Rewiring of an ancestral Tbx1/10-Ebf-Mrf network for pharyngeal muscle specification in distinct embryonic lineages

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ABSTRACT

Skeletal muscles arise from diverse embryonic origins in vertebrates, yet converge on extensively shared regulatory programs that require muscle regulatory factor (MRF)-family genes. Myogenesis in the tail of the simple chordate Ciona exhibits a similar reliance on its single MRF-family gene, and diverse mechanisms activate Ci-Mrf. Here, we show that myogenesis in the atrial siphon muscles (ASMs) and oral siphon muscles (OSMs), which control the exhalant and inhalant siphons, respectively, also requires Mrf. We characterize the ontogeny of OSM progenitors and compare the molecular basis of Mrf activation in OSM versus ASM. In both muscle types, Ebf and Tbx1/10 are expressed and function upstream of Mrf. However, we demonstrate that regulatory relationships between Tbx1/10, Ebf and Mrf differ between the OSM and ASM lineages. We propose that Tbx1, Ebf and Mrf homologs form an ancient conserved regulatory state for pharyngeal muscle specification, whereas their regulatory relationships might be more evolutionarily variable.

KEY WORDS: Development, Evolution, Fate mapping, Gene regulation, Muscles

INTRODUCTION

In vertebrates, skeletal muscle development requires one or more of the four muscle regulatory factor (MRF)-family basic helix-loop-helix (bHLH) transcription factors, MyoD, Myf5, Mrf4 (also known as Myf6) and myogenin (Block and Miller, 1992; Rudnicki et al., 1993; Braun et al., 1994; Summerbell et al., 2002; Kassar-Duchossoy et al., 2004). Muscle identity is determined by the cooperation of Myf5, Mrf4 and MyoD (Braun et al., 1994; Summerbell et al., 2002; Kassar-Duchossoy et al., 2004), whereas myogenin is required for differentiation in all parts of the head and body (Nabeshima et al., 1993; Hasty et al., 1993). By contrast, MRF-family genes play a less central role in development of skeletal muscle-like muscles in various non-chordate clades (Andrikou and Arnone, 2015; Dobi et al., 2015; Moncaut et al., 2013). The sole Drosophila MRF-family homologue, nautilus (nau), is myogenic when expressed ectopically (Keller et al., 1997), but it is only expressed in a subset of founder cell myoblasts (Michelson et al., 1990), and it is required in vivo for founder cell patterning of the somatic musculature (Wei et al., 2007). In Caenorhabditis elegans, the sole MRF homologue, hkh-1, is also restricted to a subset of muscles (Fukushige et al., 2006), indicating that its function might not always be required for muscle development (Fukushige and Krause, 2005).

The factors that specify myogenic potential upstream of the core MRF-based network in vertebrates differ between regions of the embryo, and some of these factors also have a role during invertebrate myogenesis. Specific comparisons of gene families employed in vertebrate and invertebrate myogenesis have been reviewed extensively elsewhere (Buckingham and Relaix, 2007; Ciglar and Furlong, 2009; Moncaut et al., 2013; Andrikou and Arnone, 2015). Here, we analyze the expression dynamics and functions of Tbx1 and Ebf homologues in the specification of two clonally distinct populations of pharyngeal muscle precursors in the simple chordate, Ciona. In vertebrates, Tbx1 is involved in activating MRF genes in the pharyngeal mesoderm (Kelly et al., 2004). In Drosophila, the Tbx1 homologue, Org-1, activates muscle-specific transcription factors temporally downstream of nau in several somatic muscles (Schaub et al., 2012), and acts independently of nau in alary muscle development (Boukhmati et al., 2014). collier (also known as knot), the Drosophila homolog and founding member of the COE (Collier/Olfactory-I/Early B-Cell Factor) family of transcription factors, cooperates with Nau to cause differentiation of a distinct subset of muscles from those that require Org-1 (Enriquez et al., 2012; Dubois et al., 2007). In development of Xenopus hypaxial muscles, the COE homolog Ebf3 targets MyoD and Myf5, as well as muscle-specific functional genes such as m-cadherin (Green and Vetter, 2011). Ebf3 is also a necessary co-factor to MyoD in promoting muscle-specific gene expression in mice (Jin et al., 2014). Taken together, these observations provide evidence that Tbx1, COE and MRF orthologs’ roles in myogenesis have ancient origins, although their exact functions seem to vary between species and muscle types.

As part of the sister group to the vertebrates (Delsuc et al., 2006), the simple chordate Ciona is well-positioned phylogenetically to elucidate the transition from the ‘dispensable-MRF’ mode of myogenic regulation observed in invertebrates to the MRF-dependent mode observed in vertebrates. As is the case in other invertebrates, the Ciona genome encodes a single MRF-family bHLH transcription factor. However, as is the case in vertebrates, Mrf is expressed in all committed muscle precursors: the primary and secondary tail muscles, atrial siphon muscles (ASMs) and oral siphon muscles (OSMs) (Meechel et al., 2007; Razy-Krajka et al., 2014). Another similarity with vertebrates is that Mrf expression and muscle differentiation in Ciona seem to be regulated differently depending on the embryonic origin of the muscle cells. Ciona tail muscles derive from three different embryonic lineages. In the primary muscles, which are committed first, differentiation depends on the maternal transcription factor Zic-r.a (also known as Macho-1), which, together with Tbx6b and Tbx6c, activates many muscle-specific genes during gastrulation, including Mrf (Yagi et al., 2005, 2004). In the two secondary muscle lineages, differentiation begins later in development and depends on Nodal signals from
neighboring cells (Hudson and Yasuo, 2005). Despite different regulatory inputs, Mrf regulates gene expression during differentiation of tail muscles (Kusakabe et al., 2004; Brown et al., 2007; Meedel et al., 2007; Iizumi et al., 2013). The mechanisms of differentiation in the OSM and ASM, as well as the role of Mrf in myogenesis in those contexts, have not been explored.

Oral and atrial siphon muscles derive wholly from either one of two lineages: the ASMs derive from the B7.5 lineage, whereas the OSMs derive from the A7.6 lineage (Fig. 1A). In the B7.5 lineage, Mrf is first transiently expressed in the B7.5 pair of blastomeres and in their daughter cells, the B8.9 and B8.10 founder cells. After differential fate specification of the anterior tail muscles (ATMs) versus the cardiopharyngeal progenitors (called trunk ventral cells, TVCs), Mrf expression is maintained only in the ATMs (Christiaen et al., 2008). Mrf is later re-activated downstream of Ebf specifically in ASM founder cells (ASMFs, Fig. 1B). ASMFs give rise to Mrf+ differentiating muscle cells as well as Bhlh-tun1+/Mrf- stem-like muscle precursors, which activate the orphan and tunicate-specific helix-loop-helix transcription factor Bhlh-tun1 (renamed after orphan-bhlh-1 following Stolfi et al., 2015), and cease to express Mrf in response to Notch-mediated activation of the conserved transcriptional repressor of the Hairy/Enhancer-of-split family, Hes-b (Fig. 1B). The progeny of these Bhlh-tun1-expressing cells reactivate Mrf after metamorphosis and give rise to the body wall muscles of the juvenile (Razy-Krajka et al., 2014).

The A7.6 lineage is also multipotent, but not cardiogenic. Instead, it produces blood and tunic cells, as well as stomach and gill slit epithelia in addition to OSMs (Hirano and Nishida, 1997; Tokuoka et al., 2005). Previous in situ analyses of ASM-specific gene expression indicated that most ASM-expressed genes, including the key regulators Ebf and Mrf, are also active in the OSM (Razy-Krajka et al., 2014). We sought to characterize the lineage of A7.6-derived OSM precursors, as well as regulatory mechanisms leading to the activation of a core siphon muscle program in the OSMs.

Using a defined combination of LexA, LexAop and Gal80 transgenes, we report a detailed description of the ontogeny of the A7.6 lineage, the progeny of which have previously been referred to as the trunk lateral cells (TLCs; Tokuoka et al., 2004, 2005; Imai et al., 2003; Satou et al., 2001). We identified each stereotypical division of the anterior-most descendants of the TLC, leading to the birth of two sister, fate-restricted, OSM founder cells (OSMFs), on either side of the embryo. We find that OSM commitment is defined by expression of Ebf and Tbx1/10 exclusively in the OSMFs, which initiate Mrf expression, but produce a mixed population of OSM precursors (OSMPs) expressing either Mrf or Bhlh-tun1, as is the case in differentiating or stem-cell-like ASMPs, respectively (Razy-Krajka et al., 2014). We then demonstrate that, as is the case in tail muscles, Mrf is necessary for both OSM and ASM differentiation.

Next we show that, in contrast to what has been shown in the ASM, Mrf expression in the OSM rudiment depends on the joint activities of Ebf and Tbx1/10. Thus, whereas Tbx1/10 regulates Ebf in the ASM, where Ebf is necessary and sufficient for ASM fate, Ebf is regulated independently of Tbx1/10 in the OSM, where both are required jointly for Mrf expression. Our findings reveal context-dependent rewiring of a deeply conserved kernel of muscle specification genes within a single genome.

RESULTS
LexA/LexAop is a non-toxic, efficient, penetrant and Gal80-repressible tissue-specific transgenic marker in Ciona

Many studies of gene function in Ciona are based on transient transgenesis by electroporation of plasmid DNA (Corbo et al., 1997; Christiaen et al., 2009a; Stolfi and Christiaen, 2012). Constructs carrying a reporter driven by up to 5 kb of any promoter/enhancer region derived from a gene of interest generally recapitulate endogenous gene expression. Enhancers can be dissected to identify functional elements, as well as to refine patterns of reporter expression (Wang and Christiaen, 2012). Perdurance of recombinant reporter proteins also makes such techniques useful for clonal fate mapping; however, pleiotropic enhancer activity often leads to reporter expression in more than one lineage, which makes results difficult to analyze. Therefore, we adapted the LexA/LexAop system, which is analogous to the Gal4/Gal80/UAS system (Brand and Perrimon, 1993; Suster et al., 2004; Yagi et al., 2010) for use in Ciona, in order to mark exclusively the A7.6 blastomere and its descendants throughout Ciona development.

Fig. 1. Embryonic origins of oral and atrial siphon muscles. (A) Schematic diagram of 110-cell stage embryo, tailbud embryo and larva, with the A7.6 pair of blastomeres, and their derivatives, shown in brown. The B7.5 pair of blastomeres are shown in orange. The TVCs are shown in green; the ASMs are shown in blue. (B) Schematic lineage tree of the ontogeny of the B7.5 lineage based on Satou et al. (2004), Stolfi et al. (2010), Wang et al. (2013) and Razy-Krajka et al. (2014).
The A7.6 cell is born before gastrulation (stage 10, 112-cell; Hotta et al., 2007). At that point, it begins to express zygotic transcripts that were not previously expressed elsewhere in the embryo, including Hand-related (Hand-r) (renamed after Notrc/Hand-like according to current guidelines for the nomenclature of genetic elements in tunicates; Fig. S1B) (Imai et al., 2003; Shi and Levine, 2008; Stolfi et al., 2015). The proximal enhancer of Hand-r recapitulates endogenous expression in A7.6 and anterior trunk endoderm (Davidson and Levine, 2003; Woznica et al., 2012) (Fig. 2; Hand-r(-622/-1)>H2B::mCherry). We confirmed expression of Hand-r>H2B::mCherry in the OSM by co-electroporating embryos with Isl>unc76::GFP, which we herein use strictly as a marker of ASM and OSM (Fig. 2A) (note that it also labels certain neurons in the central nervous system, as described in Stolfi et al., 2010). Endodermal expression of Hand-r>H2B::mCherry is distinguishable anatomically as small nuclei in a compact configuration internal to the larva (asterisks in Fig. 2A,B). We infer that larval cells marked by Hand-r>H2B::mCherry, but excluded from the endoderm, are derived from A7.6 blastomeres, and will give rise to OSM (marked by Isl>unc76::GFP), blood cells, tunic cells, stomach cells and the first gill slit epithelium after metamorphosis (Hirano and Nishida, 1997; Tokuoka et al., 2005).

We used this Hand-r enhancer to drive the LexA::hinge::Gal4AD (‘LHG’) transcriptional activator, which consists of the DNA binding domain from the bacterial LexA protein connected to the trans-activation domain of the yeast Gal4 protein, (Yagi et al., 2010). By electroporating combined Hand-r>LHG and LexAop>H2B::mCherry plasmids together with Isl>unc76::GFP to mark siphon muscles (Fig. 2B), or Nkx2-1>hcCD4::GFP to mark the endoderm (Fig. 2C) (Ristoratore et al., 1999; Gline et al., 2015), we confirmed that the LexA/LexAop system driven by the Hand-r proximal enhancer recapitulates Hand-r>H2B::mCherry expression in the OSM, endoderm and scattered mesenchyme (compare Fig. 2A-C). In order to eliminate endodermal expression, we used the early endoderm-specific enhancer from Nkx2-1 to express Gal80, which can inhibit the transactivator function of Gal4AD (Nkx2-1>Gal80; Suster et al., 2004). By adding Nkx2-1>Gal80 to the Hand-r>LHG;LexAop>H2B::mCherry combination of transgenes, we observed a complete loss of H2B::mCherry expression in the endoderm (Fig. 2D). Therefore, the combination of Hand-r>LHG; Nkx2-1>Gal80;LexAop>GFP constructs (hereafter referred to as LexO(A7.6)>>GFP for simplicity) drives transgene expression specifically in the A7.6 lineage and its descendants. These results demonstrate the efficiency and specificity of the LHG/Gal80/LexAop system to achieve refined lineage-specific transgene expression in Ciona.

**The detailed A7.6 origins of oral siphon muscles**

In order to understand the embryonic context in which siphon muscle specification occurs, we determined the clonal origins of the fate-committed OSM precursors. Using a combination of staged *in situ* hybridization and reporter gene analysis, we describe the entire A7.6 lineage through 8.5 h post-fertilization (hpf) (stage 17, initial tailbud I; Fig. S2) (staging after Hotta et al., 2007), and of every OSM progenitor through 24 hpf (stage 26+; Fig. 3; Figs S1, S2).

Shortly after birth, A7.6 cells zygotically upregulate several genes, including Hand-r and MyT (Imai et al., 2003; Shi and Levine, 2008) (Fig. S1B,L). Hand-r is generally required for A7.6-derived trunk lateral cells (TLCs) fates (Imai et al., 2003), but neither the ontogeny of the TLCs nor the expression dynamics of Hand-r after gastrulation have been reported. We found that the A7.6 cell first divides anteroposteriorly during gastrulation, and that only the anterior daughter maintains Hand-r expression, while downregulating MyT expression (Fig. 3A,A′; Fig. S1C,M). Hand-r expression in the anterior progeny of A7.6 is preserved through two rounds of cell division, so that by 8.5 hpf there are four Hand-r+ cells in the TLC, which comprises a total of eight cells (Fig. 3G; Fig. S1D-F). At 8.5 hpf, only the single anterior-most of these four Hand-r+ cells in the lineage activates Ebf (Fig. 3B,B′; Fig. S1H). Because Ebf is required for ASM development in the B7.5 lineage (Stolfi et al., 2010; Razy-Krajka et al., 2014), we regarded this cell as the most likely OSM progenitor and focused our clonal analysis
after 8.5 hpf only on the Ebf+ anterior-most TLC, the descendants of which we hereafter refer to collectively as anterior TLCs (aTLCs) (Fig. 3B,B′,G; Fig. S1A,I).

Between the neurula and pre-hatching larva stages (~8.5 hpf to 16 hpf), anterior TLCs follow stereotyped division patterns and gene expression dynamics (Fig. S2). By 11 hpf, the anterior-most Hand-r+Ebf+ aTLC has divided once and both daughter cells maintain Hand-r and Ebf expression. At 13 hpf, Tbx1/10 is activated exclusively in the most anterior of the Hand-r+Ebf+ aTLCs (Fig. 3C′; Fig. S2J). By 16 hpf, the Tbx1/10+;Ebf+;Hand-r+ cell has divided once (Fig. 3C,C′) and Hand-r and Ebf both cease to be expressed in the other, more posterior, aTLC. Therefore, by 16 hpf, only the anterior-most two of the TLCs co-express Hand-r and Ebf expression. At 15 hpf (Fig. 3E; Fig. S3), within the A7.6 lineage, we found that after 8.5 hpf only on the TLCs are the sole fate-restricted OSM founder cells, we characterized the expression dynamics of the siphon muscle marker Isl>YFP beginning at 15 hpf (Fig. 3E; Fig. S3). Within the A7.6 lineage, we found that Isl>YFP was first expressed in the two anterior-most TLCs at 16 hpf (Fig. 3D; Fig. S3A,A′). Subsequently, each cell divided once between 18 hpf and 22 hpf, giving rise to four cells on either side of the larva (Fig. S3A−D, with close-ups of the same data shown in A′−D′). These YFP+;mCherry+ cells migrate to form a ring underneath the oral ectoderm (also known as stomodeum or oral siphon placode; Christiaen et al., 2005), where at 24 hpf the complete OSM rudiment will contain eight cells, all of which are derived from the Hand-r+Ebf+;Tbx1/10+ cell, which we now refer to as the OSM founder cell (OSMF; Fig. 3G; Fig. S3D,D′).

To further confirm that derivatives of the Hand-r+Ebf+;Tbx1/10+ OSMF cell are fate-restricted OSM precursors, we analyzed the expression dynamics of Mrf and Bhlh-tun1 in the A7.6 lineage. The ASMP markers Mrf and Bhlh-tun1 are also expressed in the OSM ring in 28 hpf swimming larvae (Razy-Krajka et al., 2014); we thus reasoned that the earliest expression of these genes would also be limited to the OSMF. We found that Mrf first turned on at 18 hpf in the most anterior pair of TLCs (Fig. 3F; Fig. S3E), which we infer to be the same Hand-r+Ebf+;Tbx1/10+;Isl>YFP+ pair. Between 18 and 22 hpf, in young swimming larvae, these cells continue to divide and a subset starts expressing the marker of stem-cell-like siphon muscle precursors, Bhlh-tun1 (previously known as orphan-bhlh-1; Razy-Krajka et al., 2014) at the expense of Mrf. This results in the production of a small heterogeneous group of Mrf+Bhlh-tun1− and Mrf−Bhlh-tun1+ cells (Fig. S4F−H). Taken together, these observations indicate that, at 18 hpf, the two anterior-most TLCs, which express Hand-r, Ebf, Tbx1/10 and Isl>unc76::Venus, and produce both Mrf+Bhlh-tun1− and Mrf−Bhlh-tun1+ cells, are the A7.6-derived fate-restricted OSM precursor cells (Fig. 3G).

Mrf is required for siphon muscle differentiation, but not early morphogenesis
Mrf is necessary for differentiation of Ciona tail muscles, and is sufficient to activate expression of muscle-specific genes in the entire vegetal half of the embryo, where maternal Zic1.a activity determines the competence for muscle differentiation (Yagi et al., 2004; Meedel et al., 2007; Izzii et al., 2013). However, the function of Mrf in development of the ASMs and OSMs has not been characterized. In order to assess whether Mrf is required for siphon muscle development, we used validated CRISPR/Cas9 reagents (Stolfi et al., 2014) to generate deletions in the first exon of the Mrf locus (Fig. S4) and assayed the expression of the siphon muscle-
specific differentiation markers Myosin regulatory light chain 4 (Mrlc4) and Myosin heavy chain 3 (Mhc3) at late larval stages (26 hpf at 18°C).

In control larvae, we detected both Mrlc4 and Mhc3 mRNA in the vast majority of OSM and ASM rings (Fig. 4). By contrast, upon ubiquitous expression of Mrf-targeting sgRNA and Ebf-driven Cas9, we observed a significant reduction of the proportions of Mrlc2- and Mhc3-expressing OSMs and ASMs (Fig. 4A-D,G). Thus, as is the case for tail muscles, Mrf function is necessary for proper gene expression during siphon muscle differentiation. In both OSMs and ASMs, Mrf turns on before crucial morphogenesis happens (e.g. collective migration and ring formation). Thus, we tested whether early siphon muscle morphogenesis occurred normally in the absence of Mrf. We used Isl>YFP to mark the committed siphon muscle precursors, because it turns on before, and thus independently of, Mrf in both ASM and OSM (Fig. S3) (Razy-Krajka et al., 2014). In control larvae, only half to two thirds of the OSMs and ASMs express the Isl>YFP transgene, because of classic mosaicism. Remarkably, the proportions of Isl>YFP+ cells in the vicinity of the oral and atrial siphon placodes did not change significantly in experimental larvae expressing Mrf-targeting CRISPR/Cas9 constructs (Fig. 4E,F). Therefore, although Mrf is required for differentiation of siphon muscles, it seems to be dispensable for early siphon muscle morphogenesis.

Newly discovered regulatory relationships between Tbx1/10 and Ebf

Having established that Mrf is a key regulator of siphon muscle differentiation in both the ASM and OSM, we sought to investigate and compare the mechanisms that activate its expression in the two distinct lineages. Because the early ASM determinants Ebf and Tbx1/10 are both also expressed in OSM progenitors, we first sought to test whether Tbx1/10 and Ebf interact functionally in specifying OSM and regulating each other’s expressions. In the B7.5/cardiopharyngeal lineage, Tbx1/10 function is required for Ebf expression, and contributes to inhibiting the heart program in the ASMs (Wang et al., 2013). However, because Tbx1/10 is not observed in the OSMF until after Ebf has been expressed for several hours (Fig. S2J,L), we ruled out a role for Tbx1/10 in the onset of Ebf expression. Instead, we reasoned that Tbx1/10 could either contribute to Ebf maintenance or be dispensable for its expression in fate-restricted OSMPs.

To evaluate these hypotheses, we first tested whether Tbx1/10 misexpression would maintain Ebf expression broadly in anterior TLCs, especially in the sisters of the OSMFs that transiently express Ebf but do not maintain it and never activate Tbx1/10 (see Fig. 3G for the clonal context and Fig. 5A for a diagram of the TLC at 16 hpf). We overexpressed Tbx1/10 throughout the A7.6 lineage and analyzed the effect on Ebf expression at 16 hpf, when it is normally expressed in the nervous system, the ASMFs and the OSMFs (Fig. 5D; Fig. S2I) (Wang et al., 2013). Upon Tbx1/10 misexpression, we observed ectopic Ebf expression neither in anterior TLC lineage (Fig. 5B,E), nor in the posterior TLCs (data not shown). Thus, Tbx1/10 misexpression is not sufficient to maintain Ebf expression in the anterior TLC lineage, nor cause ectopic Ebf expression. This is in contrast to the situation observed in the B7.5 lineage, where Tbx1/10 misexpression caused robust ectopic Ebf activation in the second heart precursors (Wang et al., 2013).

**Fig. 4. Mrf is required for siphon muscle differentiation, but not morphogenesis.**

(A-D) Micrographs of 26 hpf larvae under control conditions (A,B) or with Mrf mutated (C,D) showing expression of Mrlc4 or Mhc3. Siphon muscle-specific gene expression is lost when Mrf is mutated. (E,F) 26 hpf larvae electroporated with the siphon muscle marker Isl>YFP, which turns on before Mrf, showing that ASM and OSM precursors still migrate to their respective placodes in the absence of functional Mrf. (G) Bar plot showing percentage of larvae of each condition expressing each gene or reporter. Mrf mutated, n=97 for Mrlc4 in situ hybridization; n=98 for Mhc3 in situ hybridization; n=223 for Isl>YFP. With Mrf mutated, n=113 for Mrlc4 in situ hybridization; n=198 for Isl>YFP. ***P<0.00001 by χ² analysis with continuity correction; ns, not significant (P>0.05). White arrowhead, OSM; open arrow, ASM. Scale bars: 20 μm.
In the B7.5 lineage, RNAi-mediated loss of Tbx1/10 function abolished Ebf expression in the ASMF (Wang et al., 2013). To test whether Tbx1/10 is required for the maintenance of Ebf expression in the OSMF, we used CRISPR/Cas9-based tissue-specific mutagenesis to disrupt Tbx1/10 function specifically in the A7.6 lineage (see Materials and Methods and Fig. S5). We found that loss of Tbx1/10 function in the TLCs did not alter Ebf expression in the OSMPs at 16 hpf (Fig. 5C,G). Taken together, these results indicate that, in contrast to its function in the B7.5-derived ASMF, Tbx1/10 is not involved in either activating or maintaining Ebf expression in the A7.6-derived OSM precursors.

Next, because Ebf expression in OSM progenitors precedes that of Tbx1/10 by about 5 h, we sought to evaluate a potential role for Ebf in activating Tbx1/10 in the OSMF. Ebf misexpression throughout the A7.6 lineage failed to cause ectopic Tbx1/10 expression in any part of the TLC population. As Ebf is expressed more broadly than Tbx1/10, this data further suggests that Tbx1/10 expression requires additional, Ebf-independent and aTLC-specific inputs (Fig. 5C,G). Taken together, these results indicate that, in contrast to its function in the B7.5-derived ASMF, Tbx1/10 is not involved in either activating or maintaining Ebf expression in the A7.6-derived OSM precursors.

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Tbx1/10 and Ebf are both required together and act in parallel to specify the OSM fate

We have shown that, although ASM and OSM both express Mrf in the siphon muscle founder cells, some upstream regulators of Mrf expression in B7.5-derived ASMP are deployed differently in the A7.6 lineage. The divergent regulatory relationship between Ebf and Tbx1/10 in A7.6 opens the possibility that their respective functions in Mrf activation also differ between the ASM and OSM. We first tested whether loss of either Ebf or Tbx1/10 function throughout the A7.6 lineage inhibited Mrf expression in 24 hpf larvae (Fig. 6). In control conditions Mrf was co-expressed with LexO(A7.6)>>H2B:mCherry in the OSM of 92% of larvae (Fig. 6A,D). Targeting Tbx1/10 specifically in the TLC (G) significantly reduced the proportion of transfected larvae expressing Mrf. We therefore conclude that the OSM precursors uniquely require Ebf to express Tbx1/10, whereas Ebf expression is independent of Tbx1/10 expression. This is in stark contrast to the regulatory relationship between Tbx1/10 and Ebf in the ASM, where Tbx1/10 is required for Ebf expression, and misexpression of Tbx1/10 was sufficient to cause ectopic Ebf expression in the cardiopharyngeal mesoderm (Wang et al., 2013).

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**Context-specific wiring of a conserved siphon muscle differentiation kernel**

Having established the parallel requirement for Ebf and Tbx1/10 upstream of Mrf in OSM specification, we sought to test whether this regulatory architecture also governs ASM specification. Previous work suggested that Ebf is necessary and sufficient for ASM specification in the B7.5 lineage, but because Tbx1/10 expression is transiently maintained in the ASMF after activating Ebf, it might also act in parallel to Ebf in regulating Mrf (Stolfi et al., 2010; Wang et al., 2013; Razy-Krajka et al., 2014). In order to determine whether Ebf is sufficient for ASM fate specification in the absence of Tbx1/10, we designed a strategy using CRISPR/Cas9 to mutate Tbx1/10 in the B7.5 lineage, while using a minimal B7.5-lineage-specific Tbx1/10 enhancer to restore Ebf expression specifically in the STVCs and their progeny (Fig. 1B; Fig. S7). We first verified that this strategy was viable by showing that STVC-lineage-specific expression of a Tbx1/10>5-GFP construct was not affected by Tbx1/10 mutations (Fig. S7B,C).

In control 22 hpf larvae, there are four ASM precursors (ASMP), with the outer ASMP expressing Mrf and the inner ASMP expressing Bhlh-tun1 (Razy-Krajka et al., 2014) (Fig. 1D, Fig. 8A). In the B7.5 lineage, RNAi-mediated Tbx1/10 inhibition abolishes Ebf expression in the ASMF (Wang et al., 2013) and Ebf activates ASMP-specific expression of Mrf and Bhlh-tun1 (Razy-Krajka et al., 2014). Consistent with this, we found that B7.5-lineage-specific targeting of Tbx1/10 with CRISPR/Cas9 significantly abolished Ebf>5-GFP expression, as well as Mrf and Bhlh-tun1 expression the ASMF, likely due to loss of Ebf expression (Fig. 8C; Fig. S7B). Because the Tbx1/10 enhancer drives transgene expression in the ASMF as well as the second heart precursors (SHP; see Fig. 1B), and Ebf is a potent activator of the ASMF program (Stolfi et al., 2010; Razy-Krajka et al., 2014), we expected to see the effects of Ebf overexpression expanded to the SHP. Indeed, electroporation of Tbx1/10>5-Ebf with control guide RNA caused an expansion of Mrf and Bhlh-tun1 expression to the SHP (Fig. 8B,E). We then used Tbx1/10>5-Ebf to rescue Ebf expression in Tbx1/10-mutated embryos and found that Ebf alone was able to rescue expression of Mrf and
Bhlh-tun1 in the ASMP, as well as cause ectopic expression of these genes in the SHP (Fig. 8D,E). These data indicate that Tbx1/10 function is dispensable for ASM specification downstream of Ebf. Therefore, whereas Ebf can regulate Mrf without Tbx1/10 in the ASM, its function as an activator of Mrf in the OSM depends on co-expression with Tbx1/10.

Fig. 7. Effect of Ebf or Tbx1/10 gain-of-function on expression of Mrf. (A–D) In situ hybridization showing Mrf mRNA in 24 hpf larvae under control conditions (A), when overexpressing Ebf specifically in the TLC (B), when overexpressing Tbx1/10 (C), or when overexpressing Ebf and Tbx1/10 together (D). When overexpressing Ebf, TLC derivatives tend to cluster to the dorsal midline when expressing ectopic Mrf (white arrow in C); when overexpressing both Ebf and Tbx1/10, TLC derivatives that express ectopic Mrf cluster near the ASM placode (white arrow in D). (E) Bar chart showing proportion of larvae in each condition in which we observed ectopic Mrf expression. Each bar represents pooled scores from two biological replicates, with error bars showing estimated standard error and sample sizes indicated. **P<0.002, ***P<0.00001, by χ² analysis with continuity correction. White arrowhead, OSM; open arrow, ASM. Scale bars: 25 μm.

Fig. 8. Ebf is an independent regulator of siphon muscle fate in the B7.5 lineage. (A–D) Close-up of ASM (marked with open arrowheads), and first heart precursors (FHP, marked with white arrows) and second heart precursors (SHP, marked with white arrowheads), derived from the B7.5 lineage, revealing Mrf mRNA (blue) and Bhlh-tun-1 mRNA (green), with the B7.5 lineage marked by Mesp>H2B:mCherry. (A,B) Larvae under CRISPR control condition either expressing Tbx1/10>LacZ (A) or overexpressing Ebf with Tbx1/10>Ebf (B). In B, note that expression of Mrf and Bhlh-tun-1 has expanded to the SHP, due to earlier Tbx1>Ebf expression in the secondary TVCs. (C,D) Larvae with Tbx1/10 mutated specifically in the B7.5 lineage, and expressing Tbx1/10>LacZ (C) or Tbx1/10>Ebf (D). Note that in C there is a complete loss of Mrf or Bhlh-tun-1 expression, whereas in D, Mrf and Bhlh-tun-1 show wild-type expression patterns in the ASM, and are also expressed in the SHP. Scale bars: 25 μm. (E) Bar chart showing percentage of larvae expressing Mrf or Bhlh-tun-1 in ASMP or SHP under indicated conditions. Each bar represents the pooled scores from two biological replicates, with error bars showing estimated standard error and sample sizes indicated. ***P<0.00001, by χ² analysis with continuity correction. (F) Schematic diagram comparing core regulatory interactions upstream of morphogenesis and Mrf-driven differentiation in ASM and OSM. In black, documented shared expression of Hand-r, Ebf and Tbx1/10 in cells that give rise to, among other tissues, siphon muscles, where Hand-r might be involved in activation of the core common regulators Tbx1/10 and Ebf, as indicated by green dashed arrows. Distinct regulatory relationships in place in ASM versus OSM are indicated by orange and brown arrows, respectively. The shared direct input from Ebf to Mrf in both ASM and OSM is indicated by the solid green arrow. The dashed green arrows from Ebf and Tbx1/10 to morphogenesis are inferred from our results that mutagenesis of Mrf affects differentiation, but not morphogenesis of both ASM and OSM (see Fig. 4).
DISCUSSION

In this study, we analyzed two clonally distinct muscle groups of the same subtype to examine context-dependent control of commitment to myogenesis. As the atrial siphon muscles (ASMs) and the oral siphon muscles (OSMs) converge on virtually identical differentiation programs, we tested whether the single MyoD homologue, Mrf, is necessary for differentiation in both contexts, and whether the mechanisms that control Mrf expression differ between the two lineages. The regulatory mechanisms upstream of Mrf expression in the ASMs founder cells of the basal chordate Ciona have been described (Stolfi et al., 2010; Wang et al., 2013; Razy-Krajka et al., 2014), but very little was known about the origins of the OSMs or the mechanisms of OSM determination. We present a detailed description of the developmental origins of the OSMs. We also show that Mrf is required for differentiation of both OSMs and ASMs in Ciona, but that the regulatory mechanisms upstream of Mrf expression in the ASMs are different from those that activate Mrf in the OSMs, albeit using the same conserved regulatory factors Ebf and Tbx1/10.

Ebf, Tbx1/10 and Mrf define an ancient regulatory state for muscle specification

In the B7.5 lineage, Tbx1/10 activates Ebf, which activates Mrf (Stolfi et al., 2010; Wang et al., 2013; Razy-Krajka et al., 2014). In mice, Tbx1 regulates Myf5 and MyoD in the myogenic branchial arch mesoderm (Sambasivan et al., 2009), and is required for formation of striated esophageal muscles, both of which derive from Mesp+ cardiopharyngeal mesoderm (Gopalakrishnan et al., 2015). Indeed, it has been proposed that the Mesp+ B7.5 lineage is homologous to the vertebrate cardiopharyngeal mesoderm (Stolfi et al., 2010; Wang et al., 2013; Kaplan et al., 2015; Diogo et al., 2015). By contrast, we show that Tbx1/10 has no role in activating or maintaining Ebf expression in the OSM lineage (Fig. 5), where it is instead required in cooperation with Ebf to activate Mrf (Fig. 6, Fig. 7). These specific regulatory interactions are in fact more similar to those documented in the branchiomerically mesoderm of vertebrates (Sambasivan et al., 2009, 2011). Moreover, we show that Tbx1/10 is not needed for Mrf expression in the B7.5 lineage, where Ebf can activate Mrf even when Tbx1/10 is absent (Fig. 8).

COE (Collier/Olf1/Ebf)-family genes are emerging as deeply conserved regulators of myogenesis in bilateria. The myogenic function of Drosophila collier was first reported for its role in the specification of a hemisegmentally repeated abdominal muscle subtype (Crozatier and Vincent, 1999), where it is regulated by and required in cooperation with nau/MyoD for DA3 muscle differentiation (Enriquez et al., 2012; Dubois et al., 2007). This domain is notably distinct from that of org-1/Tbx1 action (Schaub et al., 2012; Boukhatmi et al., 2014). Two COE homologs in vertebrates, EbF2 and EbF3, regulate Myf5 and MyoD expressions in the somitic and branchiomeric muscles of Xenopus (Green and Vetter, 2011). In the mouse, EbF1 and EbF3 interact with MyoD in the developing diaphragm to activate muscle-specific gene transcription (Jin et al., 2014). It has also been shown that Ebf genes are expressed in the pharyngeal arches of the developing chick in the same domain as Tbx1 (El-Magd et al., 2014), indicating that a role for Ebf-Tbx1 interactions in the pharyngeal mesoderm might be conserved from tunicates to vertebrates. EbF2 and EbF3 are also expressed in the somites of chicken embryos, where they might be involved in skeletal development (El-Magd et al., 2013). In Amphioxus, the single homolog of Tbx1 is expressed in the developing branchial arches (Mahadevan et al., 2004), and although neither Ebf nor MyoD expression have been reported in branchial arches of Amphioxus, Tbx1, Ebf and MyoD homologues are all expressed in somitic mesoderm (Mahadevan et al., 2004; Mazet et al., 2004; Schubert et al., 2003; Urano et al., 2003). Taken together, these diverse observations point to deep evolutionary origins for the myogenic functions of Tbx1/10, COE and Mrf orthologs in bilaterians. However, the three classes of regulators clearly co-exist in vertebrate pharyngeal muscles and ascidian siphon and body wall muscles. Therefore, we propose that the co-expression of Tbx1/10, COE and Mrf homologs defines an ancient regulatory state that is associated with pharyngeal/head muscle specification in tunicates and vertebrates.

Flexibility of regulatory linkages between stably co-expressed myogenic determinants

Our results demonstrate that the determinants Ebf, Tbx1/10 and Mrf can be stably co-expressed to promote myogenesis, whereas their regulatory linkages and relative expression dynamics might differ between independent lineages. Even in vertebrates, where MRF-family genes have been uniquely co-opted to the forefront of all skeletal myogenesis, MRF genes are regulated by, and work in concert with, other transcription factors to activate muscle-specific gene expression. We show here that two conserved regulators, Tbx1/10 and Ebf, both operate upstream of Mrf, though according to different regulatory logic in ASM versus OSM.

Why would the temporal deployment and regulatory logic of two such deeply conserved muscle-regulatory genes differ so greatly to each context? Specification of the siphon muscle founder cells in both the A7.6 lineage and the B7.5 lineage are instances of binary fate choices, the outcome of which is Mrf expression and commitment to a siphon muscle identity. However, the alternative fates and embryonic context differ between the two lineages. Therefore, the rewiring of the Ebf-Tbx1/10 interactions upstream of Mrf might reflect larger network constraints specific to the A7.6 and B7.5 lineages, respectively.

The shared need for either Tbx1/10 or Ebf in siphon muscle specification likely reflects the fact that Ebf and Tbx1/10 regulate other aspects of ASM and OSM development besides Mrf expression and terminal differentiation. For instance, the complete siphon muscle developmental program also includes the maintenance of a Notch-dependent undifferentiated stem-cell-like state (Razy-Krajka et al., 2014), and the morphogenetic gene batteries for siphon-muscle-precursor-specific cell behaviors. Mrf loss-of-function altered neither the latter, nor Ebf-dependent Islet-YFP expression, indicating that Ebf governs these siphon-muscle-specific features independently of Mrf (Fig. 8E).

Tbx1/10 and/or Ebf might also contribute to regulating terminal differentiation genes in concert with Mrf. Such cooperation between COE and MyoD to activate muscle-specific differentiation genes has already been demonstrated in mouse (Jin et al., 2014). Extensive cis-regulatory analyses indicated that terminal muscle differentiation genes require combined inputs from Mrf, Tbx6 and an unknown CREB/ATF factor(s) during primary tail muscle differentiation in Ciona (Kusakabe et al., 2004; Brown et al., 2007; reviewed in Wang and Christiaen, 2012). Similarly, combinatorial inputs from Tbx1/10, Ebf, Mrf and/or possibly other factors, might drive gene expression during terminal differentiation. In this context, co-expression of the main regulatory genes defines a conserved myogenic regulatory state regardless of their relative timing and co-dependence.
Materials and Methods

Animals and electroporation

Gravid Ciona robusta, formerly classified as C. intestinalis Type A (Brunetti et al., 2015), adults were obtained from M-REP, San Diego, CA. Collection of gametes, fertilization, dechorionation, and electroporation of zygotes were all performed as described previously (Christiaen et al., 2009b). Animals were electroporated with 10-60 μg of plasmid DNA and raised at 18°C. Care and use of animals complied with all relevant institutional and national animal welfare laws, guidelines and policies.

Cloning of unary transgenic enhancers

Enhancers were amplified from genomic DNA using specific primers shown in Table S1 and cloned into backbones containing reporters using standard molecular cloning procedures.

Cloning of LexA/LexAop and Gal80 constructs

The LHG coding sequence and LexAop enhancer sequences were taken from pDPPuntB-LHG and p28-pJFRC19-13xLexAop2-IVS-myr–GFP (Yagi et al., 2010). The LHG coding sequence was amplified using specific primers and adding restriction sites NotI and EcoRI (NotI-LHG-F: 5’-AAAGGCGGCCGCAACCATGAAAGCGTTAACGGCCAG-3’; EcoRI-LHG-R 5’-TTTAGAATCTTACCTCTTTTTGTTGTTG-3’) and cloned downstream of the Hand-r(-622/-1) enhancer. The 13xLexAop enhancer along with the Drosophila melanogaster HSP70 basal promoter was excised from the pJFRC19-13xLexAop2-IVS-myr–GFP vector using AscI and NotI restriction enzymes and cloned into a backbone containing H2B:mCherry and subsequently subcloned using standard molecular cloning methods.

Gal80 was amplified from Tubp-Gal80, a gift from Liqun Luo (Addgene #17748; Lee and Luo, 1999) using specific primers and adding NotI and EcoRI restriction sites (NotI-Gal80-F 5’-AAAGGCGGCCGCAACCATGAAAGCGTTAACGGCCAG-3’; EcoRI-Gal80-R 5’-AAAGAATTCTTATACCTCTTCTTTTTTTGTTGTTG-3’).

A7.6 lineage analysis and in situ hybridization

To obtain the initial data regarding timing and orientation of cell divisions within the A7.6 lineage, we visualized the A7.6 lineage in cells samples fixed every 15 min starting at 5 hpf (~stage 10), and applied Conklin’s nomenclature (Conklin, 1905) to identify and name every cell within the A7.6 lineage up to 8.5 hpf (~stage 17; Fig. S1). For early stages, we marked the A7.6 lineage by the co-expression of Hand-r>H2B:mCherry and MyoT>unc76:GFP transgenes, which overlap exclusively in the A7.6 lineage, because mCherry proteins generated by the LexA/LexAop transgenes, which overlap exclusively in the A7.6 lineage. Our inferences about the clonal relationships between the A7.6 lineage up to 8.5 hpf (~stage 17; Fig. S1).

In situ hybridization was performed using published probes for Hand-r and Bhlh-nan1, Mrxl-4, Muc3 (Razy-Krajka et al., 2014), Ebf (Stolfi et al., 2010) and Tbx1/10 (Wang et al., 2013).


Supplemental Materials and Methods

Table S1. Primers used in amplification of Hand-related and MyT enhancers from genomic DNA

<table>
<thead>
<tr>
<th>Gene Model ID</th>
<th>Citation</th>
<th>Oligo Name</th>
<th>Oligo Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH2012:KH.C1.274</td>
<td>A7.6 expression reported in (Imai et al. 2004; Shi and Levine 2008)</td>
<td>MyT(-3271)AsclF:</td>
<td>5’-AAAGGCGCGCCCTTTTTCGTGCAAGACTG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MyT(+37)NotIR:</td>
<td>5’-AAAGGCGCGCTTTTTTCTTGTCGCAAAGTGG-3’</td>
</tr>
</tbody>
</table>

Genome editing using CRISPR

Single guide RNAs against *Mrf* were taken from Gandhi et al. (2016). We mutated the *Mrf* locus using the ubiquitous, early Ef1α enhancer driving Cas9 (Eef1a1>nls::cas9::nls; Stolfi et al. 2014), along with the RNA-polIII-dependent U6 promoter to supply single guide RNAs targeting the *Mrf* coding region at bp208-226 and bp1182-1200 of the *Mrf* genomic locus, each of which has an estimated cutting efficiency of about 33% (Supplemental Figure S4A; U6>sgMrf.226 and U6>sgMrf.1182; Gandhi,
et al., 2016). By combining them, we generated large deletions in the first exon of Mrf, which we were able to observe as a secondary band when amplifying genomic DNA by PCR using specific primers (Supplemental Figure S4B), as well as in Sanger sequencing of PCR product (Supplemental Figure S4C).

Single guide RNAs against Tbx1/10 were designed using CRISPRdirect (Naito et al. 2015), but eliminating putative targets that fell on SNPs documented in the published genome. Complementary oligonucleotides of each (N)21-GG target were synthesized by Sigma-Aldrich, St. Louis, Missouri, USA, and cloned downstream of the U6 RNA polymerase III promoter according to the methods described in (Stolfi et al. 2014). We tested a total of nine putative sgRNAs (“+” or “-” indicates whether the PAM was on the + or – strand; the number after the period indicates the nucleotide number on the coding sequence of the first base-pair targeted; all loci are represented graphically in Supplemental Figure S5A:

<table>
<thead>
<tr>
<th>Target Locus</th>
<th>Sequence (PAM in bold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sgTbx1.303(+)</td>
<td>TTGCGGCTTCCGCTCCGTTGG</td>
</tr>
<tr>
<td>sgTbx1.422(-)</td>
<td>ACGGGAACGTCAAGGAAGG</td>
</tr>
<tr>
<td>sgTbx1.558(+)</td>
<td>CGAAAGATTTGGGCGCGTGG</td>
</tr>
<tr>
<td>sgTbx1.673(+)</td>
<td>TTCCAGTGCAAGTGCTCGG</td>
</tr>
<tr>
<td>sgTbx1.783(+)</td>
<td>TGCACCGCTTCCACCTCGG</td>
</tr>
<tr>
<td>sgTbx1.835(+)</td>
<td>CCAGACTCGCCAGCAAAAGG</td>
</tr>
<tr>
<td>sgTbx1.982(+)</td>
<td>TGCACCGCTTCCACCTCGG</td>
</tr>
<tr>
<td>sgTbx1.971(-)</td>
<td>AGCACAACATGAAACCGAGG</td>
</tr>
<tr>
<td>sgTbx1.1067(+)</td>
<td>CAGCTTATACAAATCACAGG</td>
</tr>
</tbody>
</table>

We verified that sgRNAs directed cutting of genomic DNA by electroporating embryos with the ubiquitously-expressed Eef1a>nls::Cas9::nls and 25µg each of a single
U6>sgTbx1 plasmid. We extracted gDNA from 17hpf larvae, amplified the targeted region using specific primers, and TOPO-cloned the PCR products into pCRII vectors for sequencing. We found that when electroporated individually, sgTbx1.303 and sgTbx1.558 produced the most efficient cutting, with 2/6 and 4/8 sequenced clones, respectively, showing mutations in the targeted region. However, when paired, sgTbx1.303 and sgTbx1.558 produced a large deletion in 6/8 clones sequenced, however, this deletion was not large enough to discern by PCR run on a 1% agarose gel (Fig. S2B). For all tissue-specific manipulations of Tbx1/10 function, we used

LexO(A7.6)>>nls:cas9:nls;U6>sgTbx1.303;U6>sgTbx1.558, with 25µg of Hand-r>LHG, 25µg of LexAop>nls:Cas9:nls, and 25µg each of U6>sgTbx1.303 and U6>sgTbx1.558, referred to as LexO(A7.6)>>sgTbx1 throughout the text.

References


Supplementary Data

Figure S1 – Embryonic development of the trunk lateral cells (TLC) 5.5hpf to 8.5hpf. (A) Cartoon of divisions within the A7.6 lineage at 5.5hpf, 6hpf and 8.5hpf. Each cell is named according to the scheme developed by Conklin (Conklin 1905). Boxes indicate the regions shown in close-up in micrographs. (B-H) Embryos of various stages, electroporated with MyT>H2B:mCherry and showing expression of Hand-related (B-F), MyT (G-K), or Ebf (G, H) mRNA by in situ hybridization. Note the faint expression of H2B:mCherry in two adjacent endoderm cells beginning at 5.5hpf, presumably derived from A7.5, the sister of A7.6, thus
reflecting an occasional early onset of transgene expression in the mother A6.3. These A6.3-derived cells are marked with a Roman numeral “x” wherever they appear. This staining was accounted for in all observations, and did not interfere with our ability to identify A7.6-derived cells. (I) Schematic diagram of all TLC divisions from 5hpf to 8.5hpf, with gene expression patterns mapped on to each cell and color-coded. Blue = hand-related; Red = MyT Green = Ebf. Scale bars=25µm. Newborn A7.6 blastomeres express Hand-r and MyT. However, following a seemingly symmetrical division along the antero-posterior axis, the anterior daughter (A8.12) preferentially maintains Hand-r expression, while the posterior daughter maintains MyT. The respective anterior and posterior domains of Hand-r and MyT are maintained during one more round of subsequent divisions, which occurs synchronously to produce 4 TLC on each side of the embryo by 6hpf, the two anterior of which express Hand-r and the two posterior of which express MyT. These four cells are named, anterior-to-posterior, A9.24/23/22/21. At around 7.5 hpf, the A9.23 cell re-activates MyT, and then divides. At 8.5hpf, only the anteriormost TLC, called A10.48, which is Hand-r+, turns on Ebf. We refer to all cell derived from the A10.48/47 pair as the “anterior TLC” and the rest of the TLC as the “posterior TLC.”
Figure S2 - Embryonic development of the trunk lateral cells (TLC) 8.5hpf to 16hpf. First cartoon in A, and panels B and F are the same data shown in Supplemental Figure S1F and H, to emphasize continuity of A10.48 cell and to show the anterior TLC in the whole-embryo context. Panels C-E and G-K show only the anterior TLC,
which are boxed in the cartoons in A. (B-K) Close-up of anterior TLC (descendants of A10.48/47 cell pair only) marked with LexO(A7.6)>>H2B:mCherry showing expression of Hand-r (B-E), Ebf (F-I) and Tbx1/10 (J, K) at 8.5hpf, 10hpf, 13hpf, and 16hpf. Arrowheads mark the A10.48 cell; large white arrows mark A11.96, the OSM founder cells (OSMF); small white arrows mark the daughters of A11.96, named A12.192/191, which we call the OSM precursor cells (OSMP). (L) Schematic diagram of cell divisions and gene expression in the anterior TLC 10-16hpf. Blue = hand-related; Green = Ebf; Red = Tbx1/10; lighter versions of each color indicates lower levels of gene expression. Scale bars = 25μm.

Figure S3. Development of the OSM precursors after larval hatching. (A-D) Larvae electroporated with LexO(A7.6)>>H2B:mCherry; Isl>unc76::GFP showing initiation of Isl>unc76::GFP expression in the OSMP at 16hpf (A), followed by cell divisions, anterior migration of the OSMP, and ring formation around the stomodeum by 24hpf (B-D). Boxed
regions in A-D are shown close-up in A’-D’. (E-H) Close-up of OSMP in 18-24hpf larvae labeled with LexO(A7.6)>>H2B:mCh and with *Mrf* (blue, long arrows) and *Orphan-bHLH-1* (green, white arrowheads) mRNA revealed by *in situ* hybridization. Scale bars = 10μm.

Figure S4 – Validation of CRISPR sgRNAs targeting *Mrf* Exon 1. (A) Schematic diagram of the *Mrf* genomic locus. Location of oligonucleotides used to amplify *Mrf* from control and mutated gDNA are shown in pink (F: 5’ – ATGACGTCACAAAAACGGACG – 3’; R: 5’ – cgcttaagggctcgtacg – 3’), with the expected sizes of the respective amplicons indicated. (B) 1% Agarose gel showing the result of amplifying *Mrf* Exon 1 using the stated primers from control or mutated gDNA. Note the secondary band in the CRISPR lane that corresponds to
the expected 300bp length of DNA from which the region between sgMrf.226 and sgMrf1182 excised.

Figure S5 - Validation of sgRNAs to target Tbx1/10 exons. (A) Schematic of the Tbx1/10 locus, with exons shown as orange blocks, and introns as orange lines. The approximate locations of each tested sgRNA are shown, with sgRNAs targeting the (+) strand marked above the schematic, and sgRNAs targeting the (-) strand marked below the schematic. (B) Alignment of sequencing results from TOPO-cloning of PCR products from genomic DNA of 16hpf larvae electroporated with Ef1α>nls:Cas9:nls;U6>sgTbx1.303;U6>sgTbx1.558. Of eight clones sequenced, six were alleles carrying large deletions between pos.303 and pos.558, while two did not align to the Tbx1/10 locus at all.
Figure S6. Complete expression pattern of Tbx1/10 mRNA at 16hpf, with TLC marked by LexO(A7.6)>>H2B:mCherry. En=endoderm; STVC=secondary TVC; OSMF=OSM founder cells, i.e. A12.192/191. Scale bars = 10μM.
Figure S7 Validation of sgTbx1.303 and sgTbx1.558 in the B7.5 lineage. Since RNAi experiments indicated that Tbx1/10 is necessary for Ebf expression in the ASMF, we first tested the efficacy of CRISPR/Cas9-based genome editing of Tbx1/10 in the B7.5 lineage by electroporating fertilized eggs with Mesp>nls:Cas9:nls and either U6>sgControl or U6>sgTbx1.303;U6>sgTbx1.558. (A) JGI genome browser snapshot of the conservation between C. intestinalis and C. savignyi of noncoding sequences upstream of Tbx1/10. Below the snapshot, the regions used to drive the Tbx1>GFP and Tbx1>Ebf constructs are indicated with a red bar. (B) Bar graph showing that Tbx1/10 knockdown abolishes Ebf>GFP, but not Tbx1>GFP expression. Bars represent the percent of larvae co-expressing Mesp>H2B:mCherry and Ebf>GFP (black bars) or Tbx1>GFP (grey bars) in the ASM at 24hpf.
in control vs. Tbx1/10 loss-of-function conditions. (C) Bar graph showing that under Tbx1/10 loss-of-function conditions, Tbx1>Ebf can rescue Ebf>GFP expression in the ASM. Bars represent the percent of larvae co-expressing Mesp>H2B:mCherry and Ebf>GFP in the ASM at 24hpf, in control and Tbx1/10 loss-of-function conditions with either Tbx1>LacZ (i.e. no rescue of Ebf expression) or Tbx1>Ebf. Compared to Tbx1/10 loss-of-function with Tbx1>LacZ, Tbx1>Ebf led to a 64% recovery of larvae expressing Ebf>GFP in the ASM (Chi-Squared test, p=8.6E-04).