Developmental Cell, Vol. 5, 865–876, December, 2003, Copyright ©2003 by Cell Press

Cadherin-Mediated Differential Cell Adhesion Controls Slow Muscle Cell Migration in the Developing Zebrafish Myotome

Fernando Cortés,[1,2] David Daggett,[1,2] Robert J. Bryson-Richardson,[1,3] Christine Neyt,[1,3] John Maule,[1] Phillipe Gautier,[1] Georgina E. Hollway,[1,3] David Keenan,[1,3] and Peter D. Currie[1,2,3,*]

[1]Comparative and Developmental Genetics Section
MRC Human Genetics Unit
Edinburgh EH4 2XU
United Kingdom

Summary

Slow-twitch muscle fibers of the zebrafish myotome undergo a unique set of morphogenetic cell movements. During embryogenesis, slow-twitch muscle derives from the adaxial cells, a layer of paraxial mesoderm that differentiates medially within the myotome, immediately adjacent to the notochord. Subsequently, slow-twitch muscle cells migrate through the entire myotome, coming to lie at its most lateral surface. Here we examine the cellular and molecular basis for slow-twitch muscle cell migration. We show that slow-twitch muscle cell morphogenesis is marked by behaviors typical of cells influenced by differential cell adhesion. Dynamic and reciprocal waves of N-cadherin and M-cadherin expression within the myotome, which correlate precisely with cell migration, generate differential adhesive environments that drive slow-twitch muscle cell migration through the myotome. Removing or altering the expression of either protein within the myotome perturbs migration. These results provide a definitive example of homophilic cell adhesion shaping cellular behavior during vertebrate development.

Introduction

The differential adhesion hypothesis proposed by Steinberg (1963) has been the prevalent theory of classical cell adhesion biology for almost 40 years, all the more remarkable for the fact that is was postulated prior to the identification of the first cell adhesion molecules. This model provides an explanation of cell motility behaviors as a consequence of cell sorting and relies on the supposition that different cellular cohorts possess differing levels of affinity for each other. Accordingly, cell populations that were initially mixed can undergo a process of "sorting out" into compartmentalized populations. Support for this theoretical model has come from landmark studies, which revealed that when specific members of a particular class of cell adhesion molecule, the classical cadherins, are expressed within cultured cells, cell sorting can result (Steinberg and Takeichi, 1994; Nose et al., 1988; Friedlander et al., 1989). Classical cadherins form a large family of transmembrane proteins that are thought to mediate cell-cell interactions, primarily as homophilic adhesion molecules, linking the membrane to the cytoskeleton through the intermediary of cytoplasmic catenins (for a review, see Yagi and Takeichi, 2000; Tepass et al., 2002). In vitro, differences in cell sorting can be achieved both by the expression of different levels of the same cadherin as well as via the expression of different cadherin family members (Steinberg and Takeichi, 1994; Nose et al., 1988; Friedlander et al., 1989; Miyatani et al., 1989; Duguay et al., 2003).

In mammals, over 20 different classical cadherin molecules have now been isolated (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/5/6/865/DC1), with individual family members being expressed in both distinct and overlapping patterns within specific tissues. Consequently, cadherins have been postulated to control a number of tissue-specific, morphogenetic events including cell migration and sorting, the separation of tissue layers, epithelial to mesenchymal transitions, and aggregation and dispersal of cells, although definitive evidence for specific functional roles remains elusive (reviewed in Tepass et al., 2002). Of particular interest has been the regional expression of classical cadherins within the developing nervous system, where compartment boundary formation and neuronal cell sorting are both thought to be controlled by differential cadherin expression (Fishell et al., 1993; Inoue et al., 2001; Price et al., 2002). However, there remains only a single, genetically defined, example of the requirement for cadherins in cell migration and sorting during the development of any organism, that of the differential levels of *Drosophila* E-cadherin (DE-cadherin) driving border cell migration during *Drosophila* oogenesis (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998).

The zebrafish embryo has proved a valuable model system in which to examine questions of cellular morphogenesis and migration, due in the main to the optical clarity of the embryo and the battery of sophisticated cell labeling techniques that are available. This has enabled researchers to examine cell behaviors at single-cell resolution within the intact embryo. In particular, cell movements within the embryonic myotome, the structure that generates the majority of the muscles of the embryo, have been particularly well characterized (reviewed in Brennan et al., 2002). These studies have revealed a surprisingly dynamic array of cellular behaviors underlying the final formation of different muscle fiber types.

All vertebrate muscle cells are composed of two basic fiber types that express either fast or slow-twitch varieties of myosin heavy chain (MyHC). Zebrafish embryonic slow-twitch muscle cells are derived from the adaxial cells, a single-cell layer of paraxial mesodermal cells immediately flanking the notochord (Devoto et al., 1996). Prior to segmentation, these cells initiate expression of the myogenic regulator factor (MRF)-encoding genes *myoD* and *myf5* and exhibit a cuboidal, epithelial-like

*Correspondence: p.currie@victorchang.unsw.edu.au
[2]These authors have contributed equally to this work.
[3]Present address: Victor Chang Cardiac Research Institute, 384 Victoria Street, Darlinghurst, Sydney 2010, Australia.

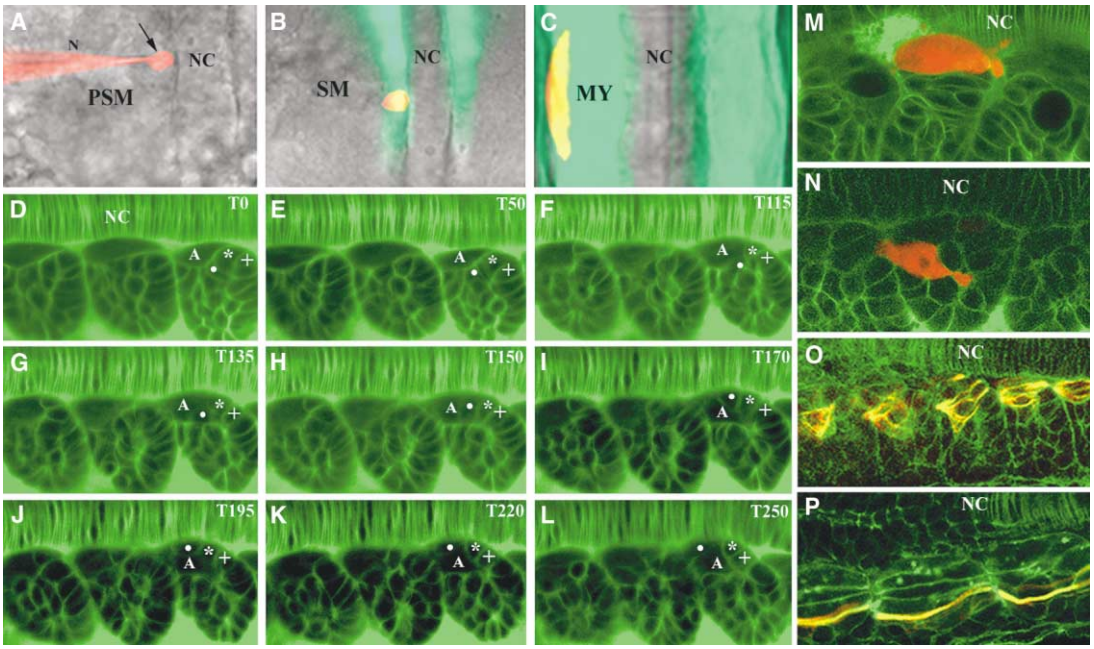

Figure 1. Morphological Characterization of Cell Behaviors during Slow Muscle Migration

(A) Iontophoretic injection of high molecular weight rhodamine dextran into a single caudal adaxial cell of a two-somite stage zebrafish embryo transgenic for the muscle-specific α-actin promoter GFP transgene (Higashijima et al., 1997). Both the needle electrode (N) and the labeled cell (arrow) are shown.

(B) By the seven-somite stage, the labeled adaxial cell has initiated GFP expression, in line with the precocious differentiation of the adaxial cell compartment within the PSM as a whole.

(C) By the end of somitogenesis (26-somite stage), the labeled cell has traversed, in a medial to lateral direction, the entire extent of the forming myotome, to form a single, subcutaneous, elongated, and differentiated muscle fiber.

(A–C) Dorsal view, anterior to the top. PSM, presomitic mesoderm; SM, somitic mesoderm; MY, myotome; NC, notochord.

(D–L) Selected confocal time-lapse images of a bodipy ceramide-stained zebrafish embryo at the 20-somite stage (full time-lapse series in Supplemental Movie 1). Yolk extension somites are shown at the dorsoventral level of the notochord. T, time in minutes from the commencement of the time-lapse analysis. Individual cells located with the caudal aspect of the somite are denoted by +, *, and •, and tracked through individual frames. A, adaxial cells.

(M) Premigratory adaxial cells have been injected with rhodamine dextran (red) within a 20-somite stage embryo treated with bodipy ceramide (green). Yolk extension somites are shown at the level of the notochord.

(N) Migrating rhodamine dextran-labeled adaxial cell showing the triangular and irregular morphology that migrating cells adopt.

(O) Twenty-somite stage embryo stained for phalloidin (green) to mark actin and cell shape and the slow muscle-recognizing antibody F59 (red). Single image from a confocal reconstruction (provided in Supplemental Movie 2) of caudal yolk extension somites at the level of the notochord. The irregular triangular shaped morphology of newly migrating adaxial cells is also evident in this analysis.

(P) Image from the same confocal reconstruction in (O), but from more rostral somites in which adaxial cell migration is more advanced. Adaxial cells have flattened into a fiber-like appearance, and immediately medial fast muscle progenitors have also begun to elongate. Fast muscle progenitors ahead of the migrating adaxial cells retain their rounded appearance.

(D–P) Dorsal views, anterior to the left.

morphology that identifies the first cellular compartment within the presomitic mesoderm (PSM; Weinberg et al., 1996; Coutelle et al., 2001). Midway through segmentation, while still in contact with the notochord, all adaxial cells differentiate as slow MyHC-expressing cells. Dye labeling experiments have revealed that shortly after adaxial cells initiate differentiation, the majority migrate from their medial position to traverse the entire extent of the myotome, coming to lie at its most lateral surface, forming a subcutaneous layer of slow-twitch muscle (Devoto et al., 1996; Blagden et al., 1997; see Figures 1A–1C). A subset of the adaxially derived slow muscle cells, termed the muscle pioneer cells (MPCs), fail to undergo this migration and remain notochord associated (Hatta et al., 1991; Ekker et al., 1992). Fast-twitch muscle cells, by contrast, differentiate specifically from the lateral aspect of the somite, completing their differentiation after slow-twitch muscle migration is complete (Devoto et al., 1996; Blagden et al., 1997).

Despite a detailed understanding of the processes that specify slow muscle progenitors (reviewed in Brennan et al., 2002), there is little understanding of how the movements of these cells are controlled. The coordinated, directional migration of differentiating slow muscle cells through the entire extent of the forming myotome remains a striking and little understood morphogenetic event. Here we describe the cellular and molecular basis for slow-twitch muscle cell migration and reveal that differential cell adhesion, generated by dynamic reciprocal waves of expression of N- or M-cadherin within the zebrafish myotome, propagates the lateral migration of slow muscle cells.

Results

Morphological Characterization of Cell Behaviors during Slow Muscle Migration Suggests a Functional Role for Differential Cell Adhesion

In order to understand the mechanistic basis of slow muscle migration, we examined the cell behaviors that occur during this process within the forming zebrafish myotome. Time-lapse analysis of 20-somite stage embryos stained with the vital interstitial fluorescent cell marker bodipy ceramide was carried out on yolk extension (caudal) somites during a 5 hr period, prior to and during the initiation of adaxial migration. This analysis revealed that lateral fast muscle progenitors in the caudal region of each somite move medially to take up positions flanking the notochord. Concomitant with this initial movement of lateral somitic cells, the elongated morphology of differentiated adaxial cells is transformed, as cell bodies adopt a triangular shape and are displaced toward the anterior edge of each somite (Figures 1D–1L; Supplemental Movie 1). Contact between the notochord and the rostrally displaced adaxial cells is ultimately broken upon the invasion of medially migrating lateral cells into the anterior-medial quadrant of the somite (Figures 1J–1L). This displacement of adaxial cells from their notochordal juxtaposition renders them morphologically indistinguishable from surrounding fast muscle progenitors within bodipy ceramide-stained embryos. Therefore, to allow the definitive identification of adaxial cells after migration initiation, we iontophoretically labeled adaxial cells with the lineage tracking dye rhodamine dextran within bodipy ceramide-stained embryos and observed their lateral movements using time-lapse confocal microscopy. This analysis revealed that migrating slow muscle cells retained their irregular triangular morphology as migration continued, with lateral fast muscle progenitors progressively being displaced medially relative to slow muscle movement (Figures 1M and 1N).

In order to understand the three-dimensional relationship of migrating slow cells to the lateral myotomal substrate through which they are migrating, wild-type embryos were fixed at the 20-somite stage and costained for slow MyHC and F-actin to mark cell shape (Figures 1O and 1P). At this embryonic stage, the entire range of slow muscle migratory cell behaviors, from initiation of migration to the postmigratory phase, can be visualized by examining somites of differing developmental stages, which are present at different rostrocaudal levels within a single embryo. Serial confocal sections were taken through the entire dorsoventral extent of the developing myotome, positioned at several different rostrocaudal levels of the embryo. These images were consequently rendered in three dimensions (3D; reconstruction is animated in Supplemental Movie 2; selected images from individual Z stacks are shown in Figures 1O and 1P) and analyzed for cell morphology. This analysis confirmed the existence of the different morphological transitions that migrating slow muscle cells underwent, as initially revealed in our time-lapse analysis. It further revealed that as slow cells approached a distance of five to six cell diameters from the midline, they underwent a final morphological transition from a triangular morphology to that of an elongated fiber (Figure 1P). After this transition, fast progenitors, medial to migrating slow fibers, themselves take on an elongated appearance. Fast muscle progenitors that lie lateral to the newly elongated slow muscle cells retain a rounded appearance until slow fibers have moved past them, when they in turn begin to elongate.

n- and *m-cadherin* Are Expressed in a Dynamic and Reciprocal Manner and Are Associated with Slow Muscle Migration

The relative behavior of the myotomal subpopulations described above could potentially be explained by slow and fast muscle progenitors possessing different adhesive properties. We therefore screened known cell adhesion molecules to identify those that were differentially expressed within the myotome during slow muscle migration. We identified two such molecules with reciprocal expression profiles. By a combination of cDNA screening, mining of the zebrafish genomic sequence, and RACE strategies, we characterized the full-length open reading frame of zebrafish *m-cadherin* gene (Supplemental Figure S1A). By comparison to the zebrafish genome project, we were also able to define the exon/intron boundaries of the *m-cadherin* locus (Supplemental Figure S1B). Phylogenetic analysis confirmed that we had identified a true zebrafish ortholog of *m-cadherin*, a gene that had previously only been identified in mammalian species (Supplemental Figure S1B). In situ hybridization with *m-cadherin* antisense RNA revealed that it is first expressed early during somitogenesis, exclusively within adaxial cells (Figures 2A and 2C). Expression of *m-cadherin* expands laterally through the forming myotome during late somitogenesis, with the most lateral extent of its expression always initiating one or two cell diameters in front of the migrating slow cells (Figures 2D, 2E, 3A, and 3C–3E). At the same time as lateral expansion of *m-cadherin* expression begins, expression is downregulated within the nonmigratory MPCs and remains so even after migration is complete (Figures 2E and 2F). This lack of *m-cadherin* expression with MPCs is more evident within embryos homozygous for the mutation *uboot*, which exhibit an expansion of MP-like cells (Figure 3K; P.D.C., unpublished observations; Roy et al., 2001). In these embryos, expression of *m-cadherin* becomes reduced in a greater medial segment of the somite, corresponding to the extent of this MPC-like expansion.

By contrast, we found that expression of a second classical cadherin, *n-cadherin*, occurs in a reciprocal pattern. *n-cadherin* mRNA is initially globally expressed throughout the somite, in a domain that encompasses adaxial cells (Figures 2G–2J, 3B, and 3H; Bitzur et al., 1994). Coincident with the initiation of slow cell migration *n-cadherin* expression is lost medially, although it remains expressed within MPCs (Figures 2I, 2J, 3B, and 3F–3H). The reduction of *n-cadherin* in a medial to lateral wave corresponds exactly with the onset of slow muscle migration, with the most medial aspect of its expression corresponding to the position of the migratory slow cells (Figures 3B and 3F–3H). By the end of somitogenesis, after migration of slow cells is complete, *n-cadherin* mRNA is localized exclusively to postmigratory slow cells and MPCs (Figures 2J and 3J). Therefore, as a

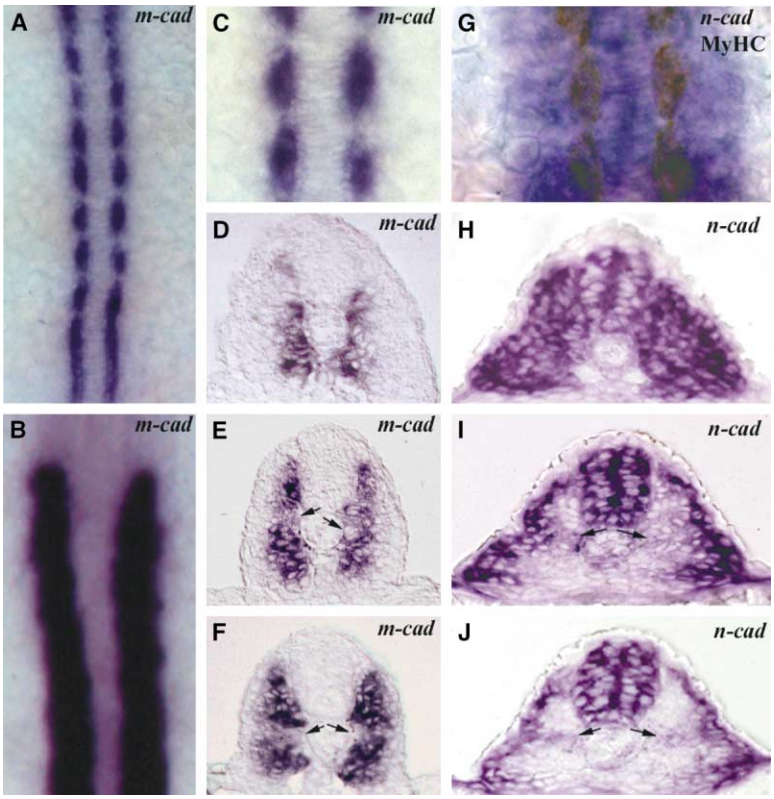

Figure 2. Expression of *m-* and *n-cadherin* during Development of the Myotome

(A) Dorsal view, anterior to the top, of an eight-somite stage zebrafish embryo stained with an antisense mRNA probe to *m-cadherin*. At this stage, *m-cadherin* expression is restricted to the adaxial cells.

(B) Similar view as in (A) but of a 26-somite stage embryo, revealing that *m-cadherin* expression now extends to the entire extent of the myotome.

(C) High-magnification view of an eight-somite embryo stained with an *m-cadherin* antisense probe illustrating the adaxial restriction.

(D–F) Serial cross-sections, caudal to rostral ([D] to [F]), of a 20-somite stage zebrafish embryo in which an expansion of *m-cadherin* expression from medial to lateral can be seen. Arrows note the loss of expression within the muscle pioneer cells adjacent to the notochord, simultaneous with the expansion of expression from the midline.

(G) Eight-somite stage embryo (dorsal view at the level of the notochord, anterior to the top) stained for *n-cadherin* expression (blue) and an anti-MyHC antibody (A4.1025, brown) to mark the adaxial cells. At this stage *n-cadherin* is expressed throughout the somite, inclusive of the adaxial cells.

(H–J) Serial cross-sections, caudal to rostral ([H] to [J]), of a 20-somite stage zebrafish embryo in which a retraction of *n-cadherin* expression occurs in a medial to lateral wave, directly correlated with the expansion of *m-cadherin* expression. Arrows mark the retention of *n-cadherin* expression in the non-migratory muscle pioneer cells.

consequence of the reciprocal expansion and contraction of *m-* and *n-cadherin* expression, respectively, a zone of overlapping expression of the two genes is maintained ahead of migrating slow cells (Figures 3I and 3J).

Cadherin-mediated adhesion is proposed to occur primarily in a homophilic manner, with cadherins on one cell interacting with like molecules expressed on an adjacent cell. Experimental evidence for this model comes from cell aggregation assays where cells transfected in vitro with either E- or P-cadherin (Nose et al., 1988) or R- versus B-cadherin (Duguay et al., 2003) segregate from each other when mixed. Furthermore, cells differentially expressing discrete levels of a single cadherin have also been shown to adhere preferentially to each other in vitro (Friedlander et al., 1989). These experiments have led to the suggestion that both qualitative and quantitative changes in cadherin expression can promote cell-sorting behaviors.

Invoking these principles, the expression profiles of *n-* and *m-cadherin* within the zebrafish myotome suggest a model whereby differential adhesive environments could direct the lateral movement of slow muscle cells to the body wall in the manner that we have documented. The profiles of *n-* and *m-cadherin* suggest that the adaxial cells uniquely possess a high level of both cadherins, prior to and throughout their migration. At the onset of migration, the *m-cadherin* expression domain expands laterally from the midline, overlapping with *n-cadherin* expression up to three cell diameters ahead of the adaxial cells (Figures 3A–3J). Consequently, the adaxial cells would have the highest homophilic attraction for cells immediately lateral to themselves with whom they would share nearly identical adhesive properties within a narrow M- and N-cadherin expression "zone." This dynamic zone of high N- and M-cadherin levels is propagated as a medial to lateral wave (see below). Therefore, the continuously M- and N-cadherin-expressing adaxial cells would preferentially sort within it, driving slow muscle cell migration to the lateral extent of the myotome. Reciprocally, as lateral cells downregulate *n-cadherin* expression, they in turn would sort to the medial zone that contains cells that have already lost *n-cadherin* expression.

Loss of Function of *n-* and *m-cadherin* Reveals a Requirement for Their Activity in Slow Cell Migration

Our model would predict that removal of the function of either of the bimodal cadherin components should have a severe effect on slow muscle migration. As we had identified a full-length open reading frame for the *m-cadherin* gene, and defined the exon/intron boundaries of the entire *m-cadherin* locus (Supplemental Data), we designed two nonoverlapping "translation-blocking" antisense morpholino oligonucleotides to the upstream region of *m-cadherin* as well as a third "splice site-blocking" oligonucleotide directed against the donor site of exon4. When injected, all the morpholino oligonucleotides produced a similar phenotype, in which

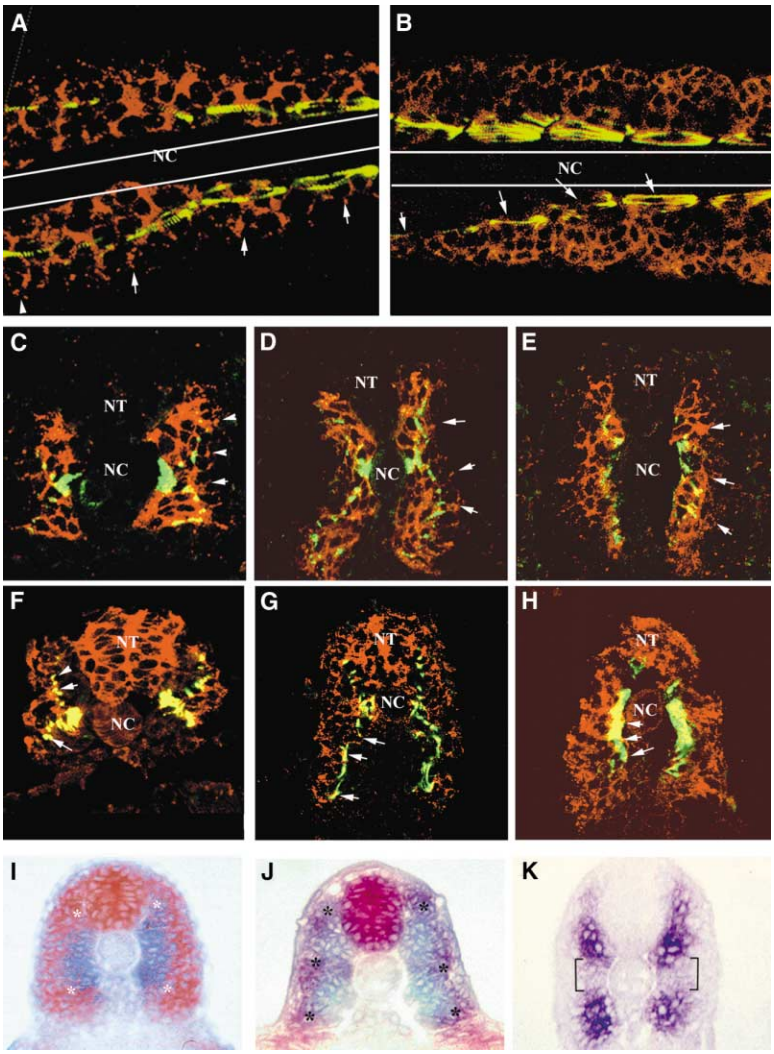

Figure 3. The Expression Profiles of *m-* and *n-cadherin* Correlate with Slow Cell Migration

(A and B) Single confocal sections of 20-somite stage zebrafish embryos, dorsal view at the dorsoventral level of the notochord (NC), which has been costained either for *m-cadherin* (red, [A]) or *n-cadherin* (red, [B]) mRNA and anti-slow MyHC-recognizing antibody (F59, green). Arrows represent the extent of the expanding *m-cadherin* expression domain (A) and retracting *n-cadherin* expression (B).

(C–E) Selected serial cross-sections, rostral to caudal ([C] to [E]), of a 20-somite stage zebrafish embryo stained for *m-cadherin* (red) and slow MyHC (green). Arrows indicate the lateral extent of the *m-cadherin* expression domain.

(F–H) Selected serial cross-sections, rostral to caudal ([F] to [H]), of a 20-somite stage zebrafish embryo stained for *n-cadherin* (red) and slow MyHC (green). The most medial extent of *n-cadherin* expression (marked with arrows) is always found within the migrating adaxial cells regardless of the mediolateral position of these cells.

(I and J) Selected serial sections (caudal to rostral) of a 20-somite stage zebrafish embryo that has been costained for *m-cadherin* mRNA (blue) and *n-cadherin* (magenta). Asterisks mark the region of overlap between the two expression profiles.

(K) In situ hybridization of an antisense *m-cadherin* probe to a homozygous mutant *uboot* embryo (22-somite stage), sectioned at the level of a yolk extension somite. These embryos exhibit an expansion of the *m-cadherin*-negative domain corresponding to the expansion of MPC-like cells present at this stage (compare to Figure 2F). NT, neural tube.

the migration of slow muscle cells were grossly inhibited, but did so to varying degrees. Oligo M1, designed to provide antisense hybridization over the ATG of the *m-cadherin* mRNA, resulted in a higher percentage of embryos in which migration of the slow muscle cells was inhibited (83%, n = 73; Figures 4D–4F) and to a greater extent than oligo M2, which was targeted against a region upstream of the *m-cadherin* ATG (61%, n = 57; Figures 4G–4I). As the weaker phenotype could also be generated by the injection of lower concentrations of the M1 morpholino (data not shown), we surmised that the less severe phenotype generated by M2 injection resulted from less efficient targeting of the M2 antisense oligonucleotide and a consequent partial knockdown of M-cadherin function. This was consistent with our analysis of embryos injected with the splice-blocking morpholino SM1, which generated a similar phenotype to M2-injected embryos (Figure 4M). Morpholinos directed against splice site sequences have been used previously to inhibit splicing, resulting in either missplicing or degradation of targeted mRNAs, and can consequently be used to estimate the efficacy and specificity of targeted knockdown (reviewed in Draper et al., 2001). Therefore, we were able to determine that SM1 injection specifically reduced the levels of *m-cadherin* transcript but did not eliminate the transcript completely (Figure 4N).

We have characterized the phenotypes of morpholino-injected embryos by generating 3D reconstructions of slow MyHC staining (Figure 4; Supplemental Data). A four-somite width view was reconstructed utilizing over 100 single confocal scans encompassing the entire myotome from embryos injected with different oligonucleotides (M1, n = 12; M2, n = 10). Analysis of these three-dimensional renderings revealed that, despite there being similar numbers of slow fibers present within injected and uninjected embryos (average number of fibers present within individual myotomes: wild-type, 23.93 ± 1.17 SD; M1-injected 19.70 ± 1.48 SD), a large number of slow cells failed to reach the lateral aspect of the myotome (average number of fibers present at the lateral surface of the myotome: wild-type, 20.18 ± 1.42 SD; M1-injected, 5.41 ± 1.81 SD; M2-injected, 12.23 ± 2.40 SD). Cells were found instead to remain at their medial origin or lie in a position perpendicular to the migratory trajectory; that is, aligned mediolaterally within the myotome instead of the usual anterior-posterior alignment (see Figures 4D–4I for selected reconstructed images and specific confocal sections; animated movies of reconstructions are supplied as

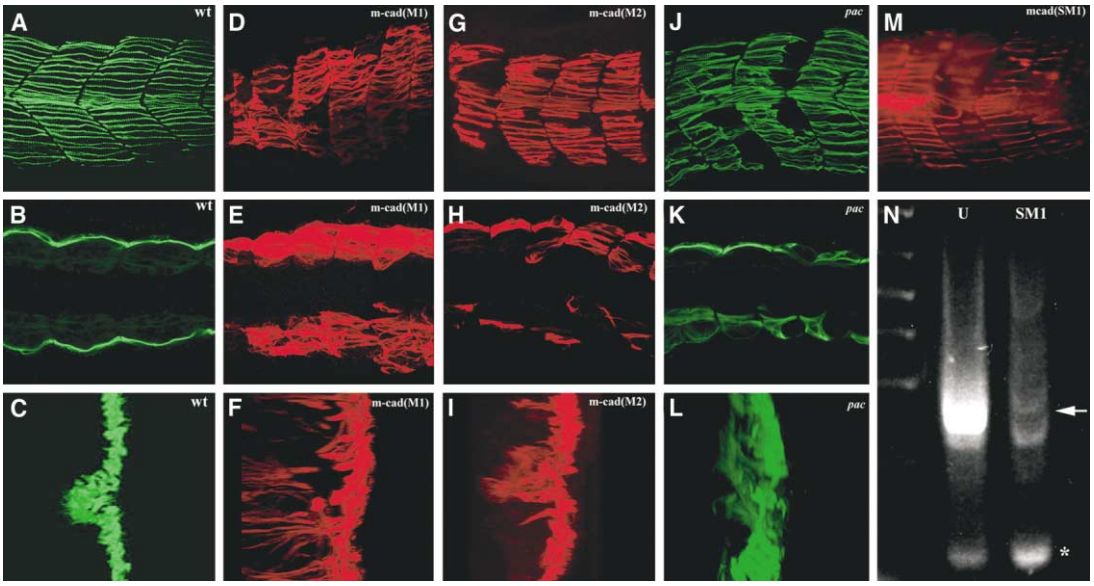

Figure 4. Removal of *n-* or *m-cadherin* Function Results in Altered Slow Muscle Migration

Single frames of a 3D reconstruction in lateral (A, D, G, and J) and cross-sectional (C, F, I, and L) views encompassing a four-somite width view of a 26-somite stage embryo.

(B, E, H, and K) Single confocal scans at the dorsoventral level of the notochord taken from the same image stacks used to make the respective reconstructions.

(A–L) All are incubated with an antibody that recognizes slow MyHC (F59).

(A–C) Wild-type embryonic pattern of slow MyHC.

(D–F) Embryos that have been injected with the morpholino antisense oligo M1 against the ATG region of *m-cadherin* (mcad[M1]) exhibit a highly penetrant and reproducible defect in slow cell migration (D and E). Fibers fail to migrate to the lateral surface of the myotome and are often seen to elongate or are bent in a direction perpendicular (medial to lateral) to normal fiber orientation (F).

(G–I) Embryos that have been injected with the morpholino antisense oligo M2, against a region upstream of the ATG region of *m-cadherin* (mcad[M2]) exhibit a similar, but less severe, phenotype to M1-injected embryos. Slow cells again fail to migrate correctly, leading to gaps in the lateral palisade of slow muscle fibers.

(J–L) Embryos homozygous for the *n-cadherin* mutation *pac*^{tm101a} exhibit a near identical phenotype to M1-injected embryos, with similar gaps in the final pattern of slow fibers.

(M) Staining of SM1 morpholino-injected embryos for slow MyHC (red) results in an identical phenotype to that produced in M2-injected embryos.

(N) RT-PCR of cDNA derived from mRNA of uninjected (U) and SM1-injected (SM1) embryos. Injection of the SM1 morpholino results in a specific knockdown of *m-cadherin* transcript (arrow) but not of a transcript encoding actin (asterisk).

Supplemental Movies 3 and 4 for M1-injected embryos, and Supplemental Movie 5 for M2-injected embryos). Nonmigratory and misaligned fibers were also detected at single-cell resolution, within *m-cadherin* morpholino M1-injected embryos, via the iontophoretic labeling of individual adaxial cells with lineage tracking dyes and the consequent time lapse of their cellular morphogenesis (Figures 5A–5F). Embryos maintain this deficit in slow cell migration up to 4 dpf, the latest stage of development at which injected embryos were examined (data not shown). Injection of control oligonucleotides of unrelated sequence (n = 103) produced no such phenotype. We conclude that M-cadherin function is required for the correct migration of slow muscle cells.

Mutations within the *n-cadherin* open reading frame have recently been identified in *parachute* (*pac*) homozygous mutant embryos (Lele et al., 2002). *pac* mutants exhibit ectopically delaminating cells within the dorsal aspect of the hindbrain, and defects in retinal laminae and neural outgrowth, but no defects within the myotome of these embryos have previously been reported (Lele et al., 2002; Erdmann et al., 2003; Masai et al., 2003). We examined the *n-cadherin* mutant allele *pac*^{tm101a} for defects in slow muscle migration. *pac*^{tm101a} results from the generation of a premature stop codon, just prior to the fourth extracellular domain of *n-cadherin*, and is thought to represent a null allele (Lele et al., 2002). Again, using confocal reconstruction to chart the position of slow muscle cells within the myotome, we found that *pac*^{tm101a} homozygotes (clearly identifiable via their hindbrain defect) exhibited a highly penetrant and reproducible reduction of slow muscle at the lateral extent of the myotome despite possessing a near normal number of total slow fibers within affected myotomes (n = 56, average lateral slow fiber number 12.17 ± 2.90 SD, average total fiber number 20.53 ± 1.21 SD; Figures 4J–4L; Supplemental Movie 6). The majority of somites possessed altered fiber arrangements with individual muscle cells missing from the stereotypical palisade of postmigratory slow muscle fibers, which was more severe in posterior somites at the 26-somite stage. The reduction of lateral fiber number was also clearly evident up to 4 dpf, the latest point up to which *pac*^{tm101a} homozygotes were examined (data not shown). Furthermore, injection of antisense morpholinos to the *n-cadherin* ATG produced a similar but noticeably less severe reduction in slow muscle migration (n = 30).

The myotomal phenotypes found in *pac*^{tm101a} mutants

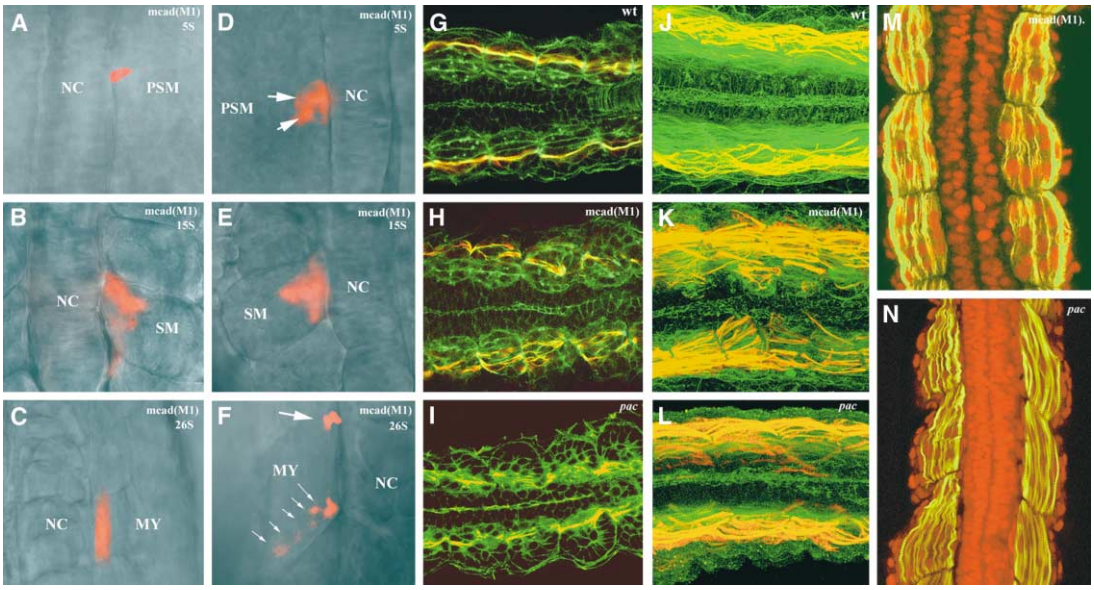

Figure 5. Removal of N- or M-Cadherin Results in Altered Migration of Slow-Twitch Muscle without Affecting Myotomal Integrity

(A–F) Iontophoretic injection of rhodamine dextran into adaxial cells of M1 morpholino-injected embryos reveals slow muscle cells fail to migrate (A–C) or become misaligned within the differentiated myotome (D–F). In (A), a single adaxial cell is labeled that undergoes the normal morphogenetic transition to a triangular morphology (B), which elongates but fails to migrate from the midline (C). In (D), two adjacent adaxial cells have been labeled (arrows) and these similarly adopt the normal triangular morphology (E), but become aligned mediolaterally (small arrows) or obliquely (large arrow) within the differentiated myotome (F). 5S, five-somite stage; 15S, 15-somite stage; 26S, 26-somite stage.

(G–I) Single confocal scans of embryos stained for slow MyHC (red, F59) and actin (green, phalloidin) within 20-somite stage wild-type (G), M1 morpholino-injected (H), and *pac*^{tm101a} homozygote ([I], more caudal view than [G] and [H]) embryos reveals no disruption to myotomal integrity, prior to and during slow muscle cell migration.

(J–L) Dorsal view of 3D reconstructions projecting through the entire dorsoventral extent of a four-somite width view of wild-type (J), M1-injected (K), and *pac*^{tm101a} homozygote (L) 26-somite stage embryos. All embryos have been stained for slow MyHC (red, F59) and actin (green, phalloidin). The altered slow MyHC fiber migration does not affect general myotomal morphology within M1-injected or *pac*^{tm101a} homozygote embryos.

(M and N) Confocal sections showing embryos stained with MyHC (green) and counterstained with propidium iodide (red) to mark nuclei positions within the myotome of *m-cadherin* M1 morpholino-injected ([M], caudal somites) and *pac*^{tm101a} homozygote ([N], rostral somites) embryos. Nuclei positioning provides a sensitive indicator of myotomal integrity, which is unaltered in these embryos.

are identical to defects found in embryos injected with the *m-cadherin* M2 morpholino or low concentrations of the M1 morpholino (compare Figure 4G to Figure 4J). In all cases large gaps were found in the lateral pattern of slow fibers, resulting from the absence or altered migration of slow muscle cells. Injection of the *m-cadherin* M1 morpholino into *pac*^{tm101a} mutants did not lead to a more dramatic loss of slow fiber than in M1-injected morpholinos alone ($n = 45$). This suggests that N-cadherin activity was not redundant to that of the M-cadherin function. We note, however, that database searches have revealed the existence of several possible *n-cadherin* genes, other than that initially reported and mutated in *pac*^{tm101a} mutants (Bitzur et al., 1994; Lele et al., 2002; P.G. and P.D.C., unpublished observations). This is in contrast to *m-cadherin*, where only a single match could ever be found within zebrafish and *Fugu* databases. This raised the possibility of redundancy acting at the level of *n-cadherin*. Genetic redundancy would explain the relative lack of severity of the *pac*^{tm101a} phenotype both in comparison to the *m-cadherin* M2 morpholino phenotype as well as the phenotype of *n-cadherin* knockout mice, which possess severe cardiac or somitic epithelization defects (Radice et al., 1997; see below) not evident in *pac*^{tm101a} homozygotes.

Despite the clear association of zebrafish slow muscle cell migration with the respective expansion and retraction of *m-* and *n-cadherin* expression, it remained possible that defects in myotome ontogeny could occur through nonspecific defects in myotomal structure, which would relate to an early somite epithelial defect. However, no generalized defects in myotomal structure could be detected prior to, during, or after completion of migration of slow muscle migration structure within *m-cadherin* morpholino-injected and *pac*^{tm101a} homozygotes utilizing a number of different criteria (Figures 5G–5N; Supplemental Movies 7–9). We therefore conclude that expression of both N- and M-cadherin are required specifically for the correct, coordinate migration of slow muscle cells, rather than for general myotomal integrity.

Ectopic Expression of N-Cadherin Disrupts the Patterning of Slow Muscle Fibers

A further prediction of our model is that altering the balance of cadherin in advance or within migrating slow cells would disrupt the ability of cells to sort according to the levels and type of cadherin that they express. This would have as its consequence a possible failure or perturbation in slow cell migration. In order to examine this question, we undertook two different types of experiment. First, we expressed N-cadherin ectopically in

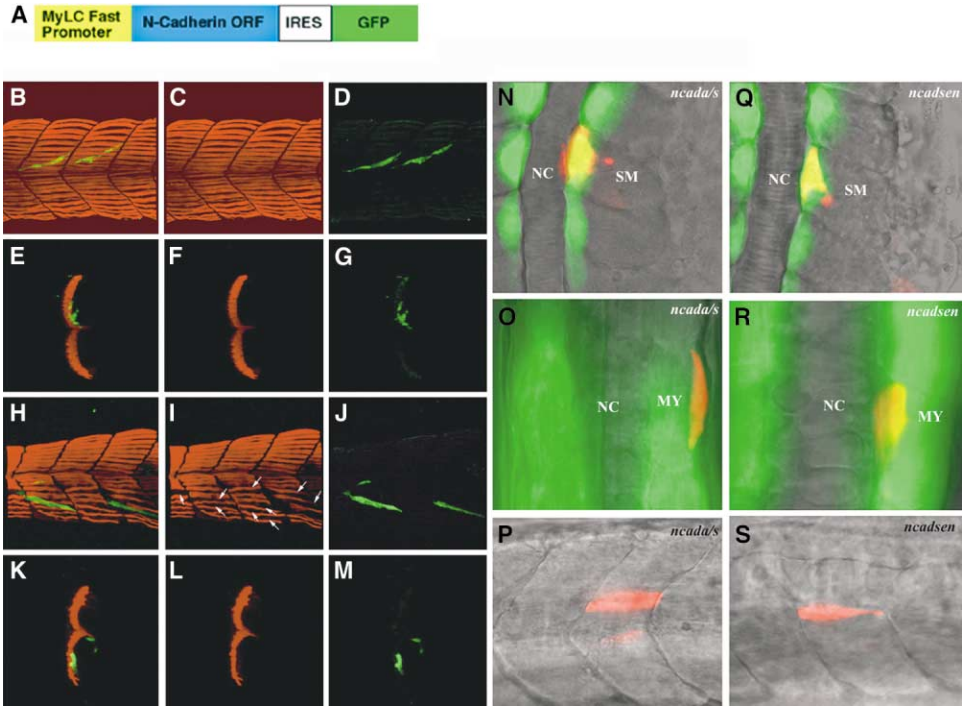

Figure 6. Overexpression of N-Cadherin Perturbs Slow Muscle Migration

(A) N-cadherin can be ectopically expressed via use of the fast muscle-specific promoter of the *Fast Myosin light chain 2* gene (MyLC fast; Xu et al., 1999; Ju et al., 2003) and visualized by the simultaneous expression of GFP (green), which is translated from a bicistronic message through the utilization of an intervening internal ribosome entry site (IRES) element.

(B–G) Injection of the "empty" construct that contains the MyLC2 fast-specific promoter and GFP alone does not alter slow muscle migration.

(H–M) Injection of the MLC2F-Ncad-GFP construct results in missing fibers (arrows) from the final stereotypical palisade of postmigratory slow muscle (red). (B–D) and (H–J) are selected lateral views, anterior to the left, of a 3D confocal reconstruction projected over four somites. (E–G) and (K–M) are cross-sectional views, anterior out of the page, dorsal to the top of the same 3D confocal reconstructions.

(N–S) Injection of mRNA encoding the antisense strand of *n-cadherin* into the premigratory adaxial cell of a 15-somite stage embryo (N) does not perturb migration to the lateral myotome evident at the 26-somite stage (O and P). Injection of mRNA encoding the sense strand of the *n-cadherin* gene into the adaxial cell of a 15-somite stage embryo (Q) results in a block in lateral migration of adaxial cells (R and S). Red, rhodamine dextran lineage tracking dye marks the injected cell; green, GFP derived from muscle-specific α-actin promoter GFP transgene. (N, O, Q, and R) Dorsal view, anterior to the top.

(P and S) Lateral views of the rhodamine channel only to reveal the normal, elongated morphology of injected cells.

front of the migrating slow cells via the use of a lateral, fast muscle-specific promoter derived from the zebrafish myosin light chain 2 fast gene (MLC2f; Xu et al., 1999; Ju et al., 2003). The expression of this gene and promoter is activated prior to slow muscle migration within the lateral, fast muscle-specific domain of the myotome. We placed the *n-cadherin* open reading frame under the control of the MLC2f gene regulatory elements and upstream of the GFP ORF (referred to as MLC2F-Ncad-GFP; Figure 6A). Simultaneous cadherin/GFP translation could be achieved within the bicistronic message via use of an internal ribosome entry site (IRES) placed upstream of GFP. IRES elements have been previously shown in zebrafish to result in efficient translation of GFP, which faithfully delineates the expression of the bicistronic message (Fahrenkrug et al., 1999; Wang et al., 2000). DNA injection into the early blastomeres of the zebrafish embryo results in a highly mosaic inheritance of DNA within transgenic clones. Therefore, injection of the MLC2F-Ncad-GFP construct into zebrafish embryos resulted in small clones of cells, comprised most often of single cells, expressing GFP and N-cadherin ahead of the migrating slow cells (Figures 6H–6M). We hypothesized that providing a fixed and high level of N-cadherin within lateral fast muscle progenitors would, when the expanding wave of M-cadherin expression reached the position of the ectopic N-cadherin expressing clone, generate an environment that may mimic the high N- and M-cadherin zone containing the adaxial cells. As levels of N-cadherin within the clones would not be spatially and temporally altered they may "trap" adaxial cells in a fixed homophilic environment, thus halting their lateral-ward migration, and result in gaps within the final stereotypical palisade of postmigratory slow muscle fibers. An examination of embryos injected with the MLC2F-Ncad-GFP construct revealed gaps in the final slow muscle cell pattern that were correlated with GFP-positive cells (Figures 6H–6M; 32%, n = 25; Supplemental Movie 11). Curiously, gaps could often extend to neighboring somites, with muscle fibers missing directly appositional to the gaps present in the myotomes in which MLC2F-Ncad-GFP expression was evident, suggesting some role for cadherins in coordinating adhesion between muscle fibers in adjacent myotomes. None of these effects were evident upon injection with a construct driving GFP alone from the MLC2F promoter (Figures 6B–6G; n = 32; Supplemental Movie 10).

A second experimental approach, aimed at dissecting

the importance of the levels of specific cadherin molecules within migrating slow cells, was to directly inject *n-cadherin* mRNA into adaxial cells, just prior to their migration. Direct iontophoretic injection has previously been shown to be an efficient mechanism for delivery of mRNA into zebrafish cells in vivo (Dorsky et al., 1998). By providing a "spike" in N-cadherin expression, we aimed to produce single adaxial cells that expressed a uniquely high level of N-cadherin within an otherwise unaltered myotome. We hypothesized that such cells would no longer possess homophilic attraction with migrating adaxial cells or the N-/M-cadherin zone into which they were sorting. Consequently, these cells would fail to correctly migrate to the lateral surface of the myotome. As predicted, injection of *n-cadherin* capped sense mRNA into premigratory adaxial cells, dramatically altered the ability of these cells to migrate. Injected cells remained medial, within two to three cell diameters of the midline (Figures 6Q–6S; 85%, n = 13). No such alteration in migration was evident in adaxial cells injected with mRNA derived from the antisense strand of the *n-cadherin* gene (Figures 6N–6P; n = 9). From the results of these two separate analyses, we have concluded that the levels of individual cadherins expressed within cells of the myotome are important for correct slow cell migration.

Discussion

We have analyzed the morphogenetic and molecular mechanisms by which slow MyHC-expressing muscle cells undergo a directional lateral-ward migration within the zebrafish myotome. Time-lapse analysis of myotomal cells, prior to and during slow cell migration, suggests a possible role for differential cell adhesion in the control of migration. We have identified cell adhesion molecules that are expressed within the zebrafish myotome and revealed that zebrafish *n-* and *m-cadherin* are expressed in a dynamic and reciprocal fashion that correlates with slow muscle cell migration. Loss of the expression of either cadherin from myotomal cells inhibits its migration. Furthermore, altering cadherin expression, either in migrating slow muscle cells themselves or within cells through which slow muscle cells must migrate, results in aberrant migration. We conclude that differential cell adhesion, driven by dynamic and reciprocal expression of members of the classical cadherin family within the myotome, is responsible for coordinating the migration of zebrafish slow muscle cells (see Figure 7).

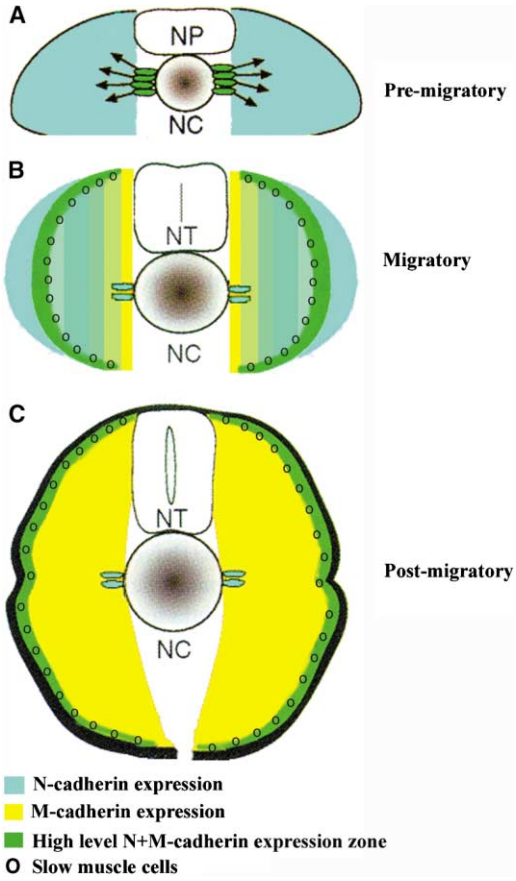

Figure 7. Model of Slow Muscle Cell Migration in the Zebrafish Embryo

(A) Adaxial cells initially express a high level of both N- and M-cadherin, with N-cadherin expression also extending throughout the somite.

(B) Migration proceeds through the dynamic expansion and retraction of M- and N-cadherin, creating a unique homophilic environment for adaxial cells that traverse the myotome to generate cell sorting of differentiating slow muscle cells. MP cells lose M-cadherin expression but retain N-cadherin, and thus they do not sort into the migratory domain.

(C) Postmigratory expression of N-cadherin is restricted to the adaxial cells including the MP cells, and M-cadherin is expressed throughout the myotome with the exception of the MP cell.

N- and M-Cadherin Function in Mammalian Cell Adhesion and Migration

Numerous studies, carried out both in vivo and in vitro, have pointed to multiple roles for N- and M-cadherin in the development of the mouse embryo. In the case of M-cadherin, its predominance of expression within developing and regenerating mammalian muscle has led to a number of hypotheses about its function. Chief among these is the suggestion that M-cadherin may be essential for the fusion of myoblasts to form multinucleated myofibers, a view that is supported by results of in vitro studies (Kaufmann et al., 1999; Zeschnigk et al., 1995). Furthermore, within mature, postnatal muscle fibers of the mouse, and within fully differentiated fibers in vitro, M-cadherin expression is largely lost. In vivo, however, M-cadherin expression remains associated with a subset of quiescent satellite cells (muscle-specific stem cells), and has long been postulated to control the adherence and positioning of satellite cells along a fiber. The only nonmuscle-related site of M-cadherin expression is within the cerebellum of adult mice at a structure termed the contactus adherens. The contactus adherens forms specialized adherence junctions in the granular cell layer of the cerebellar glomerulus, and the expression of M-cadherin within these cells has led to speculation on its role in coordinating junction formation (Rose et al., 1995; Bahjaoui-Bouhaddi et al., 1997).

Surprisingly, however, knockout studies have so far failed to provide any evidence of a role for M-cadherin in myoblast fusion or adhesion, as homozygous *m-cadherin*

mutant mice possess no discernible phenotype (Hollnagel et al., 2002). A detectable upregulation of N-cadherin within tissues that would normally express M-cadherin has been suggested to be responsible for the lack of phenotypic consequences of M-cadherin removal. However, as myotomal fiber positioning has not been examined in any detail within *m-cadherin* knockout mice it would be of interest, in light of the results reported here, to determine whether the final pattern of the embryonic myotome is perturbed.

By contrast, *n-cadherin* mutant mice do possess a number of well-characterized developmental defects that appear to result from a lack of cell adhesion in specific tissues (Radice et al., 1997). Analysis of mouse embryos chimeric for N-cadherin-expressing and -non-expressing cells, as well as the results of in vitro studies, have provided additional evidence for both a cell adhesive and migration-promoting role for N-cadherin (Kostetskii et al., 2001; Tran et al., 1999; Dufour et al., 1999). While such studies implicate N-cadherin function in the maintenance of tissue integrity in different regions of the mouse embryo, including the somites, none of the defects so far studied in *n-cadherin* null mice have been shown to result from a migratory, or cell-sorting, abnormality within a specific subpopulation of cells. Furthermore, an analogous role to that demonstrated in the control of skeletal muscle fiber organization in zebrafish cannot be ruled out, as early defects in somite epithelization preclude an examination of this question.

In contrast to these analyses, our study points to a specific requirement for both N- and M-cadherin in the cell sorting of different myotomal cell types. It is possible that N- and M-cadherin could play similar roles during the formation of the amniote myotome, but no correlation between these genes and fiber type morphogenesis has yet been reported. Although amniote fast and slow-twitch muscle cells do not lie in spatially separable populations but are peppered throughout the myotome, recent studies have revealed the possible existence of chick primary "pioneer" myoblasts. These cells express both slow and fast MyHC, which differs from later born cells that express only fast MyHC and intersperse between pioneer fibers (Kahane et al., 1998; Kalcheim et al., 1999). These findings support the hypothesis that the evolutionary origin of amniote myotome formation might be reflected within the simplified muscle cell arrangement present in zebrafish. However, it must be acknowledged that amniote myotome formation remains controversial, with several different models in evidence (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000).

Slow Muscle Cell Morphogenesis during Migration and the Role of Cadherin-Mediated Adhesion

Our time-lapse analyses of slow muscle cell migration within the zebrafish myotome have cataloged the stereotypical nature of the morphological transitions that slow cells undergo during their medial to lateral migration. We have further revealed that the molecular basis for these morphological transitions resides in the differential deployment of cadherins during the ontogeny of the myotome. However, one unexplained feature of our experimental analysis is that overexpression of N-cadherin ahead of the migrating slow cells results in an absence of slow muscle fibers from the stereotypical lateral slow muscle palisade, rather than a detectable prevention of their migration and consequent altered positioning in the myotome. Why slow fibers that encounter a fast myocyte expressing an altered level of cadherin do not simply fail to migrate past this point remains unclear. Clearly, the loss of function of *n-* and *m-cadherin* results in such a phenotype, where large cohorts of slow muscle cells fail to migrate from the midline—most dramatically illustrated in the *m-cadherin* M1 morpholino-induced phenotypes. One possible explanation may lie in the phenotype of the zebrafish mutation *uboot* (*ubo*). In this mutation, differentiating slow muscle cells are specified normally but fail to migrate from the midline. Consequently, they fuse with adjacent fast muscle precursors, turn off slow MyHC expression, and begin to express fast MyHC (Roy et al., 2001). It may be that when individual slow muscle cells are similarly held within the fast muscle progenitors domain for an extended period, such as by an inability to find a homophilic environment, that they are triggered to fuse with surrounding fast muscle cells. These cells would consequently downregulate slow MyHC and initiate a fast muscle-specific gene expression profile. Our experiments overexpressing N-cadherin within individual adaxial cells also appears to support such a conclusion, as these cells remain localized within the fast muscle domain and are not lost from the postmigratory myotome.

Collectively, our results define a role for classical cadherin-mediated adhesion in the coordinated migration of slow MyHC-expressing myocytes within the zebrafish myotome. The dynamic and reciprocal expression of two different cadherin molecules within the zebrafish myotome and their precise correlation with a cell migratory event establishes a paradigm for the regulation of cell migration by cadherins. Altering or removing the expression of either cadherin within the myotome perturbs slow muscle cell migration. These results provide a definitive example of adhesion-mediated cell sorting controlling the direction of cellular migration. It also provides clear in vivo evidence that both qualitative and quantitative changes in cadherin expression can mediate cell sorting, confirming results of earlier in vitro studies. It is perhaps the strongest illustration to date that the differential adhesion hypothesis can be applied to the morphogenesis of complex tissues in vivo.

Experimental Procedures

Zebrafish Strains

Wild-type embryos of the AB strain homozygous for the pigment mutation golden were used in all stainings and manipulations. The *parachute* (*pac*^{tm101}) and *uboot* (*ubo*^{p39}) were a gift of H.-G. Frohnhöfer at the Tübingen Stock Center.

Bodipy Ceramide Staining, Iontophoretic Injections, and Time-Lapse Analysis

Bodipy ceramide labeling was performed essentially as described (Cooper et al., 1999) with time-lapse images captured on a Zeiss LSM510 inverted confocal microscope. Single-plane capture was performed over the course of 4–6 hr at 5 min intervals, and the

captured images were exported as a Quicktime movie file. For iontophoretic labeling, embryos were stained for bodipy ceramide as described above and allowed to develop to the two-somite stage and injected utilizing methods previously described (Devoto et al., 1996). For mRNA injections, capped mRNA, synthesized using previously described methods (Currie and Ingham, 1996), was added to the diluted rhodamine dextran to a concentration of 0.2 μg/ml and iontophoretically injected into untreated, 15-somite stage embryos as described above. Dextran/mRNA-injected embryos were agarose mounted at the 26-somite stage and imaged by simultaneous DIC and fluorescence imaging utilizing a digital camera (Hamamatsu).

Gene Cloning and Alignments

The full-length N-cadherin cDNA was a gift of B. Geiger (Weizmann Institute of Science, Israel). An EST with homology to the 3′ end of amniote *m-cadherin* genes was identified from the zebrafish EST database. Hidden Markov modeling of the known amniote *m-cadherin* genes was used to identify upstream exons of the zebrafish *m-cadherin* gene present with the zebrafish genomic sequence. Sequences up to exon 2 could be isolated in this manner, and the intervening exonic sequence was amplified from cDNA pools. A RACE strategy was utilized to isolate exon 1 sequences and complete the *m-cadherin* ORF, which was subsequently confirmed as being expressed by RT-PCR (GenBank accession number AY443033).

Morpholino Design and Injection

Two separate antisense morpholino oligonucleotides (Gene Tools, Pilomath) were designed against the 5′ region of the *m-cadherin* ORF. Oligo M1 was designed to overlap the start ATG of *m-cadherin* (5′-CCGCCAAAACCACCACTGCCTTCAT-3′), and Oligo M2 was designed to hybridize to 5′ untranslated region four base pairs upstream of the ATG (5′-AGCCTTTTTGATCCTCAATGACCAC-3′). All oligonucleotides were diluted in water to 1 mM and injected as described (Currie and Ingham, 1996). Oligo M1 phenocopied Oligo M2 at 0.2 mM injection concentration. The splice site-directed morpholino, SM1 (5′-TGATTATCTTACCTGGAACGGGAAAA-3′), was designed to overlap exon 4's splice donor site. If produced, exon skipping of exon 4 would result in an in-frame stop codon within exon 5 of any aberrantly spliced mRNA. Injections were performed as described (Blagden et al., 1997).

Gene Expression

In situ and antibody stains were performed essentially as described (Currie and Ingham, 1996; Blagden et al., 1997). F59 antibody was a gift from F. Stockdale (Stanford University). Fluorescein-labeled phalloidin (Molecular Probes) was diluted 1:300 and used to costain in a standard whole-mount antibody protocol (Blagden et al., 1997). Three-dimensional reconstruction of individual myotomes was performed on a laser scanning confocal microscope LSM510 (Carl Zeiss) using software supplied by the manufacturer. To determine morpholino-mediated transcript knockdown, RT-PCR was carried out on cDNA created from thirty 20-somite stage embryos each of wild-type and SM1-injected embryos using AMV reverse transcriptase as described by the manufacturer (Invitrogen).

Acknowledgments

We are grateful to F. Stockdale and B. Geiger for providing reagents. We are indebted to the Tübingen Stock Center, in particular H.-G. Frohnhöfer and C. Nusslein-Volhard for zebrafish strains. We thank S. Devoto for thoughtful discussions, and R. Harvey and N. Hastie for comments on the manuscript. We thank members of the Harvey lab for technical help in manuscript preparation. F.C. was supported by an EMBO long-term fellowship, D.D. by a Burroughs-Wellcome fellowship, and G.E.H. is supported by a Royal Society/NH and MRC Howard Florey postdoctoral fellowship. P.D.C. was supported by the Medical Research Council, UK.

Received: June 5, 2003
Revised: June 16, 2003
Accepted: October 24, 2003
Published: December 8, 2003

References

Bahjaoui-Bouhaddi, M., Padilla, F., Nicolet, M., Cifuentes-Diaz, C., Fellmann, D., and Mege, R.M. (1997). Localized deposition of M-cadherin in the glomeruli of the granular layer during the postnatal development of mouse cerebellum. J. Comp. Neurol. *378*, 180–195.

Bitzur, S., Kam, Z., and Geiger, B. (1994). Structure and distribution of N-cadherin in developing zebrafish embryos: morphogenetic effects of ectopic over-expression. Dev. Dyn. *201*, 121–136.

Blagden, C.S., Currie, P.D., Ingham, P.W., and Hughes, S.M. (1997). Notochord induction of zebrafish slow muscle is mediated by Sonic hedgehog. Genes Dev. *11*, 2163–2175.

Brennan, C., Amacher, S.L., and Currie, P.D. (2002). Somitogenesis. Results Probl. Cell Differ. *40*, 271–297.

Cooper, M.S., D'Amico, L.A., and Henry, C.A. (1999). Confocal microscopic analysis of morphogenetic movements. Methods Cell Biol. *59*, 179–204.

Coutelle, O., Blagden, C.S., Hampson, R., Halai, C., Rigby, P.W., and Hughes, S.M. (2001). Hedgehog signalling is required for maintenance of *myf5* and *myoD* expression and timely terminal differentiation in zebrafish adaxial myogenesis. Dev. Biol. *236*, 136–150.

Currie, P.D., and Ingham, P.W. (1996). Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. Nature *382*, 452–455.

Denetclaw, W.F., Jr., and Ordahl, C.P. (2000). The growth of the dermomyotome and formation of early myotome lineages in thoracolumbar somites of chicken embryos. Development *127*, 893–905.

Denetclaw, W.F., Jr., Christ, B., and Ordahl, C.P. (1997). Location and growth of epaxial myotome precursor cells. Development *124*, 1601–1610.

Devoto, S.H., Melancon, E., Eisen, J.S., and Westerfield, M. (1996). Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. Development *122*, 3371–3380.

Dorsky, R.I., Moon, R.T., and Raible, D.W. (1998). Control of neural crest cell fate by the Wnt signalling pathway. Nature *396*, 370–373.

Draper, B.W., Morcos, P.A., and Kimmel, C.B. (2001). Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown. Genesis *30*, 154–156.

Dufour, S., Beauvais-Jouneau, A., Delouvee, A., and Thiery, J.P. (1999). Differential function of N-cadherin and cadherin-7 in the control of embryonic cell motility. J. Cell Biol. *146*, 501–516.

Duguay, D., Foty, R.A., and Steinberg, M.S. (2003). Cadherin-mediated cell adhesion and tissue segregation: qualitative and quantitative determinants. Dev. Biol. *253*, 309–323.

Ekker, M., Wegner, J., Akimenko, M.A., and Westerfield, M. (1992). Co-ordinate embryonic expression of three zebrafish engrailed genes. Development *116*, 1001–1010.

Erdmann, B., Kirsch, F.P., Rathjen, F.G., and More, M.I. (2003). N-Cadherin is essential for retinal lamination in the zebrafish. Dev. Dyn. *226*, 570–577.

Fahrenkrug, S.C., Clark, K.J., Dahlquist, M.O., and Hackett, P.B., Jr. (1999). Dicistronic gene expression in developing zebrafish. Mar. Biotechnol. (NY) *1*, 552–561.

Fishell, G., Mason, C.A., and Hatten, M.E. (1993). Dispersion of neural progenitors within the germinal zones of the forebrain. Nature *362*, 636–638.

Friedlander, D.R., Mege, R.M., Cunningham, B.A., and Edelman, G.M. (1989). Cell sorting-out is modulated by both the specificity and amount of different cell adhesion molecules (CAMs) expressed on cell surfaces. Proc. Natl. Acad. Sci. USA *86*, 7043–7047.

Godt, D., and Tepass, U. (1998). *Drosophila* oocyte localization is mediated by differential cadherin-based adhesion. Nature *395*, 387–391.

Gonzalez-Reyes, A., and St Johnston, D. (1998). The *Drosophila* AP axis is polarised by the cadherin-mediated positioning of the oocyte. Development *125*, 3635–3644.

Hatta, K., Bremiller, R., Westerfield, M., and Kimmel, C.B. (1991). Diversity of expression of engrailed-like antigens in zebrafish. Development *112*, 821–832.

Higashijima, S., Okamoto, H., Ueno, N., Hotta, Y., and Eguchi, G. (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. Dev. Biol. *192*, 289–299.

Hollnagel, A., Grund, C., Franke, W.W., and Arnold, H.H. (2002). The cell adhesion molecule M-cadherin is not essential for muscle development and regeneration. Mol. Cell. Biol. *22*, 4760–4770.

Inoue, T., Tanaka, T., Takeichi, M., Chisaka, O., Nakamura, S., and Osumi, N. (2001). Role of cadherins in maintaining the compartment boundary between the cortex and striatum during development. Development *128*, 561–569.

Ju, B., Chong, S.W., He, J., Wang, X., Xu, Y., Wan, H., Tong, Y., Yan, T., Korzh, V., and Gong, Z. (2003). Recapitulation of fast skeletal muscle development in zebrafish by transgenic expression of GFP under the mylz2 promoter. Dev. Dyn. *227*, 14–26.

Kahane, N., Cinnamon, Y., and Kalchiem, C. (1998). The origin and fate of pioneer myotomal cells in the avian embryo. Mech. Dev. *74*, 59–73.

Kalcheim, C., Cinnamon, Y., and Kahane, N. (1999). Myotome formation: a multistage process. Cell Tissue Res. *296*, 161–173.

Kaufmann, U., Kirsch, J., Irintchev, A., Wernig, A., and Starzinski-Powitz, A. (1999). The M-cadherin catenin complex interacts with microtubules in skeletal muscle cells: implications for the fusion of myoblasts. J. Cell Sci. *112*, 55–68.

Kostetskii, I., Moore, R., Kemler, R., and Radice, G.L. (2001). Differential adhesion leads to segregation and exclusion of N-cadherin-deficient cells in chimeric embryos. Dev. Biol. *234*, 72–79.

Lele, Z., Folchert, A., Concha, M., Rauch, G.J., Geisler, R., Rosa, F., Wilson, S.W., Hammerschmidt, M., and Bally-Cuif, L. (2002). Parachute/n-cadherin is required for morphogenesis and maintained integrity of the zebrafish neural tube. Development *129*, 3281–3294.

Masai, I., Lele, Z., Yamaguchi, M., Komori, A., Nakata, A., Nishiwaki, Y., Wada, H., Tanaka, H., Nojima, Y., Hammerschmidt, M., et al., (2003). N-cadherin mediates retinal lamination, maintenance of forebrain compartments and patterning of retinal neurites. Development *130*, 2479–2494.

Miyatani, S., Shimamura, K., Hatta, M., Nagafuchi, A., Nose, A., Matsunaga, M., Hatta, K., and Takeichi, M. (1989). Neural cadherin: role in selective cell-cell adhesion. Science *245*, 631–635.

Nose, A., Nagafuchi, A., and Takeichi, M. (1988). Expressed recombinant cadherins mediate cell sorting in model systems. Cell *54*, 993–1001.

Price, S.R., De Marco Garcia, N.V., Ranscht, B., and Jessell, T.M. (2002). Regulation of motor neuron pool sorting by differential expression of type II cadherins. Cell *109*, 205–216.

Radice, G.L., Rayburn, H., Matsunami, H., Knudsen, K.A., Takeichi, M., and Hynes, R.O. (1997). Developmental defects in mouse embryos lacking N-cadherin. Dev. Biol. *181*, 64–78.

Rose, O., Grund, C., Reinhardt, S., Starzinski-Powitz, A., and Franke, W.W. (1995). Contactus adherens, a special type of plaque-bearing adhering junction containing M-cadherin, in the granule cell layer of the cerebellar glomerulus. Proc. Natl. Acad. Sci. USA *92*, 6022–6026.

Roy, S., Wolff, C., and Ingham, P.W. (2001). The u-boot mutation identifies a Hedgehog-regulated myogenic switch for fiber-type diversification in the zebrafish embryo. Genes Dev. *15*, 1563–1576.

Steinberg, M.S. (1963). Reconstruction of tissue by dissociated cells. Science *141*, 401–408.

Steinberg, M.S., and Takeichi, M. (1994). Experimental specification of cell sorting tissue spreading, and specific spatial patterning by quantitative differences in cadherin expression. Proc. Natl. Acad. Sci. USA *91*, 206–209.

Tepass, U., Godt, D., and Winklbauer, R. (2002). Cell sorting in animal development: signalling and adhesive mechanisms in the formation of tissue boundaries. Curr. Opin. Genet. Dev. *12*, 572–582.

Tran, N.L., Nagle, R.B., Cress, A.E., and Heimark, R.L. (1999). N-Cadherin expression in human prostate carcinoma cell lines. An epithelial-mesenchymal transformation mediating adhesion with stromal cells. Am. J. Pathol. *155*, 787–798.

Wang, X., Wan, H., Korzh, V., and Gong, Z. (2000). Use of an IRES bicistronic construct to trace expression of exogenously introduced mRNA in zebrafish embryos. Biotechniques *29*, 814–816.

Weinberg, E.S., Allende, M.L., Kelly, C.S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O.G., Grunwald, D.J., and Riggleman, B. (1996). Developmental regulation of zebrafish *MyoD* in wild type, no tail and spadetail embryos. Development *122*, 271–280.

Xu, Y., He, J., Tian, H.L., Chan, C.H., Liao, J., Yan, T., Lam, T.J., and Gong, Z. (1999). Fast skeletal muscle-specific expression of a zebrafish myosin light chain 2 gene and characterization of its promoter by direct injection into skeletal muscle. DNA Cell Biol. *18*, 85–95.

Yagi, T., and Takeichi, M. (2000). Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. Genes Dev. *4*, 1169–1180.

Zeschnigk, M., Kozian, D., Kuch, C., Schmoll, M., and Starzinski-Powitz, A. (1995). Involvement of M-cadherin in terminal differentiation of skeletal muscle cells. J. Cell Sci. *108*, 2973–2981.