours of development. *rols6* reaches maximum levels between 12-16 hours of development and remains at high levels up to 24 hours (Fig. 4B). Therefore, the expression of *rols7* coincides with the time course of the process of fusion between founder/precursor cells and FCMs, while maximum levels of *rols6* occur at later stages.

Expression of the *rols7* starts at the extended germ band stage in progenitor/founder cells and persists during fusion to myofibers of the body wall and pharyngeal muscles

The characterization of the P-element-induced alleles $rols^{P1027}$ and $rols^{P1729}$ provided strong evidence that the identified gene is responsible for the *rols* phenotype. Furthermore *rols* mRNA is present during the time of myoblast fusion in embryonic mRNAs. However, the RT-PCR experiments do not resolve the tissue(s) in which the particular transcripts are expressed. Therefore we used in situ hybridization to analyze the cellular transcript distribution during embryogenesis with specific probes for *rols7* and *rols6* (see Materials and Methods). *rols7* expression is first observed at the extended germ band stage in a few mesodermal cells per segment (Fig. 6C) and in the head region where the precursors of the pharyngeal muscles arise (Fig. 6D). Transient expression of *rols7* is observed in the visceral mesoderm during germband retraction (Fig. 6D) and then vanishes. The functional significance of *rols7* expression in the visceral mesoderm remains to be clarified.

During germband retraction, myogenesis proceeds and several cells express *rols7* in every segment. The precursors formed at stage 13 accumulate *rols7* mainly around one nucleus (Fig. 6E,F). The mRNA is of very low abundance so that colocalization experiments with other markers were not of sufficient quality. Therefore, we compared the expression pattern of *rols7* to that of Mef2 which is expressed throughout embryogenesis (Nguyen et al., 1994; Lilly et al., 1995; Bour et al., 1995; Taylor, 1995) and with rP298. In these experiments we used rP298 to identify muscle founder cells. Only a few myoblasts are rP298 positive (Fig. 6H) when compared to Mef2 in all nuclei of myoblasts (Fig. 6G). By contrast, the *rols7* expression (Fig. 6E) is very similar to the expression pattern of rP298 (compare Fig. 6E with 6H) in the somatic mesoderm, indicating that *rols7* is expressed in progenitor/founder cells up to the muscle precursor cells.

The pharyngeal musculature is also syncytial and we show that the development of these muscles is also dependent of *rols* (Fig. 1H). Previously, we have shown that *sticks and stones* mutants show fusion defects in the development of the pharyngeal musculature (Paululat et al., 1995) (EMS alleles previously named *rost*) (Paululat at al., 1999; Bour et al., 2000). Our expression analysis indeed revealed two distinct cell populations in the clypeolabrum at late stage 12 (Fig. 6K-N) that later invaginate to form the pharyngeal muscles. *rols7* and rP298 show overlapping expression patterns in the ventrally localized mesodermal cells of the clypeolabrum (Fig. 6K,M), while *sns* transcripts, which mark FCMs, are localized in the dorsally adjacent mesodermal cells (Fig. 6N). As the *rols7* pattern overlaps with the rP298 pattern (reflecting *duf* expression), we suggest that also the pharyngeal musculature consists of founder cells and fusion competent cells.

rols6 exhibits a distinct transcript profile. At the extended germ band stage, *rols6* is expressed strongly in the invaginating endoderm (Fig. 6I). At stage 14 and later, *rols6* is expressed in ectodermal cells of the head region (Fig. 6L) and the

developing malpighian tubules are stained weakly (Fig. 6J). Furthermore, a stripe-like expression pattern is observed in the ectoderm at stage 16 (data not shown). The expression pattern of *rols6* is thus very similar to the enhancer trap pattern observed in $rols^{P1729}$ in the apodemes and in $rols^{P1027}$ in the endoderm (Fig. 6A,B). This distinct distribution shows that *rols7* is transcribed during development of the *Drosophila* pharyngeal and body wall musculature. As only *rols7* is detected in myoblasts during fusion and ceases beyond stage 14, we propose that this is the relevant transcript. Comparison with the rP298 staining indicates that *rols7* is expressed starting with progenitors/founders up to muscle precursor cells during myoblast fusion.

Rols is required in muscle precursor cells for the progression of muscle fusion

The expression profile of *rols7* suggests that it may act at the level of muscle founder or precursor cells. The analysis of the mutant phenotype reveals the appearance of elongated mini-muscles containing more than one nucleus in several cases, which was shown in detail for dorsal muscle 1. In order to test the hypothesis that Rols acts on the precursor level, we examined the fusion competence of *rols* mutant cells in a wild-type background with a cell transplantation strategy adapted from Klapper et al. (Klapper et al., 2001a). The wild-type host embryos contain a *daGAL4* construct, which is expressed in all cells, while the transplanted cells of the *rols* mutant contain an UAS-*lacZ* gene. Thus *rols* mutant cells in a wild-type background will express only the reporter β-galactosidase after successful cell fusion with the wild-type host cells. One might expect that successful fusion is dependent on the transplanted cell type. As we transplant ventral mesodermal cells at the cellular blastoderm, they might develop either to founders or to fusion-competent cells the last being the far more abundant cell type. If Rols is indeed required in muscle precursor cells – as suggested by its expression and mutant phenotype – but not in FCMs, wild-type precursor cells of the host embryo should be able to recruit FCMs from *rols* mutants to form myofibers that express β-galactosidase. However, transplanted donor cells that develop into precursors should not be able to recruit further host myoblasts for fusion.

We chose the deficiency *Df(3L)BK9* as a null allele for *rols*. As we transplant mutant mesodermal cells into wild-type embryos, the loss of *rols6* in the endoderm and *semaphorin 5c* in the ectoderm as well as the loss of other genes localized in the deficiency should not influence this assay. After transplantation the recipient embryos were allowed to develop until 3rd instar larval stage and the muscle pattern was examined with respect to myotube development.

From a total of 191 transplantations, 119 embryos (62%) reached the third larval instar. Of these larvae, 53 showed a clone derived from the transplanted cell descendants, which had fused to host cells. In 11 cases, the clones derived from homozygous *rols* donors and were found in the musculature (Fig. 7). We compared these clones at the morphological level with 42 clones derived from control donors (hetero- or homozygous blue balancer donors) with regard to size, shape and correct attachment. The 11 clones derived from homozygous *rols* embryos were found in the ventral, lateral and dorsal body wall muscles of third instar larvae, and they demonstrate that at least a population of *rols* mutant cells is able to fuse with host cells to multinucleate myotubes (Fig. 7A-C). These data show that *rols* mutant cells can participate in fusion. As these large muscle clones are abundant, we suggest that in these clones *rols* mutant FCMs fuse with wild-type founders. Moreover we found the clones at the same positions in the thoracic and the abdominal segments and approximately in the same size (Fig. 7A) as the control clones. In the case of transplanted *rols* mutant cells, we found in five larvae small muscle-like structures referred as mini-muscles (Fig. 7B-D) or compact, not elongated muscle-like cells (Fig. 7B, arrowhead). These mini-muscles represent only a small part of the observed clones and contain two to five nuclei. We consider these mini-muscles as precursor cells formed by a *rols* mutant founder and fusion competent cells of the donor. In one case, we observe two of these mini-muscles close to each other. We interpret the appearance of duplicated mini-muscles as evidence for two precursor cells derived from asymmetric cell division of a progenitor. On the basis of these observations, we conclude that the mini-muscles can be hybrid precursor cells derived from a *rols* mutant founder (with *UAS-lacZ*) and one to four cells of the wild-type host (carrying *daGAL4*). Therefore we suggest that Rols acts in muscle precursors to recruit further FCMs. With *rols*-deficient cells, we found that nearly 50% of the host embryos contain mini-muscles. We detected in four cases of the 43 control transplantations (in about 10% of the embryos) with hetero- or homozygous balancer embryos similar defects and assumed that this is probably a result of a dosis effect or may be due to incompatibility of homozygous balancer cells in the mosaic clones.

In summary, the ultrastructural data, the immunostaining of founder and precursor cells as well as the transplantation assays clearly show that *rols* is required for proceeding from the precursor level towards multinuclear myotubes. This correlates convincingly with the immunohistological localization of the Rols protein at the cell borders as shown by Menon and Chia (Menon and Chia, 2001).

DISCUSSION

The *rolling pebbles* gene encodes two distinct transcripts with independent transcription initiation sites: *rols7* and *rols6*. *rols7* starts to be expressed in muscle progenitor cells but is detected still in muscle precursors until stage 14. The encoded proteins Rols7 and Rols6 share several putative protein-protein interaction domains as a RING-finger motif, ankyrin and a TPR repeat. Rols7 is distinguishable by 309 unique N-terminal amino acids, Rols6 by 79 unique N-terminal amino acids. We focus on the possible role of Rols7 during myoblast fusion.

Muscle precursor cells are correctly determined in *rols* mutants

An important question is at which cellular level *rols* is essential. The early enhancer trap pattern of rP298, which reflects the expression of the *duf* gene, is normal in *rols* null mutants, suggesting that muscle progenitors and founders are specified correctly. However, after stage 14, the number of rP298-positive cells decreases significantly. Electron microscopic analysis of $rols^{AD328}$ shows cells with three nuclei, presumably muscle precursor cells. Further phenotypical analysis of *rols* mutants revealed small myofiber-like structures

with only a few nuclei also at later stages of myogenesis. These muscle precursors stretch and contact the epidermis, as it has been observed in other fusion mutants (Paululat et al., 1999b). From the presence of muscle precursors in *rols* mutants we deduce that *rols* is not essential for their formation. It is possible that another protein compensates for *rols* function in these early fusion steps leading to precursor formation. Further on, muscle precursors attract additional myoblasts for fusion in the wild type. In the *rols* deficiency allele these precursor cells form the small myofiber-like cells and seem to attract some fusion-competent cells, as we observed filopodia of fusion-competent cells directed towards these myofiber-like cells; however, they do not fuse to them.

rols is required only in muscle precursor cells for recruitment and fusion with FCMs

In in situ hybridization studies, we detect *rols7* RNA only in muscle progenitor descendants, implying that Rols7 is essential in the precursor cells but not in the surrounding myoblasts. However, the *rols7* transcript is rare and we would not have detected a tenth of the mRNA level in FCMs. We therefore established an in vivo system to determine the functional competence of muscle precursor cells and fusion-competent cells by transplanting *rols*-deficient cells into wild-type host embryos. The majority of the transplanted cells formed multinucleated myofibers with normal shape and attachment to the epidermis. This clearly showed that transplanted fusion-competent cells from *rols*-deficient embryos (these are far more abundant than muscle founder cells) behave like wild-type cells in the establishment of multinucleated myofibers, and Rols is not required in myoblasts for fusion to wild-type precursor cells. By contrast, we observed mini-muscles with two to five nuclei, which originate from fusion between transplanted and host cells. Based on these results, we concluded that the muscle progenitor cells are correctly determined in *rols* mutants and that they divide to two founder cells, which is supported by our observation that occasionally two precursor cells are located close to each other in the host embryo. These precursor cells represent heterokaryons of $rols^{-}$ and $rols^{+}$ nuclei; nevertheless, they behave like Rols-defective precursors. Thus, the recruited $rols^{+}$ nuclei obviously do not complement for the $rols^{-}$ nucleus. As a consequence, we observe mini-muscles instead of normal sized myotubes. It is well known that the muscle progenitors and founders are determined by intrinsic components like transcription factors and extrinsic factors as signaling molecules from the overlaying ectoderm (Baker and Schubiger, 1995; Baylies et al., 1998). This integration of determining factors is probably missing in $rols^{+}$ nuclei of the mini muscles, and we suggest that this is the reason that they are unable to activate *rols* and thus to complement the *rols*-deficient founder cell nucleus. Determined founder cells recruit one to three myoblasts for muscle precursor cell formation. After this step, development to myofibers is interrupted in *rols* mutant cells in the wild-type background. These data are confirmed by our observation of cells with up to three Eve-positive nuclei characteristic for dorsal muscle 1 in the analyzed *rols* deficiency and our ultrastructural analysis, showing cells with three nuclei in *rols* mutants.

We conclude from these observations that the first phase of myoblast fusion leading to muscle precursor cells with two to four nuclei, is unaffected in *rols* mutants. Another indication of a two step fusion process might be the observation that Eve-positive precursors for dorsal muscle 1 are formed in a strong *sns* allele (Paululat et al., 1999a). For the second phase of fusion leading to multinucleated myofibers, Rols7 is essential in muscle precursor cells.

Comparison with the onset of the *rols7* mRNA detection already at progenitor/founder stage to the analysis of the mutant phenotype and the transplantation assays shows biological activity of Rols in the precursor cells with two to four nuclei. This correlates well with a change in protein distribution from uniform cytoplasmic to membrane associated during fusion. This reorganization is dependent on Duf (Menon and Chia, 2001). Our data obtained with the rP298 enhancer trap line suggest that the early activity of Duf is independent of Rols, while the maintenance of expression is dependent on the Rols-mediated fusion steps.

Integration of *rolling pebbles* into the cascade of myoblast fusion relevant genes

As described in detail in the introduction myofiber formation is prefigured by the specification of progenitor cells out of an equivalence group. After asymmetric division of a progenitor cell two founder cells are determined that lead to two distinct myofibers, or to one myofiber and one founder cell set aside to form an adult muscle during metamorphosis. Subsequently myoblast fusion starts. On the morphological level precursor cells with two to three nuclei can be distinguished (Bate, 1990), then fusion proceeds by recruiting additional myoblasts resulting in a mature myofiber. It has recently been shown that *duf* is essential for this first step of fusion (Ruiz-Gómez et al., 2000) as well as *mbc* (Carmena et al., 1998), while other fusion mutants (e.g. *blow)* allow precursors to form but stop at subsequent steps of fusion (Paululat et al., 1999b). Our analysis enables us to integrate *rols* into this cascade. We show that precursor cells are often correctly determined in *rols* mutants but that they are not able to recruit additional myoblasts for fusion. We started to analyze this feature in detail by the analysis of individual muscles first by Eve expression in dorsal muscle 1 in *rols*-deficient embryos. For this muscle, we can clearly show that founder cells start to fuse to mini-muscles of two to four nuclei, presumably precursor cells. Thus *rols* should be positioned downstream of *duf*, where even the muscle precursors are not formed. However, *duf* may have additional functions later in the fusion process. The transplantation assays furthermore demonstrate that *rols* function is restricted to the muscle precursor cells and not needed in FCMs. The phenotypic analysis at the EM level revealed that *rols* mutants neither form a prefusion complex nor electron-dense plaques, which shows that *rols* is required earlier than *blow* and *sns*. In agreement with these data, Menon and Chia (Menon and Chia, 2001) have shown that the Rols protein distribution is not affected in *sns* and *blow*, but is clearly dependent on *duf*.

rols encodes a molecule with several protein-protein interaction domains that mediate a signal between precursors and FCMs

Both Sns and Duf belong to the immunoglobulin superfamily and have been inferred to mediate cell adhesion, Sns in FCMs and Duf in muscle progenitors and founders. Duf furthermore attracts myoblasts to founders. Whether *duf* is also involved in

the cell fusion process itself is not known, as the mutations available so far are null mutants. Rols clearly belongs to a new class of molecules that are essential for fusion. In stage 13 embryos, cell adhesion between muscle precursor cells and fusion-competent cells initially looks like wild-type but cells appear to lose contact. After cell adhesion, we would expect that a signaling mechanism acts between both cell types. Rols may mediate directly or indirectly a signal after recognition and adhesion between precursor cells and fusion-competent cells, leading to later steps of fusion. In the absence of these later steps, adhesion may not be maintained. Although the prefusion complex and the subsequent steps are symmetrically exhibited by both cell types, it might be expected that a specific signal from the precursor cell to the fusion-competent cells is essential to initiate such a process (Fig. 8). Ankyrin repeats are especially predestinated to mediate further steps in a signal transduction cascade on the precursor site. The RING-finger structure is a further candidate for protein-protein interaction in the precursor cell. Proteins with such a domain have been found in many organisms, and some of them are involved in the ubiquitin-mediated protein-degradation pathway as they function as E3-ubiquitin ligases (Zheng et al., 2000). However, comparison of the RING-finger sequence present in Rols to a *Drosophila Cbl* homolog reveals no agreement of otherwise very conserved amino acids between human, *C. elegans* and *D. melanogaster Cbl* homologs. Therefore, a role for Rols in an ubiquitination pathway driven protein degradation is quite unlikely, but cannot be completely excluded. Taken together, the isolation and characterization of *rols*, the very specific phenotype of mutants analyzed so far and its extremely interesting distribution during the fusion process points to a specialized function exclusively on the side of the muscle precursor cell. So the search for possible interacting partners besides *Drosophila* Titin (Menon and Chia, 2001) will probably lead to further insights into the molecular mechanisms of the fusion process.

We thank W. Janning, A. Nose, the *Drosophila* Stock Centers in Bloomington and Umea for fly stocks, our colleagues M. Frasch, D. Kiehart and H. T. Nguyen for antibodies, and S. Abmayr for *sns* cDNA. We thank very much Sree Devi Menon and Bill Chia for communication and exchange of manuscripts prior to publication. We are indebted to R. Klapper and W. Janning for communication of methods before publication and for supply of the *daGAL4* strain. We thank Heike Sauer for excellent secretarial assistance, Peter Seum, Miriam Findt and Dominik Helmecke for technical assistance, and Thomas Kremser for technical advice. This research was supported by the Deutsche Forschungsgemeinschaft to R. R.-P. (Re 628/10-3 and 10-4, SFB 397), the Kerckhoff-Stiftung and the Fonds der Chemischen Industrie. A. M. M. is an Associate investigator of the Howard Hughes Medical Institute. This research was also supported by NSF grant (IBN-0077727) to J. B. S.

REFERENCES

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al. (2000). The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185-2195.

Baker, R. and Schubiger, G. (1995). Ectoderm induces muscle-specific gene expression in *Drosophila* embryos. *Development* **121**, 1387-1398.

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

Bate, M. and Martinez Arias, A. (1993). *The Development of* Drosophila melanogaster. Cold Spring Harbor Laboratory, Plainview, NY: Cold Spring Harbor Laboratory Press.

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

Blatch, G. L. and Lassle, M. (1999). The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. *BioEssays* **21**, 932-939.

Bour, B. A., O'Brien, M. A., Lockwood, W. L., Goldstein, E. S., Bodmer, R., Taghert, P. H., Abmayr, S. M. and Nguyen, H. T. (1995). *Drosophila* MEF2, a transcription factor that is essential for myogenesis. *Genes Dev.* **9**, 730-741.

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.

Buff, E., Carmena, A., Gisselbrecht, S., Jiménez, 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.

Burchard, S., Paululat, A., Hinz, U. and Renkawitz-Pohl, R. (1995). The mutant *not enough muscles* (*nem*) reveals reduction of the *Drosophila* embryonic muscle pattern. *J. Cell Sci.* **108**, 1443-1454.

Buttgereit, D., Paululat, A. and Renkawitz-Pohl, R. (1996). Muscle development and attachment to the epidermis is accompanied by expression of β3 and β1 tubulin isotypes, respectively. *Int. J. Dev. Biol.* **40**, 189-196.

Carmena, A., Bate, M. and Jimenez, 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. (1998). Combinatorial signaling codes for the progressive determination of cell fates in the *Drosophila* embryonic mesoderm. *Genes Dev.* **12**, 3910-3922.

Davies, S. A., Goodwin, S. F., Kelly, D. C., Wang, Z., Sözen, M. A., Kaiser, K. and Dow, A. T. (1996). Analysis and inactivation of *vha55*, the gene encoding the vacuolar ATPase B-subunit in *Drosophila melanogaster* reveals a larval lethal phenotype. *J. Biol. Chem.* **48**, 30677-30684.

Doberstein, S. K., Fetter, R. D., Mehta, A. Y. and Goodman, C. S. (1997). Genetic analysis of myoblast fusion: *blown fuse* is required for progression beyond the prefusion complex. *J. Cell Biol.* **136**, 1249-1261.

Dressel, U., Thormeyer, D., Altincicek, B., Paululat, A., Eggert, M., Schneider, S., Tenbaum, S. P., Renkawitz, R. and Baniahmad, A. (1999). Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. *Mol. Cell. Biol.* **19**, 3383-3394.

Erickson, M. R., Galletta, B. J. and Abmayr, S. M. (1997). *Drosophila myoblast city* encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization. *J. Cell Biol.* **138**, 589-603.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M. (1987). Characterization and localization of the even-skipped protein of *Drosophila*. *EMBO J.* **6**, 749-759.

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

Frasch, M. and Leptin, M. (2000). Mergers and acquisitions: unequal partnerships in *Drosophila* myoblast fusion. *Cell* **102**, 127-129.

Freemont, P. S. (2000). RING for destruction? *Curr. Biol.* **10**, R84-R87.

Galletta, B. J., Niu, X. P., Erickson, M. R. S. and Abmayr, S. M. (1999). Identification of a *Drosophila* homologue to vertebrate *crk* by interaction with MBC. *Gene* **228**, 243-252.

Gisselbrecht, S., Skeath, J. B., Doe, C. Q. and Michelson, A. M. (1996). *heartless* encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the *Drosophila* embryo. *Genes Dev.* **10**, 3003-3017.

Goubeaud, A., Knirr, S., Renkawitz-Pohl, R. and Paululat, A. (1996). The *Drosophila* gene *alien* is expressed in the muscle attachment sites during embryogenesis and encodes a protein highly conserved between plants, *Drosophila* and vertebrates. *Mech. Dev.* **57**, 59-68.

Holz, A., Meise, M. and Janning, W. (1996). Adepithelial cells in *Drosophila melanogaster*: origin and cell lineage. *Mechanisms of Development* **62**, 93-101.

Hooper, J. E. (1986) Homeotic gene function in the muscles of *Drosophila* larvae. *EMBO J.* **5**, 2321.

Khare, N., Fascetti, N., DaRocha, S., Chiquet-Ehrismann, R. and

Baumgartner, S. (2000). Expression pattern of two new members of the semaphorin family in *Drosophila* suggest early functions during embryogenesis. *Mech. Dev.* **91**, 393-397.

Kimmerly, W., Stultz, K., Lewis, S., Lewis, K., Lustre, V., Romero, R., Benke, J., Sun, D., Shirley, G., Martin, C. and Palazzolo, M. (1996). A P1-based physical map of the *Drosophila* euchromatic genome. *Genome Res.* **6**, 414-430.

Klapper, R. (2000). The longitudinal visceral musculature of *Drosophila melanogaster* persists through metamorphosis. *Mech. Dev.* **95**, 47-54.

Klapper, R., Heuser, S., Strasser, T. and Janning, W. (2001a). A new approach reveals syncytia within the visceral musculature of *Drosophila melanogaster*. *Development* **128**, 2517-2524.

Klapper, R., Stute, Ch., Schomaker, O., Strasser, T., Janning, W., Renkawitz-Pohl, R. and Holz, A. (2001b). The formation of syncytia within the visceral musculature of the *Drosophila* midgut is dependent on *duf*, *sns* and *mbc*. *Mech. Dev.* (in press).

Leiss, D., Hinz, U., Gasch, A., Mertz, R. and Renkawitz-Pohl, R. (1988). β3-*tubulin* expression characterizes the differentiating mesodermal germ layer during *Drosophila* embryogenesis. *Development* **104**, 525-531.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B. M., Schulz, R. A. and Olson, E. N. (1995). Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*. *Science* **267**, 688-693.

Luo, L., Liao, Y. J., Jan, L. Y. and Jan, Y. N. (1994). Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev.* **8**, 1787-1802.

Meise, M. and Janning, W. (1993). Cell lineage of larval and imaginal thoracic anlagen cells of *Drosophila melanogaster* as revealed by single-cell transplantations. *Development* **118**, 1107-1121.

Menon, S. D. and Chia, W. (2001). *Drosphila* Rols7: A novel multidomain protein essential for myoblast fusion that localises at fusion sites and recruits D-Titin in response to the myoblast attractant Dumbfounded. *Dev. Cell* (in press).

Nguyen, H. T., Bodmer, R., Abmayr, S. M., McDermott, J. C. and Spoerel, N. A. (1994). *D-mef2*: a *Drosophila* mesoderm-specific MADS box-containing gene with a biphasic expression profile during embryogenesis. *Proc. Natl. Acad. Sci. USA* **91**, 7520-7524.

Nolan, K. M., Barrett, K., Lu, Y., Hu, K. Q., Vincent, S. and Settleman, J. (1998). Myoblast city, the *Drosophila* homolog of DOCK180/CED-5, is required in a Rac signaling pathway utilized for multiple developmental processes. *Genes Dev.* **12**, 3337-3342.

Nose, A., Isshiki, T. and Takeichi, M. (1998). Regional specification of muscle progenitors in *Drosophila*: the role of the *msh* homeobox gene. *Development* **125**, 215-223.

Paululat, A., Burchard, S. and Renkawitz-Pohl, R. (1995). Fusion from myoblasts to myotubes is dependent on the *rolling stone* gene (*rost*) of *Drosophila*. *Development* **121**, 2611-2620.

Paululat, A., Breuer, S. and Renkawitz-Pohl, R. (1999a). Determination and development of the larval muscle pattern in *Drosophila melanogaster*. *Cell Tissue Res.* **296**, 151-160.

Paululat, A., Holz, A. and Renkawitz-Pohl, R. (1999b). Essential genes for myoblast fusion in *Drosophila* embryogenesis. *Mech. Dev.* **83**, 17-26.

Ruiz-Gómez, M. (1998). Muscle patterning and specification in *Drosophila*. *Int. J. Dev. Biol.* **42**, 283-290.

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.

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.

San Martin, 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.

Schultz, J., Milpetz, F., Bork, P. and Ponting, C. P. (1998). SMART, a simple modular architecture research tool: Identification of signalling domains. *Proc. Natl. Acad. Sci. USA* **95**, 5857-5864.

Schultz, J., Copley, R. R., Doerks, T., Ponting, C. P. and Bork, P. (2000). SMART: A Web-based tool for the study of genetically mobile domains. *Nucleic Acids Res.* **28**, 231-234.

Spradling, A. C., Stern, D., Kiss, I., Roote, J., Laverty, T. and Rubin, G. M. (1995). Gene disruptions using P transposable elements: an integral component of the *Drosophila* genome project. *Proc. Natl. Acad. Sci. USA* **92**, 10824-10830.

Taylor, M. V., Beatty, K. E., Hunter, K. and Baylies, M. K. (1995) Drosophila MEF2 is regulated by twist and is expressed both in the primordia and differentiated cells of the embroynic somatic, visceral and heart musculature. *Mech. Dev*. **50**, 29-41.

Taylor, M. V. (2000). Muscle development: molecules of myoblast fusion. *Curr. Biol.* **10**, R646-R648.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**, 81-85.

Wodarz, A., Hinz, U., Engelbert, M. and Knust, E. (1995). Expression of *crumbs* confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*. *Cell* **82**, 67-76.

Zheng, N., Wang, P., Jeffrey, P. D. and Pavletich, N. P. (2000). Structure of a c-CBL-UcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* **102**, 533-539.

Zipursky, S. L., Venkatesh, T. R., Teplow, D. B. and Benzer, S. (1984). Neuronal development in the *Drosophila* retina: monoclonal antibodies as molecular probes. *Cell* **36**, 15-26.