The winged helix transcription factor Foxc1a is essential for somitogenesis in zebrafish

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Previous studies identified zebrafish foxc1a and foxc1b as homologs of the mouse forkhead gene, Foxc1. Both genes are transcribed in the unsegmented presomitic mesoderm (PSM), newly formed somites, adaxial cells, and head mesoderm. Here, we show that inhibiting synthesis of Foxc1a (but not Foxc1b) protein with two different morpholino antisense oligonucleotides blocks formation of morphological somites, segment boundaries, and segmented expression of genes normally transcribed in anterior and posterior somites and expression of paraxis implicated in somite epithelialization. Patterning of the anterior PSM is also affected, as judged by the absence of mesp-b, ephrinB2, and ephA4 expression, and the down-regulation of notch5 and notch6. In contrast, the expression of other genes, including mesp-a and papc, in the anterior of somite primordia, and the oscillating expression of deltaC and deltaD in the PSM appear normal. Nevertheless, this expression is apparently insufficient for the maturation of the presumptive somites to proceed to the stage when boundary formation occurs or for the maintenance of anterior/posterior patterning. Mouse embryos that are compound null mutants for Foxc1 and the closely related Foxc2 have no morphological somites and show abnormal expression of Notch signaling pathway genes in the anterior PSM. Therefore, zebrafish foxc1a plays an essential and conserved role in somite formation, regulating both the expression of paraxis and the A/P patterning of somite primordia.

[Key Words: Forkhead; somite formation; morpholino antisense oligonucleotide; Danio rerio]

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the Notch pathway [Jiang et al. 2000]. Opposing the segmentation clock is a hypothetical wave front activity that proceeds posteriorly, slowing and then halting the oscillation cycles and inducing somite maturation. When anterior PSM cells receive this putative wave front signal, they give rise to bands or cohorts of cells one somite wide with the same gene expression profiles. Signaling within and between these stabilized cohorts of cells subsequently refines the anterior and posterior domains of the presumptive somite. Formation of these stripes is manifested in domain-specific expression of genes such as mouse Mesp1 and Mesp2 (Takahashi et al. 2000), zebrafish mesp-a and mesp-b [Durbin et al. 2000; Sawada et al. 2000a], and mouse Delta-like1 (Dll1) [Bettenhausen et al. 1995] or ephrinB2 [Holder and Klein 1999; Holder et al. 2000]. The helix-loop-helix transcription factor Mesp appears to be important for the anterior–posterior regionalization of somite primordia, and this process is thought to be essential for boundary formation [Durbin et al. 2000; Sawada et al. 2000b; Takahashi et al. 2000]. Formation of the boundary between the posterior of the forming somite and the anterior of the next-to-be-formed presumptive somite involves the correct expression in the mesoderm of a number of evolutionarily conserved genes, as well as signals, not yet identified, from the ectoderm [Sosic et al. 1997; Correia and Conlon 2000]. Among the mesodermally expressed genes are members of the ephrin and Eph receptor gene families, encoding components of the Eph cell communication pathway. A role for EphA4 and ephrinB2 in somite boundary formation has been demonstrated in zebrafish. Both genes are expressed in alternating anterior and posterior domains in somite primordia, and overexpression of dominant negative constructs of Eph receptors and ephrins results in strong defects in boundary formation [Durbin et al. 1998, 2000]. Inactivation of function of the basic-helix-loop-helix [bHLH] transcription factor paraxis in the mouse embryo impairs epithelialization and formation and maintenance of morphological boundaries, but does not affect the initial specification and patterning of the somite primordia within the anterior PSM [Johnson et al. 2000]. Therefore, several genetic pathways appear to act in parallel within the PSM to orchestrate the formation of morphologically distinct somites.

Roles in somitogenesis have been postulated for several bHLH genes, both those in the Notch signaling pathway such as mesp, and hes/her1 (Tkakke and Campos-Ortega 1999; Takahashi et al. 2000) and Notch-independent genes such as paraxis [Johnson et al. 2000]. However, little is known about the function of other classes of transcription factors. We have shown previously that genes encoding two evolutionarily conserved forkhead/winged helix transcription factors, foxc1a and foxc1b, are expressed in the PSM and somites. Based on this evidence, we proposed that these closely related genes play a role in somite formation and differentiation [Topczewska et al. 2001]. In the present study, we tested this proposition by taking advantage of a recently established and effective targeted gene morpholino “knockdown” technology [Nasevicius and Ekker 2000]. The data we obtained provide the first evidence of an essential role for the forkhead gene foxc1a in zebrafish somitogenesis. Significantly, our data indicate that Foxc1a is essential for the formation of the most anterior somites, whereas the requirement for most other genes becomes manifested later, for posterior somites. Analysis of gene expression in the anterior PSM of foxc1a-morpholino-injected embryos suggests that Foxc1a protein is a key component of the complex molecular circuitry that is centered on the Notch pathway, and initially establishes and then stabilizes the anterior/posterior [A/P] domains of the presumptive somites. In the absence of Foxc1a protein, A/P patterning of somite primordia is not completed, boundaries and epithelial somites are not formed, and presumptive somites fail to complete their maturation.

Results

**Antisense morpholino-modified oligonucleotides specific for foxc1a, but not foxc1b, inhibit formation of somites**

To test the hypothesis that foxc1a and foxc1b are required for somitogenesis, we blocked translation of the respective mRNAs using antisense morpholino-modified oligonucleotides [MOs]. Recent reports have shown that MOs injected into early embryos inhibit the translation of specific mRNAs even many hours later, mimicking a null mutant phenotype [Nasevicius and Ekker 2000]. For each foxc1 gene we designed two different MOs, one against 5′ untranslated sequences [MO-1], the other against sequences overlapping the translation start site [MO-2]. The same phenotypes were observed when these MOs are injected either singly or in combination, confirming the specificity of our results.

Coinjection of MO-1 and MO-2 against foxc1a at a total concentration of ~6 ng/embryo inhibited the formation of morphologically distinct anterior somites without significantly affecting either the rate of development of the embryos or their overall size [Fig. 1A–D]. Confocal microscopy after whole-mount staining with β-catenin antibody also showed no evidence of segmental organization of the paraxial mesoderm cells [Fig. 1H,I]. Both the severity and penetrance of the observed phenotype depended on the dose of MOs. As shown in Table 1, ~80% of the embryos injected with ~6 ng of foxc1a-MOs lacked anterior somites. The inhibition was usually seen up to the stage when control embryos have developed 6–7 somites, after which there was a gradual recovery of somitogenesis with morphologically distinguishable somites forming at the anterior of the PSM. Embryos injected with higher doses of foxc1a-MO (~10 ng) displayed a more extensive loss of somites [up to nine], but they also showed a general delay in development. Lower doses caused only partial loss of somites and a more rapid recovery of somitogenesis.

Different combinations of two MOs against foxc1b did
not inhibit somite formation at any concentrations tested (2–10 ng/embryo, Table 2) but did affect the development of the head mesoderm, including the presumptive branchial arch region where the gene is strongly expressed (Topczewska et al. 2001).

**Foxc1a-MOs specifically inhibit synthesis of Foxc1a protein**

To determine the effectiveness of MOs in blocking the translation of foxc1a, we used an affinity-purified, polyclonal rabbit antibody raised against a mouse Foxc1 peptide. The sequence of the peptide used for immunization differs by two and three amino acids from the corresponding predicted sequences of zebrafish Foxc1a and Foxc1b, respectively (see Materials and Methods). Western blot analysis showed that the antibody recognizes a protein made in COS cells transfected with a mouse Foxc1 expression vector (Fig. 1L, column g) or transfected with zebrafish foxc1a [Fig. 1L, column a] but not foxc1b expression vector [data not shown]. The antibody also reacts with a protein in extracts of wild-type zebrafish embryos (at the 5-somite stage; Fig. 1L, column c) and embryos injected with zebrafish foxc1a synthetic RNA [data not shown], further confirming antibody specificity. The difference in electrophoretic mobility...
between the zebrafish and mouse proteins may be due to differences in their overall predicted amino acid sequences (476 residues vs. 553, respectively). Moreover, the zebrafish protein lacks long stretches of alanine, glycine, and serine residues that may decrease the mobility of the mouse protein (Topczewska et al. 2001).

To study subcellular localization of Foxc1a protein in the intact embryo, we used immunohistochemistry and confocal microscopy. As shown in Figure 1G and H, during the segmentation period Foxc1a protein is localized in the nuclei of cells in the PSM, but not in the tail bud. Significantly, protein expression in the PSM appears uniform, even in the anterior region where foxc1a transcript levels are higher in two distinct stripes [Fig. 2A]. These stripes overlap with the transcription domains of mesp-b gene in the most anterior cells of S-1 and S-2 (where S0 is the forming somite), but are broader and extend more posteriorly [Fig. 2B]. Foxc1a-positive nuclei were also observed in adaxial cells, which are organized into a single epithelial-like layer on either side of the notochord and give rise to slow muscles [Fig. 1H] [Devoto et al. 1996]. The newly formed epithelial somites, including dorsal and ventral layers, intersomitic border cells, and the few internal mesenchymal cells also accumulate Foxc1a in the nuclei. When young somites start to differentiate, immunoreactivity declines in most of the cells but remains high in migrating adaxial cells and presumptive sclerotome cells [Fig. 1G, data not shown].

Immunohistochemistry showed the complete absence of Foxc1 protein in MO-injected embryos at the time when un.injected embryos are at the 5-somite stage [Fig. 1I]. In contrast, adaxial cells of injected embryos stained strongly with antibody against Myf5, indicating that the MOs do not inhibit expression of an unrelated protein [Fig. 1J,K]. Interestingly, in MO-injected embryos there appears to be somewhat more Myf5 protein-positive cells than in the wild-type, and they extend more than one cell diameter from the notochord [Fig. 1J,K], but this effect has not been quantitated or explored further.

The absence of Foxc1a protein at the 5-somite stage in MO-injected embryos was also confirmed by Western blot analysis [Fig. 1L, column b]. However, protein was detected in extracts of MO-injected embryos collected at the stage when wild-type embryos have reached the 12-somite stage [Fig. 1L, columns d and e]. Taken together, these results indicate that foxc1a-MOs antisense oligo-

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### Table 1. The effect of foxc1a-specific MOs on the development of wild-type embryos

| Observed phenotypes                  | Total amount of foxc1a-MOs [1 + 2] injected |
|--------------------------------------|---------------------------------------------|
|                                      | 4 ng | 6 ng | 10 ng |
| Partial loss of somites             | 78%  | 12%  | 2%   |
| No somites                          | 9%   | 77%  | 24%  |
| No somites, retarded development    | 0%   | 2%   | 59%  |
| Not affected                        | 13%  | 9%   | 15%  |
| Total embryo number                 | 102  | 127  | 113  |

Results from two independent experiments carried out for the purpose of establishing dose response. All subsequent experiments for gene expression studies used 6 ng foxc1a-MOs [1 + 2] per embryo.

### Table 2. The effect of foxc1b-specific MOs on the development of wild-type embryos

| Observed phenotype                  | foxc1b-MOs injected |
|-------------------------------------|---------------------|
|                                     | MO-1 | MO-2 | MO-1 | MO-2 |
| 4–6 ng                              | 100% | 100% | 100% | 100% |
| 6–8 ng                              | 100% | 100% | 100% | 100% |

Embryos with somites 100% 100% 100% 100%
Total embryo number 42 38 30 26

Results from two independent experiments.
nucleotides specifically inhibit the translation of Foxc1a. Moreover, the failure of early somites formation is accompanied by loss of Foxc1a protein, and the subsequent recovery of somitogenesis is associated with its resynthesis.

**The inhibitory effect of foxc1a-MO on segmentation is selective**

To test whether the effect of foxc1a-MO is specific for somitogenesis, we assayed the expression of a number of genes normally expressed in somites and other tissues. As shown in Figure 2C–F, the expression of no tail [ntl] in the forming notochord, krox20 in rhombomeres 3 and 5, and pax2.1 in the midbrain–hindbrain boundary, otic primordia, and pronephric ducts were all unaffected compared to wild-type embryos at the 5-somite stage. However, expression domains of deltaC and pax2.1 in the pronephric primordia, which are located lateral to somites 2 to 4 [Smithers et al. 2000] were missing in MO-injected embryos, even though the duct primordia were unaffected [Fig. 2C,D]. This is consistent with the fact that only primordia of the pronephros and not the ducts express foxc1a [Topczewska et al. 2001] and suggests that the gene is specifically required for the development of the pronephros. In addition, the expression of myoD was strongly reduced in the somite region of MO-injected embryos, but not in the adaxial cells [Fig. 2G–J].

**Table 3. The effect of mouse synthetic foxc1 RNA and rescue, modified foxc1a RNA on the foxc1a-MO phenotype**

| Gene analyzed | No expression | Partial expression | Wild-type expression | Total no. of injected embryos |
|--------------|---------------|--------------------|----------------------|-------------------------------|
| deltaC       | 86%           | 8%                 | 6%                   | 140                           |
| paraxis      | 87%           | 5%                 | 8%                   | 117                           |

Results from three independent experiments. Embryos were fixed at the 5 somite stage and examined by whole amount in situ hybridization for deltaC and paraxis.

*No expression in region of first 5 somites.

*One to three stripes of deltaC or segments of paraxis expression.

*5 bands of expression.

**The foxc1a-MO induced phenotype can be rescued**

Further evidence of the specificity of the foxc1a-MO phenotype comes from rescue experiments with the mouse homolog, Foxc1, which differs from zebrafish foxc1a in the nucleotide sequence targeted by MO. Preliminary experiments indicated that zebrafish embryos are very sensitive to the dose of zebrafish or mouse Foxc1 RNA injected and that high levels impair gastrulation [data not shown]. This finding is consistent with the observation that mouse embryos are sensitive to the gene dosage of Foxc1 and Foxc2 [Kume et al. 2001]. A series of different concentrations of mouse Foxc1 RNA was therefore injected together with 6 ng of foxc1a-MOs. At a dose of about 10 pg of Foxc1 RNA, partial rescue of morphological somites and recovery of expression of deltaC and paraxis was observed compared to embryos injected with foxc1a-MO alone [Figs. 1E, 3; Table 3]. Because mouse synthetic Foxc1 RNA only weakly suppressed the MO phenotype, another construct was prepared in which the 5’ UTR sequences of zebrafish foxc1a mRNA recognized by foxc1a-MOs was deleted. Injection of this modified foxc1a RNA together with foxc1a-MO [10 pg of RNA and 8 ng of MOs] increased by 60% the frequency of embryos with morphologically distinguishable somites compared to MOs injection alone [n = 84; Fig. 1D,E].

**Figure 3.** Mouse synthetic Foxc1 RNA can partially rescue deltaC and par expression in MO-injected embryos. (A–C) deltaC expression. (D–F) par expression. (A,D) wild-type embryos; (B,E) foxc1a-MO-injected embryos; (C,F) foxc1a-MO-injected embryos coinjected with mouse Foxc1 RNA.

**Loss of morphological somites in MO-injected embryos is accompanied by loss of molecular markers in paraxial mesoderm**

Although microscopy analyses [Nomarski and confocal] have shown that there is no morphological segmentation...
in foxc1a-MO-injected embryos, we nevertheless asked whether genes associated with anterior or posterior somite identity are expressed in the anterior of the PSM of MO-injected embryos. Anterior markers tested were deltaD, notch6, ephA4, and papc, and the posterior markers were deltaC, notch5, ephrinB2, and myoD (Figs. 2,4,5; data not shown). In all cases, no stripes of RNA expression were seen in foxc1a-MO-injected embryos in the region where somites should have been present. These results suggest that Foxc1a is required for somitogenesis and functions prior to the formation of morphological somites.

Figure 4. Effect of inhibiting Foxc1a protein synthesis on expression of deltaC, notch 5, notch 6, mesp-a, and mesp-b. (A) Wild-type embryo hybridized with probe for deltaC. Only one example of the variable pattern of expression is shown for the wild-type. (B–D) Representative foxc1a-MO-injected embryos showing normal variation in pattern of deltaC expression. (E,F) Wild-type at 5-somite stage and MO-injected embryos hybridized with probe for notch5 and (G,H) for notch6. Arrowheads indicate PSM domains of notch genes; S-1 indicates presumptive somite posterior to forming somite. (I,L) Double color in situ hybridization with myoD and mesp-b probe in wild-type and MO embryos at 5-somite stage, and (K–N) the same with mesp-a and mesp-b probes at 5-somite stage. (K,M) Viewed under visible light. [L,N] The same embryos viewed with rhodamine filter. Note strong expression of myoD in the adaxial cells and complete absence of mesp-b transcripts in MO-injected embryos.

Delta genes show normal expression in the presomitic mesoderm of foxc1a-MO injected embryos

The models of somitogenesis postulate that cells in the PSM oscillate from one mutually exclusive state to another. This is manifested in the zebrafish embryo as periodic waves of expression of her1, deltaC, and deltaD that appear to sweep anteriorly from the tail bud (Jiang et al. 2000; Keller 2000; Kerszberg and Wolpert 2000; Pourquie 2000b). An example of this dynamic behavior is the characteristic pattern of broad and narrow stripes of deltaC gene expression seen in the PSM of a population of wild-type embryos all examined at the same stage. As shown in Figure 4A–D, the wild-type pattern of deltaC expression is also seen in a group of MO-injected embryos, and transcripts did become localized into tight bands in the presumptive somite region. However as mentioned before, the striped expression of deltaC was not maintained in the region where the somites should have been present. Taken together, these results suggest that the first step of segmentation, establishment of segmental prepattern, is not affected by
Foxc1a inhibition and that synchronized oscillatory behavior does occur in the PSM of MO-injected embryos.

Some aspects of the anterior/posterior identity of cells in the anterior presomatic mesoderm are affected in MO-injected embryos

Before epithelial somites are formed, precise A and P domains are established in the somite primordia, a step that is thought to require Notch-dependent signaling [Buchberger et al. 2000; Johnson et al. 2000; Sawada et al. 2000a; Takahashi et al. 2000]. Studies in both mouse and zebrafish have identified Mesp proteins as specific bHLH transcription factors required for effecting this Notch signaling in the anterior PSM. Both mesp-a and mesp-b genes are expressed in overlapping domains in the anterior of the somite primordia (S-1 and S-2) [Fig. 4I–N; Durbin et al. 2000; Sawada et al. 2000a]. In addition, a transient band of mesp-b expression is seen in the forming somite, S0. We have consistently observed that the expression of mesp-b is strongly reduced in MO-injected embryos in S0, S-1, and S-2 [Fig. 4I,J]. In contrast, the expression of mesp-a appears to be unaffected. This result is clearly illustrated in experiments in which the same MO-injected embryos were hybridized simultaneously with probes for the two genes, one labeled with DIG and the other with fluorescein [Fig. 4K,N]. Our data suggest a differential requirement of Foxc1a protein for expression of mesp-a and mesp-b. Additionally, the expression of protocadherin C (papc), which is localized in the same regions of the PSM (S-1 and S-2) as the mesp genes was not affected in MO-injected embryos [Fig. 5; data not shown].

We next examined the effect of absence of Foxc1a protein on the expression of two genes encoding Delta receptors, notch5 and notch6 [Fig. 4E–H]. Both genes are segmentally expressed in the PSM of wild-type embryos. In addition, notch5 and notch6 transcripts are present in the posterior and anteromedial parts of the formed somites, respectively [Bierkamp and Campos-Ortega 1993; Westlin and Lardelli 1997]. This segmented expression was strongly down-regulated in MO-injected embryos, but weak, uniform expression of notch6 persisted throughout the PSM. These observations indicate that only an incomplete A/P polarization of somite primordia is established in the absence of Foxc1a protein.

Expression of Ephrin2B and eph4A, required for formation of somite boundaries, is abnormal in MO-injected embryos

Because the formation of intersomitic boundaries requires intercellular signaling mediated by cell surface molecules of the Eph/ephrin family [Durbin et al. 1998, 2000], we examined the expression of ephrinB2 and ephA4 in MO-injected embryos. As described earlier, stripes of expression of both genes were absent from the region where somites should be present [Fig. 5A,B; data not shown]. To assess expression in the anterior PSM, double staining using papc and ephrin-B2 probes was carried out at the 3–4-somite stage (Fig. 5C,D). While the papc expression domains located in S-1 and S-2 were still present in MO-injected embryos, the stripes of ephrin-B2 RNA were lost. This analysis shows that the expression of Eph/ephrin signaling molecules is perturbed in MO-injected embryos, and this may account, at least in part, for the defect in somite border formation.

Expression of paraxis depends on foxc1

Mouse embryos lacking the bHLH gene paraxis [par] fail to form epithelial somites, although segmentation and initial anteroposterior specification of somite primordia do occur [Burgess et al. 1996]. As shown in Figures 2E,F and 5E,F, par expression is severely down-regulated in both the PSM and paraxial mesoderm of MO-injected embryos. As in the case of mesp-a, this effect is selective, because myoD expression in adaxial cells of injected embryos was normal or even elevated [Fig. 5E,F].

The down-regulation of par expression in MO-injected embryos raised the possibility that Foxc1a protein is a positive regulator of par gene. To test this hypothesis, we injected capped synthetic foxc1a RNA (40–60 pg) into one cell stage zebrafish embryos. As shown in Figure 5G,H, the overexpression of foxc1a induces premature transcription of par at 40% epiboly, whereas during normal development, expression of par RNA was first detected at about 65% epiboly [Shanmugalingam and Wilson 1998]. These results show that foxc1a is both essential and sufficient for par expression.

The phenotype of foxc1a-MO-injected embryos is more severe than that of fss mutants

Several zebrafish mutants have been described that are defective in somitogenesis. In every case except fused somites (fss), the anterior somites develop normally, before defects are seen in the formation of more posterior somites [van Eeden et al. 1996]. In contrast, fss mutants, like foxc1a-MO-injected embryos, lack all anterior somites. Despite this similarity, fss and foxc1a affect somitogenesis differently, because par is not down-regulated in fss as observed for MO-injected embryos [Fig. 6]. We also compared the expression of foxc1a in fss and MO-injected embryos. Our data indicate that the striped pattern of foxc1a expression is lost in the anterior PSM of fss mutants [Fig. 6A,C]. Similarly, no striped expression of foxc1a was seen in MO embryos, where inhibition of foxc1a translation resulted in uniform expression of the gene throughout the PSM and into the region where somites should have been present. We conclude that foxc1a acts in parallel to the fss genetic pathway as an essential factor for the complete anterior–posterior patterning of the PSM and the formation of epithelial somites.

Discussion

The findings reported here establish the forkhead/
winged helix transcription factor, Foxc1a, as a novel and necessary component of the molecular circuitry underlying somitogenesis in the zebrafish. Moreover, taken together with the new findings in the mouse described in the accompanying paper (Kume et al. 2001), it appears that this function of Foxc1, the requirement of the gene for par expression, and its involvement with elements of the Notch signaling pathway have all been evolutionarily conserved.

The conclusion that Foxc1a is required for zebrafish somitogenesis is based on findings with embryos in which synthesis of the protein is specifically inhibited by morpholino antisense oligonucleotides [Nasevicius and Ekker 2000]. These embryos lack, at least up to about the 6–7-somite stage, morphological somites, segmented paraxial mesoderm, and expression in the somite region of genes characteristic of anterior and posterior somite cell fates. Several lines of evidence argue that the inhibitory effect of the foxc1a-MOs is specific and not due to a general toxicity. First, MOs block the synthesis of Foxc1a protein but not that of Myf5. Second, the inhibition of somite formation is dose-dependent, and a similar phenotype is observed with two separate foxc1a-MOs, each targeted against different regions of the foxc1a RNA. In contrast, somitogenesis proceeds normally in embryos injected with MOs targeted to the closely related gene foxc1b. Third, differentiation of other tissues continues on schedule in the absence of somite formation. Fourth, spontaneous recovery of somitogenesis in MO-injected embryos at around the 7-somite stage is accompanied by reexpression of the Foxc1 protein. One explanation for this recovery is that, as shown in Figure 6, the level of foxc1a RNA increases significantly in foxc1a-MO-injected embryos, probably due to negative feedback by Foxc1a protein on the transcription of its own gene. Consequently, MO oligonucleotides may become saturated. Finally, the phenotype of MO-injected embryos can be reversed by injection of either mouse Foxc1 or zebrafish foxc1a synthetic RNA, which lacks the sequences against which the MOs are directed.

**Figure 6.** Phenotypic differences between MO-injected embryos and fss mutants. (A,D) Wild-type embryos, (B) foxc1a-MO injected embryo, and (C,D) fss embryos. (A–C) In situ hybridization with foxc1a probe. Note loss of striped expression of foxc1a in the anterior PSM of fss mutant (D,E) par probe. Note that expression of par is not down-regulated in fss embryos.

**Foxc1a protein is not required for the segmentation clock**

Analysis of gene expression in foxc1a-MO-injected embryos shows that the dynamic expression of deltaC, deltaD, and her1 genes in the PSM resembles that of wild-type embryos. Moreover, the anterior boundaries of the delta gene expression stripes are sharp and not diffuse, as seen in embryos with mutations in the Notch signaling pathway (Jiang et al. 1998; Holley et al. 2000). These observations suggest that the absence of Foxc1a protein does not affect the oscillation of cells in the PSM from one state to another. Nor does it affect the proposed synchronization of these oscillations between neighboring cells. Although notch6 expression [Westlin and Lardelli 1997] is strongly down-regulated in MO-injected embryos, and is no longer expressed in stripes in the anterior region, there is still weak uniform expression throughout the PSM. This weak expression may be sufficient to enable synchronization of oscillations in the PSM to occur. Alternatively, other Notch genes such as notch1a may be engaged in this process and be unaffected by the absence of Foxc1a.

**Foxc1a is required for correct A/P patterning of presumptive somites in the anterior presomitic mesoderm**

For morphological boundaries to be generated between forming somites, it appears necessary that each presumptive somite be subdivided into stable anterior and posterior domains (Durbin et al. 2000). Observations initially made in the mouse have indicated a crucial role for Notch/Delta signaling and the basic-loop-helix transcription factor, Mesp2, in establishing this A/P patterning [Takahashi et al. 2000]. Thus, Mesp2 in the anterior half of the presumptive somite is thought to inhibit the up-regulation of the Dll1 gene in response to Notch activation, while the absence of Mesp2 expression in the posterior domain allows Dll1 induction to proceed. Moreover, Mesp2 is thought to act in an autoregulatory loop with Notch, being both up-regulated by Notch signaling and required for Notch gene expression. A similar role has been suggested for zebrafish mesp-b gene (Sawada et al. 2000). We found that the expression of one mesp gene, mesp-a, is unaffected in MO-injected embryos, in both the posterior PSM where it is expressed diffusely and in the stripe of high-level expression in the anterior region of the presumptive somites (S-1 and S-2) [Fig. 4]. In contrast, transcripts for the mesp-b gene, normally localized exclusively in the most anterior part of S-1 and S-2, are completely absent in MO-injected embryos. This deficit is accompanied by the absence of the striped expression of notch6 and notch5 genes. We conclude that in zebrafish, the activity of mesp-b is required for correct patterning of the presumptive somites. Moreover, the loss of expression of only one mesp gene, in this case mesp-b due to the absence of Foxc1a protein, is enough to prevent the completion of somite formation, possibly in part because of the down-regulation of notch 5 and notch6.
Among the several zebrafish mutations with defects in somitogenesis, fss has the closest phenotype to that of foxc1a-MO-injected embryos, because in both sets of embryos all anterior somites are missing [van Eeden et al. 1996]. However, there are significant differences between the two phenotypes. For example, the fss mutation completely disrupts normal expression of both mesp-a and mesp-b during the segmentation period [Sawada et al. 2000a]. In other fss-type mutants such as aei, mesp-b is expressed in a diffuse “salt and pepper” pattern in the paraxial mesoderm similar to that seen for deltaC in the same mutants [Jiang et al. 2000], whereas expression of mesp-a is very reduced and limited mostly to adaxial cells. Based on these results, Sawada et al. (2000a) proposed that the two mesp genes are differentially regulated, a conclusion supported by our own results. Another difference between the phenotype of fss and foxc1a-MO-injected embryos is seen in the expression of the protocadherin gene, pape. In fss mutants, the down-regulation of both mesp genes is associated with strongly reduced expression of pape in the anterior PSM [Jiang et al. 2000]. In contrast, pape is still expressed normally in foxc1a-MO-injected embryos in the PSM, suggesting that the remaining activity of mesp-a is sufficient to drive pape expression even though segmentation does not proceed to completion. Expression of mesp-a and pape in the anterior of the presumptive somites also suggests that the hypothetical wave front activity, postulated to be disrupted in fss mutants, functions in the absence of Foxc1a activity, and is able to partially stabilize the anterioposterior patterning of the anterior PSM.

**Foxc1a is required for expression of ephrinB2 and its receptor ephA4 during somite formation**

The formation of intersomitic boundaries requires the expression of Ephrins and their receptors, and manipulation of ephrin signaling genes in zebrafish disturbs somite differentiation [Durbin et al. 1998]. We found that expression domains of ephrinB2 and its receptor ephA4 are strongly down-regulated in the anterior PSM of foxc1a-MO-injected embryos. This result is significantly different from that seen in fss mutant embryos, where the posteriorly expressed ephrinB2 is expanded, and fss-like mutants in which ephrinB2 and ephA4 are expressed in a salt and pepper pattern [Durbin et al. 2000]. The strong phenotype of MO-injected embryos compared to that of fss-type mutants raises the possibility that Ephrins and Eph receptors are regulated by Foxc1a directly, or that completed A/P specification of somite primordia is required for ephrinB2 and ephA4 expression. The observed lack of ephrinB2 and ephA4 expression in our experiments may be one of the reasons why somite boundaries are not formed and further somitic mesoderm differentiation is arrested.

**Foxc1a is required for paraxis expression**

Analysis of mouse mutants has shown that the gene paraxis, encoding a bHLH transcription factor, is required for the formation of epithelial somites but not for segmentation of paraxial somites [Burgess et al. 1995, 1996]. Recent evidence suggests that in the absence of Par in the mouse, Notch signaling and expression of Mesp2, EphrinB2, and EphA4 in the PSM are initially normal. However, intersomitic boundaries fail to form, and the A/P polarity of the anterior PSM is not maintained in the segmented mesoderm [Johnson et al. 2000]. Par axis expression does not depend on the Notch signaling pathway in the mouse [Johnson et al. 2000], and is maintained in fss mutant embryos [Fig. 6E]. In contrast, in foxc1a-MO-injected embryos and in mouse embryos lacking both Foxc1 and Foxc2 par axis expression is strongly down-regulated. Further, we have shown that injection of foxc1a RNA into the zebrafish embryo leads to ectopic and premature par expression, suggesting that par is a direct or early downstream target of the Foxc1a transcription factor. Nevertheless, the phenotype of foxc1a-MO-injected embryos is more severe than that of par null embryos and affects the A/P patterning polarity of the anterior PSM. We conclude that loss of Par axis function cannot be the primary and sole defect in MO-injected embryos, although it may still contribute to the lack of border formation and epithelialization of somites.

**Comparison of the roles of foxc1 genes in mouse and zebrafish somitogenesis**

Recent evidence in the mouse presented in the accompanying paper indicates that the two closely related winged helix transcription factors, Foxc1 and Foxc2, function combinatorially, and that inactivation of both sets of alleles is required to completely disrupt somite formation and the prepatterning of the anterior PSM [Kume et al. 2001]. However, our present results suggest that there are differences in the role of foxc1 genes in somitogenesis between mice and zebrafish. First, foxc1b, although transcribed in the same pattern as foxc1a in paraxial mesoderm and probably required for head mesoderm development, cannot substitute for the absence of foxc1a in somite formation. Second, if a homolog of mouse Foxc2 exists in the zebrafish, then it, too, does not compensate for the absence of Foxc1a. It therefore appears that the completion of somite formation in the zebrafish embryo is more dependent on the level of foxc1 gene expression compared to the mouse embryo. Inhibiting Foxc1a protein synthesis alone is sufficient to uncover an evolutionarily conserved requirement for this class of transcription factor in the maturation of the presumptive somites.

**Materials and methods**

**Fish embryos**

Fish were maintained as described [Solnica-Krezel et al. 1994]. The mutant allele used was fused somites (fss<sup>m774</sup>; Driever and Fishman 1996).
**In situ hybridization**

Whole-mount in situ hybridization was performed essentially as described [Thissie et al. 1993]. For two-color in situ hybridization, the digoxigenin- and fluorescein-labeled antisense probes were used simultaneously. After the first color reaction, the alkaline phosphatase-conjugated antibody was inactivated by washing three times for 5 min at room temperature first with 0.1 M glycine-HCl (pH 2.2), 0.1% Tween 20 and then with PBST (PBS at pH 7.4, 0.1% Tween 20). Embryos were then incubated with antifluorescein antibody for 2 h at room temperature or overnight at 4°C. After intense washing in PBST (6 times every 15 min at room temperature), followed by two more washes with 0.1 M Tris-HCl (pH 8.2, 0.1% Tween), the second color reaction was performed with Fast Red (Roche) in 0.1 M Tris (pH 8.2), 0.1% Tween, with the addition of 0.4 M NaCl. Depending on the probe, the reaction was continued for a few hours to overnight. For photomicrography, the embryos were mounted flat in 50% glycerol.

**Antibody against mouse Foxc1 protein**

A peptide from the C-terminal domain of mouse Foxc1 protein was used to raise a polyclonal rabbit antiseraum, followed by peptide-affinity purification [Research Genetics]. The differences between the predicted mouse and zebrafish protein sequences are underlined: Foxc1, AYPGQQQNFHSVREM sequences are underlined: Foxc1, AYPGQQQNFHSVREM

**Whole-mount immunocytochemistry**

Embryos were fixed in 4% paraformaldehyde, 4% sucrose, 3 mM CaCl\(_2\), and PBS (pH 7.4) overnight at 4°C and permeabilized in acetone for 7 min at −20°C. Blocking was performed using PBST buffer with 5% goat serum, 0.2% BSA, and 2% DMSO. Incubation with primary antibody was overnight at 4°C followed by intense washing in PBST buffer at room temperature. For immunofluorescence microscopy, the secondary antibody was conjugated with Cy-3 (Jackson Labs) or Alexa Fluor 488 (Molecular Probes). Incubation was performed at room temperature for 2–3 h. In double staining experiments, the samples were incubated with the Foxc1 antibody diluted 1:200 overnight at 4°C and subsequently with β-catenin monoclonal antibody (Sigma) diluted in blocking solution 1:500 for 4 h at room temperature.

**Transient transfection of COS cells**

This procedure was performed using lipofectamine reagent according to the manufacturer’s protocol [Life/Technologies-GIBCO BRL].

**Western blot**

Embryos were dechorionated and washed in Danieau buffer, and the yolks were removed manually. Tissues from 60 embryos were boiled in 60 μL of Laemmli sample buffer (Laemmli 1980) for 5 min. Following centrifugation, the supernatant equivalent of 20 embryos was electrophoresed on a 10% polyacrylamide gel according to Sambrook et al. [1989]. Proteins were electroblotted onto Zeta-Probe membranes [Bio Rad], and antigenic proteins were detected using affinity-purified Foxc1 antiseraum at 1:1000 dilution and an ECL kit [Amersham].

**Confocal microscopy**

Flat mounted embryos were observed initially using epifluorescence with a Zeiss Axioskop microscope and subsequently using a Zeiss LSM410 Confocal Laser Scanning Inverted Microscope with 20x or 40x Neofluar objectives [facility supported by NIH grants CA68485 and DK20593]. For the Alexa 488 and Cy-3 labels, excitation was at 488 and 543 nm, respectively.

**Microinjection of zebrafish embryos**

Capped mRNA was synthesized by in vitro transcription of linearized plasmid (MessageMachine kit, Ambion). RNA (10–100 pg) was injected into the yolk of one- to two-cell stage embryos.

**Morpholino**

Morpholino antisense oligonucleotides [Gene-Tools] were designed according to the manufacturer’s suggestions. Two different oligonucleotides specific for foxc1a and two for foxc1b were used for injections, as follows: foxc1a-MO-1, 5’-GT CAAGAAGCTGAAGCATACCAACA-3’; foxc1b-MO-2, 5’-CCTGATGACTGCTCTCCAAAAACCG-3’; foxc1b-MO-1, 5’-GCATGCTACCCCTTTCTTTCGTAAC-3’; foxc1b-MO-2, 5’-AAGTGAAATGAACTATGCAGACG-3’.

Morpholino oligonucleotides were diluted in 1× Danieau buffer, and between 2 and 10 ng was injected into the yolk of embryos at the 1–8 cell stage.

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The winged helix transcription factor Foxc1a is essential for somitogenesis in zebrafish

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