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A key role of *Pox meso* in somatic myogenesis of *Drosophila*

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The Pax gene *Pox meso* (*Poxm*) was the first and so far only gene whose initial expression was shown to occur specifically in the anlage of the somatic mesoderm, yet its role in somatic myogenesis remained unknown. Here we show that it is one of the crucial genes regulating the development of the larval body wall muscles in *Drosophila*. It has two distinct functions expressed during different phases of myogenesis. The early function, partially redundant with the function of *lethal of scute* [*l(1)sc*], demarcates the 'Poxm competence domain', a domain of competence for ventral and lateral muscle development and for the determination of at least some adult muscle precursor cells. The late function is a muscle identity function, required for the specification of muscles DT1, VA1, VA2 and VA3. Our results led us to reinterpret the roles of *l(1)sc* and *twist* in myogenesis and to propose a solution of the '*l(1)sc* conundrum'.

KEY WORDS: *Drosophila*, *Pox meso*, Pax gene, *lethal of scute* conundrum, Somatic myogenesis, Muscle progenitors, Muscle patterning

INTRODUCTION

The development of the complex pattern of the larval body wall muscles of *Drosophila* provides an excellent paradigm of how a final pattern is established through precise genetic control (reviewed by Bate, 1993; Baylies et al., 1998). Each of the abdominal hemisegments A2-A7 has 30 identifiable individual muscles (Bate, 1993) that develop from the somatic mesoderm. This process is initiated when the invaginated mesoderm migrates dorsolaterally under the ectoderm (Beiman et al., 1996; Gisselbrecht et al., 1996) and is prepatterned by the segmentation genes (Lee and Frasch, 2000; Riechmann et al., 1997): the product of *sloppy paired* (*slp*), whose activity is maintained by the ectodermal Wingless (Wg) signal, restricts high levels of the bHLH transcription factor Twist (Twi) to the mesodermal regions below the posterior portions of the ectodermal parasegments (Baylies et al., 1998). These high levels of Twi function as a myogenic switch, separating the posterior somatic and cardiac mesoderm from the anterior visceral mesoderm and fat body (Baylies and Bate, 1996; Dunin Borkowski et al., 1995). When the dorsal migration of the mesoderm is complete, these metamerically repeated Slp or high Twi domains are further subdivided along the dorsoventral axis by the ectodermal signal Dpp (Staehling-Hampton et al., 1994). This signal restricts transcription of *tinman* (*tin*) to the dorsal mesoderm, where its homeodomain protein specifies heart and dorsal somatic mesoderm (Azpiazu and Frasch, 1993; Bodmer, 1993; Bodmer et al., 1990; Frasch, 1995). However, the determinant of the non-dorsal somatic mesoderm remains largely unknown. It appears that Pox meso (Poxm) expression is restricted to the ventral part of the high Twi domain by Dpp (Staehling-Hampton et al., 1994) to define the lateral and ventral somatic mesoderm anlage. The characterization of the role of *Poxm* in somatic myogenesis is therefore expected to fill an important gap in our understanding of the gene network regulating this process.

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Soon after this subdivision of the mesoderm, the proneural gene *lethal of scute* [*l(1)sc*] begins to be expressed in at least 19 promuscular clusters of cells within the high Twi domain (Carmena et al., 1995). From these clusters, muscle progenitors are singled out by lateral inhibition through Notch (N) and Ras signaling and are specified by the expression of muscle-identity genes (Buff et al., 1998; Carmena et al., 1995; Carmena et al., 1998a; Carmena et al., 2002; Michelson et al., 1998; Stathopoulos et al., 2004). Cells not singled out begin to express the zinc finger protein Lame duck (Lmd; also known as Minc), which specifies them as fusion-competent myoblasts (FCMs) (Duan et al., 2001; Ruiz-Gómez et al., 2002). The progenitors divide to generate different muscle founders, a muscle founder and an adult muscle precursor, or a founder and a cell producing either two adult muscle precursors or two pericardial cells (Carmena et al., 1995; Carmena et al., 1998b; Jagla et al., l998; Nose et al., 1998; Ruiz Gómez and Bate, 1997; Ruiz-Gómez et al., 1997). Each founder forms an individual syncytial muscle precursor by fusing with neighboring FCMs. One of the key steps in muscle pattern formation is the specification of a muscle founder by the expression of a specific set of muscle identity genes (Bate, 1990; Bour et al., 2000; Dohrmann et al., 1990; Ruiz-Gómez et al., 2000; Rushton et al., 1995). Although an increasing number of these genes have been identified in recent years, the mechanisms that activate their transcription are still poorly understood. Hence, it is important to identify the genes whose products directly regulate the muscle identity genes.

In this study, we describe the functional characterization of the *Poxm* gene. *Poxm* belongs to the Pax gene family whose members encode transcription factors, including a paired domain (Bopp et al., 1989) (reviewed by Noll, 1993). The temporal and spatial expression patterns of *Poxm* and its loss- and gain-of-function phenotypes reported here demonstrate that it is required for most ventral and lateral abdominal muscles to develop properly in all segments and for the activation of muscle identity genes. In addition, *Poxm* acts itself as muscle identity gene in a few muscles and thus plays a dual role in somatic myogenesis.

MATERIALS AND METHODS

Generation of transgenic flies

To generate transgenic *Poxm-Gal4* lines, an 8.4 kb *Eco*RI fragment (most distal *Eco*RI fragment of P106, see Fig. S1A in the supplementary material) or a 1.8 kb *Xba*I-*Bam*HI fragment (from P111, see Fig. S1A in

the supplementary material; *Bam*HI site 93 bp upstream of third upstream *Eco*RI site in Fig. 3E) of the *Poxm* upstream region was cloned into a pBlueScript pKS$^+$ vector with an altered polylinker that included two *Not*I sites. These fragments were removed by *Not*I digestion and inserted into the *Not*I site of the P-element vector pDA188 (kindly provided by Konrad Basler; K. Basler, unpublished) to produce the *Poxm8.4-Gal4* and *Poxm1.8-Gal4* constructs used to generate the corresponding transgenic lines. To generate *Poxm-lacZ* lines, the 1.8 kb and 8.4 kb fragments mentioned above were removed by *Not*I digestion and inserted together with the *Poxm* promoter/leader region (–333 to +700, a *Not*I-*Kpn*I fragment, generated by PCR from a genomic DNA clone) between the *Not*I and *Kpn*I cloning sites of the pWZ.1 P-element vector (Gutjahr et al., 1994). To produce transgenic *UAS-Poxm* lines, the 2.5 kb full-length *Poxm* cDNA, P29c1, was inserted into the *Eco*RI site of the pUAST vector (Brand and Perrimon, 1993).

To generate transgenic *um1-2-Poxm* lines, an *Xba*I-*Xho*I genomic fragment, extending from the upstream *Xba*I site to the 5′ leader (Fig. 3E), and the adjacent 2.5 kb *Xho*I-*Pst*I *Poxm*-cDNA fragment of P29c2, extending from the leader to the 3′ trailer beyond the first poly(A) addition site (see Fig. S1A in the supplementary material), were cloned between the *Xba*I and *Pst*I sites of the P-element vector PW6 (Klemenz et al., 1987).

For germline transformation, these constructs, all verified by DNA sequencing, were coinjected with the transposase carrying plasmid P(Δ2-3) into w^{1118} or *y w* embryos. Three to five independent lines of each construct were established and analyzed.

Immunohistochemistry and microscopy

To produce an anti-Poxm antiserum, a *Poxm*-cDNA fragment encoding the 234 amino acids C-terminal to the paired-domain was cloned between the *Bam*HI and *Eco*RI sites of the pGEX-3X GST-fusion vector (Pharmacia). The fusion protein was produced in bacteria, purified, and used for immunization of rabbits as described previously (Gutjahr et al., 1993a). Antiserum was collected, affinity-purified, and used at a 1:10 dilution for histochemical detection of Poxm as described (Gutjahr et al., 1993a). The purified anti-Poxm antiserum is free of any crossreactivity with embryonic antigens as verified in homozygous $Poxm^{R361}$ embryos.

The following primary antisera were also used: rabbit anti-MHC [myosin heavy chain (Kiehart and Feghali, 1986)], rat anti-Slou (Carmena et al., 1995), rabbit anti-Twi (Roth et al., 1989), rat anti-L(1)sc (provided by Ana Carmena, Instituto de Neurociencias, Alicante, Spain), rabbit anti-β-galactosidase (Cappel), rabbit anti-Tin (Yin and Frasch, 1998), and rabbit anti-GFP (Medical & Biological Laboratories, Nagoya, Japan). Embryos were fixed and stained as described previously (Gutjahr et al., 1993a).

Muscle patterns were visualized after staining with anti-MHC (or with anti-β-galactosidase, when expressed under indirect control of *Poxm*) under bright-field microscopy by a Zeiss Axiophot. The fluorescent signals of double-labeled embryos were amplified by tyramide signal amplification (TSA; kits #12 and #25 from Invitrogen), and embryos were analyzed by a Leica SP1 confocal microscope.

Fly stocks

The following fly stocks were used. Oregon-R (Munich). *Df(3R)dsx*$^{D+R5}$*/TM3, Sb* (Baker and Wolfner, 1988). *Df(3R)dsx*$^{M+R29}$*/TM3, Sb* (Deák et al., 1997). *UAS-lacZ* (Bloomington stock 1777). *24BGal4* (Bloomington stock 1767). *UAS-GFPnls* (Bloomington stock 4775). *w*; Df(3R)159/TM3, Sb P{ry*$^+$*; hb-lacZ}*. w^{1118}; $Poxm^{R361}$ *red/TM3, Sb Ser P{w*$^+$*; hb-lacZ}*. *y w*; *Poxm8.4-Gal4/TM6B*. *y w*; *Poxm1.8-Gal4* (2nd chromosome). *y w*; *um1-2-Poxm*; $Poxm^{R361}$ *red/TM3, Sb P{ry*$^+$*; hb-lacZ}*. *UAS-Poxm* (3rd chromosome). *Df(1)sc*^{19}*/FM7, P{ry*$^+$*; ftz-lacZ}*. *Df(1)sc*^{19}*/FM7, P{ry*$^+$*; ftz-lacZ}*; $Poxm^{R361}$ *red/TM3, Sb Ser P{w*$^+$*; hb-lacZ}*. *w*; l(3)S028206b*^{S028206b19}*/TM3, Sb* (Deák et al., 1997). *w*; P{Mhc-tauGFP}/TM6B* (Chen et al., 2003). *P{PZ}rP298*; *ry*^{506} (Nose et al., 1998). *w*; *lmd*^{1}*/TM6B* (Duan et al., 2001). *Dmef2*$^{22\text{-}21}$*/CyO* (Bour et al., 1995). *y w*; *Poxm1.8-lacZ* (3rd chromosome). *y w*; *Poxm8.4-lacZ* (3rd chromosome).

RESULTS

Structure of the *Poxm* gene and its predicted protein sequence

The *Poxm* gene has been cloned on the basis of its homology to the paired box of the *paired* (*prd*) and *gooseberry* (*gsb*) genes (Bopp et al., 1986), and was mapped to chromosomal band 84F11-12 (Bopp et al., 1989). It extends over more than 20 kb that include two exons and many cis-regulatory elements located in the upstream region and the large intron (see Fig. S1A in the supplementary material) (Bopp et al., 1989). The Poxm protein, predicted from the longest open reading frame, consists of 370 amino acids and includes a paired domain close to its N terminus and an octapeptide in its C-terminal moiety (see Fig. S1B in the supplementary material) (Bopp et al., 1989; Noll, 1993). Except for its first 10 base pairs, the open reading frame is encoded entirely by exon 2. The paired domain of Poxm belongs to the Pax1/9 class (Bopp et al., 1989; Noll, 1993) and displays 88% identity and 92% similarity to mammalian Pax1/9-type paired domains (Fu and Noll, 1997).

Expression of Poxm in the somatic mesoderm during myogenesis

In agreement with earlier results (Bopp et al., 1989), Poxm protein is localized in the nucleus and first detectable in the somatic mesoderm at early stage 10 (Fig. 1A,B). During stage 10, Poxm becomes expressed in segmentally repeated mesodermal ‘stripes’ underlying the ectodermal parasegments 2-14, in the cephalic mesoderm, the proctodeal anlage and a group of ectodermal cells in the clypeolabrum, which presumably corresponds to part of the esophageal anlage (Fig. 1C). At this stage, the posterior boundaries of mesodermal Poxm coincide with those of ectodermal Gsb (Bopp et al., 1989), which largely coincide with the parasegmental borders (Gutjahr et al., 1993b). Consistent with these calibrations along the anteroposterior axis and those of others (Riechmann et al., 1997), we find that Poxm is expressed in cells of the high Twi domain in the ventral and lateral mesoderm (Fig. 2A-C). Since *Poxm* is repressed in the dorsal portion of each segment by the ectodermal signal Dpp (Staehling-Hampton et al., 1994), the number of Poxm-expressing cells is reduced with decreasing distance from the dorsal margin, thus forming a triangular pattern (Fig. 1D). At this stage, Tin expression is not yet completely restricted to the dorsal mesoderm (Fig. 2D). Whereas high levels of Tin in the dorsal region and Poxm are expressed in complementary patterns, Poxm is coexpressed with low Tin levels in the ventral and lateral regions (Fig. 2D-F). During stage 11 Poxm is restricted to fewer cells, some of which will form subsets of muscle progenitors and cells of the promuscular clusters (Fig. 1E,F), as evident from its partial co-localization with L(1)sc (Fig. 2G-I). During germ band retraction, Poxm disappears from the most anterior mesodermal stripe and the telson (Fig. 1E,G). By stage 12, Poxm expression is maintained only in six cells each of the abdominal segments A1-A7 (Fig. 1H), identified as founders of muscles DO3, DT1 and VA1-VA3, and as ventral adult muscle precursor (VaP) by double-staining of Poxm and Slouch (Slou) (Fig. 2J-L). At this time, it becomes apparent that more cells express Poxm in the ventral regions of the thoracic segments than of the abdominal segments (Fig. 1G). In this study, we focus on the role of *Poxm* in myogenesis of abdominal segments A2-A7.

As myoblast fusion proceeds during stage 13, the number of Poxm-positive nuclei increases (Fig. 1I,J). These coincide with the precursors of muscles DT1 and VA1-3 (Fig. 1J,L-O), identified by double-staining of Poxm and MHC-tauGFP (Myosin heavy chain-tauGFP). During stage 15, Poxm expression begins to be reduced in the ventral clusters and is diminished in the dorsolateral region (Fig.

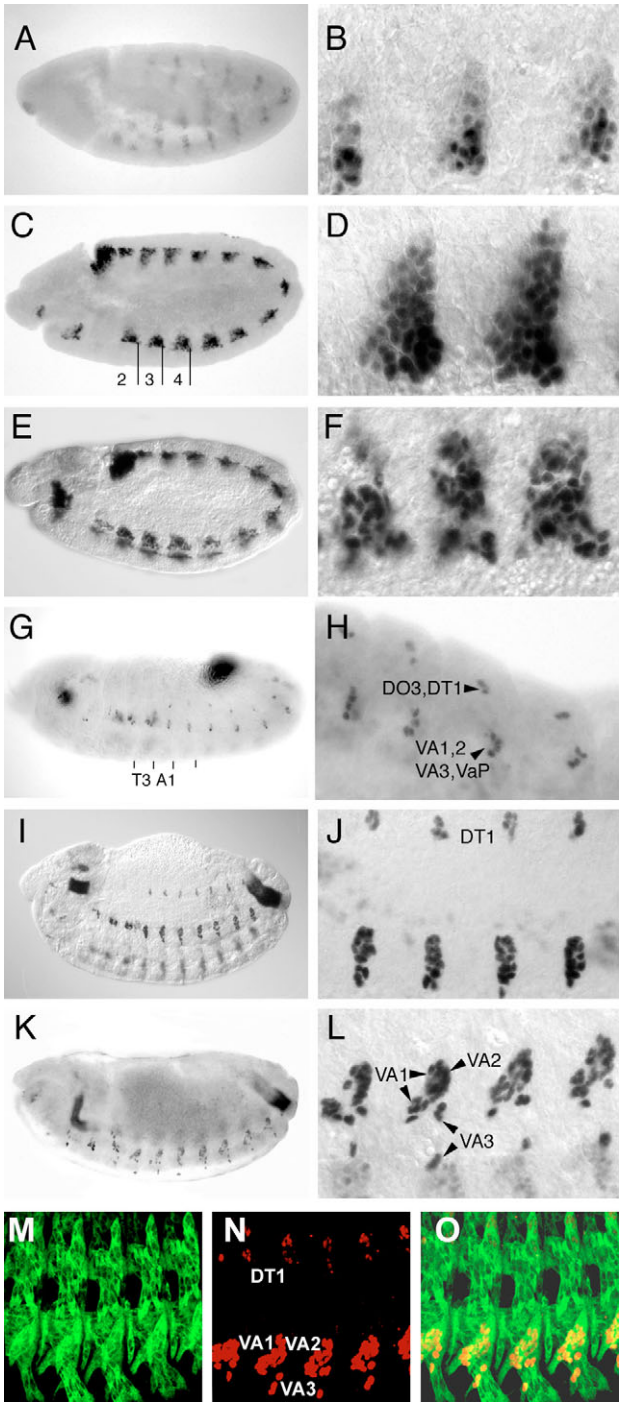


Fig. 1. Expression of Poxm protein in developing larval body wall muscles during embryogenesis. (**A-L**) Whole-mount wild-type embryos were stained with purified anti-Poxm antiserum. Entire embryos (A,C,E,G,I,K) and enlarged views of parasegments 6-8 (B,F,H), 7 and 8 (D), or 6-9 (J,L) at early stage 10 (A,B), late stage 10/early stage 11 (C,D), mid stage 11 (E,F), stage 12 (G,H), stage 13 (I,J) and stage 15 (K,L) are shown. Note that the characteristic triangular pattern of early Poxm expression (D) in parasegments 2-14 is obscured in the overviews (C,E) because not all Poxm-expressing cells are in focus. Therefore, embryos were unfolded and flattened to show enlarged views of parasegments at extended germ band stages (B,D,F) and during germ band retraction (H). Embryos are oriented with their anterior to the left and dorsal side up. At stage 12, Poxm is expressed in the founders of muscles DO3, DT1, VA1-3 and in the ventral adult precursor (VaP; H), and later in the precursors of these muscles except DO3 and VaP (J,L). (**M-O**) Muscles that express Poxm were identified by double-labeling of embryos from *P{Mhc-tauGFP}/TM6B* parents with anti-GFP and anti-Poxm to reveal the muscle pattern (M), the late Poxm expression (N) and the merged image (O). Lateral and ventral muscles in four abdominal hemisegments of a stage 15 embryo with its anterior to the left and dorsal side up are shown. Note that muscles VA1 and VA2 overlap dorsally. For muscle nomenclature, see Bate (Bate, 1993) or Fig. 4J,N.

1K), from which it disappears during stage 16. By stage 17, Poxm is no longer detectable in the mesoderm or any of its derivatives. Outside the mesoderm, particularly striking is its expression in the developing esophagus and hindgut (Fig. 1A,C), where it is maintained at high levels throughout embryogenesis (Fig. 1E,G,I,K).

In summary, Poxm expression in the ventral and lateral portions of the high Twi domain colocalizes with weak Tin expression and is complementary to the high levels of Tin in the dorsal region. Subsequently, its mesodermal expression is confined to fewer cells, some of which will form promuscular clusters and muscle progenitors. Poxm persists in some, but not all of the muscle founders derived from the Poxm-expressing progenitors and is ultimately expressed in four muscle precursors. After the formation of muscle fibers, Poxm disappears. This time course of Poxm expression in developing muscles suggests that *Poxm* functions in somatic myogenesis.

Fate of Poxm-expressing cells during early and late myogenesis

To further analyze the nature and fate of Poxm-expressing cells during early and late myogenesis, *lacZ* was expressed under the indirect control of different *Poxm* upstream regions by the use of the Gal4/UAS system (Brand and Perrimon, 1993). Because of the perdurance of β-galactosidase (β-gal) resulting from (i) the amplification and delay of β-gal inherent in the Gal4/UAS system and (ii) the considerably enhanced stability of both Gal4 and β-gal proteins as compared to that of Poxm, we can follow the fate of cells expressing Poxm during earlier embryonic stages by examining β-gal expression at later stages.

Under the control of a 1.8 kb upstream fragment of *Poxm* (Fig. 3E), β-gal is expressed in a pattern similar, but not identical, to that of early Poxm in the mesoderm (Fig. 3A), presumably because of the temporal delay in expression of the Gal4/UAS system. A similar early expression pattern is observed (Fig. 3C) when *lacZ* is expressed under the control of an 8.4 kb upstream fragment (Fig. 3E). We have also examined β-gal expression under the direct control of the 1.8 kb and 8.4 kb *Poxm* enhancers. In both cases, β-gal and Poxm are coexpressed during early embryonic stages and no ectopic β-gal is detectable (see Fig. S2 in the supplementary material).

Patterns of β-gal expression were then examined at later stages in differentiating muscles. At stage 16, the 8.4 kb fragment supports strong *lacZ* expression in muscles DT1 and VA1-3 (Fig. 3D), in agreement with late Poxm expression, which is restricted to these muscles (Fig. 1N). In addition, however, muscles VL1-4, VO1-6, frequently LT3 and LT4, and occasionally muscle SBM are labeled by β-gal, although at moderate or considerably lower intensities

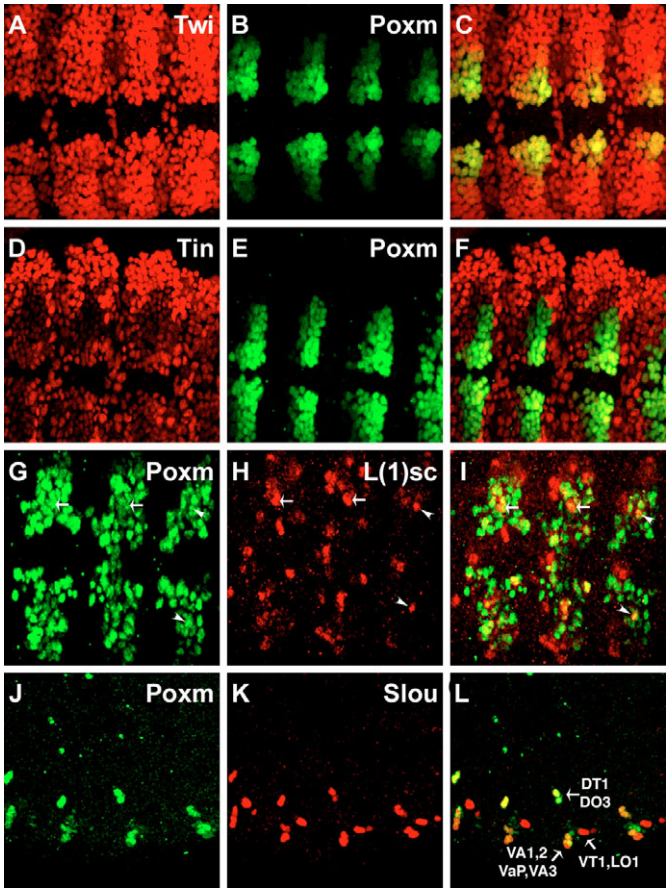


Fig. 2. Early ventral and lateral Poxm expression complementary to dorsal high Tin expression in somatic mesoderm precedes its expression in progenitors of ventral and lateral somatic muscles and in a specific subset of muscle founders. (**A-C**) Early Poxm expression is restricted to the ventral and lateral somatic mesoderm. Poxm expression (B) and high levels of Twi (A), which mark the somatic mesoderm (Dunin Borkowski et al., 1995; Baylies and Bate, 1996) can be seen to coincide in the ventral and lateral regions of the somatic mesoderm (C), as observed by confocal microscopy. A ventral view of four abdominal parasegments of a late stage 10/early stage 11 embryo, oriented with their anterior to the left. (**D-F**) Complementary expression patterns of Poxm and high levels of Tin in the somatic mesoderm. Poxm expression in the ventral and lateral regions (E) abuts high expression levels of Tin in the dorsal region (D), but coincides with lower levels of Tin (F) in an early stage 11 embryo shown in a ventral view. (**G-I**) Poxm is expressed in cells of promuscular clusters and muscle progenitors. Poxm (G) and L(1)sc (H) expression coincide in many ventral and lateral muscle progenitors (I), as observed by double-labeling of Poxm and L(1)sc. Some cell clusters and single progenitors that coexpress L(1)sc and Poxm are indicated by arrows and arrowheads, respectively. Three abdominal parasegments, oriented with their anterior to the left, are shown on both sides of the ventral midline of a mid stage 11 embryo. (**J-L**) Poxm is expressed in specific muscle founders and in the ventral adult precursor. Lateral views of four abdominal hemisegments of a stage 12 embryo double-labeled for late Poxm (J) and Slou (K) expression, oriented with its anterior to the left, revealed by confocal microscopy. In the hemisegment where muscle founders are marked by arrows, expression of late Poxm and Slou coincide (L) in the founders of muscles DO3, DT1, VA2, VA3, and in the ventral adult muscle precursor VaP, whereas Slou has disappeared from the VA1 founder that continues to express Poxm (Carmena et al., 1995; Dohrmann et al., 1990). Expression of Slou in the founders of VT1 and LO1, which do not express Poxm, is also clearly visible (Carmena et al., 1995; Dohrmann et al., 1990).

(Fig. 3D). By contrast, when *lacZ* is expressed under control of the 1.8 kb fragment, it is not detected in muscle DT1 and only at low or moderate levels in muscles VA1-3 (Fig. 3B). It follows that late Poxm expression is under the control of sequences present in the 8.4 kb but not the 1.8 kb fragment (Fig. 3E). Owing to perdurance, when expressed only under control of the early enhancer, β-gal is also observed at moderate or low levels in the ventral muscles VL1-4, VO1-6, frequently in the lateral muscles LT3, LT4, LL1, LO1, SBM and rarely in LT2 and VT1 (Fig. 3B).

These results indicate that cells expressing Poxm early during myogenesis are those from which ventral and lateral muscle progenitors are selected. However, since muscle fibers form by fusion of founders with FCMs, β-gal patterns observed in differentiating muscles may result from the perdurance of β-gal in founders or FCMs. To rule out the possibility that this perdurance is derived exclusively from expression in FCMs, we examined the expression of nuclear GFP under indirect control of the 1.8 kb fragment in lmd^{1} (Duan et al., 2001) or $Dmef2^{22\text{-}21}$ (Bour et al., 1995) mutants, in which fusion is blocked and muscle founders were marked by the *dumbfounded* enhancer trap chromosome rP298-lacZ (Nose et al., 1998). Because of the perdurance of GFP, we can follow the fate of cells expressing early Poxm by examining their expression of GFP at later stages. In lmd^{1} embryos, GFP is expressed only in the ventral and lateral portions of each segment at stage 15 (Fig. 3H). Since in the absence of myoblast fusion most founders, marked by β-gal (Fig. 3I), also express GFP at least weakly (Fig. 3J), we conclude that cells expressing Poxm early during myogenesis will give rise to most founders of the ventral and lateral muscles. In addition, Poxm is expressed early in mesodermal cells that are not selected as progenitors, as evident from the perdurance of GFP in many mesodermal cells different from founders (Fig. 3J). Similar results were obtained for $Dmef2^{22\text{-}21}$ mutants (not shown).

Isolation and characterization of *Poxm* mutant alleles

The expression patterns of *Poxm* suggest that it plays a crucial role in myogenesis. Assuming that absence of *Poxm* functions results in lethality, we screened a collection of 1,400 lethal P-element insertions on the third chromosome (Deák et al., 1997) for lack of complementation with the deficiency $Df(3R)dsx^{D+R5}$ (see Fig. S1A in the supplementary material) (Duncan and Kaufman, 1975), which uncovers *Poxm* (Bopp et al., 1989), and subsequently for complementation with $Df(3R)dsx^{M+R29}$, whose distal break point is located proximal to *Poxm*, at 84F6-7 (Baker et al., 1991). One lethal insertion, *P282*, was identified that had inserted into the neighboring gene, 5 kb downstream of the second exon of *Poxm* (see Fig. S1A in the supplementary material). Embryos homozygous for *P282* did not show any muscle defects. Imprecise excision of this P element (Robertson et al., 1988) produced a deficiency, *Df(3R)159*, whose distal breakpoint is located about 10 kb upstream of the *Poxm* transcription start site (see Fig. S1A in the supplementary material). Its proximal breakpoint maps distal to the more proximal deficiency $Df(3R)dsx^{M+R29}$, with which it complements. Embryos homozygous for *Df(3R)159* show severe defects in the larval somatic musculature.

Since *Df(3R)159* deletes, in addition to *Poxm*, at least another gene, the observed muscle phenotype might result from the absence of more than just *Poxm* functions. Therefore, eight EMS-induced embryonic lethal mutants, obtained in a screen for genes on the third chromosome affecting neuromuscular connectivity (Sink et al., 2001; Van Vactor et al., 1993), that showed defects in muscle patterning were tested for complementation with *Df(3R)159*. One of these mutants, *R361*, failed to complement and showed the same

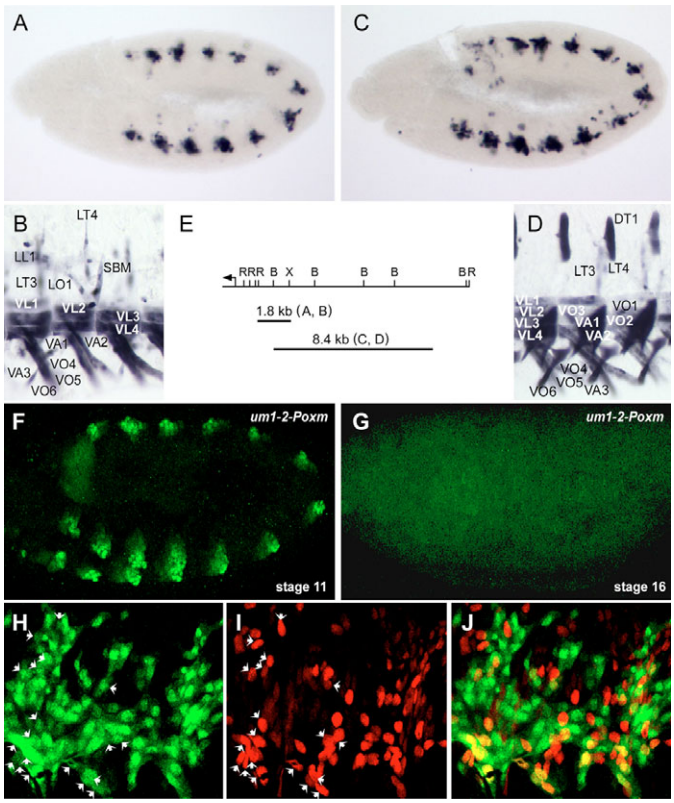


Fig. 3. Most ventral and lateral somatic muscle founders are recruited from cells expressing early Poxm. (**A-E**) Early and late Poxm expressions in the somatic mesoderm are regulated by different enhancers. Whole-mount transgenic *Poxm1.8-Gal4/UAS-lacZ* (A,B) and *Poxm8.4-Gal4/UAS-lacZ* (C,D) embryos were stained with rabbit anti-β-galactosidase antiserum. A map of the *Poxm* upstream region (B, *Bam*HI; R, *Eco*RI; X, *Xba*I), which delimits the 1.8 kb and 8.4 kb fragments used as enhancers in combination with the *hsp70* minimal promoter to drive Gal4 expression in the *Poxm-Gal4* transgenes, is shown in E. Overviews of late stage 11 embryos (A,C) and enlarged ventral and lateral views of abdominal segments A2-A4 (B) or A4-A6 (D) of stage 16 embryos are shown with anterior to the left and dorsal up. Muscle patterns (B,D) were visualized from the interior (B) or exterior (D) after staining for β-gal, by dissecting the embryos in halves along the dorsal and ventral midlines, removing tissue below the muscles, and mounting the ectoderm with the attached muscles for bright-field microscopy in a Zeiss Axiophot. The moderate to low β-gal levels observed at late stages after early activation by the 1.8 kb enhancer result from perdurance (B), whereas the high β-gal levels observed after activation by the 8.4 kb enhancer mimic late stage Poxm expression (D). For muscle nomenclature, see Bate (Bate, 1993) or Fig. 4J,N. (**F**,**G**) Absence of late Poxm expression of a *Poxm* transgene driven by the early enhancer. Homozygous $Poxm^{361}$ embryos, rescued by the *um1-2-Poxm* transgene that includes only upstream cis-regulatory sequences up to the *Xba*I site (E) and no intron, exhibit a wild-type early Poxm pattern (stage 11; F) but no late Poxm expression (stage 16; G). Confocal micrographs of embryos with their anterior to the left and dorsal side up are shown. (**H-J**) Cells expressing early Poxm give rise to most ventral and lateral muscle founders. Cells expressing early Poxm were labeled by nuclear GFP (H) and their fate was followed by confocal microscopy in *rP298-lacZ*; *Poxm1.8-Gal4/UAS-GFPnls*; lmd^{1} embryos, in which founders are marked by β-gal (I) and their fusion with FCMs is blocked. Most ventral and lateral muscle founders that are labeled by β-gal are also marked by GFP (J), many of which are marked by white arrowheads in two of the three abdominal segments of a stage 15 embryo shown in H and I.

larval muscle phenotype as *Df(3R)159*, in homozygous and transheterozygous conditions. No Poxm protein was detectable in either mutant (not shown). Sequencing of *R361* genomic DNA identified, in *Poxm*, a single point mutation, $Poxm^{R361}$, that converts a glutamine codon at position 7 of the N-terminal paired domain into an amber stop codon and hence is expected to result in a truncated N-terminal Poxm peptide of 14 amino acids (see Fig. S1B in the supplementary material). It follows that $Poxm^{R361}$ is a null allele of *Poxm*.

Loss of Poxm function causes severe disruption of the somatic muscle pattern

To investigate the effects of *Poxm* on muscle development, embryos homozygous or transheterozygous for *Df(3R)159* and $Poxm^{R361}$ were examined after visualizing their somatic muscles by staining with anti-MHC. These mutants all displayed the same severe defects in the formation of larval muscles. In our analysis, which focused on abdominal segments A2-A7, *Poxm* was considered to be required for the proper development of a specific muscle if that muscle did not form normally in a significant fraction of hemisegments in *Poxm* null mutants. It does not imply that this muscle never forms normally, as the penetrance of the phenotype may not be 100%.

In the ventral region of *Poxm* mutant embryos, usually muscles VO4-6 are absent, whereas muscles VA1-3 are still present in most segments but are poorly developed, lacking their normal shape and attachment sites (Fig. 4G,H, Fig. 5A). Further analysis revealed that muscles VL3 and VL4 are frequently abnormal or missing, whereas muscles VL1 and VL2 are occasionally or rarely affected (Fig. 5A). Also muscles VO2 and VO1 are strongly and moderately disturbed, respectively (Fig. 5A).

In the dorsolateral region, muscle DT1, in most cases, is missing or abnormal, whereas muscle DO3, which is derived from the same progenitor (Carmena et al., 1995), is mostly duplicated or abnormal and very rarely missing (Fig. 4D,E, Fig. 5A). Two additional muscles, DA3 and DO4, are occasionally abnormal, whereas the two most posterior lateral muscles, LO1 and LT4, are frequently missing and abnormal, respectively (Fig. 5A). By contrast, all dorsal muscles remain unaffected (Fig. 4A,B, Fig. 5A).

Ordering the muscles along the abscissa according to decreasing severity of their *Poxm* mutant phenotype (red bars in Fig. 5E) reveals a striking correlation with the early triangular *Poxm* expression pattern (Fig. 1D). Muscles located more ventrally or more posteriorly in a segment are always more strongly affected as compared to muscles located roughly at the same anteroposterior or dorsoventral positions, respectively (Fig. 4N). For example, muscle VL4 is affected more severely than its dorsal neighbor VL3, which is again more frequently abnormal than VL2 or VL1. Similarly, the phenotype of muscle LT4 is stronger than that of its anterior neighbors LT1-3. This phenotype suggests that it might be affected by a function that depends on a dorsoventral as well as an anteroposterior gradient, on which indeed the early *Poxm* expression pattern depends, namely on Dpp (Staehling-Hampton et al., 1994) and Wg (J.C. and M.N., unpublished), and which explains its characteristic triangular shape (Fig. 1D).

Ectopic expression of Poxm in the mesoderm generates additional muscles

To test whether Poxm can determine muscle development, we expressed it ectopically and analyzed its effect on myogenesis. *24BGal4* was used to drive expression of *UAS-Poxm* in the entire mesoderm beginning at mid stage 10 (Michelson, 1994). Ectopic Poxm produces a severely altered muscle pattern, which varies

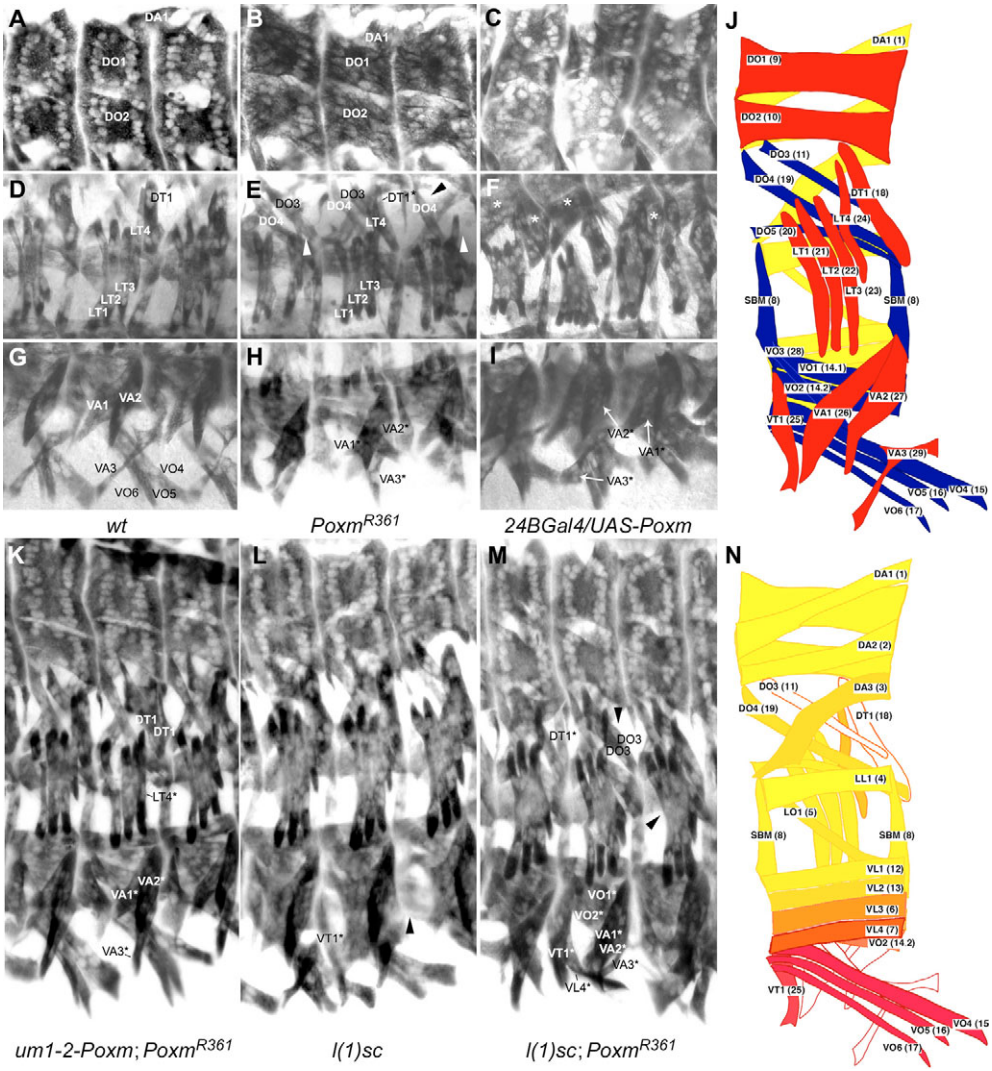


Fig. 4. Muscle phenotypes of *Poxm* and *l(1)sc* mutants. (**A-I**) Muscle phenotypes resulting from loss of, or ectopic, Poxm. Dorsal (A-C), lateral (D-F) and ventral (G-I) muscles were visualized using an anti-MHC antiserum in three abdominal hemisegments of stage 16 wild-type (A,D,G), *Poxm*^{R361} (B,E,H), and *24BGal4/UAS-Poxm* (C,F,I) embryos, oriented with anterior to the left and dorsal up. In *Poxm*^{R361} mutants, positions of missing muscles DT1 (white arrowheads) or an abnormal muscle DT1 (*) and of a missing muscle DO3 (black arrowhead) are indicated in E, ventral muscles VO4-6 are missing (H), and ventral muscles VA1-3* have lost their normal shape and attachments (H), as evident from a comparison with the wild-type ventral muscle pattern (G). Muscle VL4 is also frequently absent, as evident from inspection of a plane of focus interior to and below that shown in H. A detailed analysis of the *Poxm*^{R361} muscle phenotype is summarized in Fig. 5A. Ectopic ubiquitous mesodermal expression of Poxm (C,F,I) generates ectopic dorsal and lateral muscles (marked by asterisks in F) and enlarges some ventral muscles (arrows in I), the number and positions of which are not altered. (**J**) Schematic external view (dorsal up and anterior to the left) of larval muscles in abdominal segments A2-A7 (Ruiz-Gómez et al., 1997), with external muscles in red and more internal muscles in blue and yellow; muscles are designated and numbered according to Bate (Bate, 1993) and in parentheses according to Crossley (Crossley, 1978). (**K-M**) Muscle phenotypes of *Poxm* mutants rescued by early Poxm, and of *l(1)sc*; *Poxm* double and *l(1)sc* single mutants. Muscle phenotypes were visualized using an anti-MHC antiserum in three abdominal hemisegments of *Poxm*^{R361} embryos rescued by two copies of the *um1-2-Poxm* transgene (K), of *Df(1)sc*^{19} embryos (L), and of *Df(1)sc*^{19}; *Poxm*^{R361} embryos (M) at stage 16. Anterior is to the left and dorsal up. A detailed analysis of these phenotypes is summarized in Fig. 5B-D. Some muscles that are abnormal in shape and/or position are marked by asterisks, duplicated muscles DT1 (K) and DO3 (M) are labeled, and missing muscles VT1 (L), DT1 and LO1 (M) are indicated by black arrowheads. Ventral muscles VO4-6 that are missing in nearly all segments of *Poxm* single or double mutants (Fig. 5A,D) are also absent but not marked (M). (**N**) Schematic internal view of a hemisegment opposite to that shown in J (Ruiz-Gómez et al., 1997) of the muscle phenotype attributable to the absence of the early *Poxm* function, in which each muscle is colored in a graded fashion from red (0%) to yellow (100%) corresponding to the fraction of normal muscles observed (Fig. 5A). Muscles DO3, DT1 and VA1-3 are not colored as the contribution to their phenotype of the missing early Poxm cannot be estimated because they are also affected by the late *Poxm* function, and muscle VO3 is not colored because it has not been recorded in Fig. 5A.

among different segments and embryos. The most striking defects occur in the dorsal and dorsolateral muscles, where Poxm is normally absent or present at low levels (Fig. 4C,F). In the dorsal region, which includes four muscles in wild-type embryos (Bate, 1993) (Fig. 4A,J), ectopic muscles are generated in most segments (Fig. 4C). Ectopic muscles similar in shape and orientation to muscle DA3 occupy the dorsolateral region (Fig. 4C,F), which is largely free of muscles in wild-type embryos (Fig. 4D). Usually several muscles with abnormal shape occur at the position of muscle DT1 (Fig. 4F), whereas muscles LL1, DO4 and DO5 exhibit aberrant shapes or are missing in some segments. In addition, some of the lateral muscles are abnormally shaped. By contrast, the ventral muscles, all of which exhibited a strong early Poxm expression (Fig. 1C,D), remain largely unaffected, although some muscle fibers appear enlarged (Fig. 4I).

Poxm regulates the formation of adult muscle precursors

As adult muscle precursors derive from progenitors that also generate founders of larval muscles (Bate et al., 1991; Ruiz Gómez and Bate, 1997; Ruiz-Gómez et al., 1997), we suspected that *Poxm* also affects adult muscle precursors. To test this

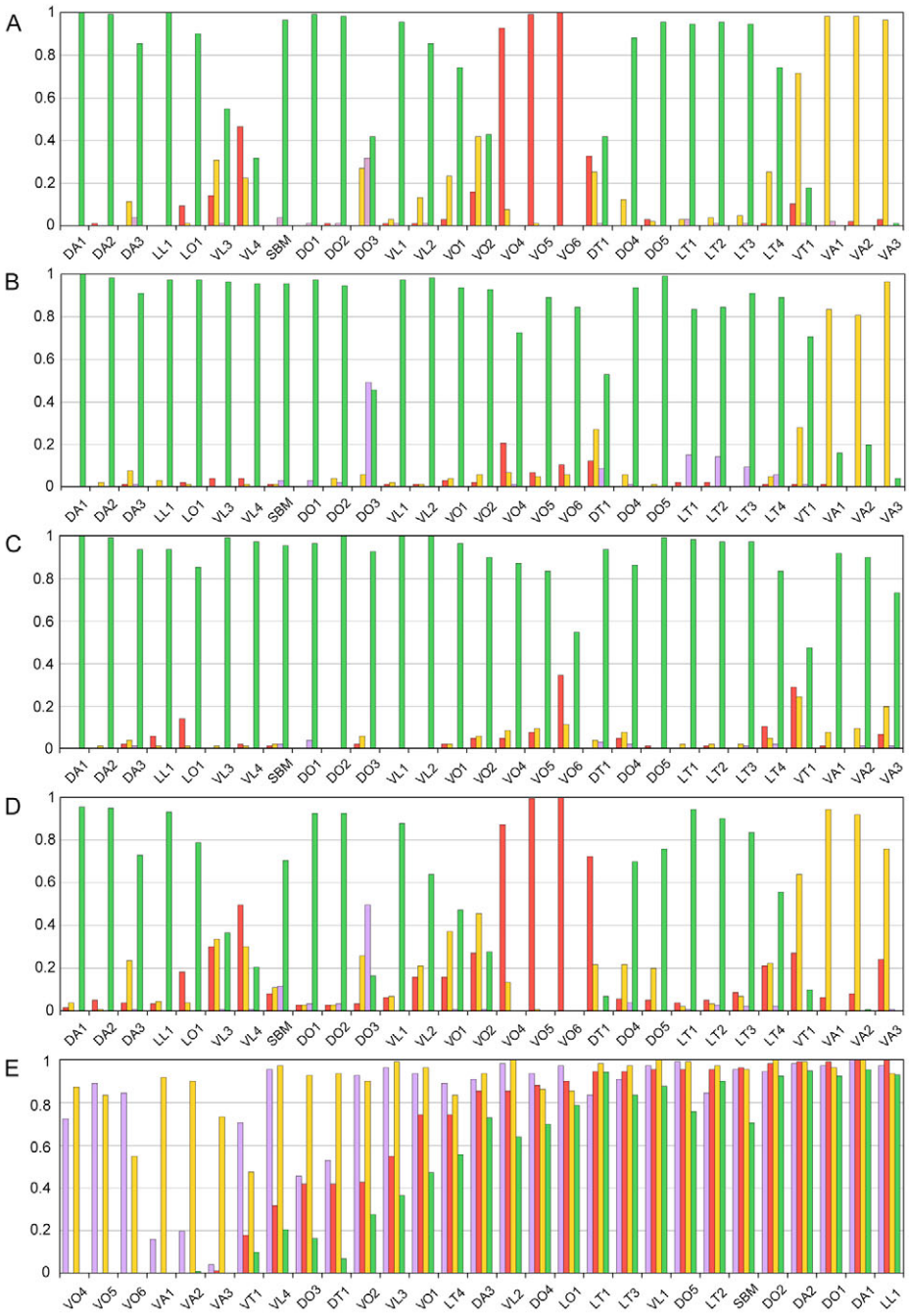
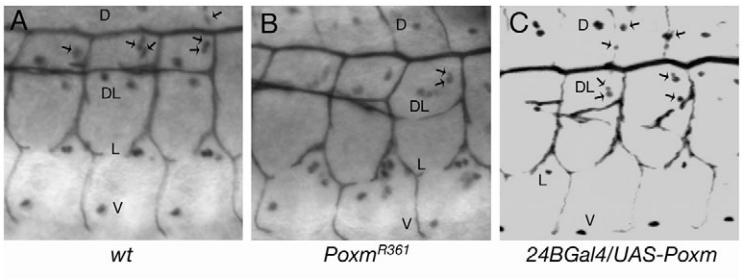


Fig. 5. Larval body wall muscle phenotypes of *Poxm* and *l(1)sc* single and double mutants, and of *Poxm* mutants rescued by early Poxm. (**A-D**) The somatic muscle patterns of $Poxm^{R361}$ embryos (A), $Poxm^{R361}$ embryos rescued by two copies of the *um1-2-Poxm* transgene (B), of $Df(1)sc^{19}/Df(1)sc^{19}$ or *Y* (C), and $Df(1)sc^{19}/Df(1)sc^{19}$ or *Y*; $Poxm^{R361}$ (D) embryos and of *y w* control embryos (not shown) were analyzed at stage 16 under bright-field optics in a Zeiss Axioplan 2 microscope after staining with anti-MHC antiserum, dissection along the ventral midline, and removal of internal tissues. Each muscle plotted on the abscissa was scored for absence (red), abnormality (yellow), duplication (purple), or wild-type appearance (green) in each of 108 (A-C) or 168 (D) hemisegments in abdominal segments A2-A7, and the resulting fractions were plotted on the ordinate. Muscles of the *y w* control embryos were usually normal in all 108 hemisegments scored, with the occasional absence, duplication or abnormality of a single muscle. (**E**) The fractions of normal muscles shown in A-D are plotted for *Poxm* mutants rescued by early Poxm (purple), *Poxm* (red) and *l(1)sc* (yellow) single mutants, and for *l(1)sc*; *Poxm* (green) double mutants, whereas muscles are ordered along the abscissa with decreasing abnormality of *Poxm* mutant phenotypes.

hypothesis, we analyzed the effects of loss-of-function and ectopic expression of *Poxm* on the expression of Twi, which is present in all adult muscle precursors but not in larval founders after germ band retraction (Bate et al., 1991). In stage 14 wild-type embryos, adult muscle precursors appear in four groups with a single precursor each in the ventral and dorsal groups (VaP and DaP) and two each in the dorsolateral and lateral groups (DLaPs and LaPs) (Fig. 6A) (Bate et al., 1991; Ruiz Gómez and Bate, 1997).

In the lateral mesoderm of embryos expressing Poxm ubiquitously, in most segments DLaPs are missing and only one of the two LaPs is present (Fig. 6C,D). The reverse situation was found in *Poxm* mutants (Fig. 6B,D). The number of LaPs increases to four to seven cells in each abdominal hemisegment, and more than two DLaPs are present in 20% of the segments. Therefore, in the lateral portions of the abdominal segments, Poxm acts to prevent the formation of supernumerary adult muscle precursors and, when ectopically expressed, can inhibit the formation of normal adult muscle precursors.

In the dorsal region, after mesodermal ubiquitous expression of Poxm, on average two DaPs instead of one are present in about half of the segments (Fig. 6C,D). This result correlates with the appearance of ectopic dorsal muscles (Fig. 4C) and hence suggests that ectopic expression of Poxm leads to the production of supernumerary adult muscle precursors and muscle founders in the region where normally only a very low level of Poxm is expressed at early embryonic stages. In embryos lacking Poxm, however, DaPs remain largely unaffected (Fig. 6B,D).



D

Genotype	Number of Hemisegments	DaP	DLaP	LaP	VaP
Poxm^{R361}	90	94 (90)	204 (180)	518 (180)	96 (90)
24B>Poxm	90	130 (90)	31 (180)	105 (180)	92 (90)

Fig. 6. Loss of Poxm and ectopic Poxm change the number of adult muscle precursors. (**A-C**) Expression of Twi in abdominal hemisegments of wild-type (A), *Poxm*^{R361} (B), and *24BGal4/UAS-Poxm* (C) stage 14 embryos was visualized by staining with an anti-Twi antiserum (anterior to the left and dorsal up). Only precursors of adult muscles and the alary cells express Twi at this stage [alary cells, marked by black arrows, are located on the segment margins in the dorsal and dorsolateral region; see Bate et al. (Bate et al., 1991)]. The adult muscle precursors are arranged in dorsal (D), dorsolateral (DL), lateral (L) and ventral (V) groups. The staining of the trachea is caused by a crossreactivity of the anti-Twi antiserum. (**D**) Analysis of number of adult muscle precursors in embryos mutant for *Poxm* or expressing ubiquitous mesodermal Poxm. The number of adult muscle precursors was analyzed in 90 hemisegments each of *Poxm*^{R361} and *24BGal4/UAS-Poxm* embryos. The total numbers for 90 hemisegments are shown for the dorsal (DaP), dorsolateral (DLaP), lateral (LaP) and ventral (VaP) group of adult muscle precursors with the numbers expected for wild-type embryos in parentheses.

In the ventral region, the number of VaPs is hardly changed not only in the presence of mesodermal ubiquitous Poxm but also in the absence of Poxm (Fig. 6B-D).

Poxm acts upstream of the muscle identity gene *slou*

Early expression of *slou*, one of the well-studied muscle identity genes (Dohrmann et al., 1990; Knirr et al., 1999; Ruiz-Gómez et al., 1997), occurs in a subset of muscle progenitors and their offspring founders, some of which also express Poxm (Fig. 1H). This raises the possibility of an epistatic relationship between these genes. Early Slou-expressing cells are arranged in three groups of muscle founders (Fig. 7A): group I will generate muscles LO1 and VT1; group II, muscles VA1-3 and the VaP; and group III, muscles DO3 and DT1 (Carmena et al., 1995; Dohrmann et al., 1990). After stage 13, Slou remains expressed only in the precursors of muscles DT1, VT1 and VA2 (Carmena et al., 1995; Dohrmann et al., 1990) (Fig. 7C,E), two of which, DT1 and VA2, also express Poxm (Fig. 1J,L). In *Poxm*^{R361} embryos, Slou protein is expressed in groups I and II, yet is absent from group III in most, though occasionally observed in more posterior, abdominal segments during late stage 12 (Fig. 7B). After stage 13, Slou is detectable only in the precursor of muscle VT1 but no longer maintained in that of VA2 in abdominal segments (Fig. 7D). Therefore, Poxm is essential for the activation of *slou* in the progenitor of muscle DT1 and for its maintenance in the precursor of muscle VA2.

In *24BGal4/UAS-Poxm* embryos, in which Poxm is ubiquitously expressed in the mesoderm, additional muscles expressing Slou were found in the dorsolateral portion of some segments (Fig. 7F), which suggests that in these cells ectopic Poxm suffices to activate *slou* and corroborates the observation that *Poxm* acts upstream of the muscle identity gene *slou*.

Early Poxm largely rescues the muscle phenotype of *Poxm* mutants

Since Poxm is expressed during early myogenesis in cells that later give rise to progenitors of most of the ventral and lateral muscles, it may play an important role in the initiation of muscle patterning. To investigate which part of the *Poxm*^{R361} muscle phenotype results from the loss of this early *Poxm* function, a transgene expressing *Poxm* only during the early myogenic stages (Fig. 3F,G), *um1-2-Poxm*, was introduced into *Poxm*^{R361} embryos. In these embryos, the phenotypes of muscles VO4-6, VL2-VL4, VO2, VO1, LO1, LT4 and VT1 are efficiently rescued (Fig. 4K; Fig. 5B,E). The only muscles affected in *Poxm* mutants (Fig. 5A) that are only slightly rescued by early Poxm (Fig. 5B) are DT1, DO3 and VA1-3, in which Poxm is also expressed during later stages in their founders and/or muscle precursors (Fig. 4K; Fig. 5B,E). These results strongly suggest that *Poxm* exerts an early function, demarcating a mesodermal domain of competence for ventral, lateral and dorsolateral somatic muscle development.

Partial redundancy of early *Poxm* and *l(1)sc* functions in somatic myogenesis

The partial penetrance of the *Poxm* muscle phenotype (Fig. 5A) suggests that the early *Poxm* function is largely redundant with that of other genes, an argument also raised to explain the weak muscle phenotype of *l(1)sc* mutants (Carmena et al., 1995). The *l(1)sc* gene encodes a bHLH transcription factor the function of which is thought to be required for the selection of muscle progenitors (Baylies et al., 1998; Carmena et al., 1995). Therefore, we examined the effect of *Poxm* and *l(1)sc* mutations on larval muscle development in single and double mutant embryos (Fig. 5A,C,D).

In agreement with earlier studies (Carmena et al., 1995), *l(1)sc* mutants exhibit a weak muscle phenotype, which deviates only slightly from that of wild-type embryos (Fig. 4L, Fig. 5C). Although

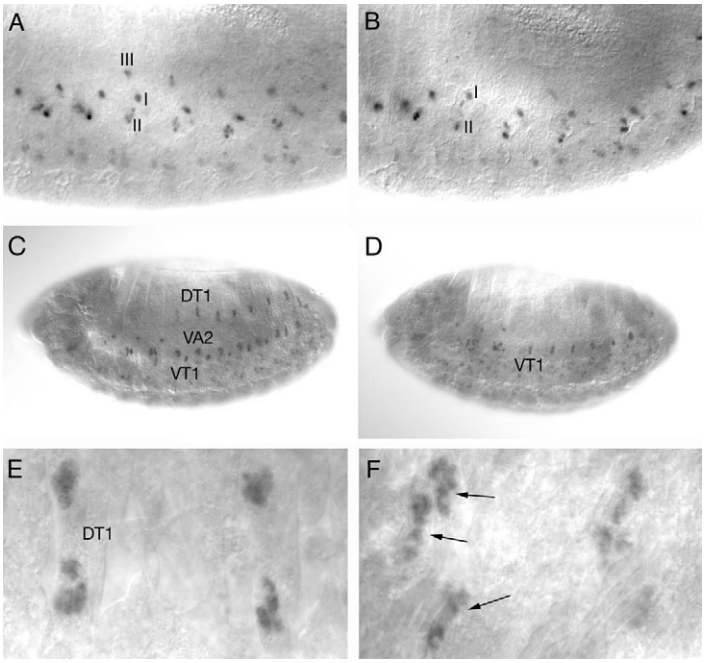


Fig. 7. Altered Slou expression in *Poxm* mutants or in the presence of ectopic Poxm. Slou expression in wild-type (**A**,**C**,**E**), $Poxm^{R361}$ (**B**,**D**), and *24BGal4/UAS-Poxm* (**F**) embryos at stage 12 (A,B), 14 (C,D) and 16 (E,F) was visualized by staining with an anti-Slou antiserum. Ventrolateral views of whole embryos (C,D) and enlarged ventrolateral (A,B) and dorsolateral (E,F) views of parasegments 4-10 (A,B) and 6-7 (E,F) are shown with anterior to the left and dorsal up. At late stage 12, Slou is expressed in three groups, I-III, of muscle founder cells of each abdominal segment of wild-type embryos (A), whereas it is expressed only in groups I and II of $Poxm^{R361}$ mutants (B). At stage 14, Slou is expressed in the precursors of dorsal muscle DT1 and of ventral muscles VT1 and VA2 of each abdominal segment of wild-type embryos (C), whereas it is detectable only in the precursor of ventral muscle VT1, but not of muscles DT1 and VA2, in $Poxm^{R361}$ embryos (D). After ectopic expression of Poxm, additional muscles express Slou (arrows in F) in a dorsolateral region where Slou is expressed only in muscle DT1 of stage 16 wild-type embryos (E).

$Poxm^{R361}$ embryos show a considerably stronger muscle phenotype, most lateral and dorsal muscles are normal (Fig. 5A). Assuming that *Poxm* and *l(1)sc* act independently in muscle development, we expect that the probability of a muscle being wild-type in $Df(1)l(1)sc^{19}/Y$; $Poxm^{R361}$ embryos is the product of the probabilities of the muscle being wild-type in the single mutants. Conversely, if we find significantly enhanced probabilities for muscle defects in double mutants, we may conclude that *Poxm* and *l(1)sc* exhibit partially redundant functions during muscle development. Applying this test to the results summarized in Fig. 5A,C,D, we find that most muscles are affected independently or nearly independently, with some notable exceptions. These concern muscles VL1-3, SBM, VO1, VO2, DT1, LT3, LT4 and VA3 that are more often absent. Some muscles are strongly affected in *Poxm* null mutants, such as muscles VO4-6 or muscles VA1-3. Among the other muscles, the more ventral and the more posterior a muscle is located within a segment, the more probable it is that it will show an enhanced phenotype in double mutants (Fig. 5E). Clearly, there is some redundancy between *Poxm* and *l(1)sc* functions in the somatic mesoderm, which is restricted largely to ventral and posterior muscles.

Late *Poxm* function specifies muscle identity

In *Poxm* mutants, only muscle DO3 is frequently duplicated (Fig. 5A). This duplication results from the transformation of muscle DT1 to DO3, as previously observed for muscles derived from the same progenitor in the absence of a muscle identity gene that is asymmetrically expressed in the two founders and muscle precursors (Knirr et al., 1999; Ruiz-Gómez et al., 1997). Thus, late expression of *Poxm* in the precursor of muscle DT1, but not of DO3, is crucial for their distinction and hence serves a muscle identity function. However, a more detailed analysis shows that muscle DT1 is missing in only two thirds (23/34) of all cases in which muscle DO3 is duplicated (see Table S1 in the supplementary material). In the remaining 11 cases, muscle DT1 is normal (4), abnormal (6) or duplicated (1). This finding suggests that the late *Poxm* function is necessary in about 10% (11/108) of all cases to prevent an additional division that generates a second founder of muscle DO3.

Absence of Poxm in their founders results in abnormal muscles VA1-3 (Fig. 5A) that cannot be rescued by the early Poxm function (Fig. 5B), which suggests that their proper specification also depends on the late function of *Poxm*.

DISCUSSION

Our results demonstrate that the development of larval body wall muscles depends on distinct *Poxm* functions during two phases. The early function of *Poxm* specifies, within the high Twi or Slp domain, a subdomain of competence for lateral and ventral muscle development, the 'Poxm competence domain' (Fig. 8). This function appears to be analogous to that of *tin*, which specifies competence for heart and dorsal muscle development in the complementary part of the Slp domain (Azpiazu and Frasch, 1993; Michelson et al., 1998; Yin and Frasch, 1998). *Poxm* and *tin* thus subdivide the posterior Slp domain into ventral and dorsal subdomains in a manner similar to the partitioning by *serpent* and *bap* of the anterior Eve domain into the ventral fat body and the dorsal visceral mesoderm anlagen (Azpiazu et al., 1996; Riechmann et al., 1998). After selection of muscle progenitors, proper development of a few muscles still depends on *Poxm*, which is expressed in muscles DT1 and VA1-3. This late function of *Poxm* participates in founder specification and muscle differentiation, as is characteristic for muscle identity genes. Finally, our findings suggest a solution to a conceptual problem of the current model of somatic myogenesis, the *l(1)sc* conundrum.

Early Poxm specifies competence for somatic myogenesis in partial redundancy with similar functions of L(1)sc

The muscle phenotype of *Poxm* mutant embryos and its rescue by early Poxm expression shows that the early *Poxm* function is crucial for the proper development of many ventral and lateral muscles (Fig. 5A,B). In addition, the generation of ectopic dorsal and dorsolateral muscles by ectopic Poxm suggests that *Poxm* has the ability to change cell fates and render cells competent for myogenesis. Therefore, we propose that early Poxm demarcates a ventral and lateral domain of competence for somatic myogenesis.

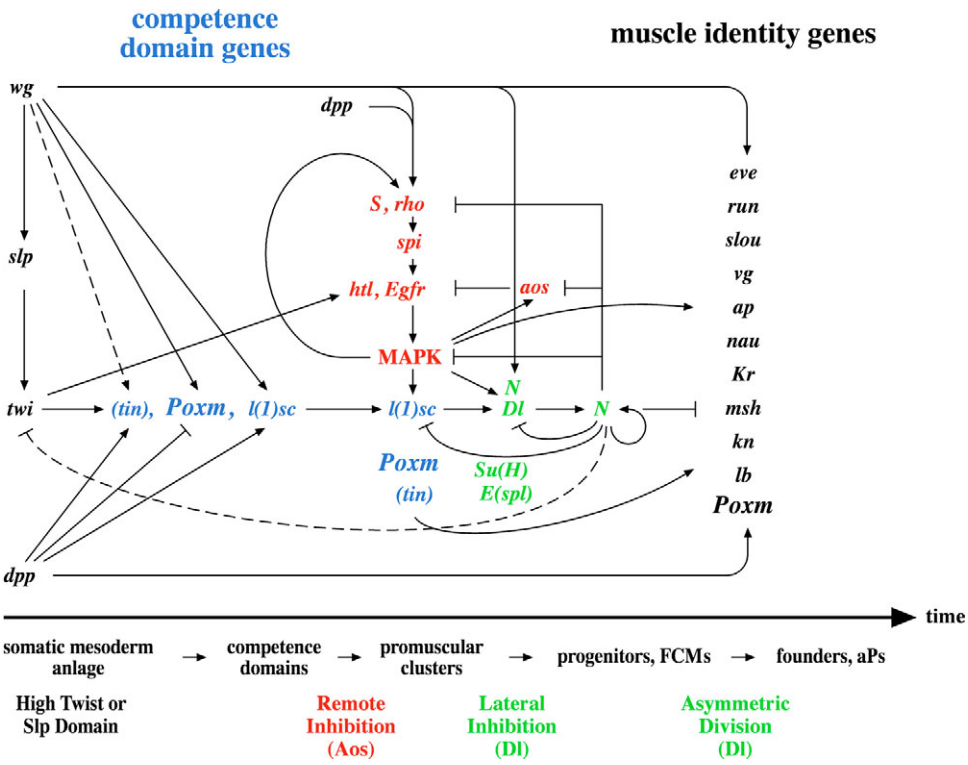


Fig. 8. Regulatory network of somatic myogenesis. The scheme depicts the interactions among the major genes and/or their products that regulate the development of larval body wall muscles from gastrulation to the specification of muscle founder cells, as explained in detail in the text. After the establishment of the high Twi domain in the mesoderm underneath the posterior portions of parasegments by Slp, domains competent for somatic myogenesis are specified by 'competence domain genes', such as *l(1)sc*, *tin* (the hypothetical role of *tin* as competence domain gene is indicated by parentheses) and *Poxm*, regulated by Twi and the ectodermal signals Wg and Dpp. These signals, in combination with the localized EGF (Spi) and FGF (Pyr, Ths) signals and through remote inhibition of Spi signaling by Aos (Freeman, 1997), determine the promuscular clusters, which express *l(1)sc* and activate the MAPK signaling pathway. Singling out of muscle progenitors and separation from fusion-competent myoblasts (FCMs) occurs by lateral inhibition, which is mediated by N signaling that is coupled to MAPK signaling through multiple feedback loops. At this stage, or subsequently, in muscle founder cells generated from progenitors by asymmetric division mediated by N signaling, muscle identity genes, such as *Poxm*, *Kr* and *slou*, are activated by the integration on their enhancers of competence domain gene products with the effectors of the mentioned signals (for references, see text). Hypothetical interactions are indicated by dashed lines.

The partial penetrance of the *Poxm* mutant phenotype implies the existence of other competence domain genes performing partially redundant functions. We have shown that Poxm and L(1)sc partially co-localize in the promuscular clusters and muscle progenitors (Fig. 2G-I). In addition, a detailed analysis of *l(1)sc* and *Poxm* single and double mutants demonstrates that their functions are partially redundant (Fig. 5A,C,D). Since the muscle phenotype of *l(1)sc*; *Poxm* double mutants still shows partial penetrance (Fig. 5D), additional competence domain genes should be expressed in the Slp domain. One of them is probably *tin*, which is initially expressed in the entire early mesoderm (Azpiazu and Frasch, 1993; Bodmer, 1993; Bodmer et al., 1990), because *tin* mutants affect muscle development in the dorsal as well as lateral and ventral Slp domain (Azpiazu and Frasch, 1993; Michelson et al., 1998). Another candidate is *D-six4*, which is required for the development of specific muscles that arise from the dorsolateral and ventral regions (Clark et al., 2006).

Thus, after the initial subdivision of the mesoderm, the high Twi domain is further subdivided by competence domain genes (Fig. 8), which specify domains that become competent to select progenitors of distinct subsets of somatic muscles and/or of myocardial and pericardial cells by responding to spatially restricted extracellular signals (Azpiazu and Frasch, 1993; Halfon et al., 2000). These competence domain genes act in a cooperative manner to determine the identities of specific muscles by regulating the expression of the muscle identity genes. When one of them is inactivated, in some cells active competence domain genes can partially compensate for the inactive gene by activating its target genes such that these sometimes, but not always, exceed the threshold levels required for normal development. Hence, muscles derived from these cells exhibit a mutant phenotype with partial penetrance. For other cells, active competence domain genes can compensate completely for the missing gene activity such that these cells will adopt the proper fate and the muscles develop normally. This illustrates that competence is not a matter of 'all' or 'nothing' for muscle development. The deeper reason for this, we deem, is that the genetic program regulating myogenesis is not organized in a hierarchical fashion but rather as a complex gene network (Fig. 8) that has an integrated function which is much more stable against mutations within the network than a hierarchical regulation would be.

Poxm is a muscle identity gene activating the muscle identity gene *slou*

Muscle identity genes usually encode transcription factors, such as Slou, Nau, Ap, Vg, Kr, Eve, Msh, Lb, Run and Kn (Bate et al., 1993; Bourgouin et al., 1992; Carmena et al., 2002; Dohrmann et al., 1990; Frasch et al., 1987; Jagla et al., 2002; Michelson et al., 1990; Knirr et al., 1999; Nose et al., 1998; Ruiz Gómez and Bate, 1997; Ruiz-Gómez et al., 1997), that are expressed in subsets of

muscle progenitors and founders and determine in a combinatorial fashion the identity of each muscle founder and its subsequent differentiation into a specific muscle of defined size, shape, attachment sites, and innervation (Baylies et al., 1998; Dohrmann et al., 1990; Ruiz-Gómez et al., 1997). We envision the activation of these genes in promuscular clusters or, after lateral inhibition, in muscle progenitors (Carmena et al., 1995; Carmena et al., 1998a) by Twi and/or the products of competence domain genes and through combinations of localized extracellular signals from the ectoderm and mesoderm (Azpiazu and Frasch, 1993; Halfon et al., 2000). During asymmetric division of progenitors, expression of a muscle identity gene may be maintained in one or both of the two sibling founders, or it may persist in the founder when division generates a founder and an adult muscle precursor. Late expression of Poxm illustrates all three cases. It is expressed in progenitors P26/27 and P29/VaP, which are derived from promuscular cluster 10 and give rise to the founders of muscles VA1 (F26) and VA2 (F27), and to the founder of muscle VA3 (F29) and the ventral adult precursor VaP (Carmena et al., 1995; Dohrmann et al., 1990). Poxm is also expressed in the progenitor derived from cluster 13, P11/18, which generates the founders of muscles DO3 (F11) and DT1 (F18). Although Poxm expression persists in F29 and F18 but not in their siblings, it is maintained in both sibling founders F26 and F27.

The late function of *Poxm* is identified as a muscle identity function by the high correlation between absence of muscle DT1 and corresponding duplication of muscle DO3 in *Poxm* mutants (Fig. 5A and see Table S1 in the supplementary material). If Poxm was the sole determinant discriminating between F11 and F18, mesodermal ubiquitous expression of Poxm would be expected to transform muscle DO3 into DT1. Our results confirm the presence of additional muscles in the region of muscle DT1. It is possible that one of these originates from a transformed F11, but it is impossible to tell whether muscle DO3 is missing (Fig. 4F) because additional muscles have been recruited.

It has been shown that in the process of muscle diversification, identity genes may repress or activate other identity genes in progenitors and founders (Jagla et al., 1998; Jagla et al., 2002; Knirr et al., 1999; Nose et al., 1998; Ruiz-Gómez et al., 1997). We found that the muscle identity gene *slou* fails to be activated in P11/18 of *Poxm* mutants. The simplest explanation of this result is that activation and maintenance of *slou* expression depend on Poxm in P11/18 and its offspring founders. In addition, *slou* expression is not maintained in F27 of *Poxm* mutants despite its initial activation in P26/27. It therefore appears that in P26/27 and its offspring F26 and F27, in addition to Kruppel (Kr) (Ruiz-Gómez et al., 1997), Poxm is necessary for the maintenance of *slou* expression. Although Poxm expression is maintained in both F26 and F27, *slou* expression is restricted to F27 because *Kr* is repressed in F26 by N signaling. Apparently, Kr is the crucial determinant that distinguishes F26 from F27, as F27 is altered to F26 in *Kr* or *numb* mutants (Ruiz Gómez and Bate, 1997; Ruiz-Gómez et al., 1997).

As *Poxm* is expressed in both F26 and F27, whereas its expression is restricted to F18 and not maintained in F11, its late expression in F26 and F27 must be regulated differently from that in F11 and F18 where it appears to be subject to asymmetric N signaling (Ruiz Gómez and Bate, 1997) repressing *Poxm* in F11.

These considerations imply that *slou* is part of the same gene network as *Poxm*, a conclusion consistent with our gene network hypothesis since, in the first test of this hypothesis, *slou* had been isolated as a PRD 9 gene on the basis of its homology to the *prd* gene (Frigerio et al., 1986).

A solution of the *l(1)sc* conundrum

The mechanism of progenitor selection from the somatic mesoderm depends on a process of lateral inhibition very similar to that of neuroblast or sensory organ precursor (SOP) selection in the neuroectoderm from proneural clusters expressing the proneural genes (Bate et al., 1993; Corbin et al., 1991). Because of this similarity, a search among proneural genes for 'promuscular' genes expressed in the somatic mesoderm was performed (Carmena et al., 1995). This search identified a single proneural gene, *l(1)sc*, a member of the *achaete-scute* complex (AS-C), that is expressed in promuscular clusters of the somatic mesoderm. It was, therefore, attractive to consider its function in myogenesis to be analogous to that of proneural genes in neurogenesis (Carmena et al., 1995; Carmena et al., 1998a). However, whereas proneural genes confer on neuroectodermal cells the ability to become neural precursors rather than epidermal cells, which is their default fate (Campuzano and Modolell, 1992), *l(1)sc* does not seem to confer on mesodermal cells the ability to undergo somatic myogenesis instead of becoming part of the visceral mesoderm, heart or fat body. When L(1)sc was expressed in the entire mesoderm from stage 8 onward, other mesodermal tissues could not be transformed into somatic mesoderm (Carmena et al., 1995), whereas a deficiency of *l(1)sc* resulted in only minor defects of somatic muscle development (Fig. 5C) (Carmena et al., 1995). In addition, as the *l(1)sc* muscle mutant phenotype can be rescued by ubiquitous mesodermal L(1)sc expression (Carmena et al., 1995), its expression in clusters is not decisive for the formation of promuscular clusters and, therefore, *l(1)sc* cannot play the decisive role in the development of larval body wall muscles that has been proposed (Carmena et al., 1995). Thus, although *l(1)sc* serves as an excellent marker for promuscular clusters, it lacks a property expected to be crucial for a promuscular gene. Are there genes that might qualify as promuscular genes and thus extend the close evolutionary relationship of progenitor selection between myogenesis and neurogenesis (Jan and Jan, 1993)?

There is indeed a gene that is homologous to proneural genes and expressed in the somatic mesoderm, in the absence of which somatic myogenesis is seriously disturbed. This gene is *twi*, whose function at stages 10 and 11 more closely corresponds to that of a promuscular gene and which, like *l(1)sc*, encodes a bHLH transcription factor. Although Twi is also expressed earlier when it is required for mesoderm specification during gastrulation, this early function can be distinguished from its later 'promuscular' function in temperature-sensitive mutants (Baylies and Bate, 1996). In these mutants, only high levels of Twi activity, necessary for the formation of the somatic mesoderm, are abolished and no normal somatic muscles develop (Baylies and Bate, 1996). Moreover, ubiquitous expression of high levels of Twi in the mesoderm blocks all other mesodermal fates, transforming them to somatic mesoderm (Castanon et al., 2001). Since the subsequent patterning of somatic muscles depends critically on the relative levels of the products of *twi* and the proneural gene *da* (Castanon et al., 2001), it seems appropriate to consider them both as promuscular genes.

In addition to its strict requirement for somatic myogenesis, the proposed promuscular function of *twi* may be subject to lateral inhibition by N signaling, in further analogy to proneural functions in neurogenesis. This is apparent from experiments demonstrating that the restriction of high Twi levels to the Slp domain during stage 9 depends on N signaling (Brennan et al., 1999; Tapanes-Castillo and Baylies, 2004), which downregulates *twi* in the mesoderm underlying the anterior regions of parasegments where Slp does not override it (Riechmann et al., 1997). Since this process acts directly

on an identified *twi* enhancer during stages 9 and 10 (Tapanes-Castillo and Baylies, 2004), it is conceivable that this enhancer also responds to N signaling during the subsequent lateral inhibition. An alternative, though not mutually exclusive, mechanism for the downregulation of *twi* implicates the Gli-related zinc finger transcription factor Lmd (Minc), whose expression is maintained by N signaling and in the absence of which *twi* is not downregulated in fusion-competent myoblasts (Duan et al., 2001; Ruiz-Gómez et al., 2002).

During lateral inhibition, loss of Twi precedes that of L(1)sc in the promuscular clusters (Carmena et al., 1995). It is therefore possible that *l(1)sc* expression in these cells also depends on high levels of Twi, i.e. on Twi homodimers (Fig. 8). Consistent with this interpretation, shifting the equilibrium between Twi homodimers and Twi-Da heterodimers in favor of the latter represses *l(1)sc* (Castanon et al., 2001). Since early Poxm expression also depends on Twi (J.C. and M.N., unpublished), *Poxm* would be similarly repressed in promuscular clusters through lateral inhibition, either indirectly by repression of *twi* and/or directly by Twi/Da heterodimers. Such a mechanism might apply generally to both competence domain genes and muscle identity genes during lateral inhibition of promuscular clusters.

Thus, *twi* satisfies two criteria considered to be crucial for a promuscular gene in analogy to those of proneural genes in neurogenesis. However, a third criterion is not fulfilled by *twi*: its expression, in contrast to that of proneural genes in the neuroectoderm, is ubiquitous rather than restricted to promuscular clusters although this criterion is not a crucial property of proneural genes (Rodríguez et al., 1990). Yet promuscular clusters from which the myogenic progenitors are selected exist, as evident from the pattern of *l(1)sc* expression (Carmena et al., 1995). These promuscular clusters depend on combinations of the long-range ectodermal signals Wg and Dpp (Lee and Frasch, 2000; Carmena et al., 1998a) and the localized activities of the EGF signal Spi in the mesoderm and the FGF signals Pyr and Ths in the ectoderm (Buff et al., 1998; Carmena et al., 1998a; Carmena et al., 2002; Michelson et al., 1998; Stathopoulos et al., 2004). These signals, together with Twi and/or products of competence domain genes depending on Twi, determine the promuscular clusters by activating specific combinations of muscle identity genes (Halfon et al., 2000) (Fig. 8). The identity of the promuscular clusters depends not only on the combination of these signals but, in the case of MAPK signaling elicited by FGF and/or EGF, also on their intensity (Buff et al., 1998). In addition, multiple positive and negative feedback loops of the coupled MAPK and N signaling networks ensure a stable selection and specification of muscle progenitors not only within, but also beyond, the limits of a promuscular cluster (Carmena et al., 1998a; Carmena et al., 2002). Such a conclusion implies that these clusters are not a priori determined, but depend on the range and intensities of the MAPK activating signals, in agreement with our assumption that it is not the expression of *l(1)sc* that determines the promuscular clusters. In fact, it may be the absence of such a priori determined clusters of equivalent cells in the somatic mesoderm that necessitates such a complex N and Ras signaling circuitry (Fig. 8).

Therefore, we propose that *twi and da*, instead of *l(1)sc*, function as promuscular genes by regulating the activities of competence domain genes, which in turn regulate the combinatorial activities of muscle identity genes and thereby specify the fates of muscle progenitors and founders (Fig. 8). It is nevertheless surprising that *l(1)sc* appears to be expressed in all promuscular clusters even though its function is not necessary in most of them. It is possible that this expression pattern is an evolutionary remnant of an atavistic promuscular function of *l(1)sc* that was later replaced by the promuscular function of *twi* on whose expression *l(1)sc* activity depends.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/22/3985/DC1

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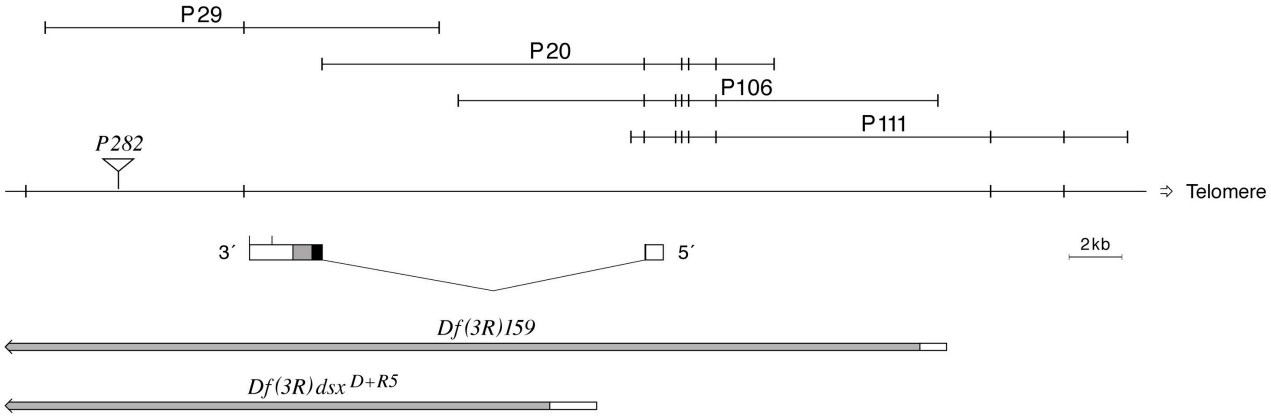
A



B

stop (*Poxm*^{R361})

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  1 MDPESQCPQYGEVNQLGGVFVNGRPLPNATRMRIVELARLGIRPCDISRQLRVSHGCVSKILARYHETGS
 71 ILPGAIGGSKPRVTTPKVVNYIRELKQRDPGIFAWEIRDRLLSEGICDKTNVPSVSSISRILRNKLGSLG
141 HQHTPGTVMGSGSSSGGGSVSSNGGQNNGTSASNNINLSNLGNPGGGPHHPHHHHHHQSAAAAASAHHVH
211 AHAHAHAHLYNSIYQPYSAAAAYSMKTPCGSPSPPQGAGGQGSVPHPHQLRSVAAAAAAAHWPSSHSVSD
281 ILAHHQAVALRASCQVGVGVGGMGGMGSTVSPLPMTPSPVAGTAGGQPLLDCEGGAGQQSPYNYYMYFQN
351 GGMHHHHHHGGMMAAGATGL 370
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Fig. S1. Structural organization of the *Poxm* gene and Poxm protein sequence. (**A**) Overlapping DNA fragments, covering the *Poxm* locus, P29, P20, P106 and P111, and isolated from a genomic library in λ phage EMBL4 (Bopp et al., 1989), are shown at the top with respect to an *Eco*RI restriction map below, in which the location of the P-element insertion P282 (Deák et al., 1997) is indicated. The locations and orientations of the two Poxm exons (Bopp et al., 1989), shown below the restriction map, were derived by sequencing of two Poxm cDNAs, P29c1 and P29c2, differing mainly in the lengths of their trailers, and of the corresponding genomic DNA, whereas the transcriptional start site was determined by 5' RACE and coincides with the 5' end of P29c1 (the GenBank accession number for the full-length Poxm cDNA P29c1, is DQ459353; for P29c2 it is DQ459354). The exons corresponding to the full-length Poxm transcript indicate the open reading frame (shaded) with the paired-domain (black), the untranslated leader and trailer (white), and two different poly(A) addition sites found in two cDNAs (vertical marks). Two deficiencies, *Df(3R)159* and *Df(3R)dsx*$^{D+R5}$, are indicated at the bottom. Whereas their proximal breakpoints map proximal to the genomic region shown, their distal breakpoints are located within the regions delimited by the open boxes. (**B**) Amino acid sequence of Poxm derived from the longest open reading frame of Poxm cDNAs. The previously published paired-domain and octapeptide (Bopp et al., 1989; Noll, 1993) are underlined, and the location of the EMS-induced amber mutation *Poxm*^{361}, Q15stop, is indicated by an arrow. The black triangle marks the position of the intron. Note that amino acids 5-370 are identical to 37-402 of the Poxm protein published by FlyBase, whereas our N-terminal amino acids deviate because the first exon has been incorrectly predicted by FlyBase.

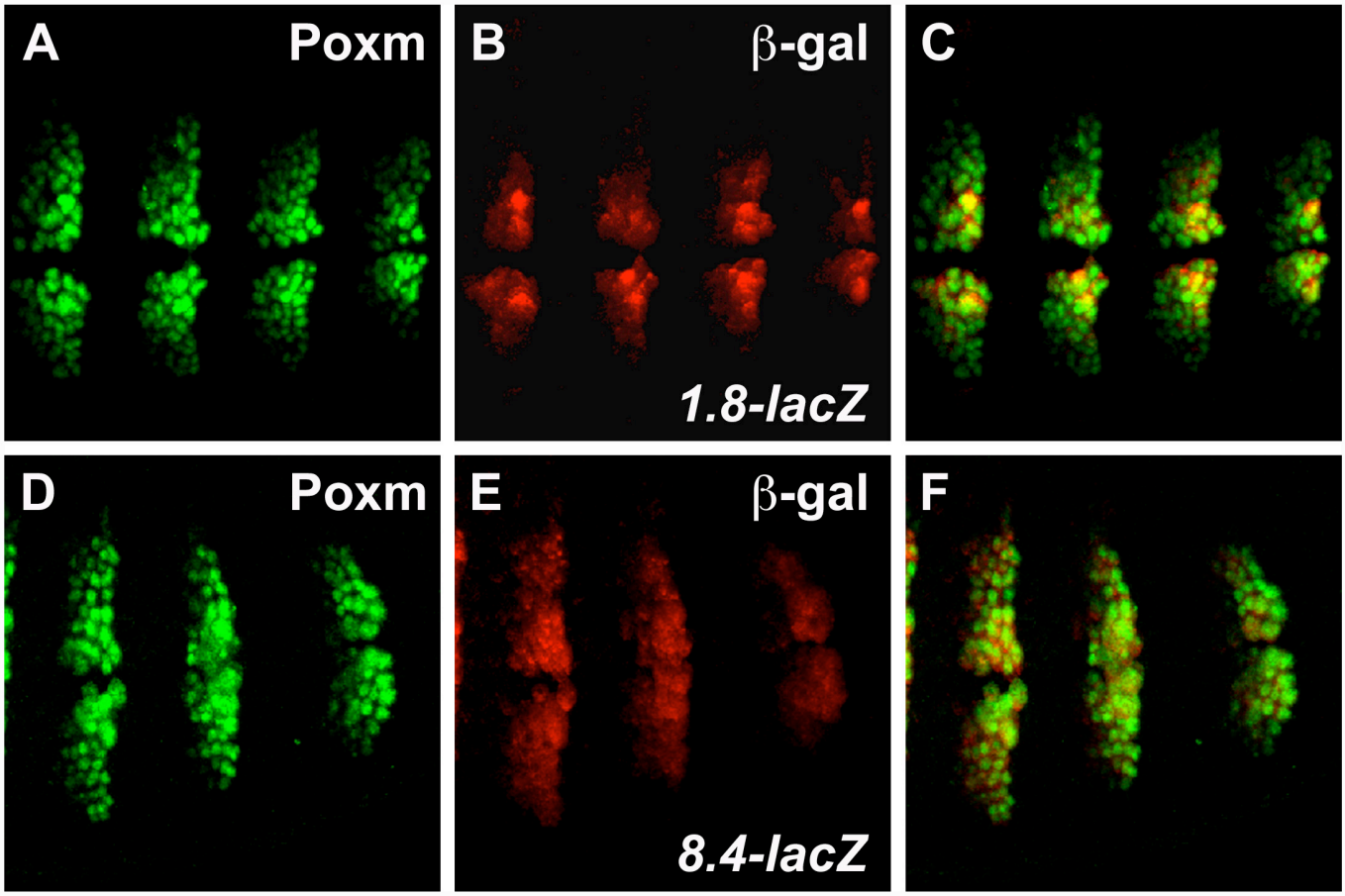

Fig. S2. ***lacZ*** **expressed under the direct control of the** ***Poxm*** **enhancers coincides with endogenous Poxm protein.** Transgenic embryos at early stage 11, expressing lacZ under the direct control of the 1.8 kb fragment (**A-C**) or the 8.4 kb fragment (**D-F**), both including the early enhancer of Poxm (Fig. 3E), were stained for Poxm (A,D) and β-gal (B,E). The two expression patterns completely coincide, and no ectopic β-gal is observed (C,F). Note that Poxm is nuclear whereas β-gal is cytoplasmic.

Poxm	
muscle 11	muscle 18
N	N
A	A
N	A
D	M
N	N
N	N
N	A
N	M
N	A
A	N
N	N
N	N
N	M
A	M
A	N
A	N
A	A
N	N
A	N
D	M
D	M
D	M
A	A
N	A
D	M
A	A
D	M
D	M
D	A
D	N
D	M
D	M
A	N
N	A
D	D
N	N
N	A
D	A
A	N
D	A
A	N
A	N
N	A
A	A
A	N
A	A
D	N
A	N
N	A
A	M
D	M
A	M
N	N
N	N
D	A
A	M

l(1)sc; Poxm	
muscle 11	muscle 18
A	M
D	M
D	M
D	M
A	M
A	M
A	A
N	N
D	A
A	A
D	A
N	A
A	A
A	M
A	A
N	M
N	M
A	A
N	M
D	M
N	A
N	A
D	M
A	A
N	A
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D	M
D	M
N	A
N	A
D	M
D	M

Poxm	
muscle 11	muscle 18
N	N
N	N
N	N
D	M
D	M
N	A
N	N
N	N
N	N
N	N
D	M
D	M
D	M
D	M
D	N
N	N
D	M
N	N
N	N
N	N
A	A
N	N
D	M
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D	M
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D	M
N	N
D	M
D	N
N	N
D	M
N	A
A	A

l(1)sc; Poxm	
muscle 11	muscle 18
N	M
N	M
N	M
N	A
D	A
N	M
A	M
A	A
M	M
M	M
A	A
D	M
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A	A
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D	M

l(1)sc; Poxm	
muscle 11	muscle 18
A	M
A	M
D	M
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D	M
D	M

Table S1. Phenotypes of muscles DO3 and DT1 in individual hemisegments of Poxm and *l(1)sc*; *Poxm* mutants. Mutant phenotypes, analyzed as described in the legend to Fig. 5, are given for muscles DO3 and DT1 of individual hemisegments, as summarized in Fig. 5A,D. Each line lists the phenotypes of a hemisegment for muscles DO3 and DT1 of $Poxm^{R361}$ mutants (left column, 108 hemisegments) and of $Df(1)sc^{19}/Df(1)sc^{19}$ or *Y*; $Poxm^{R361}$ mutants (right column, 168 hemisegments). Muscle phenotypes were classified as missing (M, red), abnormal (A, yellow), duplicated (D, purple) and normal wild type (N, green).