The regulation of mesodermal progenitor cell commitment to somitogenesis subdivides the zebrafish body musculature into distinct domains

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The vertebrate musculature is produced from a visually uniform population of mesodermal progenitor cells (MPCs) that progressively bud off somites populating the trunk and tail. How the MPCs are regulated to continuously release cells into the presomitic mesoderm throughout somitogenesis is not understood. Using a genetic approach to study the MPCs, we show that a subset of MPCs are set aside very early in zebrafish development, and programmed to cell-autonomously enter the tail domain beginning with the 16th somite. Moreover, we show that the trunk is subdivided into two domains, and that entry into the anterior trunk, posterior trunk, and tail is regulated by interactions between the Nodal and bone morphogenetic protein (Bmp) pathways. Finally, we show that the tail MPCs are held in a state we previously called the Maturation Zone as they wait for the signal to begin entering somitogenesis.

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The mesodermal progenitor cells (MPCs) are a population of undifferentiated progenitor cells that originate in the early gastrula embryo (for review, see Schier et al. 1997; Kimelman 2006). By the end of gastrulation, they have moved to the very posterior end of the embryo in a region called the tailbud. Throughout gastrulation and somitogenesis, the MPCs continuously contribute cells to the presomitic mesoderm, producing a species-specific number of segments using a complex clock and wavefront mechanism to segment the presomitic mesoderm (for review, see Tam et al. 2000; Holley and Takeda 2002; Dubrulle and Pourquie 2004). The release of cells from the MPC population into the presomitic mesoderm needs to be controlled such that a sufficient number of cells continuously enter the presomitic mesoderm to form somites, yet enough precursors remain behind to form the complete set of somites. We previously provided evidence that cells from the MPC population first enter a transitional Maturation Zone at the very posterior end of the embryo, marked by the overlapping expression of the T-box transcription factors *no tail*, *spadetail*, and *tbx6*, as they make the commitment to enter

the presomitic mesoderm (Griffin and Kimelman 2002) (this zone is not the same as the maturation front at the anterior of the presomitic mesoderm, where the somite border forms). These results suggest that the decision to enter the presomitic mesoderm involves a multistep process that is under tight regulatory control.

Several lines of evidence indicate that the zebrafish tail somites are genetically different from the trunk somites even though the mechanism of forming the trunk and tail somites is indistinguishable (Ho and Kane 1990; Halpern et al. 1993; Mullins et al. 1996; Ober and Schulte-Merker 1999; Agathon et al. 2003). One key regulator of tail formation in zebrafish as well as in *Xenopus* is the bone morphogenetic protein (Bmp) pathway (Ober and Schulte-Merker 1999; Gonzalez et al. 2000; Beck et al. 2001; Hammerschmidt and Mullins 2002; Myers et al. 2002; Agathon et al. 2003; De Robertis and Kuroda 2004; Munoz-Sanjuan and Hemmati-Brivanlou 2004). Recent studies have shown that Bmp signaling is required during early gastrulation for the specification of the tail (Pyati et al. 2005; Connors et al. 2006), suggesting that the tail MPCs might be uniquely specified at early stages of development and held in an undifferentiated state until the tail somites are ready to form.

Unfortunately, it is very difficult to study this process because the trunk and tail MPCs are intermixed at the gastrula stage and within the early tailbud (Kanki and Ho

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1997; Warga and Nüsslein-Volhard 1999), and thus it is not possible to uniquely identify the tail MPCs within an embryo until they are in the process of contributing to the tail somites. To circumvent this problem, we investigated the use of a genetic approach. Embryos lacking maternal and zygotic One-eyed pinhead (*MZoep*), an essential coreceptor for Nodal signaling (Gritsman et al. 1999), form only a small tail-like structure and do not form any trunk somites (Gritsman et al. 1999; Carmany-Rampey and Schier 2001). To determine if this structure is a true tail, we carefully compared the development of wild-type and *MZoep* embryos. In every case, *MZoep* embryos did not start forming somites until the wild-type embryos formed their first tail somite (the 16th somite), and the *MZoep* embryos continued to form somites at the wild-type rate until the wild-type embryos formed their final somite (Supplementary Fig. S1). This result indicated that *MZoep* embryos could provide a unique source of tail MPCs for analysis.

We show here that *MZoep* provides a valuable system for studying the subdivision of the zebrafish body into trunk and tail domains. We show that the trunk is subdivided into an anterior and posterior domain, and that the decision of cells to enter the anterior trunk, posterior trunk, and tail is regulated by an interplay between Nodal and Bmp signaling, as well as by a trunk-promoting signal. Analyzing the expression of marker genes, we show that *MZoep* cells are held in a state equivalent to the Maturation Zone before they are ready to contribute to the tail. We propose that Nodal and Bmp signals control a regulator, which determines when the MPCs can first leave the Maturation Zone and commit to entering the presomitic mesoderm. In this way, some cells enter

somitogenesis early and others are held in reserve, ensuring that a continuous supply of cells enter the presomitic mesoderm throughout somitogenesis.

Results

Somite fate of *MZoep* MPCs

Within the context of the *MZoep* embryo, *MZoep* MPCs are only able to contribute to the tail (Carmany-Rampey and Schier 2001). To determine if *MZoep* MPCs cell-autonomously recognize when to enter the tail, or if their fate can be changed when surrounded with MPCs entering the trunk and tail, we examined the ability of *MZoep* cells to contribute to trunk and tail somites in a wild-type background using cell transplantation. Small groups of fluorescently labeled cells were removed from the prospective mesodermal region of *MZoep* embryos at 5 h post-fertilization (hpf; 5 hpf = 40% epiboly) and transplanted into the prospective mesodermal region of wild-type embryos at the same stage (Fig. 1A). It is important to note that the transplanted cells will randomly end up in different positions across the dorsoanterior–ventroposterior axis, and thus individual cells are potentially exposed to different intercellular signaling factors in the wild-type embryos.

We generated 90 chimeric embryos and observed fluorescent *MZoep* cells in several regions including the neural tube, brain, and somites at 24 hpf when the muscle cells are easily distinguished from other cell types morphologically (Fig. 1B; data not shown). These results are generally consistent with the previous fate map of *MZoep* embryos (Carmany-Rampey and Schier 2001). To

Figure 1. Homochronic cell transplantation. (A) Schematic depiction of cell transplant experiment showing 5-hpf *MZoep* cells transplanted into a 5-hpf wild-type host embryo. (B) Representative picture of a chimeric embryo. White arrows denote the boundary between trunk and tail regions. (C) Graph showing the most anterior somite populated by transplanted *MZoep* cells. The bars in the graph are differently colored in the trunk and tail regions for clarity. (D–F) Transplants of 4-hpf *MZoep* cells into 4-hpf wild-type host embryos. Note that *MZoep* cells only populate the tail when transplanted at 5 hpf, whereas they also populate the posterior trunk when transplanted at 4 hpf.

determine the contribution of *MZoep* cells specifically to wild-type somites, we scored chimeric embryos for the most anterior somite containing a fluorescent cell, and represented this in a graphical format. Importantly, *MZoep* cells were never observed to contribute to the trunk somites (somites 1–15) (Fig. 1C).

In order to ensure that the restriction of the *MZoep* muscle cells to the tail was not an artifact of the transplantation procedure, we transplanted rhodamine (red)-labeled wild-type cells and fluorescein (green)-labeled *MZoep* cells together into wild-type host embryos at 5 hpf (Fig. 2A). Whereas red fluorescent wild-type cells were found throughout the trunk and tail somites, green fluorescent *MZoep* cells were only found in the tail somites (Fig. 2B,C). Our results demonstrate that 5-hpf *MZoep* MPCs only contribute to the tail somites, and that the *MZoep* MPCs exclusively populate the tail domain despite the presence of wild-type cells contributing to the trunk and tail somites.

Timing of commitment to the tail fate

Our cell transplantation data indicated that the *MZoep* MPCs are committed to a tail fate by 5 hpf. To determine if *MZoep* MPCs can only ever contribute to the tail somites or if this is a property they acquire during early

development, we transplanted cells from *MZoep* embryos at 3.3 and 4 hpf (high and sphere stages, respectively) into wild-type host embryos of the same stages (Fig. 1D). When *MZoep* cells were transplanted at 4 hpf and earlier, they populated both the trunk and tail (Fig. 1E,F; data not shown). These results suggest that the fates of the *MZoep* MPCs are not fixed, and that they acquire their identity as tail MPCs between 4 and 5 hpf.

What changes between 4 and 5 hpf that causes the *MZoep* MPCs to become committed to a tail fate by 5 hpf? One possibility is that the fate of the MPCs becomes fixed between 4 and 5 hpf such that the 5-hpf cells do not respond to a new environment (Fig. 3A, Model 1). A second possibility is that the prospective tail cells need to be exposed to a tail-promoting signal during the 4–5-hpf interval (Fig. 3A, Model 2). When *MZoep* cells are transplanted at 4 hpf into 4-hpf wild-type embryos, some of the MPCs would no longer be exposed to the tail signal in this model because they randomly ended up in a region of the wild-type embryo that does express the tail signal, and these cells therefore would adopt a trunk fate. A third possibility is that a trunk-promoting signal is present in wild-type embryos only prior to 5 hpf (Fig. 3A, Model 3). In this scenario, transplantation of *MZoep* cells randomly into wild-type embryos at 4 hpf would

Figure 2. Two-color cell transplantation. (A) Schematic depiction of the experiment. Five-hour-post-fertilization wild-type cells containing rhodamine dextran (red) and 5-hpf *MZoep* cells containing fluorescein dextran (green) were cotransplanted into 5-hpf wild-type host embryos. (B) A representative embryo. White arrows denote the boundary between the trunk and tail regions. (C) Graph showing the most anterior somite populated by transplanted wild-type cells (labeled in red) and *MZoep* cells (labeled in green). The anterior somite limit for *MZoep* cells in this experiment was somite 16, whereas wild-type cells were found as far anterior as somite 1. (D,E) Same as in A and B except that the donor cells and host embryo were all at 4 hpf. (F) Graph showing the most anterior somite populated by transplanted wild-type cells (labeled in red) and *MZoep* cells (labeled in green). The anterior somite limit for *MZoep* cells in this experiment was somite 9, whereas wild-type cells were found as far anterior as somite 1.

Figure 3. Heterochronic cell transplantation. (A) The three models are shown. At the *top* of each model is the hypothesis; in the *middle* is the experimental test, with a cartoon of a fish showing the predicted result based on the hypothesis; and at *bottom* is the result, either yes or no. Only Model 3 fits the data. (B) Schematic depiction of the experiment showing 5-hpf MZoep cells transplanted into 4-hpf wild-type host embryos. (C) Representative picture of a chimeric embryo. White arrows denote the boundary between trunk and tail regions. (D) Graph showing the most anterior somite populated by transplanted MZoep cells. (E–G) Transplants of 4-hpf MZoep cells into 5-hpf wild-type host embryos. Note that 5-hpf MZoep cells populate the posterior trunk and tail when transplanted into 4-hpf hosts, whereas 4-hpf MZoep cells only populate the tail when transplanted into 5-hpf hosts.

expose some of them to the trunk-promoting signal, whereas transplanting MZoep cells into wild-type embryos at 5 hpf would not expose them to the trunk promoting signal for enough time to change the fate of the MZoep cells.

To distinguish between these possibilities, we used heterochronic cell transplantation by transplanting 5-hpf MZoep cells into 4-hpf wild-type hosts (Fig. 3B) and transplanting 4-hpf MZoep cells into 5-hpf wild-type hosts (Fig. 3E). If cells lost competence to change their trunk-tail fate by 5 hpf, then transplantation of 5-hpf MZoep cells into 4-hpf wild-type embryos would result in MZoep cells only populating the tail (Fig. 3A, Model 1). Instead, we found that transplanting 5-hpf MZoep cells to earlier wild-type embryos caused some of them to populate the trunk (Fig. 3C,D). If a tail-promoting signal was disrupted by transplantation at 4 hpf, we predicted that transplantation of 4-hpf MZoep cells into 5-hpf wild-type hosts would cause the MZoep

cells to populate the trunk and tail, whereas transplantation of 5-hpf MZoep cells into 4-hpf wild-type hosts would result in MZoep cells only populating the tail (Fig. 3A, Model 2). This is not what we observed (Fig. 3C,D,F,G). Conversely, if a trunk-promoting signal was present in wild-type embryos up until 5 hpf, we predicted that transplantation of 5-hpf MZoep cells into 4-hpf wild-type hosts would cause the MZoep cells to populate the trunk and tail, whereas transplantation of 4-hpf MZoep cells into 5-hpf wild-type hosts would result in MZoep cells only populating the tail (Fig. 3A, Model 3). This exactly matches the experimental results (Fig. 3C,D,F,G). We therefore conclude that wild-type embryos express a trunk-promoting signal that MZoep cells must first encounter prior to 5 hpf to adopt a trunk fate. Moreover, since MZoep embryos lack Nodal signaling and do not form a trunk, we conclude that the trunk-promoting signal is Nodal dependent.

Nodal signaling specifies the anterior trunk

Although *MZoep* cells can contribute to trunk somites when transplanted into wild-type hosts at 4 hpf, we noticed that they never contributed to somites anterior to somite 9 in either homochronic or heterochronic transplants (Figs. 1F, 3D). This was not related to the age of the *MZoep* cells since transplantation of 3.3-hpf *MZoep* cells produced exactly the same result as a 4-hpf transplantation (data not shown). To rule out the possibility that this result was an artifact of our transplantation technique, we cotransplanted 4-hpf red fluorescent wild-type cells and 4-hpf green fluorescent *MZoep* cells into 4-hpf wild-type hosts (Fig. 2D). Whereas wild-type cells populated all regions of the embryo, the cotransplanted *MZoep* cells were not found anterior to somite 9 (Fig. 2E,F).

Our results suggest that *MZoep* cells lack an essential property that allows them to populate the anterior trunk even when exposed to the trunk-promoting signal. We reasoned that the failure of the *MZoep* cells to receive a direct Nodal signal might be the cause of this deficit, which we tested by expressing a cell-autonomous activator of the Nodal pathway (a constitutively active Alk4 receptor, *CA-Alk4*) (Chang et al. 1997) in *MZoep* embryos, and then transplanting cells from these embryos at 5 hpf into 5-hpf wild-type hosts (Fig. 4A). As shown in Figure 4, *MZoep* cells expressing the CA-Alk4 populate the most anterior somites (cf. Figs. 4B,C and 1B,C). Thus, Nodal signaling is cell-autonomously required in cells to form the anterior, but not the posterior, trunk somites.

Bmp signaling is required to establish the trunk-tail boundary

What instructs the *MZoep* cells transplanted at 5 hpf to only enter the tail domain? Studies in *Xenopus* and zebrafish have shown that Bmp signaling is important for forming a tail, and thus it was a strong candidate factor (Mullins et al. 1996; Kishimoto et al. 1997; Ober and Schulte-Merker 1999; Dick et al. 2000; Gonzalez et al. 2000; Beck et al. 2001; Hammerschmidt and Mullins 2002; Myers et al. 2002; Agathon et al. 2003; De Robertis and Kuroda 2004; Munoz-Sanjuan and Hemmati-Brivanlou 2004; Pyati et al. 2005). We investigated the role of Bmp signaling in the development of the MPCs by examining the ability of 5-hpf *MZoep* MPCs expressing a dominant-negative Bmp receptor (*dnBmpR*) (Pyati et al. 2005) to contribute to the somites in wild-type hosts using cell transplantation (Fig. 4D). Whereas uninjected *MZoep* cells contribute only to the tail (Fig. 1B,C), *MZoep* cells injected with *dnBmpR* RNA were also found in the posterior trunk (Fig. 4E,F). Conversely, whereas uninjected 4-hpf *MZoep* cells transplanted into 4-hpf wild-type hosts produced many embryos with cells in the posterior trunk (Fig. 1F), 4-hpf *MZoep* cells injected with 138 pg of *bmp2b* RNA (Kishimoto et al. 1997) transplanted into wild-type host embryos at 4 hpf had a reduced number of trunk cells (Supplementary Fig. S2A–C). We confirmed this finding by showing that the proportion of 4-hpf *MZoep* cells contributing to the trunk decreased with increasing amounts of injected *bmp2b* RNA (Supplementary Fig. S2D). We note that even at the highest doses of injected *bmp2b* RNA we

Figure 4. Regulation of *MZoep* somite commitment by Nodal and Bmp. (A) Schematic depiction of the experiment showing 5-hpf *MZoep* cells expressing a constitutive activator of the Nodal pathway (*CA-Alk4*) transplanted into 5-hpf wild-type host embryos. (B) Representative picture of a chimeric embryo. (C) Graph showing the most anterior somite populated by transplanted *MZoep* cells. Note that activation of the Nodal pathway causes the 5-hpf *MZoep* cells to enter the anterior trunk (cf. Fig. 1A–C). (D–F) Same as A–C except that the *MZoep* cells express an inhibitor of Bmp signaling (*dnBmpR*) and the donor cells and host embryos are at 5 hpf. Note that inhibiting the Bmp pathway causes 5-hpf *MZoep* cells to populate the trunk (cf. Fig. 1A–C).

were not able to convert all of the MPCs to a tail fate. This might indicate that Bmp signaling alone is not able to promote the tail fate in all MPCs, or it might be more simply due to the fact that RNA injected at the one-cell stage frequently is not incorporated into all of the embryonic blastomeres (our unpublished results). These results demonstrate that Bmp signaling plays an essential role in establishing the boundary between the posterior trunk and the tail.

Since *MZoep* cells can be converted to a trunk fate by inhibiting Bmp signaling, we suggest that Bmp inhibitory factors such as Chordin, Noggin, and Follistatin are candidates to be the trunk signal. A second means of regulating Bmp signaling in zebrafish involves Fibroblast growth factor (Fgf) signaling (Furthauer et al. 1997, 2004). Three *fgfs* are expressed in a dorsal–ventral gradient in the late blastula zebrafish embryo (Furthauer et al. 1997, 2004; Draper et al. 2003), and they have been shown to inhibit the transcription of *bmp2b*, *bmp4*, and *bmp7* during the late blastula stages, which is important for restricting *bmp* transcripts to the ventral side of the embryo (Furthauer et al. 1997, 2004). Since the *fgfs* were also candidates to be components of the trunk-promoting signal, we examined whether *fgf* overexpression in *MZoep* cells would cause them to enter the posterior trunk. As shown in Figure 5, *MZoep* cells overexpressing Fgf entered the posterior trunk when transplanted at 5 hpf (cf. Figs. 5A–C and 1A–C).

Tail progenitors are held in the Maturation Zone state during early somitogenesis

The formation of the somites from the MPCs involves progressive changes in T-box gene expression starting with an initial expression of *no tail (ntl)* (Schulte-Merker et al. 1992), progressing to the activation of *spadetail (spt)* (Griffin et al. 1998) and *tbx6* (Hug et al. 1997) in the Maturation Zone, and finally to the down-regulation of *ntl* and the activation of *tbx24* (Nikaido et al. 2002) in the early presomitic mesoderm (Griffin and Kimelman 2002; Nikaido et al. 2002). Since we found that the *MZoep* embryos formed only tail MPCs, we recognized that we could use the *MZoep* embryos to determine whether the tail MPCs are normally held in the Maturation Zone state before they are ready to contribute to the somites.

We examined the expression of each of the four T-box genes during the early stages of development. *ntl* is expressed in *MZoep* embryos during the gastrula stages, although at decreased levels compared with wild-type embryos, as previously noted (Gritsman et al. 1999), and it continues to be expressed at wild-type levels in the tailbud throughout somitogenesis, although not in the notochord, which is absent in *MZoep* (Fig. 6, cf. E–H and A–D). Similarly, *spt* and *tbx6* are expressed throughout gastrulation and somitogenesis in *MZoep* embryos (Supplementary Fig. S3). In contrast, *tbx24*, the marker of the early presomitic mesoderm (Nikaido et al. 2002), is not expressed in *MZoep* embryos until after the 12-somite stage, whereas it is expressed in wild-type embryos throughout the gastrula and somitogenesis stages (Fig. 6, cf. I–L and M–P). Since there is a delay between when cells first enter the presomitic mesoderm and when they are incorporated into a morphologically apparent somite, we suggest that the cells first expressing *tbx24* at the 12-somite stage will be the cells that end up in the first tail somite (somite 16). To confirm that the *MZoep* tail MPCs fail to enter the early presomitic mesoderm prior to the 12-somite stage, we also examined the expression of *Wnt Inhibitory Factor-1 (WIF-1)* (Hsieh et al. 1999). Like *tbx24*, *WIF-1* is expressed in the early presomitic mesoderm but not in the tailbud. Whereas *WIF-1* is expressed from the end of gastrulation through somitogenesis in wild-type embryos, it is not expressed in *MZoep* embryos until the 12-somite stage, paralleling the expression of *tbx24* (Supplementary Fig. S4). We conclude that the tail MPCs are held in the Maturation Zone state, expressing *ntl*, *spt*, and *tbx6*, but prevented from expressing markers of the early presomitic mesoderm such as *tbx24* and *WIF-1*.

Discussion

Regional development of zebrafish body

Our studies using cell transplants have demonstrated that the zebrafish body is divided into three domains, which the MPCs recognize: the anterior trunk, the posterior trunk, and the tail (Fig. 7A). When transplanted among wild-type cells that contribute to all three domains, the *MZoep* cells transplanted at 5 hpf contribute only to the tail, whereas *MZoep* cells transplanted at 4

Figure 5. Fgf overexpression causes *MZoep* cells to enter the posterior trunk. (A) Schematic depiction of the experiment showing 5-hpf *MZoep* cells overexpressing Fgf4 transplanted into 5-hpf wild-type host embryos. (B) Representative picture of a chimeric embryo. (C) Graph showing the most anterior somite populated by transplanted *MZoep* cells. Note that Fgf overexpression causes 5-hpf *MZoep* cells to enter the posterior trunk (cf. Fig. 1A–C).

Figure 6. Tail MPCs express *ntl* but not *tbx24* prior to the 12-somite stage. (A–D) Expression of *ntl* in wild-type embryos. (E–H) Expression of *ntl* in *MZoepe* embryos. (I–L) Expression of *tbx24* in wild-type embryos. (M–P) Expression of *tbx24* in *MZoepe* embryos. A, E, I, and M are animal pole views with dorsal to the top. The other panels are posterior views with dorsal to the top.

hpf contribute to the posterior trunk and tail. Intriguingly, this subdivision of the zebrafish body is also observed with several zebrafish mutants; mutants in *integrin α 5* fail to properly form just the anterior trunk somites (Julich et al. 2005), whereas *MZoepe*, *ntl*, *spt*, and strong *bozozok;chordino* mutants (Ho and Kane 1990; Halpern et al. 1993; Griffin et al. 1998; Gritsman et al. 1999; Gonzalez et al. 2000), as well as embryos with perturbed dorsal organizer formation (Ober and Schulte-Merker 1999), have phenotypes that specifically affect either the trunk or tail somites. Thus, the timing of MPC commitment to the somites is likely to reflect a basic subdivision of the zebrafish body into three genetically distinct regions.

Regulation of the zebrafish body by *Bmps* and *Nodals*

We propose that the different domains within the somites are established through Nodal and Bmp signaling (Fig. 7A). Nodal signaling within the MPCs is necessary for cells to ingress during gastrulation (Carmany-Rampey and Schier 2001), and we show here that expression of a constitutively activate Nodal receptor causes *MZoepe* cells to contribute to the anterior trunk (Fig. 4C). This result shows that the only defect in *MZoepe* embryos with regards to forming trunk MPCs is due to a defect in the Nodal signaling pathway and not due to other possible roles of Oep (Warga and Kane 2003).

At the posterior end of the embryo, we show that Bmp is necessary to establish the trunk–tail boundary since *MZoepe* cells expressing a dominant-negative Bmp receptor no longer recognize this boundary (Fig. 4F). Whereas previous studies in zebrafish have shown that Bmp signaling is necessary for tail formation (Mullins et al. 1996; Ober and Schulte-Merker 1999; Gonzalez et al. 2000; Hammerschmidt and Mullins 2002; Agathon et al. 2003; Pyati et al. 2005; Connors et al. 2006), we show that Bmp signaling plays a critical role in precisely setting the boundary between the trunk and tail domains. This is in general agreement with previous studies demonstrating that overexpression of *Bmps* promotes posteriorization of the embryo, whereas inhibition of Bmp signaling results in anteriorization (for review, see Hammerschmidt and Mullins 2002).

A second important role of Bmp signaling is to regulate the morphogenesis of cells on the ventral side of the zebrafish embryo. Whereas dorsal and lateral cells converge toward the dorsal side and extend along the future anterior–posterior axis, the ventral cells, which will contribute many of the tail MPCs (Warga and Nüsslein-Volhard 1999; Carmany-Rampey and Schier 2001), exhibit a zone of no convergence–no extension behavior, and instead migrate to the vegetal pole of the embryo where the tailbud forms at the end of gastrulation (Myers et al. 2002). The specific morphogenetic properties of the ventral cells depends on high-level Bmp signaling (Myers et al. 2002). Thus, Bmp signaling not only determines exactly when the tail cells enter the somitogenesis program, but it also controls their morphogenesis so that they will end up in the tailbud, ready to contribute to the formation of the tail somites (Kanki and Ho 1997).

Figure 7. Model for the mesodermal progenitor cell commitment regulator. (A) Schematic diagram showing the three domains within the somites. The anterior trunk region (somites 1–8) requires a direct Nodal signaling input. The posterior trunk region (somites 9–15) requires a “trunk signal” that is activated by Nodal signaling. The tail region (somite 16 and beyond) requires a Bmp signal. (B) The MPC commitment regulator. In the presence of low Nodal and Bmp signaling, MPCs begin to enter at somite 9 (S9). High levels of Nodal signaling set the regulator for entry at somite 1 (S1), whereas high levels of Bmp signaling set the regulator for entry at somite 16 (S16).

Regulation of posterior trunk formation

Our data suggest that the posterior trunk is regulated by a trunk signal, which is activated by Nodal signaling since the trunk fails to form in *MZoe*p embryos (Fig. 7A). Since *MZoe*p cells can contribute to the trunk when Bmp signaling is inhibited, we suggest that the trunk signal includes several Bmp inhibitory molecules, which are known to work in an overlapping fashion (Khokha et al. 2005). In support of this hypothesis, *MZoe*p mutants fail to express *noggin1* and *folistatin* (Ragland and Raible 2004) and have reduced expression of *chordin* (Gritsman et al. 1999; Ragland and Raible 2004), supporting a role for Nodal signaling in regulating these Bmp inhibitors.

An additional set of candidates to regulate Bmp signaling are the Fgfs, which inhibit *bmp* transcription (Furthauer et al. 1997, 1999). *fgf8* is not expressed in *MZoe*p embryos until the mid-gastrula stages, whereas *fgf3* expression is initiated but not maintained in early *MZoe*p embryos and it is not expressed again until the mid-gastrula stages (Mathieu et al. 2004) (the expression of *fgf24* in *MZoe*p has not been reported). Together with reduced or absent expression of the Bmp inhibitors, this results in a reduction of dorsal organizer gene expression and an expanded expression of *bmp2b* and Bmp-regulated genes in *MZoe*p embryos (Gritsman et al. 1999; Ragland and Raible 2004). We therefore suggest that the Nodal-dependent trunk-promoting signal present in the late blastula wild-type embryo is composed of the Bmp inhibitors (Chordin, Noggin1, and Folistatin), which bind the Bmps and prevent them from interacting with their receptors, and the Fgfs, which regulate the transcription of the *bmps*.

Our heterochronic experiments indicate that the trunk signal is only present in wild-type embryos prior to 5 hpf. Thus if 4-hpf or 5-hpf *MZoe*p cells are transplanted into 4-hpf wild-type embryos, they can contribute to the trunk, whereas if the *MZoe*p cells are transplanted into 5-hpf wild-type embryos, they only contribute to the tail. It is important to note that when the *MZoe*p cells are transplanted into wild-type embryos, they randomly enter into different positions along the dorsoanterior–ventroposterior axis. Thus, some *MZoe*p cells will end up in the ventroposterior region where they are exposed to high levels of Bmp signaling, whereas other *MZoe*p cells will end up in lateral and dorsoanterior regions where they will be exposed to much lower levels of Bmp signaling. This is likely to explain why only some of the *MZoe*p cells transplanted into 4-hpf wild-type embryos contribute to the trunk.

We do not know why *MZoe*p cells transplanted into 5-hpf wild-type embryos are not converted to a trunk fate since the Bmp inhibitors continue to be expressed in embryos after 5 hpf. One possibility is that the length of time that MPCs are exposed to the inhibitors is critical, and that they first have to see the trunk signal prior to 5 hpf. However, it is also becoming increasingly clear that the regulation of Bmp signaling in the early vertebrate embryo is quite complex (for review, see Kimelman and

Szeto 2006). An intriguing possibility is that while the inhibitors are expressed after 5 hpf, their activity is diminished, and thus they are no longer able to convert the transplanted cells to a trunk fate. Understanding whether or not the Bmp inhibitors change in potency during development will be essential to distinguish between these ideas.

Regulation of MPC commitment to somitogenesis

Our data are consistent with a model in which the MPCs contain a regulator that instructs them when to first begin entering the somites (Fig. 7B). We suggest that high-level Nodal signaling drives the cells to enter at somite 1 (the anterior trunk), whereas high-level Bmp signaling instructs cells to begin entering at somite 16 (the tail). Since cells lacking both Nodal and Bmp signaling (*MZoe*p cells expressing the dominant-negative Bmp receptor) contribute to the posterior trunk, we suggest that cells that receive low levels of Nodal and Bmp signals are programmed to enter at somite 9. There is good evidence for a gastrula-stage gradient of Bmp signaling across the dorsoanterior–ventroposterior axis and a gradient of Nodal signaling across the animal–vegetal axis (for review, see Schier and Talbot 2005; Kimelman 2006), and we suggest that these overlapping gradients determine when each of the MPCs enters into the somitogenesis program. Before the regulator tells cells to begin entering the somites, cells wait in the Maturation Zone state until they are ready to enter the presomitic mesoderm, suggesting that the regulator acts by not only preventing cells from leaving the Maturation Zone, but also by blocking the expression of a subset of genes including *tbx24* and *WIF-1*. We note that the regulator does not tell cells exactly when to enter the presomitic mesoderm, but instead specifies when they can first begin entering. We suggest that the regulator plays an important role by preventing subsets of the MPCs from entering the somite-forming program early to ensure that there is a continuous supply of MPCs throughout somitogenesis to allow all of the somites to form. In the absence of Bmp signaling, for example, the tail muscles are absent since they have been converted to a trunk fate as shown in *Xenopus* studies (Lane et al. 2004), resulting in an expansion of the trunk muscles and severely deleterious development of the embryo. Thus, an interplay between Nodal and Bmp signals provides critical information to the MPCs, resulting in the formation of distinct domains within the zebrafish body musculature.

Materials and methods*Embryos and RNA injections*

Zebrafish embryos were obtained by natural spawning of adult AB/WIK wild-type and *MZoe*p mutant zebrafish. RNAs were synthesized from Asp718 linearized CS2-*dnBmpR* (Pyati et al. 2005), CS2-*zBmp2b* (Kishimoto et al. 1997), and CS2-*fgf4* templates using the mMessage Machine Kit (Ambion) and dissolved in RNase-free sterile water. RNA (at concentrations indicated in

the text) was injected into one- to two-cell stage zebrafish embryos. Injected embryos were collected at the appropriate stages for in situ hybridization analysis.

Cell transplantation

Single and double cell transplantations were performed at the various developmental stages indicated in text. Briefly, donor embryos were labeled at the one-cell stage with 2% (10,000 MW) fluorescein dextran or rhodamine dextran (Molecular Probes) and allowed to develop to the desired stage. Cells from a donor embryo were removed using a forged micropipette and transferred to the margin of an unlabeled wild-type host embryo. The resulting chimeric embryo was allowed to develop to 24 h. The location of the labeled donor cells within the trunk and tail regions was monitored under an epi-fluorescent microscope. Images were captured using a digital camera and stored as Adobe Photoshop files for manipulation and analysis.

Scoring chimeric embryos

After cell transplantation, chimeric embryos were allowed to develop for 24 h. Under an epi-fluorescent microscope, embryos with fluorescein-labeled *MZoe*p donor cells in the developing somites were identified and taken out for further evaluation. Each of these selected chimeric embryos was scored for the most anterior somite containing a fluorescein-labeled *MZoe*p donor cell. The somite number was plotted along the X-axis, and the number of chimeric embryos with a fluorescent cell at that position was plotted on the Y-axis.

In situ hybridization

Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense RNA probes and visualized using anti-digoxigenin Fab fragment conjugated to alkaline phosphatase (Roche Molecular Biochemicals) as described (Szeto and Kimelman 2004). Riboprobes were made from DNA templates, which were linearized and transcribed with either SP6 or T7 RNA polymerases. Embryos were processed and hybridized as described (Szeto and Kimelman 2004).

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