Cell cycle progression is required for zebrafish somite morphogenesis but not segmentation clock function

Lixia Zhang, Christina Kendrick, Dörthe Jülich and Scott A. Holley*

Cell division, differentiation and morphogenesis are coordinated during embryonic development, and frequently are in disarray in pathologies such as cancer. Here, we present a zebrafish mutant that ceases mitosis at the beginning of gastrulation, but that undergoes axis elongation and develops blood, muscle and a beating heart. We identify the mutation as being in early mitotic inhibitor 1 (emi1), a negative regulator of the Anaphase Promoting Complex, and use the mutant to examine the role of the cell cycle in somitogenesis. The mutant phenotype indicates that axis elongation during the segmentation period is driven substantially by cell migration. We find that the segmentation clock, which regulates somitogenesis, functions normally in the absence of cell cycle progression, and observe that mitosis is a modest source of noise for the clock. Somite morphogenesis involves the epithelialization of the somite border cells around a core of mesenchyme. As in wild-type embryos, somite boundary cells are polarized along a Fibronectin matrix in emi1+/−. The mutants also display evidence of segment polarity. However, in the absence of a normal cell cycle, somites appear to hyper-epithelialize, as the internal mesenchymal cells exit the core of the somite after initial boundary formation. Thus, cell cycle progression is not required during the segmentation period for segmentation clock function but is necessary for the normal segmental arrangement of epithelial borders and internal mesenchymal cells.

KEY WORDS: Somitogenesis, Cell cycle, Zebrafish, emi1, Somite morphogenesis

INTRODUCTION

Somites are the segmented precursors to the axial skeleton and musculature created as the trunk and tail elongate. The periodic formation of somites is governed by the segmentation clock, which creates oscillations in gene expression in the presomitic mesoderm (PSM) (Pourquié, 2003). In zebrafish, the segmentation clock requires Notch signaling, while the amniote clocks also incorporate Wnt and Fgf signaling (Holley, 2007). It is debated whether the Notch, Wnt or Fgf pathways constitute core components of the clock, or whether they are a readout of a global clock that governs all of embryonic development (Aulehla et al., 2003; Dequeant et al., 2006; Niwa et al., 2007; Wahl et al., 2007). For instance, some models link the segmentation clock to the cell cycle oscillator (Collier et al., 2000; Mc Nerney et al., 2004; Primmett et al., 1989; Primmett et al., 1988).

Somite morphogenesis occurs as the segment boundary cells undergo a mesenchymal to epithelial transition (MET), forming a ball of cells with an epithelial surface and a core of mesenchyme (Holley, 2007). Zebrafish somite morphogenesis requires the transcription factor fused somites (fss; also known as tbx24), Eph/Ephrin signaling and integrin α5/fibronectin function (Barrios et al., 2003; Durbin et al., 1998; Durbin et al., 2000; Jülich et al., 2005a; Koshida et al., 2005a; Kragtorp and Miller, 2007; Yang et al., 1993). Double mutants between integrin α5 and the Notch pathway lead to a complete loss of MET in the paraxial mesoderm (Jülich et al., 2005a). Simultaneous loss of ephrin B2a, a ligand for ephA4, and integrin α5 leads to a synergistic defect in somite boundary morphogenesis (Koshida et al., 2005). Ema/Vasp and Fak, which function in Integrin signaling, are necessary for somite formation in Xenopus (Kragtorp and Miller, 2006). Chick somite morphogenesis is regulated by Snail2 and Cdc42, which promote mesenchymal cell morphology, and Rac1, which fosters epithelial cell morphology (Dale et al., 2006; Nakaya et al., 2004).

Emi1 is a negative regulator of the Anaphase Promoting Complex (APC) and is required for entry into mitosis in Xenopus embryos (Reimann et al., 2001). APC, an E3 ubiquitin ligase, also functions in post-mitotic cells. In Drosophila and C. elegans neurons, APC localizes to the synapse and regulates the turnover of glutamate receptors (Juo and Kaplan, 2004; van Roesel et al., 2004). In vertebrate neurons, inhibition of APC by RNA interference or overexpression of Emi1 increases axonal growth and overcomes much of the growth-inhibitory effects of myelin. In contrast to the synapse studies, virtually all of the APC is located in the nuclei of these neurons, and the axon growth phenotype appears to be due to stabilization of Id2 and SnoN (Lasorella et al., 2006; Stegmuller et al., 2006).

Here, we identify a zebrafish mutant for emi1 that ceases mitosis at the beginning of gastrulation. Using this mutant, we find that normal cell cycle progression is not required for segmentation clock function, but rather that mitosis is a modest source of noise for the
Development 135 (12) 2066 RESEARCH REPORT

Fig. 1. tiy121 mutants have a cell cycle defect. (A-D) Phosphorylated Histone H3 staining (PHH3, red) marks mitotic cells in wild-type embryos at the shield (A) and 10-somite stage (C). No mitotic nuclei are seen in tiy121 embryos at the shield (B) or 10-somite stage (D). Nuclei are stained with DAPI (blue). (E,F) Wild-type (E) and tiy121+/– (F) embryos at 30 hpf. (G,H) BrdU labeling (red) in the trunks of wild type (G) and tiy121+/– (H) at the 14-somite stage. Asterisks label the notochord. BrdU was injected into the yolk at the 8-somite stage. In C,F, anterior is left; in G and H, anterior is up.
Note that, at the 8- and 15-somite stage, the tailbud of for the clock (Horikawa et al., 2006). To test this hypothesis, we clock, it has been postulated that mitosis is actually a source of noise segmentation clock is not dependent upon the cell cycle. (Riedel-Kruse et al., 2007), our data indicate that continued oscillation of the domain of mesogenin is an localization signal. (Fig. 2C). YFP-emi1 mRNA (5 ng/μl) was injected into clusters from tiy121+/– parents. Normal PHH3 was seen in 99% (n=75) and 98% (n=141) of embryos at the shield and tailbud stage, respectively. By the 5-somite stage, 16% (n=110) showed reduced PHH3, and at the 18-somite stage, 27% (n=153) showed no PHH3. tiy121 fails to complement hi2648, a hypomorphic, retroviral allele of emi1 (Fig. 2D) (Amsterdam et al., 2004). Together, these data indicate that the tiy121 phenotype is due to perturbation of emi1. emi1 mRNA is maternally deposited (Fig. 2E), and later ubiquitously expressed in the blastula and gastrula stages (Fig. 2F). Emi1-YFP protein localizes to the nucleus but is diminished in cells undergoing mitosis (Fig. 2H-J). During segmentation, emi1 is broadly expressed, including within the somites (Fig. 2G).

Although tiy121 embryos are short, the mutant phenotype indicates that cell proliferation is not absolutely required for trunk and tail extension. However, the mutants display irregularly sized and partially fused somites and myotomes (Fig. 3A–D; see also Fig. S1 in the supplementary material). The segmentation clock creates oscillations in transcription that manifest as stripes of expression sweeping through the cells of the PSM in a wave-like fashion. We examined the expression of three oscillating genes, her1, her7 and deltaC, at the 3-, 8- and 15-somite stage and found no appreciable defect in their expression in emi1+ (Fig. 3E–J; data not shown). Note that, at the 8- and 15-somite stage, the tailbud of emi1+ embryos is smaller than normal (compare Fig. 3F and G to 3I and J, respectively). This decrease is reflected in the reduction of the domain of mesogenin expression (see Fig. S1 in the supplementary material). Although emi1 mutants undergo mitosis at the beginning of gastrulation when oscillations are first seen (Riedel-Kruse et al., 2007), our data indicate that continued oscillation of the segmentation clock is not dependent upon the cell cycle.

In contrast to models that link the cell cycle to the segmentation clock, it has been postulated that mitosis is actually a source of noise for the clock (Horikawa et al., 2006). To test this hypothesis, we examined the effect of inhibiting mitosis in embryos lacking either of the Notch ligands deltaC or deltaD. The deltaC and deltaD mutants form the first 3-5 and 7-9 somites, respectively, as the oscillating pattern of gene expression gradually breaks down, leading to the segmentation defect (Fig. 3K–N; see also Fig. S1 in the supplementary material) (Holley et al., 2000; Jiang et al., 2000; Jülich et al., 2005b; Oates et al., 2005a; van Eeden et al., 1996; van Eeden et al., 1998). This breakdown may be accelerated because of noise. Thus, if mitosis is a source of noise in the segmentation program, one would predict that the breakdown would decelerate in the absence of cell division. We assayed the expression of her1 mRNA in deltaD or deltaC morpholino-injected embryos that were either wild type or mutant for emi1 (Fig. 3N, Fig. S1 in the supplementary material). The difference between the mutants and siblings was not immediately apparent. However, upon careful categorization of the expression patterns, we found a subtle improvement in the integrity of the her1 stripes in embryos lacking emi1 compared with sibling embryos. For each trial, the more organized stripe patterns are biased towards the injected emi1 mutants, and the two more disorganized expression categories are biased towards the injected siblings. In summary, these results are consistent with mitosis being a modest source of noise in the segmentation clock.

Further examination of the segmentation defect in emi1+ embryos revealed profound abnormalities in somite morphology. Although emi1+ somites initially contain internal mesenchymal cells, these cells leave the core of the somite and at least some integrate into the epithelial somite boundary (Fig. 4A,B,D,E). We have observed other cells migrating to the lateral surface of the paraxial mesoderm. The somite boundary cells then appear to elongate and meet in the middle of each segment, creating somites solely consisting of two rows of boundary cells (Fig. 4B,E,G,I). These hyper-epithelialized somites, having no internal mesenchyme and abnormally elongated epithelial border cells, often fuse to create irregularly sized segments. The nuclei
of the boundary cells show a basal localization, as does Integrin α5-GFP clustering (Fig. 4A,B). Fibronectin matrix is also assembled along the somite boundaries (Fig. 4F,G). This maintenance of border cell polarity distinguishes the emi1–/– phenotype from that of integrin α5 and fibronectin1a mutants (Jülich et al., 2005a; Koshida et al., 2005). Ephrin B2 is localized to the cortex of the somite cells, with slightly higher levels in the posterior somite cells, and this pattern appears largely intact in emi1–/– (Fig. 4H,I; see also Fig. S1 in the supplementary material). Expression of mespb, ripply1 and tbx18, myod and deltaC is clearly segmental, although there is some aberrant expression of deltaC in the mutant embryos (Fig. 4J-M; Fig. S1 in the supplementary material). The segment polarity alterations observed in emi1 mutants are slight in comparison to those defects seen in fss and the Notch pathway mutants, and seem unlikely to be the cause of the morphological phenotype.

The morphological analysis suggests that the polarity of the somite boundary cells is maintained. In addition, the somite phenotype does not seem to follow from a defect in anteroposterior patterning of the somites. Rather, it appears that the somites in emi1–/– mutant embryos are hyper-epithelialized. The hyper-epithelialization could be due to elevated APC activity, which may affect the stability of proteins involved in regulating cell morphology independently of the cell cycle (Juo and Kaplan, 2004; Konishi et al., 2004; Lasorella et al., 2006; Stegmuller et al., 2006; van Roessel et al., 2004). To test this hypothesis, we blocked mitosis using a combination of hydroxyurea and aphidicolin (Harris and Hartenstein, 1991; Lyons et al., 2005). Addition of the compounds at the germ ring/early shield stage blocked all mitosis by the late shield stage and resulted in embryos lacking internal mesenchymal cells in their somites, strongly phenocopying emi1−/− (Fig. 4C; see also Fig. S1 in the supplementary material). These data suggest that the segmentation defect in emi1−/− mutant embryos is primarily due to the lack of normal cell cycle progression and not to a cell cycle-independent function of emi1 or APC. Note that in both mutant and drug-treated embryos, the cells and nuclei are larger than in wild type (Fig. 4). The increase in cell size, along with the decrease in cell number, might also be causally linked to the somite morphogenesis defect.

The mitotic defect in emi1−/− embryos arises after the midblastula transition (MBT). MBT initiates during the tenth cell cycle [3 hours post-fertilization (hpf)], when divisions become asynchronous and zygotic transcription commences (Kane, 1999; Kane and Kimmel, 1993). During cycles 11 and 12, the blastula forms three domains, the extra-embryonic yolk syncytial layer and enveloping layer, and the deep cells that give rise to the embryo proper (Kane, 1999). At 5.5 hpf, gastrulation starts, as most of the deep cells are in cell cycle 14 (Kane, 1999; Kane et al., 1992). emi1−/− embryos cease cell division around this time. In wild-type embryos, the cell cycle lengthens during this period, with the thirteenth, fourteenth, fifteenth

![Image](https://example.com/image.png)

Fig. 3. Cell cycle progression is necessary for somitogenesis but not segmentation clock function. (A,B) Dorsal views of anterior trunk somites in wild-type (A) and emi1–/– (B) embryos at the 15-somite stage. (C,D) Posterior trunk myotomes of wild-type (C) and emi1–/– (D) embryos at 36 hpf, lateral views. Slow muscle fibers are labeled with S58 antibodies (green). Scale bars: 30 μm. (E-J) her1 expression at the 3-, 8- and 15-somite stages in (E-G) wild-type and (H-J) emi1–/– embryos. (K-N) her1 stripe integrity was examined in emi1–/– and sibling embryos injected with morpholinos against either deltaC or deltaD. her1 expression was rated according to four categories representing increasing levels of disorganization: wild type; (K) stripes with some ‘salt and pepper’ expression; (L) stripes begin to merge; and (M) no stripes. (N) Distributions of gene expression patterns are displayed for three independent trials (x-axis). Within each gene expression category, the fraction of sibling embryos is subtracted from the fraction of mutant embryos. For example, the wild-type category in the first deltaC morpholino trial included 0.20 fraction of the mutant embryos (20%) and 0.02 fraction of the sibling embryos (2%), giving a graphed value of 0.18. In deltaC morpholino trials, the number of mutants and siblings assayed (mutants/siblings) were: 39/74, 27/79 and 16/57. For deltaD morpholino trials, the corresponding numbers were: 60/50, 28/74 and 49/90. Given the subjective nature of the expression classification, a second assayer performed an independent blind classification of the same embryos (see Fig. S1 in the supplementary material).
and sixteenth cycles averaging 54, 78, 151 and 240 minutes, respectively. During segmentation, most cells are in either cell cycle 16 or 17 (Kane, 1999). The mild elongation defect in emi1–/– is likely to be due to the fact that mitosis is normally not a great contributor to axial growth during the segmentation period. This conclusion was also reached by examining the elongation of clonal strings of cells in the CNS: the exponential lengthening of the string suggested that it was largely due to cell intercalation and not cell division (Kimmel et al., 1994). The relatively normal differentiation in emi1–/– embryos can be explained by the fact that many cells undergo a terminal differentiation during cell cycle 15, 8-10 hpf, and a major wave of differentiation occurs during cycle 16 (Kane, 1999; Kimmel et al., 1994; Kimmel and Warga, 1987). Thus, for many cell lineages, the mitotic defect in emi1 embryos does not reduce dramatically the number of cell cycles that these cells would normally undergo.

The cell cycle could in principle serve as a clock to regulate developmental timing (Johnson and Day, 2000). Experiments in ascidians have suggested that the timing of myogenesis may depend upon the number of cycles of DNA synthesis that a myogenic progenitor experiences (Satoh, 1987). However, previous cell labeling experiments indicate that zebrafish myofiber differentiation is not regulated by such a cell cycle-counting mechanism (Kimmel and Warga, 1987). Similarly, the expression of differentiation markers in the C. elegans gut occurs independently of cell cycle counting (Edgar and McGhee, 1988). The segmentation clock has been suggested to be linked to the cell cycle oscillator. Reiterated segmentation defects are seen in chick embryos after treatment with cell-cycle inhibitors, and the periodicity of this defect is equal to the cell cycle length at that stage of development (Primmett et al., 1989). Similar periodic defects were seen after a single heat shock (Primmett et al., 1988). More recently, this cell cycle model has been formalized mathematically (Collier et al., 2000; McNerney et al., 2004). In the zebrafish, a single heat shock can produce reiterated segmentation defects, but the periodicity of the defect does not correlate with the length of the cell cycle during segmentation (Roy et al., 1999). Additionally, there is no organized pattern of cell proliferation in the zebrafish tailbud (Kanki and Ho, 1997). Our data are consistent with the hypothesis that mitosis is a modest source of noise for the segmentation clock (Horikawa et al., 2006).

We thank Hiroyuki Takeda and members of the lab for comments on the manuscript. The ty121 mutant was isolated in the Tübingen 2000 Screen, and we acknowledge all participants of the consortium: F. Van Bebber, E. Busch-Nentwich, R. Dahm, O. Frank, H.-G. Fröhöfer, H. Geiger, D. Gilmour, S. Holley, J. Hooge, D. Jülich, H. Knaut, P. Kuhlman, H.-M. Maischein, C. Nüsslein-Volhard, H. Roehl, S. Schier, M. Soczewinski, A. Wehner, P. Erker, H. Habech, U. Hagner, C. E. Hennen Kaps, A. Kirchner, T. Kobizilek, U. Langheinrich, C. Metzger, R. Nordin, J. Odenthal, M. Pezzuti, K. Schollm, J. de Santana-Stamm, T. Trowe, G. Vacun, B. Walderich, A. Walker and C. Weiler. We thank Nancy Hopkins for the es5-GFP allele. We thank Joseph Wolenski for assistance with the confocal microscopy. This work was supported by the March of Dimes Foundation, research grant number 1-FY07-424, and the NICHD, R01 HD045738.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/12/2065/DC1

References


