The differentiation and movement of presomitic mesoderm progenitor cells are controlled by Mesogenin 1

Rita Fior1,2,*, Adrienne A. Maxwell3, Taylur P. Ma4, Annalisa Vezzaro5, Cecilia B. Moens4, Sharon L. Amacher3,‡, Julian Lewis6 and Leonor Saúde1,2,*

SUMMARY
Somites are formed from the presomitic mesoderm (PSM) and give rise to the axial skeleton and skeletal muscles. The PSM is dynamic; somites are generated at the anterior end, while the posterior end is continually renewed with new cells entering from the tailbud progenitor region. Which genes control the conversion of tailbud progenitors into PSM and how is this process coordinated with cell movement? Using loss- and gain-of-function experiments and heat-shock transgenics we show in zebrafish that the transcription factor Mesogenin 1 (Msgn1), acting with Spadetail (Spt), has a central role. Msgn1 allows progression of the PSM differentiation program by switching off the progenitor maintenance genes ntl, wnt5a, wnt8 and fgf8 in the future PSM cells as they exit from the tailbud, and subsequently induces expression of PSM markers such as tbx24. msgn1 is itself positively regulated by Ntl/Wnt/Fgf, creating a negative-feedback loop that might be crucial to regulate homeostasis of the progenitor population until somitogenesis ends. Msgn1 drives not only the changes in gene expression in the nascent PSM cells but also the movements by which they stream out of the tailbud into the PSM. Loss of Msgn1 reduces the flux of cells out of the tailbud, producing smaller somites and an enlarged tailbud, and, by delaying exhaustion of the progenitor population, results in supernumerary tail somites. Through its combined effects on gene expression and cell movement, Msgn1 (with Spt) plays a key role both in genesis of the paraxial mesoderm and in maintenance of the progenitor population from which it derives.

KEY WORDS: Mesogenin 1, Spadetail (Tbx16), Paraxial mesoderm

INTRODUCTION
The axial skeleton and skeletal muscles arise from the somites. As somites form from the anterior end of the presomitic mesoderm (PSM), mesoderm progenitors in the tailbud continually generate new mesoderm cells and feed them into the posterior PSM (Holley, 2007). The number of progenitors and the rate at which their progeny differentiate and move from the tailbud into the PSM must be controlled to ensure that the correct somite number is reached. Premature exhaustion of progenitors results in premature extinction of the PSM, a deficit of posterior somites, and therefore a truncated body.

Several mouse mutants with a truncated axis have been described and all but one result from lack of mesoderm (Wilson et al., 2009). The one exception carries a null mutation in the bHLH transcription factor mesogenin 1 (Msgn1). It lacks thoracic, lumbar and sacral vertebrae and skeletal muscles (Yoon and Wold, 2000), but there is no lack of mesoderm progenitors – quite the opposite: the lack of PSM tissue is accompanied by an enlarged tailbud containing an excess of cells expressing brachyury – a mesodermal progenitor marker. This suggests that in the absence of Msgn1, mesoderm progenitors that should normally emerge to become PSM remain instead in the tailbud. What, then, is the precise function of Msgn1? Do the brachyury-expressing progenitors remain in the tailbud because of a block in differentiation, cell migration, or both? To investigate this question, we characterised Msgn1 function through loss- and gain-of-function experiments combined with live imaging in zebrafish.

In zebrafish, msgn1 is expressed in a domain similar to that in mouse (Yoo et al., 2003), but its function has not been described. However, the zebrafish spadetail (spt, now termed tbx16) mutant, like mouse Msgn1 mutants, shows a large accumulation of cells expressing the brachyury-like gene no tail (ntl) in the tailbud (Griffen et al., 1998; Griffin and Kimelman, 2002). It has been proposed that Spt promotes the differentiation of tailbud progenitors by inhibiting the progenitor maintenance genes ntl and Wnt (Griffin and Kimelman, 2002; Martin and Kimelman, 2008). Spt also controls cell movement during gastrulation (Ho and Kane, 1990; Kimmel et al., 1989; Row et al., 2011), suggesting that Spt might also control motility in the tailbud. However, Spt cannot be the only factor regulating the transition of tailbud progenitors into PSM because spt null mutants still form tail somites (Griffen et al., 1998). Msgn1 is thus a candidate additional factor in zebrafish responsible for the switch from a tailbud progenitor state to a PSM state.

We show that combined loss of msgn1 and spt leads to complete failure of trunk and tail somite formation accompanied by a large excess of ntl-expressing cells in the tailbud. Using a heat shock-inducible transgenic line, we find that a pulse of msgn1 expression causes a rapid downregulation of ntl and wnt8, indicating that these two genes, which work in an intricate positive-feedback loop with each other, are themselves negatively regulated by Msgn1. This Msgn1-induced downregulation of ntl and wnt8 expression is
followed by ectopic activation of an intermediate/anterior PSM marker, *tbx24* (also known as *fsx* and now termed *tbx6*), in the tailbud, consistent with the idea that Msgn1 throws a switch that converts cells from a tailbud progenitor state into a PSM state. *msgn1* expression is itself positively regulated by the *ntl*, *Wnt* and Fgf mesoderm progenitor maintenance genes (Griffin and Kimelman, 2002; Göering et al., 2003; Wittler et al., 2007; Wang et al., 2007; Morley et al., 2009; Garnett et al., 2009) (our data), which, by activating *msgn1* in a subset of the tailbud cell population, evidently trigger these cells to embark on the PSM differentiation pathway. We show that Msgn1 drives not only the differentiation but also the migration of such cells out of the tailbud into the PSM region. By governing the flux of cells from the progenitor region into the PSM, Msgn1 helps control both the size of somites and the size and persistence of the progenitor cell population; loss of Msgn1 activity thus gives rise to additional tail somites.

**MATERIALS AND METHODS**

Zebrafish lines and heat-shock experiments

Zebrafish lines: *msgn1* [a mutant found by screening ENU-mutagenised F1 fish (Draper et al., 2004)]; *spbt105* (Kimmel et al., 1988); *nkb105* (Halpern et al., 1993); *hsp70:dkk1-GFP* (Stoick-Cooker et al., 2007); and *hsp70:dnfgr1-EGFP* (Lee et al., 2005).

For all heat-shock experiments, embryos were raised at 25°C and heat shocked at 39°C for the indicated time. *hsp70:HA-msgn1, hsp70:dkk1-GFP* and *hsp70:dnfgr1-EGFP* embryos were generated from a cross between transgenic heterozygous and wild-type fish, giving batches with an expected mean ratio of 50% transgenics to 50% wild-type siblings. *hsp70:HA-msgn1* embryos were sorted into distinct phenotypic classes after in situ hybridisation (confirmed by genotyping) and *hsp70:dkk1-GFP* and *hsp70:dnfgr1-EGFP* embryos were sorted by GFP expression.

DNA constructs

*msgn1* cDNA was amplified from a zebrafish EST (IMAGE:7286125) with primers (5′-3′) pFWecoRI (CCGGAATTCATGGCGCAAATCG-ACGTGGATG) and pRxbal (CTAGTCTAGATCATCTGCTGTCGAAGGATGCC) and cloned into the EcoRI and XbaI sites of pCS2+ and NotI/SphI was used to produce *msgn1* poly(A)-capped RNA and CtaI/T7 to produce an antisense RNA probe.

The *hsp70:HA-msgn1* transgenic was created by placing *msgn1* cDNA containing an N-terminal HA tag downstream of the *hsp70* heat-shock promoter in the pT2 vector (UAS-*hsp70*-polyA-β-crystallin promoter-CFP) using primers pFW-HA-Cla-Kozak (CCATCGATGCCCACCT-ATGGCTTCATATCCTTACGATG) and pRStuI (AAAAGGCCTTTTC-CTTTTCTTTTCTTTTTTGGAGA). The resulting PCR product was cloned in a subset of the tailbud cell population, evidently trigger these cells to embark on the PSM differentiation pathway. We show that Msgn1 drives not only the differentiation but also the migration of such cells out of the tailbud into the PSM region. By governing the flux of cells from the progenitor region into the PSM, Msgn1 helps control both the size of somites and the size and persistence of the progenitor cell population; loss of Msgn1 activity thus gives rise to additional tail somites.

### RESULTS

#### Depletion of *msgn1* and *spbt* leads to complete loss of trunk and tail somites

To uncover the role of *msgn1* in zebrafish, we examined the embryonic phenotype in loss-of-function experiments using a translation-blocking morpholino (*msgn1*MO) (supplementary material Fig. S1A). *msgn1*MO-injected embryos showed increased *ntl* expression in the tailbud when compared with control siblings (Fig. 1A’,B’), as does the mouse *Msgn1* mutant (Yoon and Wold, 2000). However, somites formed in zebrafish *msgn1* morphants (Fig. 1B’,B’), in contrast to the mouse mutant, in which somite formation is abolished. To validate the *msgn1* knockdown, we analysed a zebrafish *msgn1* nonsense mutant allele, *msgn1*fh273 (supplementary material Fig. S1B). Homozygous *msgn1*fh273 mutants are viable and have a phenotype indistinguishable from that of the *msgn1* morphants (compare Fig. 1B’ with 1F’). The mildness of the *msgn1*fh273 phenotype is not due to a maternal contribution, as no maternal *msgn1* mRNA was detected either by in situ hybridisation or by RT-PCR and maternal-zygotic and zygotic *msgn1*fh273 mutants had indistinguishable embryonic phenotypes (data not shown). Another possible explanation for the mildness of the phenotype is a second Msgn gene, which is not unlikely given the genome duplication event in teleosts. However, we could not find any evidence for such a gene duplication.

The enlarged population of ntl-expressing tailbud cells in *msgn1* morphants and mutants is similar to that seen in *spbt* mutants,
although less severe (Fig. 1C′) (Griffin and Kimelman, 2002). To test the hypothesis that Msgn1 and Spt function collaboratively, we injected msgn1MO into embryos derived from a cross of spt heterozygotes and we also generated msgn1−;spt− double mutants. In both cases, the combined loss of Msgn1 and Spt led to a complete failure of trunk and tail somite formation along with a greatly enlarged tailbud that was full of ntl-expressing cells (Fig. 1D′;H′). This severe phenotype is very similar to that described for the Msgn1 mouse mutant and strongly suggests that cells that should have emerged to form PSM remained instead in the tailbud progenitor region in an immature state. As further evidence of a shared function of msgn1 and spt, loss of one copy of spt in an msgn1 mutant or morphant background led to an enhanced msgn1 phenotype (Fig. 1G−G′; data not shown).

In wild-type embryos, the intermediate/anterior PSM is marked by expression of tbx24 throughout somitogenesis; in spt− single mutants, tbx24 expression is initially defective but is restored around the 14-somite stage, correlating with the recovery of somitogenesis at this stage in these mutants (Griffin and Kimelman, 2002) (Fig. 1C′). By contrast, the combined absence of Msgn1 and Spt leads to a sustained loss of expression of tbx24 (Fig. 1D′;H′) and mespaa (Fig. 1H′), suggesting that embryos lacking Msgn1 and Spt function fail to generate somites because their cells are unable to progress along the PSM differentiation pathway.

Our data reveal a PSM formation program that differs between zebrafish and mouse. In the mouse, Msgn1 is required for both trunk and tail somite formation, but in zebrafish Spt is required for trunk somite formation and Msgn1 is not, whereas tail somite formation depends on both Msgn1 and Spt. To establish whether Msgn1 has a similar genetic relationship with Ntl during somitogenesis, we generated msgn1−;ntl− double mutants; these did not display any enhancement of the ntl phenotype, suggesting that Msgn1 works downstream of Ntl and not in parallel to it (supplementary material Fig. S2).

Fig. 1. Msgn1 and Spt are essential for tail somite formation. (A−D,E−H) Live zebrafish embryos typical of their genotypic classes. (A′−D′,E′−H′) In situ hybridisation for ntl and cb1045 (xirp2a), myoD (myod1), tbx24 and mespaa in uninjected wild-type (wt) siblings and in the genotypes indicated. spt− mutants were derived from a heterozygous spt+ cross. msgn1− mutants, spt−;msgn1− mutants and spt−;msgn1+ double mutants were derived from a double heterozygous msgn1+;spt+ cross. Indicated is the number of embryos (n) observed with the phenotype shown in each panel, and when embryos were derived from mutant crosses the obtained n corresponded to the expected frequencies for each genotype. Asterisk indicates that wt and msgn1 mutants have an undistinguishable phenotype at the 14-somite stage.

Msgn1 regulates the transition from the tailbud maturation zone to the PSM
To determine the step of the PSM differentiation pathway at which Msgn1 acts, we analysed the msgn1 morphant phenotype and compared this with the phenotype obtained when msgn1 was overexpressed.

During normal development, mesoderm progenitors located in the dorsal tailbud region, which is known as the progenitor zone (PZ), express ntl and wnt8 (Griffin and Kimelman, 2002) (Fig. 2A, dorsal view). The progeny of these cells that are destined to become PSM move ventrally to enter a so-called maturation zone (MZ), where they express msgn1, spt and tbx6l, in addition to ntl (Kanki and Ho, 1997; Griffin and Kimelman, 2002) (Fig. 2A ventral view, 2B′). When cells reach the posterior PSM, they downregulate ntl expression but maintain expression of msgn1, spt and tbx6l (Griffin and Kimelman, 2002; Amacher et al., 2002). A little later still, as cells become displaced from the posterior to the intermediate PSM, they start to express tbx24 and will continue to do so until the somite border is completed (Nikaido et al., 2002) (Fig. 2A).

In msgn1 morphants, the tailbud domain marked by ntl and wnt8 was clearly expanded in comparison with control siblings (Fig. 2C−F′). Conversely, when msgn1 was overexpressed by mRNA injection at the one-cell stage, expression of ntl and wnt8 was severely reduced and lost prematurely (Fig. 2G−J′), followed later by a severely truncated tailbud (supplementary material Fig. S3L). Strikingly, msgn1 overexpression led also to loss of the notochord as seen both by morphology and loss of midline ntl expression (Fig. 2H,H′; supplementary material Fig. S3L).

Double fluorescent in situ hybridisation for ntl and tbx6l showed that the tailbud PZ (identified by the expression of ntl but not tbx6l) and the MZ (located more deeply and identified by ntl and tbx6l co-expression) were both expanded in the absence of Msgn1 (Fig. 2K−L′). These data suggest that in the absence of Msgn1 there is a
 reduction both in the flux of cells from the PZ state into the MZ state and in the flux from the MZ state into a PSM state: tailbud progenitors fail to progress normally through the PSM differentiation program. Consistent with this idea, when msgn1 was overexpressed, the converse effect was seen: expression of tbx24 was ectopically activated in the tailbud region (Fig. 2N), suggesting premature differentiation of mesoderm progenitors.

**Msgn1 inhibits the Wnt/Ntl/Fgf loop and promotes progression along the PSM differentiation pathway**

To clarify the dynamics of the regulatory interactions among msgn1, the tailbud progenitor marker genes wnt3a, wnt8, ntl and fgf8, and the PSM-specific genes tbx24 and tbx6l, we created a zebrafish line containing a heat shock-inducible HA-tagged msgn1 transgene (hsp70:HA-msgn1) that allowed us to activate msgn1 in a time-controlled manner (supplementary material Fig. S4A-K').

hsp70:HA-msgn1 transgenics were heat shocked during segmentation, at a stage corresponding to the time when cells located in the tailbud are fated to contribute to trunk (supplementary material Fig. S4L-N') or tail somites (Fig. 3). We observed a consistent and marked reduction of the levels of ntl and wnt8, and, to a lesser extent, of wnt3a and fgf8, in the tailbud of the hsp70:HA-msgn1 transgenics as early as 1 hour post heat shock (hpHS) (Fig. 3A-D'), suggesting that these genes are direct downstream targets of Msgn1. The effect was most rapid and striking for ntl, which was almost completely undetectable, whereas tailbud expression of wnt3a, wnt8 and fgf8 persisted at a reduced level for longer, but disappeared completely by 7 hpHS (Fig. 3H-J').

Interestingly, in embryos fixed 7 hpHS, tailbud expression of msgn1 itself was downregulated (supplementary material Fig. S4E'), suggesting that Msgn1 exerts a delayed negative feedback on its own expression. This is probably mediated through ntl, Wnt and/or Fgf genes: whereas Msgn1 has an inhibitory action on this set of genes, they themselves are known to be required for msgn1 expression, not only during gastrulation (Griffin and Kimelman, 2002; Goering et al., 2003; Morley et al., 2009) but also during segmentation/tailbud stages (supplementary material Fig. S5C-F'). Moreover, this is consistent with the subtle but distinct expansion of msgn1 mRNA expression in msgn1 loss-of-function mutants (supplementary material Fig. S5A-A').

Further insight into Msgn1 function comes from the timecourse of expression of tbx24 after ectopic activation of Msgn1 expression, which is normally restricted to the intermediate/anterior PSM region (Fig. 3E,K,L). At 2 hpHS, tbx24 expression was enhanced in its normal domain and was ectopically induced in the somites (Fig. 3E'). Strikingly, at 7 hpHS, we could detect tbx24 expression also in the tailbud and midline of hsp70:HA-msgn1 transgenics (Fig. 3K'); this expanded expression coincided with the time at which tailbud expression of ntl, wnt8, wnt3a and fgf8 was completely abolished (Fig. 3G'-J'). The implication is that, in normal development, Msgn1 drives cells along the pathway of PSM differentiation, but that the progression to an
intermediate/anterior PSM state depends on escape from the influence of Ntl/Wnt/Fgf. This conclusion is further supported by our finding that, when Wnt signalling was transiently inhibited in hsp70:dkk1 transgenics (Gmsgn1G-mRNA), ventral diving of tailbud cells can be compared to the internalisation of germ ring cells during gastrulation, where an epithelial-to-mesenchymal (EMT)-like transition takes place (Marlow et al., 2004; Solnica-Krezel, 2006). We hypothesized that Msgn1 might regulate these EMT-like movements of tailbud cells during segmentation by regulating Snail expression or activity, as the Snail transcription factor family plays a role in EMT initiation in several contexts (Solnica-Krezel, 2006). We next investigated how Msgn1 might control cell movements. The Msgn1-regulated cell movement may be mediated through negative regulation of snail1a

We next investigated how Msgn1 might control cell movements. The ventral diving of tailbud cells can be compared to the internalisation of germ ring cells during gastrulation, where an epithelial-to-mesenchymal (EMT)-like transition takes place (Marlow et al., 2004; Solnica-Krezel, 2006). We hypothesized that Msgn1 might regulate these EMT-like movements of tailbud cells during segmentation by regulating Snail expression or activity, as the Snail transcription factor family plays a role in EMT initiation in several contexts (Nieto, 2002). We investigated the expression of snail1a (also known as snail1a) – the only member of the zebrafish Snail family that is expressed in the tailbud (Blanco et al., 2007). During normal development, snail1a is strongly expressed in the MZ and fades in the PSM in a pattern that is largely complementary to, and non-overlapping with, that of msgn1 (Fig. 6A-A’). We found that, in the
absence of msgn1, snail1a expression was upregulated and expanded anteriorly (Fig. 6D,E); a similar expansion has been reported in spt mutants (Thissé et al., 1993). Conversely, when msgn1 was overexpressed by a short heat shock during segmentation, snail1a expression was severely and quickly downregulated 1 h pHS (Fig. 6G).

Since Msgn1 regulates snail1a expression during segmentation, we investigated the role of Snail1a in the control of tailbud cell movements. We followed Kaede photoconverted tailbud cells in embryos injected with snail1aMO (Blanco et al., 2007), comparing these with controls and with msgn1MO-injected embryos. In the absence of snail1a, the ventral diving movement from the superficial MZ (Fig. 6H, dot-1) was defective: the mean diving velocity was 0.05 µm/minute (s.d.=0.09, n=37 cells/4 embryos), significantly different from controls (t-test, P<10⁻⁸) (Fig. 6I,J; supplementary material Movies 5, 6). The similarity between this result and the defective ventral diving observed in msgn1MO-injected embryos (supplementary material Movie 3), in which there is an excess of snail1a expression, suggests that a balanced level of Snail1a – not too much and not too little – is crucial for properly directed ventral diving movements in the MZ.

To further explore the role of msgn1 and snail1a in A/P PSM extension, we marked and tracked Kaede photoconverted cells in the anterior and posterior PSM (Fig. 6H, dot-3 and dot-2). We converted our raw measurements of cell positions as a function of time into mean A/P velocities (V_{ap}) of the dot-2 cells relative to the dot-3 cells, reflecting the rate at which the intervening PSM tissue was extending or contracting along the A/P axis. Our data show that the A/P extension rate is significantly reduced in the absence of Msgn1 [Fig. 6K-L’, V_{ap} controls=0.15±0.05 µm/minute (dot-2 n=26 cells, dot-3 n=22 cells, 3 embryos); supplementary material Movies 7, 8 versus Fig. 6M-N’, V_{ap} msgn1MO=0.06±0.02 µm/minute (dot-2 n=22 cells, dot-3 n=22 cells, 3 embryos); t-test, P=0.0077; supplementary material Movies 9, 10] and significantly increased in the absence of Snail1a [Fig. 6O-P’, V_{ap} snail1aMO=0.27±0.05 µm/minute (dot-2 n=31 cells, dot-3 n=37 cells, 4 embryos); t-test, P<10⁻⁷; supplementary material Movies 11, 12]. Thus, Msgn1, possibly through Snail1a, controls cell movement not only at the point of origin of the paraxial mesoderm in the tailbud but also subsequently as the PSM cells mature. However, since the phenotype of loss of snail1a is very mild (supplementary material Fig. S6) (Blanco et al., 2007), it is likely that other factors are involved downstream of Msgn1.
Lack of Msgn1 leads to an increase in the number of tail somites accompanied by a reduction in their size

We have shown that, in msgn1 mutants/morphants and in msgn1 mutants lacking one copy of spt, somitogenesis is not blocked but that increased numbers of ntl/wnt8-positive progenitor cells are retained in the tailbud, suggesting that progenitor cells might persist there for an abnormally long time. If so, one might expect that somitogenesis would be prolonged, leading to an increase in the total number of somites produced. To test this, we counted the number of somites formed using the somitic boundary probe cb1045 (now termed xirp2a). Strikingly, in the absence of msgn1 or in the msgn1 enhanced phenotype (msgn1–/–;spt+/–), there was a significant increase in the number of somites formed (Fig. 7A,B,E-I). Whereas wild-type embryos produce on average 31.5 somites (s.d.=0.75, n=33), msgn1–/– mutants make 33 (s.d.=0.54, n=14; t-test, P=0.002) and msgn1–/–;spt+/– mutants make on average 33.8 somites (s.d.=0.8, n=30; t-test, P=0.00001). msgn1 morphants showed a very similar phenotype to msgn1 mutants, with an increase in the average number of somites formed (mean=32.7, s.d.=1.18, n=23; t-test, P=0.006), whereas their wild-type siblings formed on average 31.6 somites (s.d.=0.72, n=23). The additional somites, in all these cases, were tiny and appeared as an extension of somitogenesis at the extreme tail end of the embryo (Fig. 7E-H), reflecting the abnormal persistence of a small population of progenitors there. We also saw effects on the pattern of somites more anteriorly, however: in the absence of msgn1, somites in the trunk and tail regions of the embryo were on average 15% smaller than in wild-type controls (t-test, P=0.002; Fig. 7C,D). This fits with the other indications that cells were being recruited into the PSM from the tailbud at a reduced rate during formation of the trunk and tail somites. To our knowledge, our findings in msgn1 single and msgn1–/–;spt+/– mutants represent the first experimental examples of genetic perturbations that leads to an increase in somite number.

DISCUSSION

As somites are being formed from the anterior region of the PSM, mesodermal progenitors have to constantly feed new cells into the posterior PSM. The numbers of progenitors and the rate at which they differentiate and move out from the tailbud to feed the PSM have to be tightly controlled, with termination of the process only when the correct species-specific number of somites is reached. With too fast a rate of exit, or too slow a proliferation of progenitors, the PSM would be prematurely extinguished and the body axis would be truncated. How is the balance between
differentiation and progenitor maintenance achieved and how is this coordinated with cell movement? In this work, we propose that Msgn1, acting in a semi-redundant fashion with Spt, plays a crucial role in controlling these processes (Fig. 8).

**Msgn1 is required redundantly with Spt for tail formation in zebrafish**

Loss of msgn1 function in zebrafish causes a mild phenotype that is reminiscent of that observed in the mouse. In the absence of msgn1, the characteristic feature of retention of ntl/Wnt-positive progenitor cells in the tailbud is observed, although somites continue to be formed. However, when we generated msgn1/spt double mutants and morphants the tailbud was hugely enlarged and both trunk and tail somites fail to form (Fig. 1D-D’). These results reveal that spt and msgn1 act redundantly in tail somite formation in zebrafish: either gene alone is sufficient to support the tail process. Although msgn1 has been shown to be a Spt target during gastrulation (Garnett et al., 2009), our results show that msgn1 cannot depend entirely on Spt for its activation but instead must act in parallel with Spt during tail formation.

**Msgn1, like Spt, regulates the transition from the tailbud to the PSM by switching off the expression of progenitor genes**

Previous work (Griffin and Kimelman, 2002) suggested that, for PSM progenitors to progress from the tailbud to the PSM, they must downregulate progenitor markers such as ntl and wnt8, and that Spt contributes to this regulation. To investigate whether Msgn1 is acting in a similar manner to Spt, we combined loss-of-function analysis with gain-of-function studies. We found that when msgn1 is overexpressed ectopically, the ntl/wnt8-expressing progenitors are severely reduced and the anterior PSM marker tbx24 is ectopically expressed in the tailbud (Fig. 2H,H’,J,J’,N). These results strongly suggest that Msgn1 is indeed acting like Spt (Griffin and Kimelman, 2002), inhibiting expression of the progenitor markers to allow cells to progress along the PSM differentiation pathway.

When we temporally controlled Msgn1 expression using our inducible msgn1 transgenic, we observed rapid and strong downregulation of ntl and wnt8 expression (Fig. 3A‘,B’,C’), indicating that Msgn1 can rapidly inhibit the expression of these genes. Msgn1 does not have a readily identifiable repressor domain and is thought to be a transcriptional activator (Yabe and Takada, 2012), but it might exert repression via the rapid transcriptional activation of a repressor, or, for example, by dimerising with, and blocking the action of, some other bHLH family activator.

Use of the msgn1 transgenic line also allowed us to show, for the first time, that the inhibition of progenitor character is essential for progression to the next step, i.e. the activation of the intermediate PSM marker tbx24. In fact, we show that Msgn1 can only ectopically activate tbx24 in the tailbud (Fig. 3K’,L’) after a time delay that corresponds to the time required for the downregulation of ntl, Wnt and Fgf progenitor genes in the tailbud. This is further supported by the posterior expansion of tbx24 upon transient inhibition of Wnt signalling during segmentation (Fig. 4).

**msgn1 and the ntl, Wnt and Fgf genes are coupled in a negative-feedback loop**

During segmentation, Ntl, Wnt and Fgf positively regulate msgn1 expression (supplementary material Fig. S5) (Griffin and Kimelman, 2002; Goering et al., 2003; Wittler et al., 2007; Wang et al., 2007; Morley et al., 2009; Garnett et al., 2009), and we have shown that, in turn, Msgn1 represses ntl, Wnt and Fgf genes (Fig. 3), thereby establishing a negative-feedback loop.

How does this relate to the phenotypes that we see upon gain or loss of msgn1 function? Our data show that, in the absence of msgn1, progenitors are retained longer in the PZ and MZ, as shown by ntl and tbx6l double in situ hybridisation. Normally, msgn1 only starts to be expressed in the MZ and is not seen in the PZ, consistent with a role for Msgn1 in promoting the transition from the MZ to the PSM. But if Msgn1 is normally only expressed in the MZ and PSM, why does its loss cause an expansion of the PZ (Fig. 2L)? One possible explanation is based on the msgn1/ntl/Wnt/Fgf gene regulatory circuitry and the action of Wnt
have shown here, Spt and Msgn1 share partially redundant functions, suggesting that both Msgn1 and Spt might repress snail1a to complete the EMT-like movement in the tailbud.

Our results also show that Msgn1, through repression of snail1a, contributes to axis extension: in the absence of msgn1 (expansion of snail1a) axis extension is reduced, whereas when snail1a is downregulated axis extension is increased. Why would stopping a motile state be important to promote axis elongation? A possible clue comes from recent work in the chick embryo that proposed that proper axis extension is dependent on a PSM cell motility gradient, i.e. effective A/P axis extension only occurs if the PSM cells reduce their motility as they become displaced anteriorly (Bénazéraf et al., 2010). Note that in zebrafish a similar gradient of cell motility has also been described in the PSM (Mara et al., 2007), reflecting the progressive epithelialisation of somite formation. In this scenario, following the ventral diving movement, efficient termination of Snail1a activity regulated by Msgn1/Spt could be required for the progressive reduction of PSM cell motility leading to efficient A/P axis extension in zebrafish.

**Msgn1 regulates the number of somites by controlling the flux of cells out of the tailbud**

According to the clock and wavefront model (reviewed by Dequéant and Pourquié, 2008), somite size should be proportional to the number of cells entering the PSM in each oscillation cycle of the segmentation clock, and the total number of somites should be equal to the total time for which production of PSM cells continues, divided by the length of that cycle. Our data fit these expectations, if we assume that the clock continues to tick at its normal rate in our mutants and morphants. Thus, for example, in the absence of Msgn1, where there is a reduced flux of cells from tailbud to PSM, somites are abnormally small (Fig. 7C,D). Moreover, retention of cells in the tailbud in msgn1–/– and msgn1–/–; spt–/– mutants delays exhaustion of the stock of progenitors and allows somitogenesis to continue for longer than normal, leading to an increase in the final number of somites (Fig. 7A,B). To our knowledge, this is the first report of mutants that have an increased somite number. Although the effect that we see is small, our data suggest that modulation of the rate of exit of cells from the tailbud zone might be a strategy used during evolution to create the different species-specific somite numbers observed across vertebrates.

In summary, Msgn1, together with Spt, plays a central role in the production of paraxial mesoderm, controlling both a switch of cell character and cell movement, thereby propelling the transition from tailbud into PSM and driving the subsequent program of PSM differentiation.

**Acknowledgements**

We thank Stephen Wilson, David Kimelman and Randall Moon for snail1a, ntl and wnt8 plasmids, respectively; Christian Tendeng for the pT2 vector; Ben Martin for the hsp70;ntl and hsp70;caFkcatenin DNA constructs; the Yamaguchi, Feldman and Takada labs for sharing data prior to publication; Lara Carvalho, Fábio Valério, Jen St Hilaire, Deborah Weinman and Keely Yamaguchi, Feldman and Takada labs for sharing data prior to publication; Ana Margarida Cristovão, Pedro Henriques and Ana Margarida Cristovão and Pedro Henriques for technical advice; and Ana Margarida Cristovão and Pedro Henriques for technical support.

**Funding**

This work was supported by a Fundação para a Ciência e a Tecnologia (FCT) grant [PTDC/SAU-OB/101282/2008 to L.S.]; an FCT Fellowship [SFRH/BPD/28586/2006 to R.F.]; Cancer Research UK (J.L.); a National Institutes of Health/National Institute of General Medical Sciences (NIH/NIGMS) grant [GM061952] and ARRA supplement (S.L.A.) and March of Dimes grant [1-FY09-458] (S.L.A.). A.A.M. is funded by an NIH Research Supplement.
The TILLING allele was found with support from NIH grant R01 HG002995. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org lookup supp: doi:10.1242/dev.078923/DC1

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