Hedgehog regulation of superficial slow muscle fibres in *Xenopus* and the evolution of tetrapod trunk myogenesis

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Summary

In tetrapod phylogeny, the dramatic modifications of the trunk have received less attention than the more obvious evolution of limbs. In somites, several waves of muscle precursors are induced by signals from nearby tissues. In both amniotes and fish, the earliest myogenesis requires secreted signals from the ventral midline carried by Hedgehog (Hh) proteins. To determine if this similarity represents evolutionary homology, we have examined myogenesis in *Xenopus laevis*, the major species from which insight into vertebrate mesoderm patterning has been derived. *Xenopus* embryos form two distinct kinds of muscle cells analogous to the superficial slow and medial fast muscle fibres of zebrafish. As in zebrafish, Hh signalling is required for *XMyf5* expression and generation of a first wave of early superficial slow muscle fibres in tail somites. Thus, Hh-dependent adaxial myogenesis is the likely ancestral condition of teleosts, amphibia and amniotes. Our evidence suggests that midline-derived cells migrate to the lateral somite surface and generate superficial slow muscle. This cell re-orientation contributes to the apparent rotation of *Xenopus* somites. *Xenopus* myogenesis in the trunk differs from that in the tail. In the trunk, the first wave of superficial slow fibres is missing, suggesting that significant adaptation of the ancestral myogenic programme occurred during tetrapod trunk evolution. Although notochord is required for early medial *XMyf5* expression, Hh signalling fails to drive these cells to slow myogenesis. Later, both trunk and tail somites develop a second wave of Hh-independent slow fibres. These fibres probably derive from an outer cell layer expressing the myogenic determination genes *XMyf5*, *XMyoD* and *Pax3* in a pattern reminiscent of amniote dermomyotome. Thus, *Xenopus* somites have characteristics in common with both fish and amniotes that shed light on the evolution of somite differentiation. We propose a model for the evolutionary adaptation of myogenesis in the transition from fish to tetrapod trunk.

Key words: Dermomyotome, Slow muscle, MyoD, Pax3, Myf5, Engrailed

Introduction

During vertebrate skeletal muscle development, several populations of myogenic cells arise within each somite due, at least in part, to signals impinging on the somite from neighbouring tissues (Borycki and Emerson, 2000; Pownall et al., 2002). In amniotes, many myogenic cells arise from the dermomyotome, an epithelial sheet covering the lateral somitic surface, although small numbers of early myogenic cells arise in chick prior to dermomyotome formation. A large part of the somite forms sclerotome. In zebrafish, the earliest populations of myogenic cells arise from several zones of the early somite, yet no dermomyotome has been described. Instead, the outer surface of the developing somite becomes covered in a layer of specialised slow muscle fibres and sclerotome formation is late and limited (Stickney et al., 2000). To shed light on the origin of the dermomyotome and the relationship of myogenic cells populations in zebrafish and amniotes, we examined myogenesis and dermomyotome formation in *Xenopus laevis*, an anuran species in which a 'dermatome' has been described but is poorly characterised (Keller, 2000; Nieuwkoop and Faber, 1967).

In zebrafish, the medial adaxial myogenic cell population arises early in presomitic mesoderm (PSM) next to the notochord. Adaxial cells have a distinct cuboidal morphology and express the myogenic basic helix-loop-helix transcription factors *myf5* and *myod*, followed by slow myosin heavy chain (MyHC) (Chen et al., 2001; Coutelle et al., 2001; Devoto et al., 1996; Weinberg et al., 1996). Subsequently, as somite borders form, *myf5* and *myod* mark a distinct population of cells in the lateral somite (Chen et al., 2001; Coutelle et al., 2001; Weinberg et al., 1996). The fate of the medial and lateral myogenic cells is known: medial adaxial cells form slow muscle and most migrate laterally to generate the superficial slow muscle fibres that lie within the somite under the

epidermis (Blagden et al., 1997; Devoto et al., 1996). At a slightly later stage of development, lateral somitic cells give rise to the fast muscle that makes up the bulk of the myotome (Devoto et al., 1996). Subsequently, the myotome grows by the addition of further cell populations at its dorsal and ventral extremes, a situation reminiscent of the dorsomedial and ventrolateral dermomyotomal lips of amniotes (Barresi et al., 2001; van Raamsdonk et al., 1982; Veggetti et al., 1990).

Secreted signalling molecules encoded by Hedgehog (Hh) genes, which are expressed in ventral midline tissues, are required for appropriate medial slow muscle development (Barresi et al., 2000; Blagden et al., 1997; Coutelle et al., 2001; Currie and Ingham, 1996; Du et al., 1997; Lewis et al., 1999; Norris et al., 2000). Hh is not required for myogenesis of lateral fast muscle or a second wave of slow fibres formed from the dorsoventral tips of the myotome (Barresi et al., 2001; Blagden et al., 1997). Thus, in fish, the distinct contractile fibre type of successive waves of fibres has permitted elucidation of several modes of fibre formation.

In amniotes, independent regulation of several somitic muscle precursor populations has also been described although no clear-cut distinction, e.g. on the basis of fibre type, between the products of different myogenic inductions has been reported (Hadchouel et al., 2003; Kahane et al., 2001; Sacks et al., 2003). In birds and rodents, as in fish, early populations of fibres express slow MyHC, whereas some later fibres do not. As in fish, prevention of ventral midline signalling or blocking *sonic hedgehog* (*shh*) in birds eliminates markers of the earliest myogenic cells in the somite (Borycki et al., 1998; Pownall et al., 1996), while *shh* overexpression can enhance expression of *myod* and terminal muscle differentiation (Johnson et al., 1994; Kahane et al., 2001). Mice with ablated *Shh* function show reduced epaxial myogenesis at the dorsomedial lip of the dermomyotome (Borycki et al., 1999; Chiang et al., 1996). Elimination of the *Shh* and indian hedgehog (*Ihh*) genes or their receptor Smoothened in the mouse leads to more severe loss of medial myogenesis (Zhang et al., 2001), just as occurs in zebrafish. Lateral myogenesis within the somite is relatively unaffected by loss of Hh signalling. However, the parallel role of Hh in induction of sclerotome (Fan et al., 1995), which constitutes a large part of the early somite in amniotes, and conflicting views on the role of Hh in dermomyotome development makes interpretation difficult (Borycki et al., 1999; Cann et al., 1999; Huang et al., 2003; Kruger et al., 2001; Teillet et al., 1998). Actinopterygian teleost species diverged from sarcopterygian amniote ancestors over 400 Mya. Nevertheless, the similarities between amniote and fish myogenesis raise the possibility that a common system has been co-opted to different ends during evolution. To address this issue, we turned to amphibia, which, although sarcopterygian derived, diverged from amniotes over 350 Mya.

In *Xenopus laevis*, the axial musculature begins to differentiate early (Cary and Klymkowsky, 1994; Chanoine and Hardy, 2003; Hopwood and Gurdon, 1990; Hopwood et al., 1991). As in zebrafish, axial muscles are functional by 24-26 hours of development (stage 22-24, when the frog has 12-15 somites) and differentiation progresses in an anterior to posterior direction. Thus, over a number of developmental stages, anterior myotomes contain muscle cells that are more advanced than posterior myotomes. There is a major difference in fate of anterior and posterior somites in *Xenopus*: anterior (trunk) somites ultimately generate the complex vertebral and muscular structure of the metamorphosed adult, whereas posterior (tail) somites are fated to cell death (Nieuwkoop and Faber, 1967). Although the differentiation of muscle fibres starts at a very early stage, differences between muscle fibre types have not been reported until stage 35 or later (Hughes et al., 1998; Kordylewski, 1986; Schwartz and Kay, 1988). As in fish, the bulk of the late *Xenopus* embryo somite is built of large fibres, whereas, on the lateral surface of the myotome, there is a monolayer of distinct fibres. In many anurans a cell layer designated dermatome exists in this lateral location (Radice et al., 1989). We describe the pattern of differentiation of slow and fast muscle fibre types at early stages and its dependence on anteroposterior position. We show that manipulation of Hh signalling can affect the decision between fast and slow muscle formation in a manner similar to that observed in zebrafish. We go on to characterise later somite myogenesis and suggest a model for evolutionary adaptation of myogenesis in the transition from fish to tetrapod bodyplan.

Materials and methods

Embryo preparation and manipulation

Xenopus laevis embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Embryos at the two- to four-cell stage were injected with RNA prepared from pSP64T plasmid containing the full-length coding region of zebrafish *shh*. Whole-mount mRNA in situ hybridisation was performed using antisense RNA probes to *Col1a1* (Goto et al., 2000), *Xptc2* (Takabatake et al., 2000), *Pax3* (Martin and Harland, 2001), *XMyf5* (Hopwood et al., 1991), *actin* and *XMyoD* (Hopwood and Gurdon, 1990). Devitellinized embryos were exposed to 100 μM cyclopamine or solanidine or ethanol vehicle control.

Immunochemistry and histology

Antibodies used in this paper are available from Alexis, ATCC, the DSHB, Iowa and/or DSG Braunschweig, Germany. Antibody A4.1025 (IgG2a) recognises many, probably all, sarcomeric MyHCs in species from *Drosophila* to human (Dan-Goor et al., 1990). BA-F8 (IgG2b) was raised against human MyHC and reported to react with slow and cardiac MyHC in humans and mice, and BA-D5 (IgG2b) was raised against human MyHC and reported to detect slow MyHCs in rodent, chicken and zebrafish (Blagden et al., 1997; Schiaffino et al., 1989). Antibody EB165 (IgG1), raised against chicken fast MyHC, was a gift from Dr Everett Bandman (Cerny and Bandman, 1986). The muscle marker 12/101 was a gift from Dr Jeremy Brockes. Cryosections of staged embryos fixed in Dent or paraformaldehyde fixative were stained according to (Blagden et al., 1997), with the use of class or subclass-specific secondary and fluorescent tertiary reagents (Jackson ImmunoResearch). After whole-mount in situ mRNA hybridisation, embryos were cryosectioned and reacted for all MyHC with A4.1025 or anti-proliferating cell nuclear antigen (PCNA, Sigma). Analysis and photomicrography was on Zeiss Axiophot or Axiocam. For plastic sections, devitellinized embryos were fixed in 2% glutaraldehyde in amphibian Ringer solution, embedded in Epon-Araldite 812 mixture and semi-thin sections stained with crystal violet and basic fuchsin.

Results

Two muscle fibre types in *Xenopus* embryos

We screened over 20 monoclonal antibodies known to recognise specific isoforms of mammalian or avian MyHC on sections of developing *Xenopus* and found several that

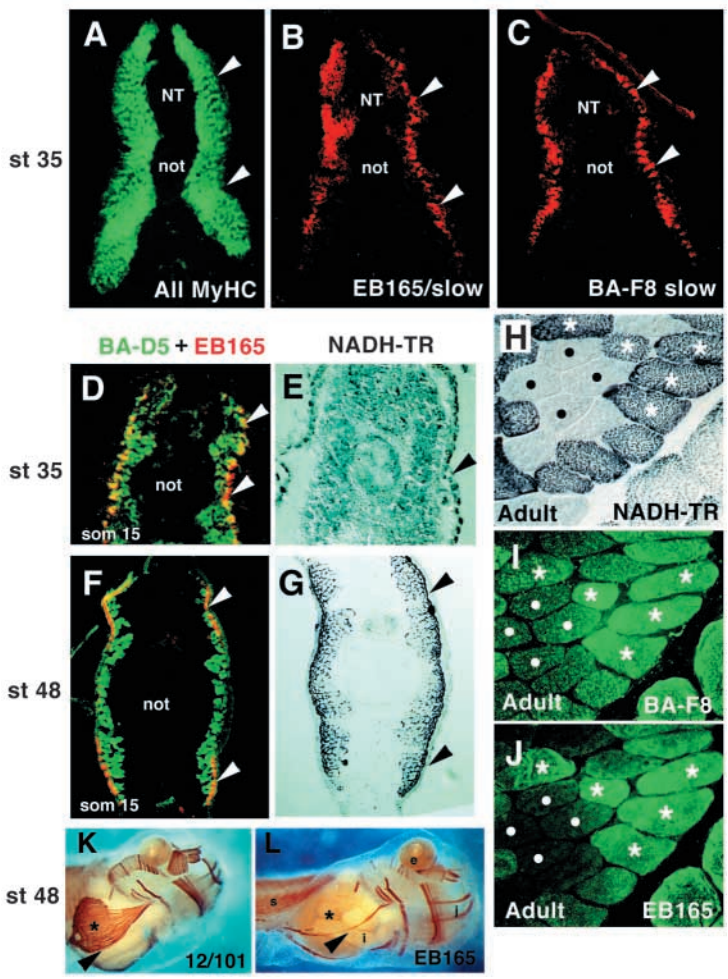

Fig. 1. Monoclonal antibodies distinguish two populations of muscle cells in *Xenopus*. Transverse cryosections of stage 35 (A-E), stage 48 trunk (F,G) and adult hindlimb muscle (H-J) stained with monoclonal antibodies to all skeletal muscle MyHC isoforms (A), MyHC of the myotomal superficial muscle fibre monolayer (B-D,F,I,J), MyHC of the deep muscle layers (D,F, green) and NADH-TR histochemistry of adjacent serial sections showing fibres with high mitochondrial enzyme content (E,G,H). (A-G) Two antibodies (BA-F8 and EB165) preferentially label the outermost muscle fibre layer (arrowheads, A-D,F). Note that the superficial layer develops oxidative metabolism (arrowheads E,G). (H-J) In adult muscle, oxidative fibres weakly express MyHC immunologically, similar to the superficial muscle layer in the larvae (asterisks), whereas glycolytic fibres show only background staining (dots). (K,L) Whole-mount immunohistochemistry reveals that a subset of all muscle fibres marked by the 12/101 muscle marker (K) express the EB165 epitope (L). Note the EB165 expression at the leading edge of the abdominal fibre layer (arrowhead) and its lack in more dorsal fibres (asterisk) at stage 48. A subset of head fibres express slow (EB165). NT neural tube; not, notochord; e, eye; i, intestine; j, jaw; s, somite.

distinguished two populations of muscle fibres in stage 35 embryos (Fig. 1). EB165 and BA-F8 react strongly with a single layer of superficial muscle fibres (Fig. 1B,C). BA-D5 reacts with the vast majority of the muscle fibres at this stage, as revealed by all MyHC antibody A4.1025 and by 12/101, a frequently-used *Xenopus* muscle marker (Fig. 1A,D; data not shown). Thus, in *Xenopus*, the 12/101;A4.1025;BA-D5 and EB165;BA-F8 antibodies distinguish two muscle fibre populations.

Slow muscle fibres are usually oxidative with abundant mitochondria. NADH tetrazolium reductase histochemistry reveals oxidative cells in the superficial location of EB165;BA-F8-reactive cells (Fig. 1E), as in zebrafish (van Raamsdonk et al., 1978). The presence of the BA-F8 slow MyHC epitope and abundant mitochondria in superficial cells suggests they are slow fibres. This expression persists through at least st48 (Fig. 1F,G). EB165 and BA-F8 also detect oxidative slow fibres weakly but preferentially in adult *Xenopus* leg muscle (Fig. 1H-J). Whole-mount staining revealed that EB165 also detects a subset of fibres in head muscles and that EB165^{+} fibres are present near the leading edge of the fibres forming from migratory muscle precursors on the abdomen (Fig. 1K,L) (Martin and Harland, 2001). So, EB165 reactivity may be transient during fibre type maturation in some fibre populations. Nevertheless, we hereafter employ BA-D5, A4.1025 and 12/101 as markers of all fibres, and BA-F8 and EB165 as markers of the superficial slow fibres. For simplicity, and by analogy with zebrafish, we hereafter refer to the fibres that do not react with BA-F8 and EB165 as ‘fast’.

Several muscle fibre populations arise sequentially

We investigated the timing of appearance of the differentiated slow and fast muscle fibre populations in *Xenopus* (Fig. 2). The earliest-formed muscle is fast, appearing in anterior somites before stage 22 (Fig. 2A,B). As in zebrafish, fast cells form the majority of medial/deep muscle in the older animal (Fig. 1F; Fig. 2K-N). By contrast, the slow fibres arise later in development, being first detected weakly in the tail tip of stage 27/28 embryos where a group of slow cells appear to span the somite from adjacent to the notochord towards the lateral somite surface (Fig. 2F, inset). At this stage, embryos have about 20 somites, and yet the older trunk somites do not have slow fibres (data not shown). By stage 35 the posterior half of the embryo, including somites 18-20, contains superficial slow cells outlining the lateral border of each somite (Fig. 2I, see also Fig. 5A). In somite 36 at the tail tip, the slow fibre markers span the somite transversely, as occurred earlier in the 20th somite (compare Fig. 2F, inset, 2J). As the functional studies below reveal, slow fibres formed prior to about stage 35 have a similar origin and we designate them ‘first wave slow fibres’. At later stages, slow superficial fibres appear in successively more anterior somites such that, by stage 48, all somites contain an outer layer of slow cells (Fig. 2K-N). This anterior extension of slow fibres roughly parallels the retraction of the gut, so that somites without underlying endodermal tissue contain slow fibres. The superficial slow fibres are detected preferentially at the dorsal and ventral extremes of the somite in stage 48 embryos, which is not the case at stage 35 (Fig. 2I-N). Functional studies below indicate that these later-formed slow fibres have a distinct

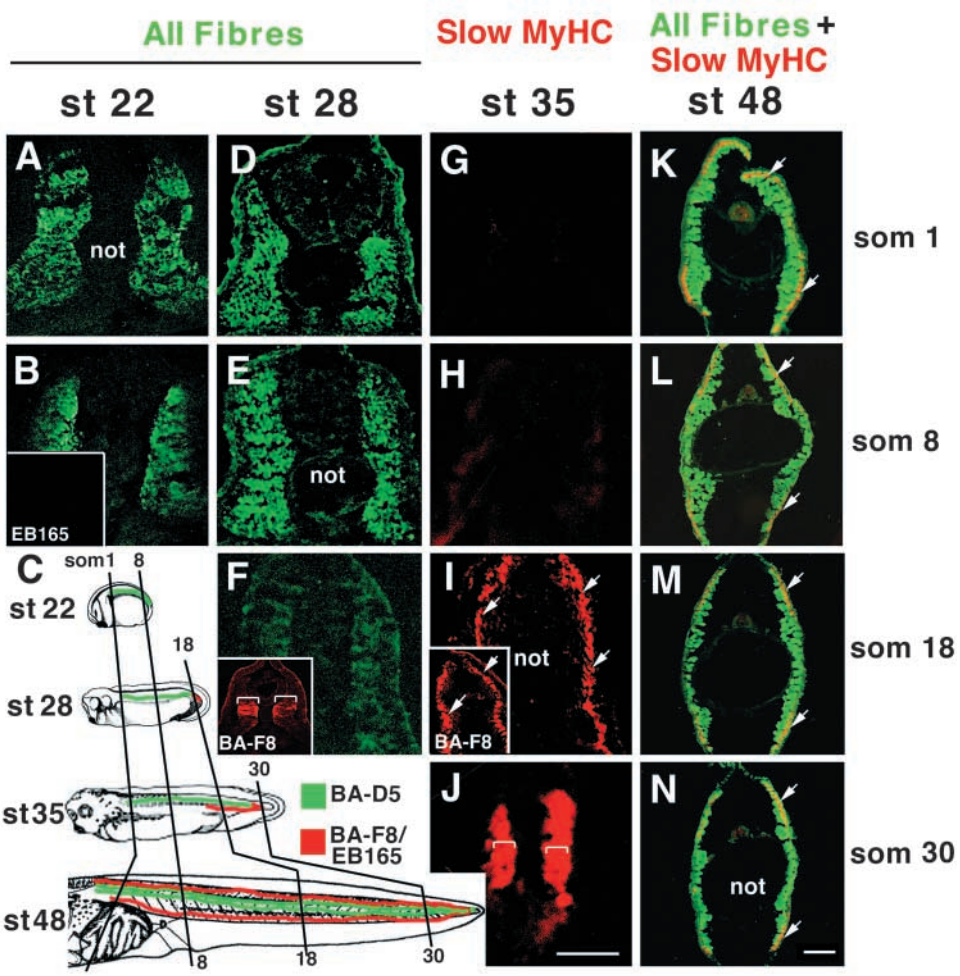

Fig. 2. Slow muscle fibres mature in a posterior to anterior wave. Embryos at successive developmental stages were serially sectioned and stained for slow (EB165, red) and all (BA-D5, green) fibres. Approximate somite number in each row of sections is indicated on the right, counting from anterior. Thus, temporal development of a somite can be followed left to right. (A,B) Only fast fibres (green) are present in the ~10 somites formed at stage 22. (C) Summary scale diagram showing the stages described with anteroposterior extent of expression of MyHC markers highlighted [modified, with permission, from Nieuwkoop and Faber (Nieuwkoop and Faber, 1967)]. (D-F) Fast expression continues anteriorly in stage 28 embryos, but in the most posterior region, the fast marker is less apparent. A few cells reactive with slow muscle marker (red) are detected medially only in the most posterior region of this ~20 somite embryo (F, inset, brackets). (G-J) By stage 35, cells in post-anal tail express slow markers in a monolayer of superficial cells (I, arrows), which is not apparent in trunk somites (G,H). In the most posterior somites of these ~36-somite embryos, slow markers appear to span the somite (J, brackets). (K-N) Fast fibres fill the somite at stage 48, as occurs earlier, and slow fibres form a monolayer at the dorsal and ventral extremes of the lateral myotome (arrows). Note that several antibodies show weak and variable crossreactivity to epidermis. not, notochord. Scale bars: in J, 75 μm for A,B,D-J; in K, 75 μm for K-N; in insets, 86 μm.

embryological origin and we designate them ‘second wave slow fibres’. Thus, because anterior (trunk) somites develop first, deep medial ‘fast’ fibres arise first during development. In more posterior (tail) somites, by contrast, first wave slow fibres arise first, initially in the medial somite and then become located more superficially as the bulk of the somite differentiates into fast muscle. Second wave slow fibres arise later in all somites.

Hedgehog signalling induces superficial slow muscle fibres

Superficial slow fibres in zebrafish arise next to the notochord and depend on axial signals carried by Hh proteins (Blagden et al., 1997; Du et al., 1997). To test the homology between the superficial slow muscle fibres of *Xenopus* and zebrafish with respect to their induction by Hh proteins, we overexpressed Shh in *Xenopus* embryos and analysed muscle fibre pattern at stage 35. *Shh*-injected, but not control uninjected or *lacZ*-injected, embryos show a mis-positioning and an increased incidence of slow muscle fibres (Fig. 3). *Shh*-injected embryos contain significantly more slow fibres/section (33.0±1.7 profiles, $P<0.01$) than controls (21.6±0.6). Both the extent and location of ectopic slow markers are patchy, with the location of ectopic slow fibres correlating with regions of co-injected *lacZ* activity (Fig. 3). However, ectopic slow fibres never exceeded a threefold increase in fibre number, nor did we detect loss of fast fibres. Strikingly, we did not observe increased slow fibres in anterior trunk somites. Thus, extra Hh signalling can augment first wave slow fibre formation, suggesting that Hh promotes formation of first wave slow fibres in *Xenopus*, as in zebrafish.

In zebrafish, notochord-derived Hh is required for adaxial cell *myf5* and *myod* expression and slow fibre formation, but not for lateral *myf5* and *myod* expression and fast muscle differentiation (Blagden et al., 1997; Coutelle et al., 2001). To test for a role of midline signals in *Xenopus* myogenesis we ablated notochord, but not neural plate which is required for lateral myogenesis (Mariani et al., 2001). Extirpation of notochord causes significant reduction of *XMyf5* expression. Yet there is no noticeable loss of *XMyoD* or actin expression, which confirms that neural plate remains intact. *XMyf5* mRNA decline is particularly noticeable in adaxial cells flanking the anterior notochord (Fig. 4A). This result raises the possibility that a midline-derived signal promotes adaxial, but not more lateral, myogenesis in *Xenopus*, just as in zebrafish.

As notochord expresses Shh, we tested the hypothesis that Hh is required to generate slow muscle. *Xenopus* embryos were exposed to the Hh-signalling antagonist cyclopamine during

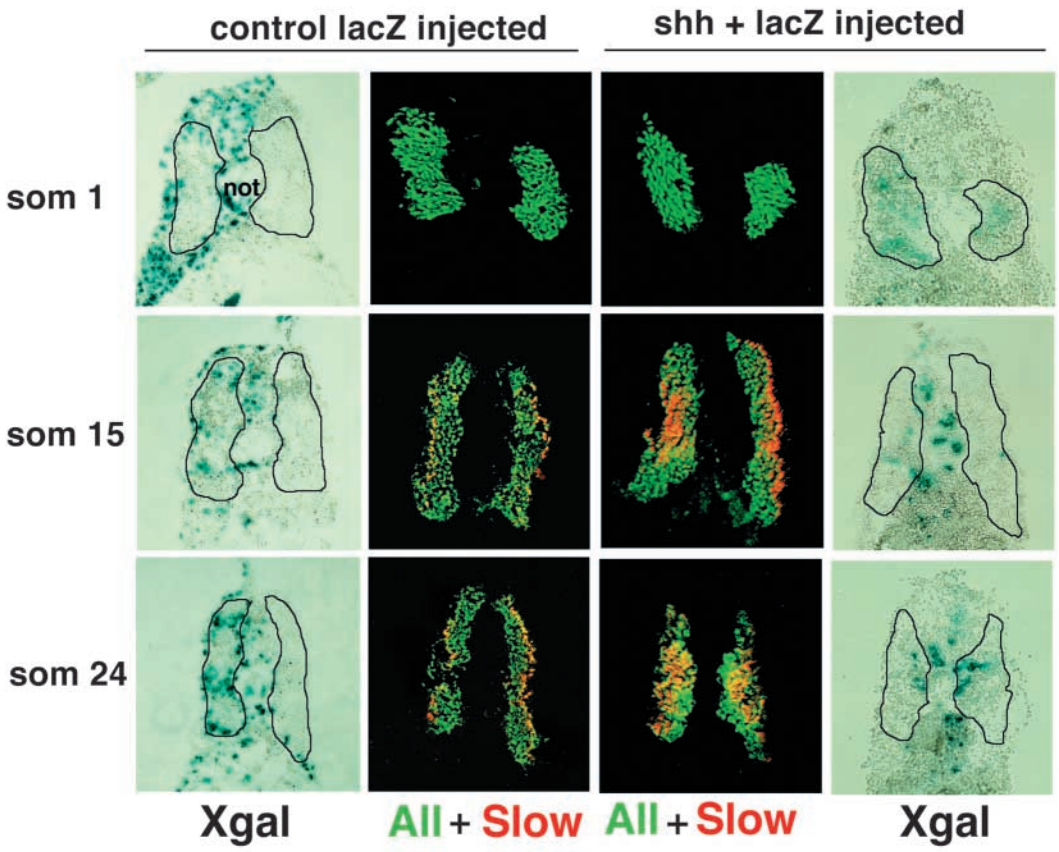

Fig. 3. Overexpression of sonic hedgehog induces ectopic slow muscle fibres. Control *lacZ* RNA, with (right panels) or without (left panels) RNA encoding zebrafish *shh*, was injected into one side of four-cell *Xenopus* embryos and the animals allowed to develop for 2 days until stage 35. Embryos were fixed, serially sectioned and stained for slow (red, EB165) and all sarcomeric (green, A4.1025) MyHCs to identify muscle fibre populations. Whereas *lacZ*-injected embryos never showed alteration in superficial slow muscle fibre number or position, either close to β-galactosidase activity or elsewhere, Shh-injected embryos frequently contained ectopic slow fibres in regions showing overexpression of β-galactosidase. Somite is outlined on X-gal panels. Despite injected RNA frequently being highest in anterior regions, ectopic slow muscle was detected posteriorly within embryos. This suggested that induction of ectopic slow fibres was more readily achieved in regions that normally contain slow superficial cells at this stage.

the period of slow myogenesis. Cyclopamine inhibits Smoothened, a required component of the Hh signalling pathway (Chen et al., 2002; Frank-Kamenetsky et al., 2002). First, we demonstrated that cyclopamine inhibits Hh signalling in *Xenopus* embryos by examining the expression of the patched gene, *Xptc2* (Takabatake et al., 2000). Expression of *patched* genes, which encode Hh receptors, is upregulated by Hh signalling (Ingham and McMahon, 2001). At stage 28, *Xptc2* is highly expressed in tailbud regions where slow muscle cells are forming next to *Xshh* expression in the notochord, and also in somite chevrons close to *banded hedgehog* expression (Ekker et al., 1995). Treatment with cyclopamine blocks *Xptc2* expression throughout the embryo at stage 13, 20, 28 and 36, whereas treatment with the related alkaloid solanidine has no effect (Fig. 4B and data not shown). Therefore, cyclopamine treatment is a specific Hh signalling inhibitor in *Xenopus*.

At early stages, cyclopamine has no effect upon medial *XMyf5* expression in trunk regions, where first wave slow muscle does not form, even though *Xptc2* expression is suppressed (data not shown). However, cyclopamine downregulates *XMyf5* expression in the PSM and nascent somites in the tail creating a ‘gap’ in tailbud expression just where untreated embryos initiate first wave slow myogenesis in the tail (Fig. 4C). Reduction of *XMyf5* is specific to the gap region at this stage, as expression in dorsal and ventral myotome of maturing somites is not affected. Strikingly, the missing *XMyf5* expression domain is the region where *Xptc2* and *XMyf5* mRNAs are high in medial cells flanking notochord (see Fig. 4B, Fig. 7G). Moreover, expression of *XMyoD* is reduced in the tail, though less markedly than *XMyf5*, but is less affected anteriorly (Fig. 4C). Thus, Hh signalling is required for normal myogenesis in the region of the first slow fibre formation in the *Xenopus* tail.

We next determined whether the cyclopamine-induced reduction in MRF expression in the medial cells of tail PSM is paralleled by later changes in slow muscle. Cyclopamine greatly reduces slow muscle formation in tail somites of stage 36 and younger embryos (Fig. 5). The related alkaloid solanidine has no effect on slow myogenesis, paralleling its inability to block Hh signalling (Fig. 4B; data not shown). No effect of cyclopamine is detected before slow muscle formation begins: medial fast muscle appears normal in trunk somites up to stage 28 (data not shown). From stage 28 onwards, however, several defects are observed in posterior tail somites, where slow myogenesis is initiated in control embryos. Live cyclopamine-treated stage 36 embryos exhibit increased spontaneous movement and a slightly thinner tail tip when viewed from the dorsal surface (data not shown). Paralleling the loss of tail slow fibres, general muscle markers such as sarcomeric MyHC and 12/101, are also lost from tail tip, although tailbud outgrowth has not ceased (Fig. 5A). This result (1) shows that blockade of Hh signalling prevents terminal differentiation and/or survival of the slow fibres, rather than changing their character; and (2) confirms that slow fibres are the first to form in tail somites. Cyclopamine-treated embryos have a small but consistent reduction in the

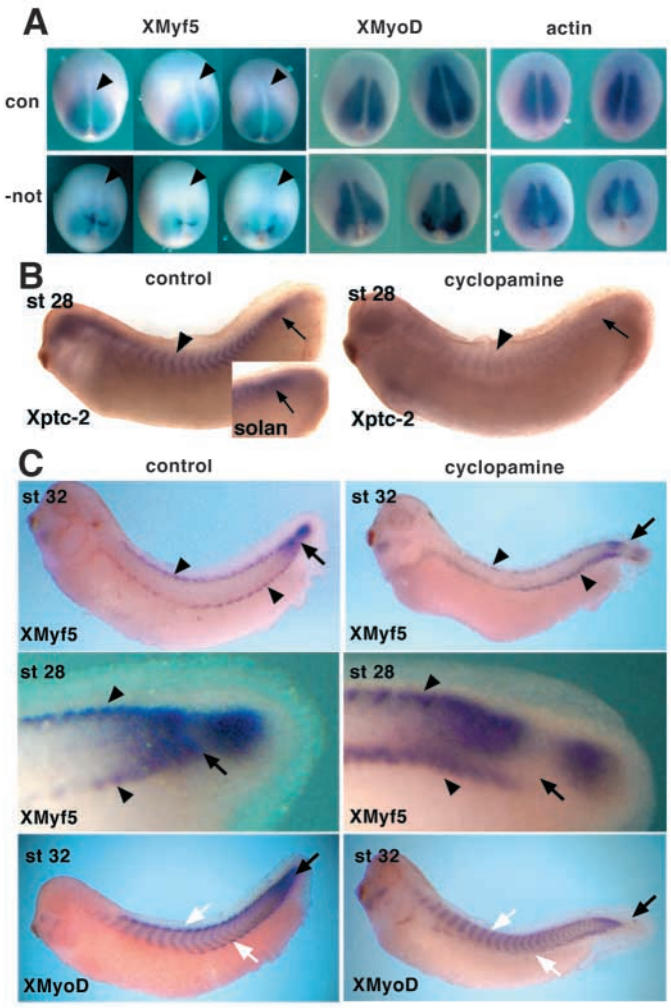

Fig. 4. Notochord and Hedgehog signalling are required for normal MRF expression. (A) Notochord was ablated at stage 13 and embryos analysed by in situ hybridisation 2 hours later for *XMyf5*, *XMyoD* and *actin* mRNA. Arrowheads indicate adaxial tissue with high *XMyf5* expression that is absent after notochord ablation. (B) *Xptc2* expression in stage 28 embryos is ablated by cyclopamine treatment, both in the first wave slow muscle-forming region (arrows) and elsewhere (arrowheads). Solanidine has no effect (inset). (C) Embryos treated with cyclopamine, or ethanol vehicle control, at stage 9 and fixed at stage 28 or 32. *XMyf5* in PSM is reduced creating a 'gap' in tail expression (arrows). However, dorsal and ventral somite borders retain *XMyf5* expression (arrowheads). *XMyoD* is reduced in tail tip (arrows), but unaffected in somitic stripes anteriorly. The dorsal and ventral somite borders fail to upregulate *XMyoD* in presence of cyclopamine (white arrows). Note reduced chevron form and dorsoventral extent of anterior *XMyoD* signal.

dorsoventral extent of musculature and a loss of chevron shape in tail somites (Fig. 5A). Defects are particularly marked in the medial somite (Table 1). Posterior muscle-containing somites beyond about somite 13 show an unusual segregation of a ventral cluster of fibres at stage 36 that parallels the altered *XMyf5* expression pattern (Fig. 4C; Fig. 5A, arrowheads). In other words, zones of remaining muscle formation in cyclopamine-treated embryos still express *XMyf5* mRNA. Slow muscle fails to form just where *XMyf5* mRNA is missing.

Table 1. Cyclopamine reduces muscle differentiation in tail somites

Experiment	Cyclopamine treatment	Analysis	Number of somites expressing 12/101*	Number of somites expressing 12/101 across entire dorsoventral extent without a gap*
1	Stage 12	Stage 36	30±1 (3)	13±2 (3)
	Control	Stage 36	32±2 (3)	32±2 (3)
	Stage 12†	Stage 48	~45 (3)	~35 (3)
	Control†	Stage 48	~50 (3)	~50 (3)
2	Stage 22-26	Stage 41	30.7±2.4 (10)	30.0±2.1 (10)
	Control	Stage 41	40.4±3.3 (18)	40.4±3.3 (18)
	Stage 22-26	Stage 48	48.6±3.2 (11)	31.3±2.1 (11)
	Control	Stage 48	53.3±2.4 (13)	50.0±2.4 (13)
3	Stage 21	Stage 47	41.7±0.7 (3)	29.7±0.6 (3)
	Control	Stage 47	46.3±2.1 (3)	37.3±0.6 (3)

Whole-mount stained embryos were scored under a Zeiss Axiophot.
Control, ethanol vehicle control slightly retards development but muscle appears normal.
*Mean±s.d. (number of embryos).
†Somite counts inaccurate because muscle was hypercontracted.

At stage 48, treated embryos have around 10 fewer somites containing muscle than controls, and the most posterior muscle-containing somites show a medial loss of muscle (Fig. 5C, Table 1). Thus, cyclopamine-treatment blocks initiation of slow muscle formation but has little if any effect on fast muscle formation. The Hh dependence of both *XMyf5* mRNA and then slow MyHC expression in tail somites defines the first wave slow fibres.

Second wave slow myogenesis is Hedgehog independent

In trunk somites, superficial slow muscle arises later than in tail somites, between stage 35-48 (Fig. 2). Examination of cyclopamine-treated embryos at stage 41 reveals that trunk slow muscle is formed, even though Hh signalling is inhibited (Fig. 5B,D). Thus, trunk slow fibre formation is Hh independent and this defines the second wave slow fibres. Despite the early lack of first wave slow fibres in tail somites, second wave slow fibre formation does occur in tail somites of cyclopamine-treated embryos (Fig. 5B,C). After cyclopamine treatment, anterior tail somites 15-30 lacked the first wave of slow muscle cells at stage 36, but by stage 41 slow fibres are present in these somites (compare Fig. 5A with 5B). First wave slow myogenesis initiates in the medial somite at the tail tip of untreated embryos and spreads outward dorsally and ventrally (Fig. 5A,B). In cyclopamine-treated embryos, by contrast, slow MyHC is initiated in the dorsal and ventral lips of the superficial myotome and appears to spread rapidly inwards to cover the surface of more anterior tail somites (Fig. 5B, arrows). Therefore, the late appearance of slow fibres is unlikely to arise from a delay in differentiation of first wave slow cells. A continuing defect in slow fibres is observed in the newly formed posterior tail somites at stage 41 and even more markedly at stage 48, indicating that effects of cyclopamine persist throughout tailbud outgrowth (Fig. 5B,C; Table 1). Our results suggest that cyclopamine ablates the first wave of slow fibres in the tail, but that a second wave of (Hh independent) slow fibre formation occurs in all somites, similar to that

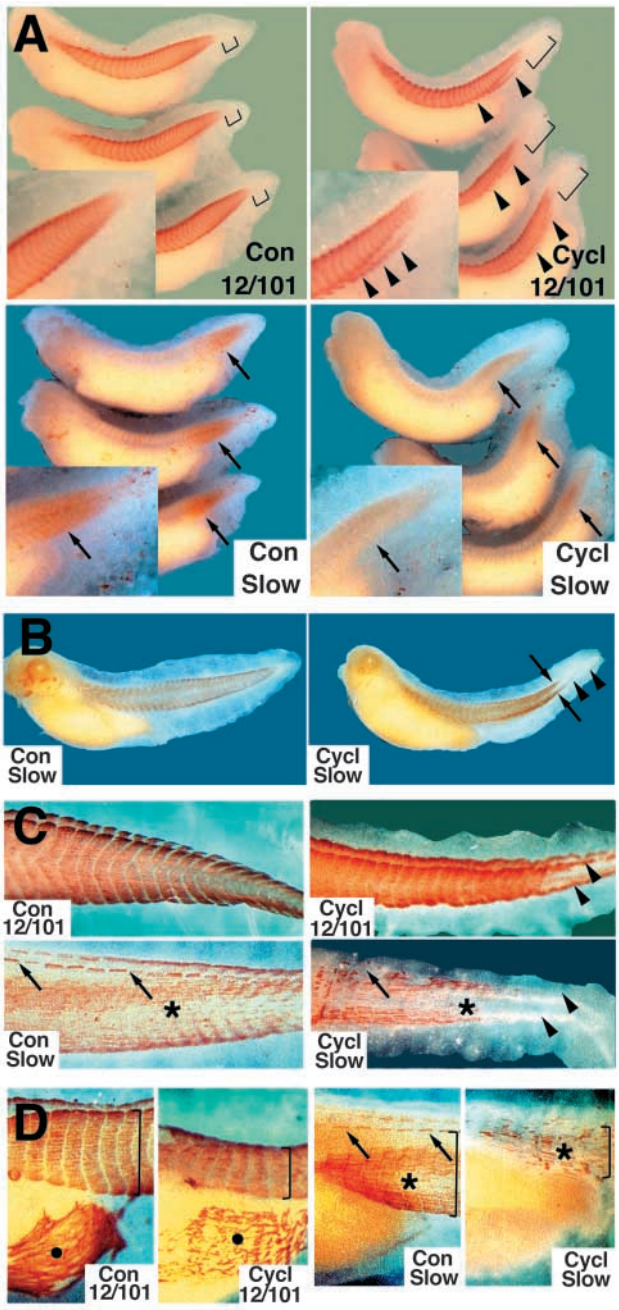

Fig. 5. Cyclopamine blocks early slow muscle formation. *Xenopus* embryos were de-vitellinized, treated with cyclopamine (100 μg/ml), or ethanol vehicle control, fixed at various stages and stained in whole mount for muscle (12/101) or slow (EB165) fibres. (A) Treatment at stage 22 leads to bent embryos with loss of posterior muscle, and severe loss of slow fibres by stage 36 (arrows). A separate group of ventral fast fibres is visible in posterior somites of cyclopamine-treated embryos (arrowheads). Posterior tissue is formed but fails to make muscle (brackets). Insets show the posterior somites at higher magnification. Note poor chevron formation. Slow muscle is greatly reduced or absent. (B) Embryos allowed to develop to stage 41 showing slow myogenesis in anterior somites of both control and cyclopamine-treated embryos, but continued posterior defects in treated embryo (arrowheads). Slow muscle initiates at dorsal and ventral extremities of the somite (arrows). (C,D) Cyclopamine treatment from stage 12 until stage 48 yields similar results. (C) Tail somites 15-30 of untreated embryos (left panels) are extensive and chevron shaped, with a ventral layer of slow fibres (asterisk), separated from a small group of slow fibres at the dorsomedial lip (arrows). Cyclopamine-treated embryos (right panels) have reduced differentiation, dorsoventral extent and less marked chevron shape. Note the initiation of fast myogenesis at dorsoventral extremity of somites in the absence of slow fibres (arrowheads) and lack of a separate row of dorsal slow fibres in the treated embryo (arrow). Slow myogenesis is reduced and commences more anteriorly than in controls (asterisk). (D) In trunk somites, cyclopamine causes reduction in dorsoventral somite extent (brackets), disorganised ventral body wall fast fibres (dot) and reduced somitic slow fibres (asterisk). Note the absence of a separate group of slow fibres at the dorsomedial lip (arrows).

reported in zebrafish (Barresi et al., 2001). In trunk somites, this second wave generates the first slow fibres of the myotome, whereas in tail somites the second wave probably augments the slow fibres already formed around the time of somitogenesis by the first wave.

Dermomyotomal myogenesis requires Hh signalling

Trunk somites are not completely insensitive to cyclopamine. Although second wave slow fibres form normally, cyclopamine treatment reduces *XMyoD* expression in dorsal and ventral edges of trunk and anterior tail somites, whereas *XMyf5* expression is normal at these locations (Fig. 4C). Subsequently, the somite is reduced dorsoventrally and there is a lack of what appears to be a transient population of slow fibres at the dorsomedial myotomal edge (Fig. 5C,D). In addition, ventral muscle fibres over the belly are aberrant, indicating disruption of ventral lip myogenesis. Section analysis of unmanipulated embryos reveals that strong *XMyoD* expression in the dorsal and ventral somite edges is associated with the dermomyotomal lips (Fig. 7E,F). Thus, Hh signalling is required for some aspects of trunk myogenesis.

Xenopus dermomyotome: a potential source of second wave slow fibres

To investigate the sources of second wave slow fibres formed in *Xenopus* somites, we prepared serial plastic sections of embryos at stages 22, 28 and 35 (Fig. 6A-F). Throughout the period, two epidermal cell layers surround the embryo as described (Nieuwkoop and Faber, 1967). At stage 22 and 28, the anterior 18 somites contain one striking specialisation, a layer of thin cells covering the lateral somite surface in some regions (Fig. 6A-C) (Blackshaw and Warner, 1976; Hamilton, 1969). As no slow fibres have yet formed in trunk somites, this layer is reminiscent of amniote dermomyotome. At stage 35, a single superficial monolayer of distinct cells is discerned both in trunk somites that lack first wave slow fibres and in tail somites that contain superficial first wave slow fibres (Fig. 6D-H). This distinct superficial cell layer is not slow muscle because it lacks MyHC and sarcomeric myofibrils (Fig. 6G,H; Fig. 7C-F). *Pax3*, a dermomyotome marker in amniotes (Bober et al., 1994; Goulding et al., 1994), is expressed in cells superficial to the differentiated muscle (Fig. 6I-K). Strikingly, however, expression is greater in trunk than in tail regions beyond about somite 12 from stage 29-34 (Fig. 6I,J). Later, at stage 37/38, *Pax3* expression increases in posterior tail somites,

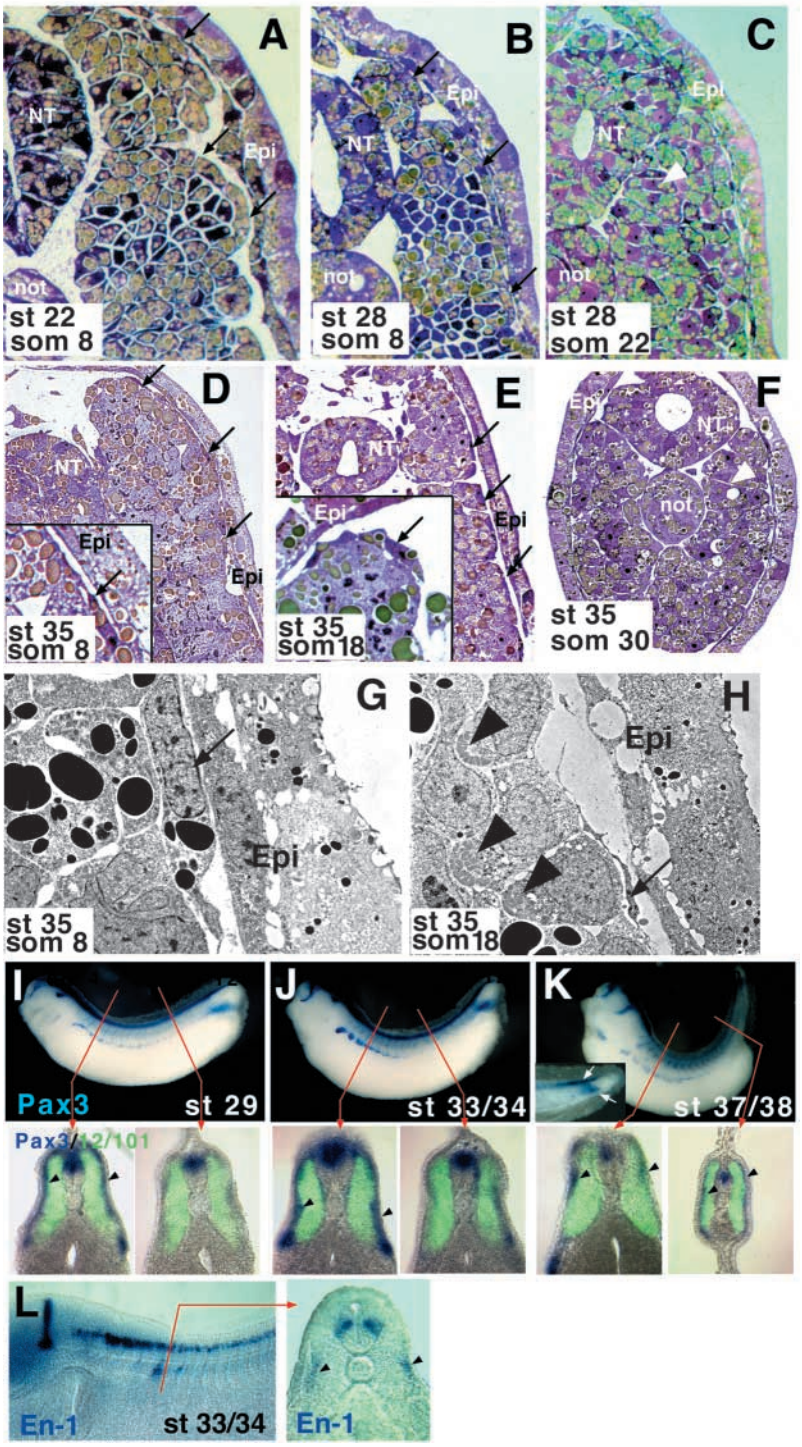

Fig. 6. Morphologically and molecularly distinct cell monolayer coats, first, trunk then tail somites. Embryos at stage 22(A), stage 28 (B,C) and stage 35 (D-F) were plastic embedded, transversally sectioned and stained with violet fuchsin. NT, neural tube; Not, notochord; Epi, epidermal bilayer. Yolk droplets appear yellow. A distinct superficial layer of cells covers trunk somites prior to superficial slow fibre differentiation (A,B,D, arrows) and anterior tail somites after slow fibre formation is initiated (E, arrows, compare with Fig. 1D). Insets show superficial layer (arrows) in middle of somite (D) and at dorsomedial lip (E) at stage 35. Note the transient lack of this layer in the nascent posterior somites present at stage 28 (C) and stage 35 (F), when single cells can be observed elongated across the somite (arrowheads). (G,H) Electron micrographs show a distinct dermomyotome (arrows) in somite 8 (G) but only spindly cells in somite 18 (H) above a layer of well-differentiated muscle with basal myofibrils (arrowheads). *Pax3* mRNA was detected by whole-mount in situ hybridisation of stage 29-38 embryos (I-K, upper panels) and *En1* mRNA marked a subset of medial cells in the superficial somite level with the notochord (L, arrowheads). Serial transverse 100 μm vibratome sections revealed that signal is superficial within the somite (I-K, lower panels, arrowheads) and non-overlapping with 12/101, a marker of differentiated muscle. Dorsal and ventral groups of cells in the tailbud express highly (K, inset, arrows). Expression persists in a complex pattern in all somites, but is consistently stronger in trunk somites anterior to about somite 12 at stage 29 (G) and stage 33/34 (H). Subsequently, *Pax3* increases in tail somites (I).

possibly in parallel with dermomyotome formation (Fig. 6E,H,K). Like amniote *Engrailed1* (Davidson et al., 1988; Davis et al., 1991), Xenopus *En1* is also expressed at stage 33, level with the notochord in the outermost layer of trunk but not tail somites (Fig. 6L). We conclude that in trunk and anterior tail somites a cell layer, which hereafter we call dermomyotome, covers the superficial surface of the somite.

To examine the differentiation status of dermomyotome further, stage 35 embryos were double stained for MRFs and MyHC. *XMyf5* and *XMyoD* are differentially expressed in *Xenopus* somites (Hopwood and Gurdon, 1990; Hopwood et al., 1991; Martin and Harland, 2001). Whereas *XMyf5* is most highly expressed at dorsomedial and ventrolateral lips of the somite, *XMyoD* labels a dorsoventral chevron across each somite (Fig. 7A,B). As in amniotes, *XMyf5* is strongly expressed at dorsomedial and ventrolateral lips of the dermomyotome (Fig. 7C,D). In addition, small groups of cells within the dermomyotome also express *XMyf5*, suggesting that myogenesis might be occurring at locations other than the lips (Fig. 7C, arrows). At a similar anteroposterior level, *XMyoD* mRNA accumulates only in the cells of the dermomyotome close to the dorsomedial lip and, unlike *XMyf5*, is also significantly expressed in MyHC-containing cells of the myotome proper (Fig. 7E,F). This expression suggests that, at the dorsomedial lip, myogenic cells in the surface layer initially express *XMyf5*, then accumulate *XMyoD* and finally terminally differentiate delaminating into the myotome proper, as occurs in amniotes. At the ventrolateral lip the structure usually appears distinct, with a ventral intense patch of *XMyf5* expression flanked medially and laterally by more weakly stained cells that are still undifferentiated. At least in trunk somites, these regions probably correspond to migratory precursors (Martin and Harland, 2001). Taken together, these data suggest the *Xenopus* dermomyotome contains several separate myogenic foci.

To further examine the fate of dermomyotomal cells in

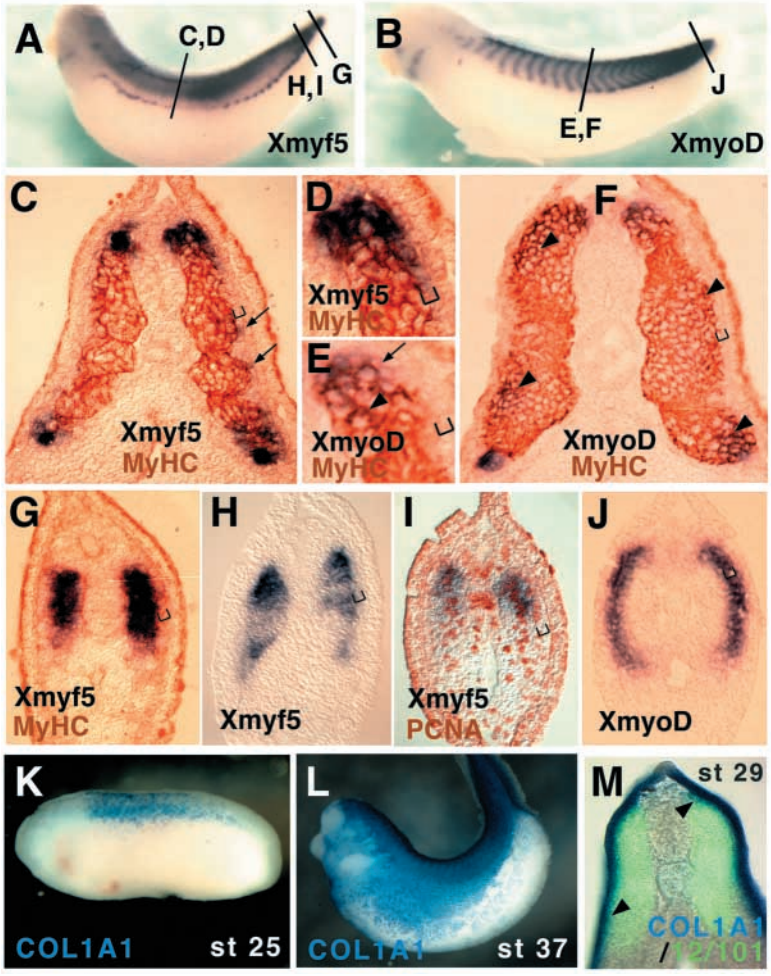

Fig. 7. *XMyoD* and *XMyf5* expression distinguish several myogenic cell populations in *Xenopus* somites. *XMyf5* (A,C,D,G-I) and *XMyoD* (B,E,F,J) mRNA was detected in whole-mount in situ hybridisation of stage 35 embryos. Sections of stained embryos at the approximate positions shown in A and B were mounted without further treatment (H,J) or after immunohistochemical staining for MyHC (C-G) or PCNA (I). (A,B) Whole-mount embryos showing the distinct expression of *XMyf5* and *XMyoD*, with section positions marked. (C-F) Trunk level sections showing that the superficial (dermomyotome, brackets) layer of the somite has distinct morphology, lacks MyHC expression and expresses *XMyf5* in dorsomedial (shown magnified in D) and ventrolateral lips, and in rare cells away from the lips (C, arrows). *XMyoD*, by contrast, is expressed within the superficial myotome (E,F, arrowheads) and in the most dorsal dermomyotomal cells (E, arrow). (G-I) Tail sections showing that *XMyf5* transcript is located medially in undifferentiated posterior tailbud (G). The outer layer of mesoderm lacks *XMyf5* (brackets). Cells with less signal appear orientated perpendicular to the notochord in slightly more anterior regions and are most obvious at dorsal and ventral somite extremes (H). The nuclei of some of these *XMyf5*-expressing cells contain PCNA (I). (J) *XMyoD* expression is primarily superficial within the somite in tail tip (bracket). (K-M) *Col1a1* is expressed in trunk regions at stage 25 (K) and more widely at stage 37 (L), and vibratome sections reveal expression in epidermis and more weakly in underlying dermomyotome (M, arrowheads).

Xenopus, we analysed *Col1a1* expression, which has been used to mark dermis (Goto et al., 2000). *Xenopus Col1a1* is expressed in the somitic regions from stage 25 and widely throughout the dorsal body at later stages when dermomyotome is mature (Fig. 7K,L). Transverse sections reveal that the dermomyotomal layer expresses *Col1a1*, as does overlying tissue of the epidermis (Fig. 7M). Thus, the stage 35 *Xenopus* trunk dermomyotome shares many characteristics with amniote dermomyotome.

Slow fibre myogenesis and migration

Comparison of MRF and MyHC expression in the tail bud provides insight into the early events in posterior muscle patterning when first wave slow and fast fibres types are generated. In *Xenopus*, the *XMyf5* expression pattern is similar to that in fish (Fig. 4C, Fig. 7A) (Coutelle et al., 2001). The most posterior *XMyf5* mRNA is abundant in a deep layer of cells adjacent to the notochord but is not detected in more superficial cells of the pre-somitic mesoderm. These *XMyf5*-expressing cells do not express MyHC (Fig. 7G). In anterior PSM, *XMyf5*-expressing cells no longer exclusively cluster around the notochord: some appear to span the somite, but *XMyf5* is still not detected in the outermost layer of cells (Fig. 7H). Some *XMyf5*-expressing cells with mediolaterally elongated nuclei express PCNA, which often marks proliferating cells (Fig. 7I). Cyclopamine prevents this anterior PSM *XMyf5* expression, but not tailbud expression (Fig. 4C). These data suggest that Hh signalling is required to maintain *XMyf5* expression in medial cells, which then become orientated mediolaterally, simultaneously losing *XMyf5*.

As *XMyf5* expression declines, *XMyoD* mRNA appears. The most posterior *XMyoD* expression is detected weakly in medial cells but within a few serial sections more anterior, is found exclusively superficially, consistent with loss of *XMyf5* and accumulation of *XMyoD* during lateral migration (Fig. 7J; data not shown). Reduction of this most posterior *XMyoD* expression is seen in cyclopamine-treated embryos (Fig. 4C). In plastic sections of this region, cells can be seen elongated mediolaterally across the somite, reminiscent of *XMyf5*-expressing cells and the slow fibres in these somites [compare Fig. 2F (inset) 2J with Fig. 6C,F and Fig. 7H]. Further anterior, *XMyoD* mRNA is present in newly differentiated superficial muscle beneath the dermomyotome (e.g. Fig. 7F). It seems likely, therefore, that the most superficial layer of cells in the most posterior tail somites contains nascent differentiating slow fibres that express *XMyoD*, but that shortly thereafter a dermomyotome arises to overlie the slow muscle.

Discussion

Our analysis of *Xenopus* myogenesis has revealed that (1) *Xenopus* early somites differentiate several distinct muscle fibre populations; (2) the common ancestor of tetrapods and

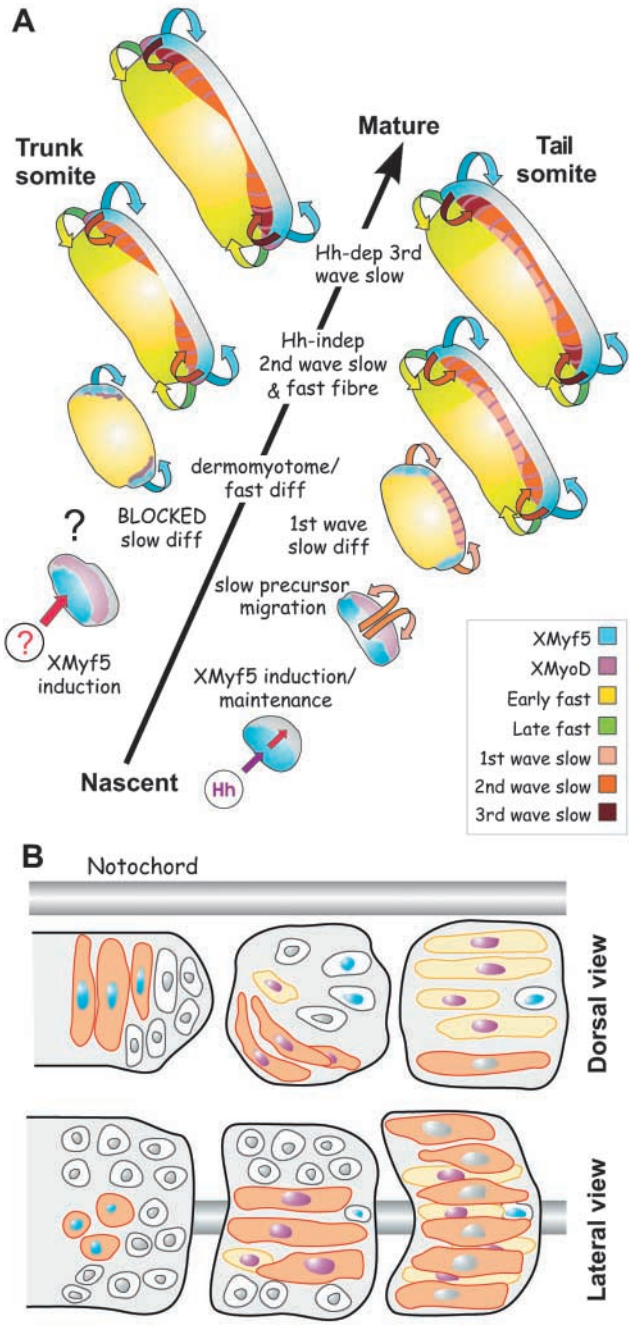

Fig. 8. Model of phases of myogenesis in trunk and tail. (A) Slow muscle is first formed in posterior somites (lower series, tail mode). Hh signalling from ventral midline acts on medial somitic cells to promote *XMyf5* expression (blue) and early slow myogenesis. These cells rapidly differentiate, express *XMyoD* (purple) and move to the superficial somite surface (orange arrows) where they elongate anteroposteriorly to make superficial slow fibres (pink). Simultaneously, most somitic cells differentiate into fast fibres, also elongating anteroposteriorly to form the bulk of somitic muscle (yellow). Undifferentiated cells form a dermomyotome (blue arrows). At later stages, a second population of slow muscle fibres (orange) is generated from dermomyotome, probably at dorsomedial and ventrolateral lips, independent of Hh signalling. In anterior somites (upper series, trunk mode), despite early notochord-dependent *XMyf5* expression (red arrow), a block on slow muscle formation prevents appearance of the first wave of slow fibres. Fast fibre formation is abundant, and precocious compared with zebrafish. However, some cells remain undifferentiated to form the superficial dermomyotome. Dorsal and ventral dermomyotomal lips continue to express *XMyf5* and *XMyoD*, reflecting their continued role as myogenic centres. Slow fibre formation is initiated from dermomyotome independently of Hh signalling. Extra fast fibres (green) probably also arise from dermomyotome at all anteroposterior levels. At even later stages Hh signalling is again required for *XMyoD* expression, somite growth and third wave slow fibre formation (dark red) at dermomyotomal lips throughout the axis. (B) How first wave slow fibre migration accompanied by terminal differentiation of fast fibres can appear like somite rotation.

teleosts developed first and second wave superficial slow and medial fast muscle fibre types in the manner first described in zebrafish – a pattern still extant in the *Xenopus* tail; (3) trunk myogenesis has undergone a striking modification during tetrapod evolution, involving a block on Hh-driven first wave slow myogenesis; (4) migration of early medial cells to the lateral somite surface may contribute to the apparent rotation of *Xenopus* somites and formation of dermomyotome; and (5) a dermomyotome similar to that of amniotes provides a source of myogenic cells during larval growth.

Muscle fibre types in *Xenopus* development

We have used antibody reagents to distinguish several fibre types in *Xenopus*. Abundant evidence shows that the superficial fibres are slow, including oxidative metabolism, early Hh dependence and labelling with BA-F8, a slow antibody in mammals. However, there is potential for confusion because BA-D5 and EB165 epitopes show a different spatial pattern in *Xenopus* to that observed in zebrafish embryos, where these antibodies also distinguish slow and fast fibres. In zebrafish, BA-D5 is first expressed in first wave slow fibres, located medially within the somite (Blagden et al., 1997). Subsequently, most of these BA-D5-reactive slow fibres migrate to a lateral and superficial position (Blagden et al., 1997; Devoto et al., 1996). In *Xenopus*, by contrast, the BA-D5 epitope is present in all/most larval muscle. Thus, the medial muscle that we have designated ‘fast’ to fit with zebrafish nomenclature, may actually have a slow contractile rate. In amniotes, all early fibres express some slow MyHC gene, regardless of their later fate (Page et al., 1992). In zebrafish, the medial myotome differentiates into EB165-reactive fast muscle (Blagden et al., 1997). In *Xenopus*, by contrast, EB165 marks slow fibres. The simplest explanation is that in *Xenopus* two epitopes characteristic of MyHC isoforms have come to be expressed in different cell populations. These observations emphasise that evolution can rapidly alter MyHC fibre type, but that MyHC markers are, nevertheless, useful in conjunction with other functional and molecular data to distinguish cell types of different developmental origin within one species.

Ancestral pattern of myogenesis: slow, quick, slow

In *Xenopus* tail somites, slow fibres arise initially in contact with the medial surface adjacent to the notochord and then become located superficially, as the bulk of the somite differentiates into fast muscle. This is what happens throughout

the body axis in zebrafish (Blagden et al., 1997; Devoto et al., 1996). Strong evidence for homology derives from (1) the mediolateral migration of slow precursors (see below); (2) the Hh-dependence of tailbud *XMyf5* and *XMyoD* expression and slow fibre formation; and (3) the generation of extra slow fibres when Hh signalling is increased. Hh dependence is also a feature of the first wave of slow myogenesis in zebrafish (Barresi et al., 2000; Blagden et al., 1997; Coutelle et al., 2001; Du et al., 1997; Lewis et al., 1999; Norris et al., 2000). Similarly, in both species, formation of deep fast fibres and a second wave of superficial slow fibres is Hh independent (Fig. 8A, tail series) (Barresi et al., 2001; Blagden et al., 1997; Du et al., 1997). The striking similarities between zebrafish and *Xenopus* in the formation of slow and fast muscle at both cellular and molecular levels suggests that the common ancestor of *Xenopus* and zebrafish (i.e. of sarcopterygian and actinopterygian fish) developed muscle in the manner observed in *Xenopus* tails and throughout zebrafish. The widespread presence of a superficial slow muscle layer in agnathans and primitive jawed fish strongly suggests that primitive vertebrates had this organisation (Flood et al., 1977). So the direct ancestor of amniotes probably generated at least three waves of somite muscle fibres: early Hh-dependent first wave superficial slow, medial fast and later Hh-independent second wave slow.

Muscle cell diversity in *Xenopus* myotome may be even greater. Our data shows that cyclopamine blocks later somite growth throughout the axis accompanied by reduction in *XMyoD* and slow MyHC expression at the dorsal somite edge (Fig 4C, Fig. 5D). This raises the possibility that additional fibre population(s) may exist that are also Hh dependent, reminiscent of the Shh-dependent epaxial myogenesis in mice (Borycki et al., 1999). These cells are indicated as third wave slow cells in our model (Fig. 8A). However, in zebrafish the first slow wave is itself composed of two cell populations: engrailed-expressing medial muscle pioneer cells and migratory superficial slow fibres (Devoto et al., 1996). In *Xenopus*, we do not find medially located pioneer-like slow fibres. Although widespread Engrailed 1-like immunoreactivity was reported in tail somites (Davis et al., 1991), we observed no *En1* mRNA in tail somites at similar stages. Although *En2* mRNA was present in brain and head, it was absent from somites (B.L.M., unpublished). Thus, we found no evidence for sub-populations of first wave cells.

First wave slow myogenesis blocked in trunk somites

In *Xenopus*, first wave (i.e. early Hh dependent) slow muscle is only formed in the tail. Why is first wave slow muscle not formed in the trunk? Lack of Hh expression, secretion or responsiveness is unlikely because expression of *Xshh* and *Xbhh* are detected throughout the axis of stage 22-35 embryos (Ekker et al., 1995; Mariani et al., 2001; Stolow and Shi, 1995). Moreover, *Xptc* genes, markers of Hh response, are upregulated flanking the notochord in trunk PSM dependent on Hh signalling (Koebernick et al., 2001; Takabatake et al., 2000) (Fig. 4B). Another possible reason for the missing first wave is that precocious fast muscle differentiation in *Xenopus* mesoderm occurs prior to Hh exposure, ensuring that the cells exposed to Hh are already committed to fast differentiation. However, we found that early Hh overexpression did not induce slow muscle in trunk somites, either at stage 22 or stage 35 (Fig. 3) (A.G., unpublished). Nor did Shh overexpression ever completely converted tail somites to slow myogenesis, as can happen in zebrafish (Blagden et al., 1997). Although generation of fast and second wave slow fibres is similar in trunk and tail somites, it seems there is a block on Hh response that prevents *Xshh* and *Xbhh* from promoting differentiation or survival of first wave slow fibres in trunk somites (Fig. 8A, trunk series).

In both fish and *Xenopus* trunk, *myf5* expression is highest close to the notochord, whereas *myod* expression persists further laterally (Chen et al., 2001; Coutelle et al., 2001; Polli and Amaya, 2002; Pownall et al., 2002; Weinberg et al., 1996). Notochord ablation prevents medial trunk *XMyf5* expression but has little effect on lateral expression of MRFs. This finding suggests the presence of two distinct *XMyf5*-expressing myogenic cell populations in trunk, with the adaxial ones being dependent on signals from notochord. However, we found that cyclopamine does not modify adaxial trunk *XMyf5* expression (M.E.P., unpublished), even though Hh is active anteriorly because *Xptc* genes are upregulated in the medial somite (Koebernick et al., 2001; Takabatake et al., 2000), and this *Xptc* expression is blocked by cyclopamine. So Hh signalling, although occurring, is not required for initial *XMyf5* expression. In mouse and zebrafish, initiation of *myf5* expression is also less sensitive to Hh removal than is *myf5* maintenance (Asakura and Tapscott, 1998; Borycki et al., 1999; Coutelle et al., 2001; Kruger et al., 2001; Teboul et al., 2003; Zhang et al., 2001). In avian trunk, MRF regulation by Hh depends on other midline/ectoderm signals (Borycki et al., 2000). We suggest, therefore, that the normal function of Hh to maintain MRF expression in adaxial cells is blocked in *Xenopus* trunk somites, paralleling loss of first wave slow fibres. It is unclear whether differences in myogenesis between amniote trunk and tail are homologous to those in *Xenopus*.

Slow fibre migration and somite rotation

Several pieces of evidence suggest that first wave slow cells migrate from adjacent to the notochord to the superficial somite surface as they terminally differentiate into slow fibres. First, MRF expression patterns suggest that medial presomitic mesoderm cells gain XMyoD as they lose XMyf5, move laterally and undergo terminal differentiation. Second, in tail tip regions slow MyHC and mediolaterally elongated nuclei are detected in single cells that span the somite from notochord to lateral surface. More anteriorly, slow fibres are located on the superficial somite surface and orientated anteroposteriorly, as are their nuclei. Third, blockade of Hh signalling leads to gap in tailbud *XMyf5* expression followed by decreased *XMyoD* expression and absence of first wave slow muscle cells. Based on *Xptc1* expression, Hh signalling acts medially (Koebernick et al., 2001). Taken together with the known migration of Hh-dependent first wave slow fibres in zebrafish (Blagden et al., 1997; Devoto et al., 1996), these data indicate that first wave slow fibres in *Xenopus* migrate to the somite surface around the time of somite formation. However, cell tracking in vivo would be required to prove this view.

The mediolaterally elongated nascent slow cells are reminiscent of the cells orientated perpendicular to the notochord in somite –I, the next somite to form (Hamilton, 1969; Keller, 2000; Youn and Malacinski, 1981a). First wave slow cells re-orientate parallel to the notochord as each

somite forms. Synchronously, underlying medial somite cells differentiate into fast muscle fibres and elongate in the same direction. Thus, the terminal differentiation of two waves of myogenic cells leads to a change from cells elongated perpendicular to the notochord to fibres orientated parallel to the notochord (Fig. 8B). Re-orientation of cells in successively older somites has been interpreted as demonstrating rotation of nascent *Xenopus* somites (Hamilton, 1969). Yet all cells do not rotate synchronously as a block (Youn and Malacinski, 1981b). Indeed, the morphology of 'rotating' *Xenopus* somite cells and migrating zebrafish slow muscle is remarkably similar (Cortes et al., 2003; Youn and Malacinski, 1981b). Our data, therefore, raise the possibility that a fish-like cell migration coincident with somite formation accounts for many of the morphological changes, rather than rotation of the entire somite. Other anuran species do not show such a somite rotation (Keller, 2000; Youn and Malacinski, 1981a). In the light of our finding, we suggest that the previous interpretation of a wholesale rotation of *Xenopus* somites should be regarded with caution until in vivo cell tracking has demonstrated which cells move where in developing somites.

Xenopus dermomyotome and the evolution of somites

Much has been written concerning the evolution of paired appendages in the transition from fish to tetrapods, but less attention has been paid to evolution of the dramatic somitic modifications required for the move to land. Our findings focus attention on the evolution of the tetrapod trunk, particularly dermomyotome. Our data show that a superficial layer of slow muscle is probably the ancestral condition of the common ancestor of teleosts and anurans. Dermomyotome has not been described in teleosts, instead their myotomes grow by polarized hyperplasia, a process by which extra muscle fibres are generated in discrete superficial somitic zones, often at dorsal and ventral extremes. In zebrafish, these zones give rise to Hh-independent slow fibres (Barresi et al., 2001) and pectoral fin musculature (Neyt et al., 2000). In this paper, we show that *Xenopus* trunk somites do not form the first wave of slow fibres but develop a dermomyotomal layer shortly after their formation.

First wave slow fibre migration and re-orientation occurs in tail somites. If we are right that this cell re-orientation accounts for the seeming 'rotation' of tail somites, then similar cell migrations probably explain the 'rotation' of trunk somites (Hamilton, 1969; Youn and Malacinski, 1981b). Such movements may carry the notochord-dependent adaxial *XMyf5*-expressing cells to the lateral somite surface. Loss of contact with midline-derived signals may explain failure of maintenance of *XMyf5* expression, as occurs in zebrafish notochord mutants (Coutelle et al., 2001). Whereas in the tail Hh drives *XMyoD* upregulation and slow fibre formation, in trunk such migratory cells may adopt another fate. Possible fates include fast muscle, myoblasts or dermomyotome. In chicken, most trunk myotomal cells arise from the medial half of nascent somites (Ordahl and Le Douarin, 1992), suggesting a medial origin of dermomyotome. If notochord-dependent adaxial cells form dermomyotome, they may not be committed to myogenesis despite *XMyf5* mRNA expression. In mice, *myf5*-expressing cells in brain do not make muscle (Daubas et al., 2000). We suggest that at all axial somite levels medial *XMyf5*-expressing cells may migrate to lie on the superficial somite surface, where they may become exposed to other signals influencing their fate. Perhaps an altered response to Hh was a key evolutionary innovation permitting these migratory cells to pursue fates other than first wave slow muscle.

Regardless of its origin, a morphologically distinct cell layer appears on the superficial surface of *Xenopus* somites that we believe constitutes the dermomyotome. This MyHC-negative layer arises shortly after somitogenesis and expresses *Pax3*, *Col1a1* and, in specific zones, *XMyf5* and *XMyoD*. These zones correspond well with the reported sites of myogenesis in the amniote dermomyotome (Ordahl et al., 2001) and with the sites of polarized hyperplasia in fish (Fig. 8A). It is important to distinguish superficial slow fibres from dermomyotome. Indeed, histology and electron microscopy (Fig. 6) suggest that the outer layer of tail somites is initially slow muscle fibres. These rapidly become covered by a layer of small 'spindly' cells, that coalesce into the dermomyotome overlying the slow fibre layer by stage 35. At the stages we examined, we found no evidence to support the earlier views that the dermomyotome is separated from the myotomal portion of the somite, nor that it forms a dermatome 'curtain' draped over the myotomes of more than one somite (Blackshaw and Warner, 1976; Hamilton, 1969). Instead, we concur with the idea that dermomyotome is also segmented (Youn and Malacinski, 1981b). As in the mouse (Davidson et al., 1988), in *Xenopus* trunk somites *En1* mRNA is restricted to the medial region of the dermomyotome. We propose this layer is the evolutionary homologue of amniote dermomyotome, generating cells for myotome growth.

It is likely that proliferative cells at the dorsal and ventral somitic lips contribute cells to the dermomyotome, as occurs in birds (Ordahl et al., 2001) (Fig. 8A). In addition, the MRF-expressing lips probably yield the second wave of Hh-independent slow muscle fibres. In trunk somites, this 'second wave' generates the first slow fibres. During metamorphosis the somites of the trunk increase in size and form numerous muscles of the back (Ryke, 1953). Perhaps failure of generation of the earliest slow fibres is related to the evolution of a pool of cells within the dermomyotome adapted to building later muscle in tetrapods (Shimizu-Nishikawa et al., 2002).

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