Convergence and extension movements mediate the specification and fate maintenance of zebrafish slow muscle precursors

Chunyue Yin, Lilianna Solnica-Krezel *

Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA

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Abstract

During vertebrate gastrulation, concurrent inductive events and cell movements fashion the body plan. Convergence and extension (C&E) gastrulation movements narrow the vertebrate embryonic body mediolaterally while elongating it rostrocaudally. Segmented somites are shaped and positioned by C&E alongside the notochord and differentiate into skeleton, fast, and slow muscles during somitogenesis. In zebrafish, simultaneous inactivation of non-canonical Wnt signaling components Knypek and Trilobite strongly impairs C&E gastrulation movements. Here we show that knypek;trilobite double mutants exhibit a severe deficit in slow muscles and their precursor, adaxial cells, revealing essential roles of C&E movements in adaxial cell development. Adaxial cells become distinguishable in the presomitic mesoderm during late gastrulation by their expression of myogenic factors and axial-adjacent position. Using cell tracing analyses and genetic manipulations, we demonstrate that C&E movements regulate the number of prospective adaxial cells specified during gastrulation by determining the size of the interface between the inductive axial and target presomitic tissues. During segmentation, when the range of Hedgehog signaling from the axial tissue declines, tight apposition of prospective adaxial cells to the notochord, which is achieved by convergence movements, is necessary for their continuous Hedgehog reception and fate maintenance. We provide direct evidence to show that the deficiency of adaxial cells in knypek;trilobite double mutants is due to impaired C&E movements, rather than an alteration in Hedgehog signal and its reception, or a cell-autonomous requirement for Knypek and Trilobite in adaxial cell development. Our results underscore the significance of precise coordination between cell movements and inductive tissue interactions during cell fate specification.

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Keywords: Non-canonical Wnt; Knypek; Trilobite; Adaxial cell; Gastrulation; Hedgehog

Introduction

The basic vertebrate body plan is established during gastrulation by a set of highly conserved morphogenetic movements, including epiboly, internalization, convergence and extension (C&E) (Keller et al., 2003; Solnica-Krezel, 2005; Warga and Kimmel, 1990). One intriguing aspect of developmental biology is to understand the connection between the morphogenetic processes of gastrulation that shape the embryo and the inductive tissue interactions that specify different cell fates. However, impaired gastrulation movements are often accompanied by early patterning defects, making it difficult to determine the consequences of defective movements on cell fate specification (Kimmel et al., 1989; Myers et al., 2002a). Large-scale genetic screens in zebrafish isolated a group of mutations disrupting C&E gastrulation movements that narrow the forming embryonic tissues mediolaterally and lengthen them anteroposteriorly (Hammerschmidt et al., 1996; Myers et al., 2002b; Solnica-Krezel et al., 1996). The C&E mutants exhibit a shortened body but normal tissue patterning during gastrulation (Myers et al., 2002b), presenting a powerful genetic tool with which to investigate the roles of gastrulation movements in subsequent cell fate specification. Molecular analyses revealed that many of the C&E mutations inactivate components of non-canonical Wnt signaling, a vertebrate equivalent of the D. melanogaster planar cell polarity (PCP) pathway that polarizes cells within the plane of epithelium (Adler, 2002; Klein and Mlodzik, 2005). During vertebrate gastrulation, non-canonical
Wnt signaling directs the mediolateral (ML) cell elongation critical for cell behaviors underlying C&E (Jessen et al., 2002; Myers et al., 2002b; Wallingford et al., 2000). "knypek" and "trilobite (tri)" encode two components of non-canonical Wnt signaling, a heparan sulfate proteoglycan of the glypican family (Topczewski et al., 2001), and a transmembrane protein homologous to D. melanogaster strabismus/van gogh and mammalian van gogh-like 2 (Jessen and Solnica-Krezel, 2004; Jessen et al., 2002), respectively. "kn" and "tri" genes act both cell-autonomously and non-autonomously to regulate the ML elongation of mesodermal cells during C&E (Jessen et al., 2002; Topczewski and LSK, unpublished). Compared to "kn" and "tri" individual mutants, "kn";"tri" double mutants show additive C&E defects (Henry et al., 2000; Marlow et al., 1998). Despite detailed analyses of the C&E movements in these mutants, the effect of the defective gastrulation movements on cell fate specification is less understood.

Segmented somites form along the notochord following gastrulation and serve as a scaffold to guide further elaboration of the vertebrate body plan (Brennan et al., 2002; pourquie, 2001). In zebrafish, trunk somites are derived from the presomatic mesoderm (PSM) that is located at the dorsolateral blastoderm margin, the blastopore equivalent of zebrafish gastrula (Kimmel et al., 1990; Stickney et al., 2000). Upon internalization, the PSM undergoes C&E movements to form anteroposteriorly elongated territories flanking the axial mesoderm (Myers et al., 2002b). We previously reported that due to defective C&E movements, the PSM formed in "kn";"tri" double mutants is extremely short anteroposteriorly and broad mediolaterally (Henry et al., 2000). Wild-type (WT) somites are composed of epithelial border cells and mesenchymal internal cells. In "kn";"tri" double mutants, although the anteroposterior (AP) intrasegmental polarity and the somitic boundaries are established normally, the somites consist exclusively of border cells (Henry et al., 2000). It has not been addressed whether the somitic derivatives develop normally in "kn";"tri" double mutants.

In zebrafish, somites give rise to sclerotome and myotome (Stickney et al., 2000), the latter being mainly composed of superficial slow muscles and medial fast muscles (Devoto et al., 1996). The precursors of slow and fast muscles are spatially segregated as early as during gastrulation (Hirsinger et al., 2004). Slow muscles originate from adaxial cells, which are specified as two patches of presomitic cells adjacent to the axial mesoderm during mid-gastrulation (Devoto et al., 1996; Thissell et al., 1993). Adaxial cells can be recognized by their distinct cuboidal morphology and expression of the myogenic basic helix–loop–helix transcription factors Myf5 and MyoD (Coutelle et al., 2001; Weinberg et al., 1996). During somitogenesis, majority of the adaxial cells migrate laterally through the somite to form a monolayer of slow muscle fibers at the surface of the myotome (Devoto et al., 1996). The migration of slow muscle cells initiates a wave of differentiation and morphogenesis of the fast muscle precursor cells, which are located more laterally in the somite (Henry and Amacher, 2004). Several studies have established that different levels and duration of Hedgehog (Hh) signaling emanating from the axial mesoderm produce different cell types in the zebrafish somite: the specification and commitment to the adaxial cell fate require high Hh activity, whereas the lateral somitic cells receiving low levels of Hh are designated to become fast muscles (Blagden et al., 1997; Coutelle et al., 2001; Hirsinger et al., 2004; Wolff et al., 2003). Concurrent with the specification of adaxial cells during gastrulation, both axial and paraxial mesoderm undergoes marked C&E movements (Glickman et al., 2003; Henry et al., 2000; Myers et al., 2002b; Warga and Kimmel, 1990). Whether the morphogenesis of the axial and paraxial tissues has an influence on the adaxial cell fate remains to be investigated.

In this study, we demonstrate that "kn";"tri" non-canonical Wnt mutants form fewer slow muscles and this reduction is a direct consequence of impaired C&E movements on the specification and maintenance of adaxial cell fate. During gastrulation, C&E affect the number of prospective adaxial cells specified by defining the AP dimension of the interface between the inducing axial and responding presomitic tissues. Convergence of prospective adaxial cells towards the notochord is essential for their fate maintenance during early segmentation, when the range of Hh signaling decreases. Our results underscore the requirement for precise coordination between gastrulation movements and local inductive signals during cell fate specification.

Materials and methods

Zebrafish maintenance, embryo generation, and staging

In this study, we demonstrate that "kn";"tri" non-canonical Wnt mutants form fewer slow muscles and this reduction is a direct consequence of impaired C&E movements on the specification and maintenance of adaxial cell fate. During gastrulation, C&E affect the number of prospective adaxial cells specified by defining the AP dimension of the interface between the inducing axial and responding presomitic tissues. Convergence of prospective adaxial cells towards the notochord is essential for their fate maintenance during early segmentation, when the range of Hh signaling decreases. Our results underscore the requirement for precise coordination between gastrulation movements and local inductive signals during cell fate specification.

Zebrafish maintenance, embryo generation, and staging

The "kn"myoD+/−, "tri"myoD+/−, and "kn"myoD+/−;"tri"myoD+/− mutant zebrafish strains were maintained as described previously (Solnica-Krezel et al., 1994; Solnica-Krezel et al., 1996). Embryos were obtained from natural spawning and morphologically staged as described (Kimmel et al., 1995). The genotypes of the embryos were judged by described morphologic phenotypes (Henry et al., 2000; Marlow et al., 1998).

In situ hybridization, immunohistochemistry, and cell proliferation assay

Antisense RNA probe synthesis for smbpc, myoD, ptc1, and α-tropomyosin was as described previously (Sepich et al., 2000). Whole-mount in situ hybridization was performed according to Thissell et al. (1993). Embryos were photographed with a Zeiss Axiohot using an Axiocam digital camera.

Whole-mount immunohistochemistry was performed as described (Topczewska et al., 2001). Monoclonal F59 antibody (a gift from FE Stockdale, Stanford University) and polyclonal human Myf5 antibody (Santa Cruz Biotech.) were used at 1:100. Polyclonal Prox1 antibody (CHEMICON) was used at 1:1000. Monoclonal anti-β-catenin antibody (Sigma) was used at 1:250. Images were acquired using the Zeiss LSM 510 laser scanning inverted microscope and processed using Velocity (Improvision).

To assess the proliferation rates of prospective adaxial cells during late gastrulation, embryos were fixed at 80% epiboly (8.5 hpf), yolk plug closure (9.5 hpf), and the tailbud (10 hpf) stages. In situ hybridization was performed prior to immunohistochemistry by using digoxigenin-labeled myoD RNA probe detected with fast red (Roche). M-phase cells were detected by polyclonal anti-phosphohistone antibody (Upstate Biotech.) at 1:1000 dilution (Chadee et al., 1995; Mahadevan et al., 1991).

Microinjections

Injections were performed at the one-cell stage as described (Marlow et al., 1998). Synthesis of the RNAs encoding membrane-GFP (the Ras membrane-
localization (CAAX) sequence fused to the carboxy terminus of green fluorescent protein (GFP) and Sth, and the injection dosages were as described previously (Krauss et al., 1993; Marlow et al., 1998; Wallingford et al., 2000).

**Time-lapse recording and analysis**

Multi-plane DIC time-lapse recordings were collected at 1.5-min intervals for 90 min from 90% epiboly (9 hpf) to the 1-somite stage (10.5 hpf) with Openlab software (Improvision) using a Zeiss Axiovert 200M. Manually dechorionated embryos were mounted in 1% low-melting-point agarose in 30% Danieau’s Buffer as described (Sepich et al., 2005). After the recordings, embryos were allowed to develop until day 1 to determine their genotypes. Object-Image (Norbert Vischer, http://simon.bio.uva.nl/object-image.html) was used to label and track cells. Data were exported to Excel (Microsoft) for calculation of the cell shape and size. The orientation and angular deviation were analyzed using VectorRose (P.A. Zippi, http://pazsoftware.com/). Statistical analyses were performed using Student’s two-tailed t-test.

The tissue shape changes were calculated as followed:

**Extension rate**

\[
\frac{(\text{Total cell No.}_{\text{column No.}})_t - \text{ap} - (\text{Total cell No.}_{\text{column No.}})_t - 0}{(\text{Total cell No.}_{\text{column No.}})_t - 0}
\]

**Convergence rate**

\[
- \frac{(\text{Total cell No.}_{\text{row No.}})_t - \text{ap} - (\text{Total cell No.}_{\text{row No.}})_t - 0}{(\text{Total cell No.}_{\text{row No.}})_t - 0}
\]

**Cell lineage tracing/incaging analysis**

Embryos were injected with fluorescein-caged dye (Molecular Probes) at the one-cell stage as described (Sepich et al., 2000). At the 5-somite stage, cells at the 3rd somite level and located within 4-cell diameters from the notochord were expressed in the slow muscle fibers that are located on either side of the notochord along the AP embryonic axis at 24 hours post-fertilization (hpf) (Fig. 1I) (Xu et al., 2000). The smbhc gene expression was discontinuous in the individual mutants (Figs. 1J, K), and strongly downregulated and disorganized in the double mutants (Fig. 1L). We examined two additional slow muscle makers in both WT and the mutants by immunohistochemistry: F59 antibody detects myosin heavy chain in amniotes and other species, and strongly labels the slow muscle fibers in zebrafish (Crow and Stockdale, 1986; Devoto et al., 1996); Prox1 antibody recognizes a homeobox protein and specifically labels the nuclei of slow muscle cells (Glasgow and Tomarev, 1998; Roy et al., 2001). Simultaneous labeling with both antibodies showed that the WT slow muscle fibers extended over the entire AP dimension of the myotome and were oriented parallel to the notochord (Fig. 1M). In the individual mutants, the slow muscle fibers were shorter and less organized (Figs. 1N, O). The slow muscle defects were significantly exacerbated in kny;tri double mutants, in which the slow muscle fibers were greatly reduced in number and highly disorganized (Fig. 1P; Table 1). Therefore, combined loss of Kny and Tri function severely disrupts slow muscle development.

**Simultaneous loss of Kny and Tri function impairs slow muscle development**

Somites in kny and tri C&E mutants have shortened AP and broadened ML dimensions (Hammerschmidt et al., 1996; Henry et al., 2000; Solnica-Krezel et al., 1996), whereas kny;tri double mutants exhibit additive defects in C&E and somite morphology (Figs. 1A–D) (Henry et al., 2000; Marlow et al., 1998). Given that kny and tri genes are continuously and broadly expressed in embryonic tissues, including the somites, throughout somitogenesis (Park and Moon, 2002; Topczewski et al., 2001), we decided to investigate whether Kny and Tri were also required for the later development of the somite. As revealed by membrane-localized GFP (mGFP) labeling (Wallingford et al., 2000) and confocal microscopy, at the end of segmentation, in WT the muscle fibers spanned the entire AP dimension of the chevron-shaped myotome and were oriented parallel to each other (Fig. 1E) (Stickney et al., 2000). In kny and tri individual mutants, the myotomes were shortened anteroposteriorly and the parallel orientation of the muscle fibers was modestly impaired (Figs. 1F, G). Strikingly, kny;tri double mutants formed very misshapen myotomes, composed of misaligned muscle fibers (Fig. 1H).

The abnormal myotome morphology in kny;tri double mutants prompted us to investigate the development of slow and fast muscle fibers within the myotome by examining the expression of cell-type specific genes. The expression of both slow and fast muscle markers was reduced and altered in the double mutants (Figs. 1I–P and Fig. S1). Because the lateral migration of slow muscle cells patterns the medial to lateral wave of fast muscle morphogenesis (Henry and Amacher, 2004), the fast muscle defects in the double mutants might be secondary to the impaired slow muscle development. Hence we focused on the analyses of slow muscles in this study. In WT embryo, slow myosin binding protein C (smbhc) gene is expressed in the slow muscle fibers that are located on either side of the notochord along the AP embryonic axis at 24 hours post-fertilization (hpf) (Fig. 1I) (Xu et al., 2000). The smbhc expression was discontinuous in the individual mutants (Figs. 1J, K), and strongly downregulated and disorganized in the double mutants (Fig. 1L). We examined two additional slow muscle makers in both WT and the mutants by immunohistochemistry: F59 antibody detects myosin heavy chain in amniotes and other species, and strongly labels the slow muscle fibers in zebrafish (Crow and Stockdale, 1986; Devoto et al., 1996); Prox1 antibody recognizes a homeobox protein and specifically labels the nuclei of slow muscle cells (Glasgow and Tomarev, 1998; Roy et al., 2001). Simultaneous labeling with both antibodies showed that the WT slow muscle fibers extended over the entire AP dimension of the myotome and were oriented parallel to the notochord (Fig. 1M). In the individual mutants, the slow muscle fibers were shorter and less organized (Figs. 1N, O). The slow muscle defects were significantly exacerbated in kny;tri double mutants, in which the slow muscle fibers were greatly reduced in number and highly disorganized (Fig. 1P; Table 1). Therefore, combined loss of Kny and Tri function severely disrupts slow muscle development.

The slow muscle defects in kny;tri double mutants might be related to the impaired C&E gastrulation movements or might reflect a general requirement for non-canonical Wnt/PCP pathway during zebrafish slow muscle development. Several genes that encode the PCP pathway components, such as wnt5,
kny, and tri, are expressed in the paraxial mesoderm during gastrulation and segmentation (Jessen et al., 2002; Kilian et al., 2003; Park and Moon, 2002; Rauch et al., 1997; Topczewski et al., 2001). Wnt11, which encodes non-canonical Wnt ligand, is expressed in the axial mesoderm during gastrulation and early segmentation, and later in the slow muscle cells as they undergo lateral migration (Makita et al., 1998). To test the requirement for these PCP components in slow muscle development, we examined pipetail/ppt and silberblick/slb mutants that are defective in Wnt5 and Wnt11 function, respectively (Heisenberg et al., 2000; Rauch et al., 1997). As judged by the morphology of the notochord and somites during somitogenesis, ppt and slb individual mutants exhibited much milder C&E defects compared to kny, tri, and kny;tri mutant embryos (Fig. S2B–C compared to Figs. 1B–D), consistent with previous reports (Heisenberg et al., 2000; Rauch et al., 1997). At 24 hpf, ppt and slb individual mutants formed normal chevron-shaped myotomes, and the number and morphology of their slow muscle fibers were not significantly altered (Fig. S2F–G). ppt;slb double mutants that lack the function of both Wnt ligands showed similar C&E phenotypes as seen in kny and tri individual mutants (Fig. S2D) (Ciruna et al., 2006; Marlow et al., 2004; Westfall et al., 2003). The myotomes formed in these mutants were shortened and the slow muscle fibers were less organized and reduced in number (Fig. S2H). These data indicate that PCP pathway itself is not absolutely essential for the establishment of slow muscle fate. However, there is a correlation between the degree of the C&E defect and the slow muscle defect: among all the PCP mutants examined, ppt and slb individual mutants exhibited the mildest C&E defects and formed slow muscle fibers with normal morphology and numbers. In ppt;slb double mutants,
kny and tri individual mutants, characterized by intermediate C&E phenotypes, there were modest disorganization and deficiency of the slow muscle fibers. Finally, kny;tri double mutants exhibited the most severe defects in both C&E and slow muscle development.

Reduced numbers of prospective adaxial cells are specified in kny;tri double mutants

To distinguish whether the deficiency of slow muscle fibers in kny;tri double mutants was due to defects occurring during gastrulation or reflected a requirement for later roles of these genes, we asked at what stage slow muscle development was affected in the mutants. At the 10-somite stage (14 hpf), zebrafish somite contains two cell types. (1) The slow muscle precursors, known as adaxial cells, which form a monolayer next to the notochord (Cortes et al., 2003; Devoto et al., 1996). The adaxial cells are labeled strongly by a human Myf5 antibody, which has been recently implicated to detect primarily MyoD protein in zebrafish (Fig. 4D) (Hammond et al., 2006; Tajbakhsh et al., 1998; Topczewska et al., 2001). (2) The lateral somitic cells, which give rise to fast muscles and express MyoD protein at much reduced levels (Coutelle et al., 2001; Devoto et al., 1996). WT embryos contained 106±6 cells in each anterior somite, 16 of which were adaxial cells (Table 1) (Devoto et al., 1996). kny;tri double mutants had fewer cells in their anterior somites (84±3 cells per somite) (Henry et al., 2000), only 7 of which were adaxial cells (Table 1). Notably, the ratio of adaxial cells to lateral somitic cells was significantly smaller in the double mutants; p<3.3×10−5). Therefore, kny;tri double mutants contain fewer adaxial cells during segmentation and such reduction is due to causes in addition to the smaller somite size.

We next asked whether defects in cell fate specification could account for the deficiency of adaxial cells in the double mutants. The prospective adaxial cells are specified at mid-gastrulation as two files of cells flanking the axial mesoderm, which is the source of the inductive signals (Hirsinger et al., 2004; Stickney et al., 2000). They can be recognized by their expression of the myogenic regulatory factor myoD gene (Fig. 2A) (Coutelle et al., 2001; Rudnicki et al., 1993; Weinberg et al., 1996). At 95% epiboly (9 hpf), the ML width and dorsoventral depth of the myoD-expression domain in kny;tri double mutants were similar to that in WT (Figs. 2A, C, Fig. S3, and data not shown), indicating that the range of the inducing signals and their reception by the PSM are normal. However, the AP length of the myoD-expression domain was much shorter in the double mutants (78% of the WT length, p<0.0001, 34 WT and 10 kny;tri embryos; Figs. 2A, C), consistent with the overall reduction of their AP embryo length due to defective C&E movements (Henry et al., 2000; Marlow et al., 1998). Since the size and shape of the double mutant prospective adaxial cells were not significantly different from WT at this stage (Table S1), we propose that fewer prospective adaxial cells are specified along the AP dimension in the mutants. These data suggest that C&E movements influence the number of adaxial cells specified by defining the size of the interface between the inducing axial and responding presomitic tissues.

The prospective adaxial cell population in kny;tri double mutants is defective in C&E movements at late gastrulation

As gastrulation continued and the tailbud formed, more myoD-expressing cells appeared in the posterior region when the nascent PSM encountered the midline tissues (Fig. 2D) (Kanki and Ho, 1997). Meanwhile, C&E movements extended the myoD-expression domain and brought more laterally positioned cells towards the midline (Fig. 2D). In kny;tri double mutants, AP extension of the myoD-expression domain was negligible (Fig. 2F). Instead, the ML width of the myoD expression was broader in the double mutants than in WT (5-cell diameters wide for WT, 7-cell diameters for kny;tri double mutants, Figs. 2D–F, and Fig. S3). Three plausible mechanisms could account for the ML expansion of the myoD expression domain: First, this expansion could be due to increased cell proliferation along the ML dimension. Second, Hh signaling range might be expanded laterally in the double mutants, resulting in the formation of ectopic numbers of prospective adaxial cells (Blagden et al., 1997; Coutelle et al., 2001). Finally, in WT the myoD-expressing cells converge medially towards the notochord, whereas such medial convergence fails to occur in the double mutants, accounting for the abnormal accumulation of myoD-expressing cells in the PSM.

To distinguish between these possibilities, we first analyzed the proliferation rate of the myoD-expressing cells at different stages during late gastrulation (Materials and methods). At all the stages analyzed, the double mutant cells exhibited lower proliferation rates compared to WT and individual mutants (Fig. 2G and data not shown), arguing against the notion that increased proliferation causes the ML expansion of the myoD expression.

Broader range of Hh signaling could induce prospective adaxial cells ectopically, accounting for the ML expansion of the myoD expression (Blagden et al., 1997). Moreover, a recent study in X. laevis indicated a requirement for PCP-related molecules in ciliogenesis that is essential for normal Hh signaling (Park et al., 2006). To test whether Hh signaling was affected in kny;tri PCP mutants, we examined the expression of patched1/ptc1 gene that encodes the Hh receptor. ptc1 expression is upregulated in response to Hh signaling and serves as a marker for the Hh responding cells (Concordet et al., 1996; Stone et al., 1996). At the end of gastrulation (10 hpf), ptc1 was expressed at high levels within a range of 4-cell diameters from the axial-PSM boundary in WT (Figs. 2H, H′). kny;tri double mutants exhibited equivalent ML dimension of the ptc1 expression (Figs. 2J, J′), indicating that the Hh signaling range in the PSM is not expanded in the double mutants.

The above results suggest that the ML expansion of the myoD expression in the double mutants is more likely caused by impaired medial convergence of this tissue. To study directly the C&E movements of the prospective adaxial cell population, we acquired Nomarski time-lapse recordings between 95%
epiboly (9 hpf) and the 1-somite stage (10.5 hpf), and monitored the movements of a small population of cells at the intermediate depth of the PSM (Figs. 3A–D). We used the first forming somitic boundary as a landmark to ensure that the analyzed cell populations were located at the equivalent AP position in each experimental embryo. The analyzed cells were located within 6-cell diameters from the axial-PSM boundary, thus representing a small portion of the prospective adaxial cells (referring to Fig. 2A and Fig. S3). During the recordings, the WT cell population underwent significant C&E as the tissue extended anteroposteriorly and narrowed mediolaterally (10 embryos; Figs. 3A, F; Movie S1). In the individual mutants, the tissue still converged and extended, but at reduced levels (9 kny mutants; 10 tri mutants; Figs. 3B, C, F). Strikingly, the cell population in kny; tri double mutants exhibited little convergence and virtually no extension (10 embryos; Figs. 3D, F; Movie S2).

Next, we tracked the positions of cells that were located 2-cell diameters away from the axial-PSM boundary at the beginning of the time-lapse. In WT, these prospective adaxial cells underwent active rearrangements, and 35% of them became adjacent to the midline by the end of the time-lapse (68 cells/10 embryos, Fig. 3A). The percentage was 24% in kny (65 cells/9 embryos, Fig. 3B), 27% in tri (68 cells/10 embryos, Fig. 3C), but only 19% in kny; tri double mutants (68 cells/10 embryos, Fig. 3D), providing direct evidence that the double mutant prospective adaxial cells are compromised in their medial convergence.

The lateral prospective adaxial cells in the double mutants fail to maintain their identity during segmentation

We next asked whether the ectopic columns of prospective adaxial cells in the double mutants maintained their fate during segmentation, when C&E movements continued to narrow and elongate the embryonic body (Myers et al., 2002b). By the 5-somite stage (11.7 hpf), the majority of the prospective adaxial cells in WT and individual mutants had converged medially and formed a monolayer of pseudo-epithelium flanking the notochord (Figs. 4A, B) (Hirsinger et al., 2004). The adaxial cell marker, MyoD protein (Hammond et al., 2006; Tajbakhsh et al.,
1998; Topczewska et al., 2001), was expressed in only one column of cells on either side of the notochord (Figs. 4A, A', B, B'). Notably, although kny individual mutants exhibited severe convergence defect during late gastrulation (Fig. 3F), such a defect was improved significantly during early segmentation so that most of the kny mutant prospective adaxial cells were located next to the notochord at the 5-somite stage (Figs. 4B, B').

In the double mutants, by contrast, MyoD protein expression was expanded mediolaterally at the 5-somite stage (Figs. 4C, C'), suggestive of persistent C&E defects in these embryos. Interestingly, among all the MyoD-expressing cells in the double mutants, only those immediately next to the notochord adopted an epithelial-like morphology (Fig. 4C).

Strikingly, at the 10-somite stage (14 hpf), we no longer observed the expansion of MyoD protein expression in kny;tri double mutants. In both WT and the individual mutants, MyoD protein was expressed throughout the somite, with the highest expression level detected in only one column of cells flanking the notochord (Figs. 4D, F). The identity of these notochord-adjacent cells as adaxial cells that adopted a slow muscle fate was confirmed by the labeling with F59 antibody (Figs. 4D', F') (Devoto et al., 1996). We quantified the numbers of MyoD-expressing cells at the 5-somite stage and the numbers of cells co-labeled by MyoD and F59 antibodies at the 10-somite stage. Within this time interval, whereas the number of cells expressing high levels of MyoD remained largely constant in WT and the individual mutants ($p > 0.08$), it was reduced drastically in the double mutants ($p < 0.0002$) (Fig. 4G).

The above data suggest that in kny;tri double mutants some prospective adaxial cells expressing MyoD during early segmentation lost their adaxial identity by the 10-somite stage. To test this hypothesis, we carried out cell tracing experiments. At the 5-somite stage, we labeled uniformly sized small cell groups that were within 4-cell diameters from the notochord by photoactivation of caged fluorescein (Figs. 4H, J) (Myers et al., 2002a), and assessed their positions at the 10-somite stage (Figs. 4I, K). The prospective adaxial cells were identified based on the MyoD protein expression. At the 5-somite stage in the WT embryos, the prospective adaxial cells were restricted to one column of the fluorescein-labeled cells juxtaposing the notochord (Fig. 4H), and only these cells exhibited strong MyoD expression at the 10-somite stage (Fig. 4I). The remaining cells within the labeled group were the lateral somitic cells that did not express MyoD at the 5-somite stage and showed weak MyoD expression at the 10-somite stage (Figs. 4H, I). In kny;tri double mutants, majority of the
Fig. 4. The prospective adaxial cells in kny;tri double mutants fail to maintain their identity during early segmentation. (A–C) MyoD protein distribution detected by the human Myf5 antibody in the embryos also labeled with mGFP. Dashed lines highlight the somite-PSM boundary. (A′–C′) show only the MyoD protein expression. (D–F) At the 10-somite stage, MyoD protein was strongly expressed in one column of adaxial cells flanking the notochord. Weak MyoD expression was detected in the lateral somitic cells at this stage. (D′–F′) At the 10-somite stage, the notochord-adjacent adaxial cells were co-labeled by MyoD and F59 antibodies. Lines in (A′–C′, F′) indicate the somite-PSM boundary. (G) Quantification of the numbers of cells expressing MyoD protein at the 5-somite stage and cells co-labeled with MyoD and F59 antibodies at the 10-somite stage. Error bars represent standard deviation. (H–K) Cell tracing analyses of the prospective adaxial cell population. (H) Within the fluorescein-labeled cell population (red) in WT embryos (n=6), only one column of cells immediately next to the notochord expressed MyoD protein at the 5-somite stage. (J) In the double mutants (n=6), at the 5-somite stage, majority of the fluorescein-labeled cells expressed MyoD. (K) At the 10-somite stage, only the column of cells flanking the notochord exhibit adaxial cell identity. (A–F, H–K) Dorsal views. NC, notochord. Scale bars: (A–F, A′–F′), 50 μm; (H–K) 20 μm.
defects in the maintenance of adaxial cell fate in Perturbed Hh signaling is not responsible for the defects in rise to fast muscle.

Prior to the 5-somite stage (Fig. 4J). Between the 5- and 10-somite stages, the overall size, shape, and cell number of the labeled cell group did not change significantly, indicating that these double mutant cells neither resumed C&E movements during segmentation, nor underwent cell death (Fig. 4K and data not shown). At the 10-somite stage, strong MyoD expression was restricted to the notochord-adjacent cells (Fig. 4K), whereas the labeled cells located more laterally exhibited much weaker MyoD expression, characteristic of the lateral somitic cell fate (Fig. 4K). Taken together, our data suggest that the laterally located prospective adaxial cells in the double mutants lose their adaxial cell identity during early segmentation and transdifferentiate into the lateral somitic cells that give rise to fast muscle.

Perturbed Hh signaling is not responsible for the defects in adaxial cell fate maintenance in kny;tri double mutants

We sought to understand the mechanisms underlying the defects in the maintenance of adaxial cell fate in kny;tri double mutants. Continuous Hh signaling is required for maintaining the adaxial cell identity (Coutelle et al., 2001; Hirsinger et al., 2004). Hence we examined ptc1 expression to assess the Hh signaling during segmentation. Interestingly, between the tailbud and the 5-somite stages, the ML range of the ptc1 expression decreased from 4-cell diameters to only 1-cell adjacent to the notochord, regardless of the embryo genotypes (Figs. 2H′–J′, Figs. 5A–C, A′–C′). Thus the double mutant prospective adaxial cells that did not converge to the notochord stopped receiving Hh signal by the 5-somite stage.

To investigate whether the double mutant cells that failed to converge were still competent to respond to Hh signaling, we injected 100 pg of synthetic sonic hedgehog/shh RNA into the double mutants to achieve ectopic Shh expression in the PSM (Krauss et al., 1993). Injection of shh RNA did not suppress the C&E defects of kny;tri double mutants (data not shown), consistent with our previous report (Marlow et al., 1998). Rather, we detected excess MyoD-expressing cells in the injected double mutants at the 5-somite stage (Fig. 5J). In contrast to the unmanipulated embryos, all of these MyoD-expressing cells maintained their adaxial cell identity and initiated slow muscle differentiation at the 10-somite stage as revealed by MyoD and F59 antibody labeling (Figs. 5D–F, J). Thus, the loss of adaxial cell fate in kny;tri double mutants is not due to an incompetence of kny;tri cells to respond to Hh signaling. We also tested whether the kny;tri double mutant cells required higher levels of Hh signaling to maintain the adaxial cell identity compared to WT. We injected different doses of shh RNA, ranging from 100 pg up to 200 pg, into the embryo (data not shown). In both WT and double mutants, upon injection of a minimum dose of 150 pg shh RNA, cells within the entire somite exhibited strong MyoD protein expression at the 10-somite stage and were strongly labeled by the F59 antibody at 24 hpf (Figs. 5G–I, G′–I′), suggesting that they were transformed into the slow muscle fate by the ectopic Hh signaling. This result indicates that the responsiveness of the double mutant prospective adaxial cells to Hh signaling is quantitatively similar to that of WT cells.

Our observations revealed a striking correlation between the decrease of Hh signaling range and the reduction of MyoD-expressing cells that occurred between the 5- and 10-somite stages in kny;tri double mutants. Hence we hypothesize that, in the WT situation, the prospective adaxial cells specified during gastrulation all converge towards the notochord to receive continuous Hh signal, allowing them to maintain their adaxial cell fate during segmentation. By contrast, in kny;tri double mutants, the laterally specified prospective adaxial cells do not converge. Therefore they reside outside of the Hh signaling range during segmentation, and consequently fail to adopt the slow muscle fate.

Cell autonomy of Kny and Tri during adaxial cell specification and C&E movements

During gastrulation and early segmentation, both kny and tri genes are expressed broadly in the zebrafish embryo, including the prospective adaxial cells (Jessen et al., 2002; Park and Moon, 2002; Topczewski et al., 2001). Their ubiquitous expression patterns and our results described above are consistent with two models for the role of these genes in adaxial cell fate specification. One possibility is that Kny and Tri act cell-autonomously during the specification and maintenance of adaxial cell fate. Alternatively, Kny and Tri function is required for the movements of prospective adaxial cells to ensure their position within the range of the inducing Hh signal. To distinguish between these two possibilities, we performed transplantation experiments (Fig. 6). Prospective somitic cells from the dorsolateral margin of the WT donor embryos and kny mutants injected with morpholino oligonucleotides (MO) targeting tri mRNA translation (Jessen et al., 2002), were co-transplanted deeply into the blastodermal margin of the WT host embryos at around 40% epiboly (4.5 hpf) (Materials and methods) (Yamashita et al., 2002). We examined the positions of the donor cells during early segmentation and found that the kny;tri-deficient donor cells were able to form adaxial cells in the WT hosts when located next to the notochord (Figs. 6A, B, the adaxial cells were recognized by their characteristic epithelial-like morphology, 4 embryos). Such position-dependent behaviors of kny;tri-deficient cells indicate that Kny and Tri activities are not required cell-autonomously for the adaxial cell fate when located in the range of inducing signals.

Interestingly, the somitic cells from the WT and kny;tri-deficient donor embryos, which were co-transplanted into the same position in the WT host embryos right before the onset of gastrulation, became segregated from each other at early segmentation (Figs. 6C, D, G). In all five WT host embryos examined, majority of the WT donor cells were localized in the medial half of the transplanted donor cell population, whereas most kny;tri-deficient donor cells were distributed in the lateral half (Figs. 6C, D, G), indicating that the WT donor cells converged more medially than the kny;tri-deficient cells and the convergence defect of kny;tri-deficient cells is cell-autonomous.
In contrast, when transplantation was performed using kny;tri-deficient hosts, cells from the WT and kny;tri-deficient donor embryos were intermingled with one another (Figs. 6E, F, G). We interpret this result to mean that when positioned in a kny;tri-deficient environment, the WT cells exhibited similar convergence defects as the kny;tri-deficient cells. Consequently, these two types of donor cells failed to segregate from each other. Taken together, Kny and Tri regulate the convergence of somitic cells both cell-autonomously and non-autonomously, consistent with our previous observations that Kny and Tri function in both cell-autonomous and non-autonomous fashions to control the ML cell elongation underlying the C&E during gastrulation (Jessen et al., 2002; Topczewski and LSK, unpublished). Moreover, these data provide direct evidence to support the notion that, in kny;tri double mutants, impaired convergence prevents the prospective adaxial cells from reaching the notochord-adjacent position, where they can receive the continuous midline Hh signal necessary to maintain their identity.

Discussion

Cell fate specification during metazoan development frequently involves inductive interactions between tissues. How such inductive interactions are coordinated with the massive tissue rearrangements during gastrulation is only beginning to be understood. Here we address the relationship between defective C&E movements and the deficiency of slow muscle precursors in kny;tri non-canonical Wnt mutants. Our study reveals that C&E movements mediate two distinct steps of adaxial cell development. First, C&E movements regulate the number of prospective adaxial cells specified during gastrulation. Second, medial convergence is essential for the maintenance of adaxial cell fate, as it ensures the juxta-notochordal
position of these cells during segmentation, when the Hh signaling range decreases. These results bring novel insights into the mechanisms underlying zebrafish slow muscle development and establish critical roles for C&E gastrulation movements in the regulation of subsequent cell fate specification events.

C&E movements regulate the size of the contact area between the inducing and responding tissues during adaxial cell specification

We uncovered a significant deficit in slow muscle fibers and their precursors in kny;tri C&E mutants. Adaxial cells divide...
once between the onset of gastrulation and the 3-somite stage, and then become post-mitotic (Hirsinger et al., 2004). At the 10-somite stage, \textit{kny;tri} double mutant somites contained only half the number of adaxial cells compared to WT (Table 1), while the difference in the proliferation rate of these cells was less than 3\% between WT and the double mutants (Fig. 2G). Therefore, we argue that although the double mutant cells undergo fewer cell divisions, such a defect is not sufficient to underlie the observed reduction of adaxial cell number.

Instead, our results indicate that fewer prospective adaxial cells are specified in \textit{kny;tri} double mutants during gastrulation (Fig. 7). Several studies demonstrated that the axial mesoderm is a source of signals that specify prospective adaxial cells in the adjacent PSM during gastrulation, and the inductive signals include, but are not limited to, Hh ligands (Blagden et al., 1997; Coutelle et al., 2001; Hirsinger et al., 2004). As a consequence of defective C&E movements, the axial mesoderm formed in \textit{kny;tri} double mutants is much shorter anteroposteriorly compared to WT at mid-gastrulation (Fig. 2) (Marlow et al., 1998), when the specification of prospective adaxial cells is thought to occur (Stickney et al., 2000). At this stage, a similar number of columns of prospective adaxial cells formed next to the axial mesoderm in both WT and \textit{kny;tri} mutants (Fig. 2). We interpret this to mean that the lateral range of the inducing signals and their reception by the PSM are normal in the mutants. We therefore attribute the specification of fewer prospective adaxial cells in the double mutants to the shortened AP interface between the axial and presomitic tissues (Fig. 7). These data demonstrate that C&E gastrulation movements play an instructive role in adaxial cell fate specification by determining the geometric proportions and dimensions of the interacting tissues.

**Medial convergence of prospective adaxial cells is crucial for their continuous reception of short-range Hh signal and fate maintenance**

Between the end of gastrulation and early segmentation, several columns of prospective adaxial cells converge medially to form a monolayer alongside the notochord (Fig. 7). Concurrently, Hh signaling range in the paraxial mesoderm decreases from 4- to 1-cell diameter (Figs. 5 and 7). In both WT and double mutants, only the notochord-adjacent prospective adaxial cells maintained the expression of adaxial cell markers and adopted the slow muscle fate, supporting the notion that continuous Hh reception is required for the commitment of adaxial cell fate during early segmentation (Coutelle et al., 2001; Hirsinger et al., 2004).

The change of Hh range is unlikely due to the downregulation of Hh expression in the notochord, as previous studies show that both \textit{shh} and \textit{echidna hedgehog/ehh} RNAs are strongly expressed in the notochord until late segmentation (Currie and Ingham, 1996; Krauss et al., 1993). However, it is intriguing that coincident with the decrease of Hh signaling range in the PSM, the prospective adaxial cells immediately adjacent to the notochord form a monolayer of pseudo-epithelium (Figs. 3A and 4A) (Devoto et al., 1996). Such a process is independent of Hh signaling (Hirsinger et al., 2004), and occurs normally in \textit{kny;tri} double mutants (Fig. 4C). Formation of the pseudo-epithelium might constrain the lateral

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**Fig. 7. A model for C&E movements mediating the specification and maintenance of adaxial cell fate.** During gastrulation, impaired C&E movements in \textit{kny;tri} double mutants shorten the AP interface between the axial and presomitic tissues, thus reducing the number of prospective adaxial cells specified in the AP dimension. During early segmentation, when the range of Hh signal decreases in the PSM, all the WT prospective adaxial cells have converged medially to reside alongside the notochord. By contrast, the lateral prospective adaxial cells in the double mutants fail to converge, stop receiving Hh signal and consequently lose their adaxial cell identity.
diffusion of Hh ligands, either by setting up a physical barrier, or by inducing the subcellular redistribution of Hh signaling components within these juxta-notochordal adaxial cells. A recent study demonstrated that the Hh interacting protein Hhip functions synergistically with the Hh receptor Ptc to limit the transduction of Hh to the adaxial cells next to the notochord (Ochi et al., 2006). It would be interesting to examine the subcellular localization of these and other Hh components in the prospective adaxial cells before and after the pseudo-epithelium is formed.

Recently, several PCP-related molecules have been shown to affect Hh signaling by mediating ciliogenesis (Park et al., 2006). Our data do not support the notion that Hh signaling is perturbed in kny;tri PCP mutants. Instead, several lines of evidence suggest that the reduction of Hh signaling range in the PSM and the C&E movements of this tissue are two independent processes. First, the formation of the monolayered pseudo-epithelium next to the notochord and the change of Hh signaling range occur normally in kny;tri double mutants (Figs. 4 and 5). Second, no C&E defects have been reported in the embryos deficient in Hh signaling (van Eeden et al., 1996). Finally, overexpression of shh RNA did not suppress the C&E defects in kny;tri double mutants (Marlow et al., 1998). However, we demonstrate that the change of Hh signaling range and the C&E movements of the prospective adaxial cell population must be coordinated to ensure the fate maintenance of adaxial cells. In kny;tri double mutants, the lateral prospective adaxial cells fail to converge towards the notochord, stop receiving Hh signal during early segmentation, and consequently transdifferentiate into the fast muscle precursors (Figs. 4 and 7). Our results reveal the significance of precisely coordinating morphogenetic and inductive events during adaxial cell fate specification.

Non-canonical Wnt/PCP pathway in adaxial cell specification and C&E movements of the paraxial mesoderm

In zebrafish, several components of non-canonical Wnt/PCP pathway are expressed in the paraxial mesoderm during gastrulation and segmentation, and a deficiency in any one of these molecules leads to C&E defects and consequently impairs somite morphology (Jessen et al., 2002; Kilian et al., 2003; Rauch et al., 1997; Topczewski et al., 2001). We provided several lines of evidence to show that PCP pathway is not absolutely essential for the specification of adaxial cell fate. First, the transplantation experiments showed that kny;tri double mutant cells, when positioned next to the notochord in the WT host embryos, were able to form adaxial cells. Second, mutants deficient in other PCP components still formed slow muscle cells. However, there is an interesting correlation between the degree of the C&E defects and the slow muscle defects: a gradation of slow muscle deficiency is observed from the mutants with the mildest C&E defect (sbi/wnt11 and ppt/wnt5 individual mutants) to those with the most severe C&E phenotype (kny;tri double mutants). These results suggest that instead of being a determinant of the adaxial cell fate, PCP pathway governs the establishment of tissue architecture to ensure the formation of the correct number of adaxial cells.

In vertebrates, PCP pathway mediates intercellular communication that is essential for the establishment of tissue polarity and effective C&E movements (Klein and Mlodzik, 2005; Myers et al., 2002b; Tada et al., 2002). Previous studies demonstrated that during gastrulation, Kny and Tri act both cell-autonomously and non-autonomously to regulate ML cell elongation, a behavior critical for directed cell migration and ML intercalation underlying C&E movements (Concha and Adams, 1998; Jessen et al., 2002; Myers et al., 2002a; Topczewski and LSK, unpublished). By performing time-lapse analyses (Fig. 3), we showed directly that the C&E of prospective adaxial cells was greatly compromised in kny;tri double mutants (Fig. 3). Our transplantation experiments further revealed the cell-autonomous and non-autonomous roles of Kny and Tri in the convergence of PSM (Fig. 6), thus confirming the direct link between impaired ML cell polarity and reduced convergence movements (Marlow et al., 2002; Topczewski et al., 2001; Wallingford et al., 2000). These results are consistent with the notion that impaired C&E movements prevent the prospective adaxial cells in kny;tri double mutants from reaching the juxta-notochordal position, where they can receive continuous Hh signal and maintain their identity.

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Appendix A. Supplementary data


References


