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

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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; McInerney 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 bbx24). 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). Ena/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 Roessel 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.
clock. Finally, we show that the cell cycle defect leads to hyper-epithelialization of the somites after the initiation of morphological segmentation.

MATERIALS AND METHODS
Zebrafish breeding, mapping and cloning
Breeding and meiotic mapping followed standard protocols (Geisler, 2002; Nüsslein-Volhard and Dahm, 2002). The coding sequence of emi1 (GenBank NM_001003869) was isolated via RT-PCR and cloned into pCS2+. This clone was used to generate sense mRNA using the Ambion SP6 Machine kit, and an antisense riboprobe using the Roche digoxigenin-labeling mix. For allele sequencing, we used an emi1 template from two independently derived tiy121 RT-PCRs. Wild-type embryos were injected with 0.5 mM emi1 morpholino targeting the splice donor of the second intron (5′-TGATTGTACCTCACATCATCT-3′).

Immunohistochemistry, in situ hybridization
Fibronectin, phalloidin, SS8 staining (Jülich et al., 2005a), and fluorescent in situ hybridization with β-catenin immunohistochemistry (Jülich et al., 2005b), were performed as previously described. All in situ hybridizations were performed with digoxigenin-labeled riboprobes. her1 and deltaC antisense probes were made from plasmid clones, as previously described (Holley et al., 2000; Holley et al., 2002). The tih18, mesogenin, mesph and ripply1 coding sequences were isolated via RT-PCR and subjected to an additional round of PCR in which a T7 promoter was added in the antisense orientation. Antisense riboprobes were then created using T7 RNA polymerase (NEB). Integrin α5-GFP (Jülich et al., 2005a) and YFP-Emi1 were visualized with rabbit anti-GFP (1:1000, Invitrogen) and anti-rabbit Alexa 647 (1:200, Invitrogen). Goat anti-EphrinB2 (1:500, R&D Systems) was paired with anti-goat Alexa 647 (1:200, Invitrogen). Rabbit anti-Phospho-Histone H3 (PHH3) antibody (1:1000, Sigma) was used with goat anti-rabbit-HRP (1:400, Invitrogen) and Fluorescein TSA (Perkin Elmer).

We analyzed her1 expression in emi1 mutant and sibling embryos injected with translation-blocking morpholinos against either deltaC or deltaD (Holley et al., 2002). Three independent trials were performed with embryos derived from different parents and injected on different days. deltaC morpholino-injected and deltaD morpholino-injected embryos were fixed in 4% paraformaldehyde (PFA) at the ~2-somite and ~5-somite stages, respectively. Embryos were co-stained for her1 expression with NBT/BCIP and for PHH3 by immunofluorescence. Absence of PHH3 staining was used to sort emi1−/− from sibling embryos.

Drug treatment and BrdU labeling
Embryos were incubated in 150 μM aphidicolin and 20 mM hydroxyurea (Sigma) in 4% DMSO, from the germ ring/early shield stage until fixation (Harris and Hartenstein, 1991; Lyons et al., 2005). Drug treatment at this stage blocked mitosis by the late shield stage, mimicking the onset of the emi1+/− phenotype. To assay for DNA synthesis, 10 mM BrdU was injected into the yolk just after the shield stage, at the 1-somite stage or at the 8-somite stage. Embryos were fixed in 4% PFA at the 14- to 15-somite stage. BrdU incorporation was visualized using a mouse anti-BrdU antibody (1:200, Sigma) and an Alexa 647-labeled goat anti-mouse antibody (1:200, Invitrogen). Embryos injected at each stage showed BrdU incorporation, indicating that endoreplication occurs continuously during late gastrulation and trunk segmentation in emi1 mutants.

RESULTS AND DISCUSSION
We identified a zebrafish mutant, tiy121, which exhibits a mitotic block (Fig. 1A-D). By the shield stage, mutant embryos cease all mitosis, as visualized by immunostaining for phosphorylated Histone H3. Despite the mitotic arrest, mutant embryos undergo gastrulation and axis elongation (Fig. 1E,F). Measurement of the distance from the otic vesicle to the tip of the tail indicates that tiy121 embryos (n=15) are on average 22% (s.d. ±3.2%) shorter than their wild-type siblings (n=17). After the mitotic block, mutant embryos continue endoreplication, as indicated by BrdU labeling (Fig. 1G,H). tiy121 embryos ultimately develop a pericardial edema and extensive necrosis in the head, and die 2-3 days post-fertilization. The relatively normal progression of early development in tiy121 embryos parallels the finding that early Xenopus development is unperturbed by the chemical inhibition of mitosis (Cook, 1973; Harris and Hartenstein, 1991; Rollins and Andrews, 1991).

We mapped tiy121, via meiotic recombination, to chromosome 13 between the simple sequence length polymorphisms (SSLP) z24268 and z55656, near a zebrafish homolog of early mitotic inhibitor 1 (emi1) (Fig. 2A). Determination of the emi1 coding sequence in the single mutant allele revealed a premature stop codon that truncates the protein prior to the F-box domain, which is likely to create an amorphic allele (Fig. 2B). Injection of a splice-blocking morpholino against emi1 recapitulates the mitotic defect through gastrulation. However, the antisense inhibition declines by the tailbud stages, and mitosis is normal by the 5-somite stage (Fig. 2C). In morpholino-injected embryos, phosphorylated Histone H3 staining (PHH3) was absent (89%) or reduced (9%) at the shield stage (n=101), was reduced in 79% of embryos at the tailbud stage (n=39), and was indistinguishable from controls at the 5-somite stage (n=37). Injection of in vitro synthesized mRNA for YFP-emi1 rescues the mitotic defect through gastrulation, but the rescue declines by the tailbud stage and is absent by the 18-somite stage.

![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 the clock (Horikawa et al., 2006). To test this hypothesis, we postulated that mitosis is actually a source of noise. Thus, if mitosis is a source of noise in 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 deaccelerate 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 somite 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

Fig. 2. tiy121 is an emi1 mutant. (A) The chromosomal location of tiy121/emi1. (B) tiy121 is a premature stop codon in emi1. NLS, nuclear localization signal. (C) Graph of the levels of mitosis in tiy121, emi1 morphants, and tiy121 embryos rescued by injection of emi1 mRNA. The level of mitosis, graphed along an arbitrary scale, was wild-type (wt) or reduced to some degree, or mitosis was absent. Morphants and mRNA-injected embryos develop normally prior to the shield stage, thus we infer that the initial level of mitosis is normal without examining PHH3 (dashed lines). hpf, hours post fertilization. (D) tiy121 and hi2648, an insertional allele of emi1, do not complement. (E-G) Expression of emi1 mRNA (E) at the one-cell stage, (F) at the sphere stage, and (G) in the most recently formed somites (arrowheads) and anterior PSM of a 12-somite stage embryo. Anterior is left in G. (H) YFP-Emi1 immunofluorescence. (I) DAPI stained nuclei. (J) Overlay of H and I. Arrows indicate a cell completing mitosis. Scale bar in H: 20 μm for H-J.
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 fibronectin1α mutants (Julich 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 Roessels 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). After 60 hpf, gastrulation starts, as most of the deep cells are in cell cycle 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
and sixteenth cycles averaging 54, 78, 151 and 240 minutes, respectively. During segmentation, most cells are in either cell cycle 16 or 17 (Kanı, 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 (Kanı, 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 analysis of the emi1 mutant indicates that cell cycle progression is not required for zebrafish segmentation clock function. Conversely, our data are consistent with the hypothesis that mitosis is a modest source of noise for the segmentation clock (Horikawa et al., 2006).

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Supplementary material
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References

Fig. 4. Cell cycle inhibition leads to somite hyperepithelialization. (A-C) Integrin α5-GFP (red) labels the cell cortex and clusters along somite borders in (A) wild-type (wt), (B) emi1−/− and (C) aphidicolin-hydroxyurea-treated embryos. Embryos were at the 12-somite stage. Somites 2-4, 3-5 and 3-5 are shown, respectively. (D-E) 12-somite-stage emi1−/− embryo labeled with membrane-localized cherry (red) to visualize the cell cortex. The same somites at the beginning (SI and SII) and end (SIV and SV, respectively) of a timelapse are shown. The somite borders are indicated by dashed lines. Somites initially have internal mesenchymal cells, but after three somite cycles have passed, the internal mesenchymal cells (asterisk in D) have moved to the surface of the somite (asterisk in E). (F,G) Fibronectin (Fn) matrix (red) forms along the borders in (F) wild type and (G) emi1−/−. Embryos were at the 12-somite stage. Somites 6-7 and 3-5 are pictured. (H,I) Ephrin B2 expression (green) shows a graded, segmental distribution in (H) wild type and (I) emi1−/−. The lateral membranes of the posterior (p) somite border cells show higher levels of Ephrin B2 than do the lateral membranes of anterior (a) border cells. Embryos were at the 12-somite stage. Shown are somites 5-6 and 4-5. (J,K) Segmental expression of myod (blue) in (J) wild type, somites 3-8, and (K) emi1−/−, somites 4-9, in 10-somite-stage embryos. Nuclei are labeled with DAPI (blue) in A-C,F-I. In D and E, nuclei are labeled with nuclear-GFP (green). β-catenin labels the cell cortex in J,K (yellow) and L,M (red). In all panels, anterior is up.


