

## Axial Muscle Development in Fish

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### Abstract

In the axial musculature of adult fish two major populations of muscle fibres lay anatomically separated: 1) a triangular red zone at the level of the horizontal septum composed of red fibres and 2) the largest part of the myotome that is filled with the white muscle bulk.

The first locomotion of newly hatched fish larvae is probably powered by the red fibres and results in continuous stationary activity. Stimulation of the larvae results in burst-swimming which is probably powered by the mass of white fibres. During embryogenesis the two muscle compartments arise from muscle precursors in the segmental plate. Medial cells also known as adaxial cells will migrate radially away from the notochord and become the superficial layer of red cells. The population of lateral presomitic cells in the segmental plate remains deep in the myotome and differentiate into white muscle fibres.

By large scale genetic screens 43 genes that are required for the differentiation and maintenance of the axial musculature are being identified in *Danio rerio*. Here we discuss the phenotype of mutant fish that are involved in muscle development at the level of myotome differentiation, paraxial mesoderm segmentation and somite patterning. Signaling events leading to myogenic precursor cell specification and formation of muscle fibres are being unraveled and the first results begin to generate an insight in the molecular pathways involved in muscle development of fish. Moreover epigenetic factors that determine the final shape of the axial musculature are discussed.

**Key words:** development, fibre types, fish, musculature, somites.

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In the over 20.000 named fish species, the structure of the lateral body musculature appears highly uniform. The segmented structure of the lateral body musculature consists of myotomes separated by myosepts that in the adult muscle form a complex pattern of anteriorly and posteriorly pointing cones. The contracting muscle fibers attached between two parallel myosepts transmit forces to the body axis that cause bending of the body and result in undulatory movement.

It is assumed that axial muscle architecture is very old and has not undergone any relevant changes during the last 300 million years [16]. Moreover it is accepted that molecular and cellular processes that act during ontogeny and morphogenesis of these structures are also highly conserved. Therefore studies on *Danio rerio* or the zebrafish that are unraveling the molecular pathways involved in muscle development are highly relevant for understanding fish muscle development and even vertebrate muscle development in general, since some of these features will certainly have been passed on to the terrestrial

vertebrates. This makes *Danio rerio* an important model organism for all vertebrates.

The ability to perform both embryological and genetic analysis makes the zebrafish a powerful tool for the study of pattern formation and morphogenesis during development [14]. Zebrafish eggs are fertilized externally, and, due to their transparency, living embryos can be observed with a dissection microscope. One pair of fish can produce up to 200 transparent, synchronously developing embryos per week. Embryos can be monitored throughout somitogenesis and formation of the notochord, brain and eyes which all take place within 24 hours after fertilization. The accessibility of the zebrafish embryos permits embryological manipulation. Visualization of individual cells can be enhanced by the use of vital dyes that label membranes or nuclei, allowing for the analysis of individual cell fate. The ability to easily examine the morphology of the zebrafish embryo also facilitates the isolation of mutants defective in embryonic and larval development and recently large-scale genetic screens have

## Axial muscle development in fish

been completed. Mapping project underway in several laboratories using polymerase chain reaction (PCR) based polymorphism will help to positional clone mutants and genetically map specific mutations.

Myotomes, the building blocks of the axial musculature, arise from somites that are repeated epithelial structures within the mesoderm. These somites derive from the unsegmented, paraxial mesoderm located just lateral to the axial mesoderm or notochord. The zebrafish somite gives rise to sclerotome and myotome the latter being the major component of the somite. In the zebrafish only ventral medial cells give rise to sclerotome. Whereas in the mouse and chick the sclerotome is the major component of the somite. Using Nomarski optics, several structures can be distinguished within the somite *in vivo*. The transverse myosepta that form the anterior and posterior borders of the myotomes and the horizontal myoseptum which divides the somite in dorsal and ventral compartments. The dorsal compartment gives rise to the epaxial musculature and the ventral part to the hypoaxial muscle mass. Adjacent to the notochord a group of 20 cuboidal cells per somite called adaxial (figure 2) cells can be distinguished from the more irregularly shaped lateral presomitic cells [17]. These are the first cells to express *MyoD* [24]. Later *MyoD* is expressed in the posterior half of each somite. After formation of the somite borders some of the adaxial cells elongate to span the anterior-posterior length of the somite, and the cells stack such that the nuclei align medially along the anterior-posterior midline of the somite. Edges of stacked adaxial cells meet at the somite boundaries between adjacent somites. Three to six of the 20 adaxial cells per somite become *Engrailed*-expressing muscle pioneers [11], which are the first cells to develop myotomal morphology. The muscle pioneer cells elongate in the middle of the somite near the future horizontal septum, which itself is also thought to be a derivative of the adaxial cells. *MyoD* and *Engrailed* are thus markers of early differentiation of myotome cells since they recognize subpopulations of cells with a distinct cell fate.

Like in other vertebrates axial musculature of fish is composed of three different muscle fiber types. Slow, fast and intermediate fibers (figure 1) in the fish are localized in specific regions of the myotome [22]. The slow muscle fibers are localized to the lateral periphery in a wedge centered on the horizontal myoseptum. Fast fibers constitute the majority of the axial musculature and intermediate fibers are located in between the slow and fast fibers. First locomotion of newly hatched larvae is probably powered by the red fibres and results in continuous stationary activity. Stimulation of the larvae results in burst-swimming which is probably powered by the mass of white fibres.

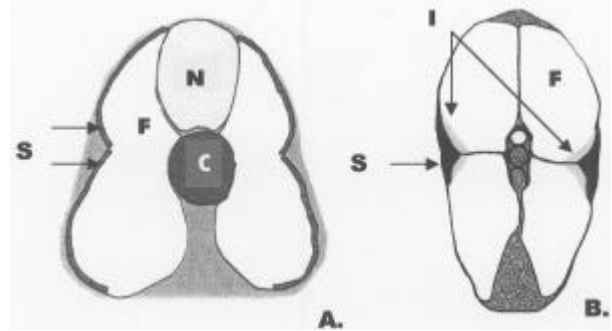


Figure 1. Schematic drawing of fibre type distribution in cross sections of A. zebrafish pre-hatching larva; B trunk myotome of an adult zebrafish. Neural tube (N); Notochord (C); slow or red muscle fibres (S); intermediate muscle fibres (I); white or fast muscle fibres (F).

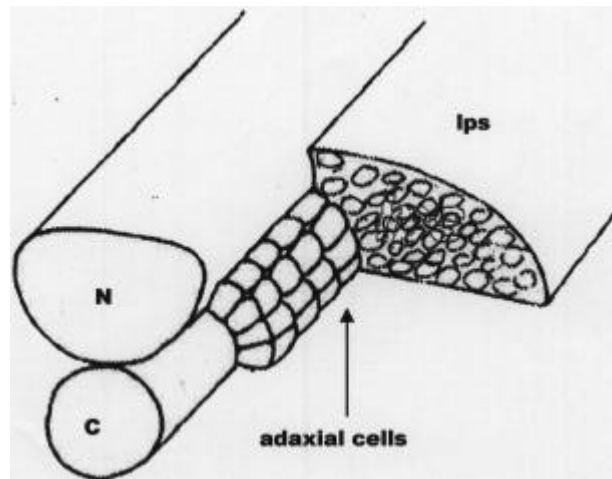


Figure 2. Schematic drawing of adaxial cells and lateral presomitic cells on one side of the notochord (N) in a zebrafish embryo prior to somite formation (after Devoto et al. [6]).

Zebrafish mutant analysis (see for a review of these mutants Holley & Nüsslein-Volhard [12]) have so far recognized 43 loci (genes) that are involved in paraxial mesoderm segmentation ( $n = 5$ ), somite patterning and differentiation ( $n = 19$ ), myotome differentiation and muscle development ( $n = 18$ ). The latter can be considered the muscle mutants in strict sense since they were recognized on basis of altered motility.

### Muscle Development Mutants

Muscle mutants were detected on basis of reduced motility. Upon the application of a tactile stimulus wild-type larvae, between 48-60 hours postfertilization, swim away from the source of the stimulus. Applying this simple motility test in a large scale genetic screening of zebrafish a total of 166 mutants with primary locomotion defects were identified [10]. Mutants that showed general degeneration within 5 days or visible defects in brain or ear development were not considered. Among the 166 motility

## Axial muscle development in fish

mutants 63 mutants have no or reduced motility combined with a reduction of muscle striation. Phenotypic analysis of these mutants suggest that in these mutants specific functions in myoblast differentiation, myofibril organization and maintenance of muscle tissue are impaired. The 103 mutations that caused no visible defects in trunk muscle development comprise a diverse collection of behavioral mutants and are considered to affect neuronal development.

The 63 mutants with suggested muscle development defects were further classified into 4 phenotypic groups (Table 1) and upon complementation analysis 18 genes were recognized.

Mutations in three genes, *frozen* (*fro*), *sloth* (*slo*) and *fibrils unbundled* (*fub*) result in immotile embryos. The phenotype becomes visible around 20 hours when muscle contractions become apparent in wild-type embryos. Mutants for the above genes fail to hatch from the chorion due to complete absence of motility. Birefringency of somatic muscle is strongly reduced or completely absent. Upon histological examination at 36 hours mutant *slo*, *fro* and *fub* embryos clearly lack organized muscle fibers. In *slo* and *fro*, the myonuclei remain round resembling nuclei of younger less differentiated myoblasts. In *fub* myonuclei elongate like in wild-type embryos. Mutations in the *fub* gene had been described earlier [9] and the gene is understood to act during myofibrillogenesis rather than in cellular differentiation of muscle cells.

*Snail1* expression [17] in the adaxial cells and myotome as well as *MyoD* expression is normal in *slo* and

*fro* embryos, indicating that early specification of muscle cell fate is normal. *fro* and *slo* genes apparently act somewhere after paraxial mesoderm differentiation but before myofiber formation.

Embryos mutant for *turtle* (*tur*), *buzz-off* (*buf*), *faulpelz* (*fap*), *slow motion* (*slw*), *schnecke* (*sne*), *hermes* (*hem*), *duesentrieb* (*dus*), *mach two* (*mah*), *slop* (*slp*), *jam*, or *slinky* (*sky*) hatch from their chorion but show low-speed escape response. In these mutants birefringency is reduced. Histological examination of the muscle tissue from these mutants muscle fibers at 72 hours reveal essentially normal myofibrils like in wild-type embryos. Some of the mutants have more obvious defects, in sections of three mutants muscle fiber organization was significantly altered. *Buf* embryos have unaltered muscle mass but less ordered sarcomeres than in wild type embryos.

In *slp* embryos, the number of muscle fibers is reduced to 80% of that in wild-type muscle, whereas *dus* embryos have only a few recognizable fibers per myotome. *Slp*, *jam* and *sky* embryos share an additional phenotype. At 48 hours of development the heart beat frequency of these embryos is lower than in wild type. The heart ventricles are smaller and blood accumulates on the yolk.

Embryos mutant for *sapje* (*sap*), *softy* (*sof*), *schwammerl* (*sml*), or *runzel* (*ruz*) have normal motility for the first 96 hours, but from then on they become slower and birefringence is reduced as the muscle mass begins to degenerate. Lesions become obvious in somitic segments. In *sap* and *sof* larvae these lesions are focal, restricted to small areas of the myotome. In *ruz* embryos degeneration is spread over entire myotomes. Other late developing structures like liver or cartilaginous jaw appear normal in these mutants, indication that the phenotype is not due to general degeneration processes.

The characteristic phenotype of these 4 groups of muscle development mutants (Table 1) become apparent sequentially, suggesting that the various genes involved act in consecutive steps of muscle development. *Slo* and *fro* embryos appear arrested in their differentiation program. In mice targeted mutations in *myogenin* result in embryos with similar defects in trunk muscle. Myoblasts are detectable and some seem to have started with myofibril synthesis, but the majority of myoblasts do not form myotubes. So *slo* and *fro* may well be defect in myoblast fusion or, like *fub*, they may be involved in early stages of myofibrillar organization.

Mutations in group 2 and 3 cause a reduction in muscle fiber number or muscle fiber organization. Reduced birefringency in these mutants reflects defects in fiber formation and fiber arrangement.

Muscle maintenance is negatively affected by mutations in the fourth group of mutants. Later onset of the mutant phenotype in this class may indicate a role for the genes involved in late differentiation or in mainte-

Table 1. Muscle mutants with affected locomotion.

Phenotype	Gene	Abbreviation
I. immotile / reduced striation	<i>sloth</i>	<i>slo</i>
	<i>frozen</i>	<i>fro</i>
	<i>fibrils unbundled</i>	<i>fub</i>
II. reduced motility / reduced striation	<i>turtle</i>	<i>tur</i>
	<i>buzz-off</i>	<i>buf</i>
	<i>faulpelz</i>	<i>fap</i>
	<i>slow motion</i>	<i>slw</i>
	<i>schnecke</i>	<i>sne</i>
	<i>hermes</i>	<i>hem</i>
	<i>duesentrieb</i>	<i>dus</i>
	<i>mach two</i>	<i>mah</i>
III. reduced motility/reduced striation plus heart defect	<i>slop</i>	<i>slp</i>
	<i>jam</i>	<i>jam</i>
	<i>slinky</i>	<i>sky</i>
IV. reduced striation, somite degeneration	<i>sapje</i>	<i>sap</i>
	<i>softy</i>	<i>sof</i>
	<i>schwammerl</i>	<i>sml</i>
	<i>runzel</i>	<i>ruz</i>

## Axial muscle development in fish

nance of trunk muscle tissue. There are obvious parallels to human diseases with muscle wasting.

### Slow and Fast Muscle Type Differentiation

As to the development of the three fiber types, ultrastructural analyses by Waterman [23] described the first myofibre structures in the medial part of the myotome. It was concluded that the fast fibers were the first to develop since in the adult fish the fibers in the medial part of the myotome are fast fibers. Using antibodies to slow myosin v. Raamsdonk et al. [20] demonstrated that at 18 hours the medial fibers had characteristics of slow fibers and that later at the same location fibers had characteristics of fast fibers. By 24 hours the most lateral fibers expressed slow myosin (figure 1). Between 18 and 24 hours a lateral shifting band of slow myosin expression could be observed in consecutive stages of the developing myotome. These results were interpreted as a transition in the fibers from an initial slow to a fast phenotype in the future fast fibers and a relatively late maturation of the lateral layer of slow (red) muscle fibers. Immobilization of larvae during this period resulted in a more spotty like expression of slow myosin in the myotome and it was inferred that although immobilization does not inhibit formation of slow and fast fibers, it does affect the rate of conversion from the initially slow to the fast fiber phenotype of the muscle mass. Suggesting a role of muscle activity on this process.

More recently labeling experiments of the most medial cells, the so called adaxial cells, demonstrated that these cells undergo elongation to span the anterior posterior length of each somite and then migrate laterally through the somite to form the lateral layer of slow fibers [6]. Apparently the future slow fibers originate from the early *MyoD* expressing adaxial cells. Interestingly in the trout (*Oncorhynchus mykiss*) two nonallelic *MyoD* genes with distinct spatiotemporal patterns of expression have recently been described [5]. During somite formation *TMyoD1* expression is present in adaxial cells of both presomitic mesoderm and forming somites. *TMyoD2* initiates later and is expressed in the posterior half of the somites.

The position of the adaxial cells along the notochord points to a specification of these cells and its derivatives by signals from this tissue. The *Engrailed* expressing subgroup of these adaxial cells also undergo elongation and were demonstrated to migrate in the region of the future horizontal septum [6]. These *Engrailed* expressing cells remain in close contact with the notochord through their medial surface also after migration. Prolonged contact with the notochord suggests again a role of signaling from the notochord that renders the muscle pioneers different from the rest of the adaxial cells. It is not clear whether the *Engrailed* expressing muscle cells ultimately form a different subset of muscle fibres. Overexpression of *sonic hedgehog* (*shh*) promotes the formation of slow

muscle at the expense of fast muscle [2]. With an *in vitro* culture system Norris et al. [15] demonstrate that exogenous sonic hedgehog peptide can control the binary cell fate choice of zebrafish myoblasts. Signaling from the notochord by *ssh*-like molecules is known to occur during segmentation and patterning of the somites. Therefore we also discuss here shortly mutations that affect somite patterning and segmentation (Table 2) since these processes eventually effect muscle development and fibers type differentiation in the fish.

### Segmentation Mutants

In the zebrafish, the *fss*-type mutants (*fused somites*, *beamter*, *deadly seven*, *after eight* and *white tail*) have defects in both the segmentation and epithelialization of the somitic mesoderm. The paraxial mesoderm forms normally, but epithelial somites fail to form properly. *Fss* affects the formation of all somites while, in *beamter* embryo's only the first three to four somites form. In *deadly seven*, *after eight* and *white tail* embryos the first seven to nine somites form. Surprisingly each of these mutants except *white tail*, is homozygous viable. Some segmentation persists in these embryos since vertebrae are not fused and irregular somite boundaries form later during embryogenesis. Double mutant combinations

Table 2. Somite segmentation and patterning mutants.

Phenotype	Gene	Abbreviation
I. severely reduced somite segmentation, adaxial cells present	<i>fused somites</i>	<i>fss</i>
	<i>beamter</i>	<i>bea</i>
	<i>deadly seven</i>	<i>des</i>
	<i>after eight</i>	<i>aei</i>
	<i>white tail</i>	<i>wit</i>
II. notochord signaling affected, adaxial cells absent, u-shaped somites	<i>you</i>	<i>you</i>
	<i>you-too</i>	<i>yot</i>
	<i>sonic-you</i>	<i>syo</i>
	<i>chameleon</i>	<i>con</i>
	<i>u-boot</i>	<i>ubo</i>
	<i>choker</i>	<i>cho</i>
III. defect in early notochord formation, loss of horizontal septum	<i>iguana</i>	<i>igu</i>
	<i>floating head</i>	<i>flh</i>
	<i>bozozok</i>	<i>boz</i>
	<i>no tail</i>	<i>ntl</i>
	<i>momo</i>	<i>mom</i>
IV. defect in late notochord development, abnormal dense somites	<i>doc</i>	<i>doc</i>
	<i>dino</i>	<i>din</i>
	<i>sleepy</i>	<i>sly</i>
	<i>grumpy</i>	<i>gup</i>
	<i>bashful</i>	<i>bal</i>
	<i>dopey</i>	<i>dop</i>
	<i>sneezy</i>	<i>sny</i>
<i>happy</i>	<i>hap</i>	
	<i>gno</i>	<i>gno</i>

## Axial muscle development in fish

between the *fss*-type mutants does not exhibit a stronger morphological phenotype indicating that somite border formation nor later forming irregular somite borders are due to genetic redundancy among *fss*-type genes. Stacking of adaxial cells occurs in *fss* mutants in the absence of somite borders.

Adaxial cells are missing in mutants that have no notochord such as *no tail*. In the *you*-type mutants adaxial cells are also missing, like in *you too* (*yot*), these mutants however do possess a morphologically normal notochord. *You too/fss* double mutants lack all somite boundaries (and all evidence of polarity in the myotome), indicating that the activity of the *you*-type genes is required for the formation of irregular boundaries within the myotome of the *fss*-embryo and is consistent with the hypothesis that adaxial cells are responsible for the formation of the boundaries.

Anterior somites in the zebrafish are clearly different from the more posterior ones, as they appear to form more rapidly, develop more synchronously and adaxial cells rearrange simultaneously. Differentiation into myotome and sclerotome proceeds in the absence of or reduction of segmentation and epithelialization, since *fss*-type mutants are viable and fertile.

### Somite Patterning and Differentiation Mutants

After segmentation, the somite is patterned into a dorsal myotome and a ventral sclerotome compartment. So far no dermatome cell lineage could be demonstrated in the zebrafish. Patterning occurs in interaction with the surrounding tissues. The neural tube and surface ectoderm have dorsalizing effects while the floor plate and notochord are involved in ventralizing the somite. The ventralizing activity of these latter structures appears to be encoded by members of the *hedgehog* family. *Sonic hedgehog* (*shh*) is expressed in both the floor plate and the notochord (Krauss et al., 1993) [13], *tiggy-winkle hedgehog* (*twhh*) is restricted to the floor plate and ventral midline of the neural tube while *echidna hedgehog* (*ehh*) is expressed in the notochord [4, 7]. Overexpression of *shh* induces both myotome and sclerotome. Even in the absence of *shh* some sclerotome is still formed in the zebrafish, indicating that part of the sclerotome is specified independently of *shh*. *Shh* expression moreover affects patterning of the myotome itself (see above 'slow and fast muscle type differentiation'). Misexpression of *shh* leads to an increased *MyoD* expression and increased *Engrailed* expression in the muscle pioneer cells. Candidates for dorsalizing activity are members of the *Bmp* and *Wnt* family of signaling molecules. *Shh* and *Wnt* family members are supposed to synergize in specifying myotome cells. An antagonist of hh signaling, protein kinase A (PKA) represses the formation of *shh* inducible structures. Patched the putative *hh* re-

ceptor is expressed in adaxial cells and this expression can be repressed by PKA [3].

In mutants lacking the notochord, the adaxial cells, the muscle pioneers and the horizontal myoseptum are all absent. In these mutants wild-type notochord cell transplantation populates the notochord and rescue the formation of each of these structures. The *floating head* (*flh*) mutant is defective in the specification of the chondrosoderm and have no chorda and no tail. In *flh* mutant fish, the expression of *brachyury* in the axial mesoderm is confined to the posterior most region. Trunk somites are fused. Such a phenotype is also found in *bozozok* embryos and *momo* (*mom*). In both *flh* and *mom*, *MyoD* is expressed in the adaxial cells. Apparently this expression does not require a differentiated notochord. *Engrailed* expression in the pioneers however is lacking. *No tail* and *doc* mutant embryos lack both the notochord and the tail. The *no tail* mutant is due to a mutation in the zebrafish homologue of the mouse gene *Brachyury*. The somites that do form in *no tail* mutants lack most *MyoD* expression in the adaxial cells, muscle pioneers and the horizontal septum. *Doc* mutants exhibit a slight reduction in the adaxial *MyoD* expression and fail to express *Engrailed* in the region lacking the notochord. Other mutants, *sleepy*, *bashful*, *grumpy*, *sneezy*, *dopey*, *happy* and *gno*, defining seven other genes have been isolated in which the notochord is undifferentiated and the somites appear as dense disorganized structures. These notochord mutants demonstrate clearly the importance of the notochord for somite patterning and differentiation. The phenotypes in these mutants are likely due to a loss or reduction of *shh* expression. For example the *Engrailed* expression in the posterior somites of *doc* embryos is found there where a few vacuolated notochord cells express *shh*.

The *you*-type group of notochord mutants share the same somite phenotype with the other notochord mutants, but they possess an apparently normal notochord. The six genes in the *you*-type group of mutants are required for horizontal septum formation. These mutants, *you*, *you-too* (*yot*), *sonic-you* (*syu*), *chameleon* (*con*), *u-boot* (*ubo*), *choker* (*cho*) and *iguana* (*igu*), have U-shaped somites. *Yot* mutants do not form any adaxial cells and like-wise no adaxial *MyoD* expression is observed, no somitic *Engrailed* expression is seen and no horizontal myosepta are formed. Also the more lateral *MyoD* expression is effected in that full expression in the medial anterior domain is not achieved and expression in older somites is not maintained. Phenotypes of *syu*, *con* and *you* mutants are less severe in that some *MyoD* expression is seen in the adaxial cells and the number of *Engrailed* expressing cells is reduced in *con* and *you* embryos but lost in *syu* mutants. In *ubo* embryos *MyoD* expression is unaffected and in this case an increased number of cells express *Engrailed* at reduced

## Axial muscle development in fish

levels. *Cho* embryos have reduced horizontal myosepta but appear to have normal muscle pioneer cells. *Igu* embryos lack the horizontal myoseptum in the posterior somites. *Sonic-you* is the zebrafish homologue of *shh* and it may well be that the *you*-type genes encode additional components in the *hh* signaling pathway.

Myosepts must necessarily be oriented oblique to the axis since myosepts perpendicular to the axis would impede the effective shortening of the muscle fibers [18]. This oblique orientation also described as the chevron shape pointing rostrally, is effected by several mutants; *you*, *you-too* (*yot*), *sonic-you* (*syu*), *chameleon* (*con*), *u-boot* (*ubo*), *choker* (*cho*) and *iguana* (*igu*). Loss or the reduction of the number of adaxial cells results in the development of U-shaped somites. This may as well be a consequence of lack of slow muscle fibres that power the first muscle movements. Immobilized embryos also fail to develop the oblique orientation of the myosepts [19].

### Conclusions

Phenotype genotype correlation in the mutants described above is underway, and positional cloning will uncover the encoded genes. The analysis of the temporal and functional regulation between the various signals governing somitogenesis and differentiation of the muscle compartment are certainly a challenge.

From the mutants described above we can not learn, however, whether there are also later-acting genes that affect the development of the more complex architecture of the axial musculature with its spiral orientation of muscle fibers and the cone-shaped myosepts. Moreover there are certainly important epigenetic factors that determine the final shape of the axial musculature. When lateral body movement is prevented, the shape of development of the somites in embryos and young larvae becomes reversed. When immobilization is terminated, larvae resume their movements and the shape develops again to that of the age control group. During development of the somite differences in shortening between lateral and medial muscle fibers, might cause differences in longitudinal growth of the muscle fibers and the oblique fiber arrangement may thus be a consequence of these differences in growth. In immobilized embryos the longitudinal growth of the muscle fibers is decreased and also the difference in growth rate among fibers is diminished in all fibers. These effects of immobilization point to the conclusion that locomotion itself has an effect on the morphogenesis of the somites and the myotomes [19]. In other words hyperplasia of the axial musculature and mechanical forces that act on the different structures in the myotome may well have a shaping effect on the myotome and will contribute to its final architecture.

The adult pattern of superficial red fibers, deep white fibers and a zone of intermediate fibers between the slow and fast fibers develops from the larval inner white and superficial red muscle zones and has been corre-

lated with the development of gill respiration [1, 8]. When gill respiration is not yet realized the larvae rely for oxygen supply on diffusion through the body surface. In combination with the high activity of the respiratory mitochondrial enzymes present in the red fibers it has been suggested that the red superficial fibers have a major role in respiration and that their role in swimming is negligible [8]. The stationary tail beats of the larvae in the yolk sac are powered by the red muscle fibers, while rapid burst swimming like the escape response upon touching is attributed to the mass of white fibers. Only after gills mature in the free swimming larvae an increase in swimming activity can be observed.

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## Axial muscle development in fish

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