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

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Summary Statement

1. We adapted the heterologous LexA/LexAop system in Ciona, and used it to characterize
2. rewired myogenic programs in separate embryonic lineages.
ABSTRACT

Skeletal muscles arise from diverse embryonic origins, yet converge on common regulatory programs involving muscle regulatory factor (MRF)-family genes. Here, we compare the molecular basis of myogenesis in two separate muscle groups in the simple chordate Ciona: the atrial and oral siphon muscles. Here, we describe the ontogeny of OSM progenitors and characterize the clonal origins of OSM founders to compare mechanisms of OSM specification to what has been established for ASM. We determined that, as is the case in the ASM, Ebf and Tbx1/10 are both expressed and function upstream of Mrf in the OSM founder cells. However, regulatory relationships between Tbx1/10, Ebf and Mrf differ between the OSM and ASM lineages: while Tbx1/10, Ebf and Mrf form a linear cascade in the ASM, Ebf and Tbx1/10 are expressed in the inverse temporal order and are required together in order to activate Mrf in the OSM founder cells.
Introduction

In Vertebrates, differentiation of skeletal muscle during development relies on the deployment of a core myogenic network comprising the MRF-family of bHLH transcription factors, MyoD, Myf5, and Mrf4 (Block and Miller 1992; Rudnicki et al. 1993; Braun et al. 1994; Summerbell, Halai, and Rigby 2002; Kassar-Duchossoy et al. 2004). However, the exact relationships between these myogenic genes in activating expression of the muscle-specific differentiation program, as well as the factors that specify myogenic potential upstream of the core MRF-based network, differ between regions of the embryo (Sambasivan et al. 2009; Harel et al. 2009; Adachi et al.; Buckingham & Rigby 2014). For example, while Pax3 is an important muscle determinant in somitic lineages, Tbx1 determines branchiomereric muscle, and Pitx2 is an activator of the core myogenic network in extraocular muscles (Harel et al. 2009; Sambasivan et al. 2009).

Research in Drosophila, C. elegans, sea urchins, Amphioxus, and non-model systems including Spiralians, Cnidarians, and brachiopods has shown that MRF-family bHLH transcription factors have roles in muscle development across Deuterostomes, and even throughout Bilaterians (Andrikou et al. 2015; Buckingham & Rigby 2014; Schaub et al. 2012; Ciglar & Furlong 2009; Zhang et al. 1999). Moreover, there is evidence, discussed below, that upstream regulatory factors responsible for MRF expression and muscle development may be conserved across long evolutionary distances. In particular, the COE (Collier/Olfactory-I/Early B-Cell Factor) family of transcription factors has been implicated in striated muscle development in Drosophila (Crozatier and Vincent 1999), Ciona (Stolfi et al., 2010; Razy-Krajka et al. 2014), Xenopus (Green and Vetter 2011) and chick (El-Magd et al., 2013, 2014a, 2014b). Homologs of the T-box transcription factor Tbx1 also seem to have a role in muscle identity in Drosophila (Schaub et al. 2012), Ciona (Wang et al. 2013), mice (Aggarwal et al. 2010) and humans (H. Yagi et al. 2003).
In the basal chordate *Ciona*, distinct muscle groups also arise from different blastomeres in the early embryo, and the single MRF-family gene in the *Ciona* genome, called *Mrf*, is expressed in all non-cardiac muscle precursors (Meedel, Chang, and Yasuo 2007; Razy-Krajka et al. 2014). In the B7.5 lineage, *Mrf* is first transiently expressed in B7.5 and in the B8.9 and B8.10 founder cells, before induction of the cardiopharyngeal progenitors (called trunk ventral cells, TVCs) (Christiaen et al. 2008). *Mrf* is later re-activated downstream of *Ebf* specifically in the atrial siphon muscle (ASM) founder cells. ASMFs give rise to *Mrf*+ differentiating muscle cells as well as *Bhlh-tun1+/Mrf*- stem-like muscle precursors, which cease to express *Mrf* in response to Notch-mediated lateral inhibition. The progeny of these *Bhlh-tun1*-expressing cells reactivate *Mrf* after metamorphosis in order to give rise to the body wall muscles of the juvenile (Razy-Krajka et al. 2014).

The oral siphon muscles (OSM) derive from a distinct lineage, named A7.6, which is not cardiogenic but instead also produces blood and tunic cells, as well as stomach and gill slit epithelia (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*, were 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. Because no A7.6-specific reporter transgene was available, we applied the heterologous, repressible transgenic systems Gal4/UAS (Brand and Perrimon 1993), LexA/LexAop (R. Yagi, Mayer, and Basler 2010), TrpR/tUAS (Suli et al. 2014), and QF/QS (Potter et al. 2010) and systematically evaluated their efficacy, specificity and toxicity for use by multiplexed electroporation in *Ciona* embryos. We identified a Gal80-repressible form of LexA/LexAop
(R. Yagi, Mayer, and Basler 2010) to be the most efficient, specific and innocuous approach
to specifically mark the A7.6 lineage.

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 has
previously been referred to as the trunk lateral cells (TLC; Tokuoka et al. 2004; Imai et al.
2003; Tokuoka et al. 2005; 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 (OSMF), 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 both activate Mrf,
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. Next we
demonstrate 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, while Tbx1/10
regulates Ebf in the ASMF where Ebf acts as a master-regulator of ASM fate, Ebf is regulated
independently of Tbx1/10, and both are required jointly for Mrf expression in the OSM. Our
findings demonstrate context-dependent rewiring of a deeply conserved kernel of muscle
specification genes within a single genome.

Results

Heterologous binary systems for lineage-specific transgene expression in Ciona

Many genetic studies in Ciona are based on the use of transient transgenesis by
electroporation of plasmid DNA (Corbo, Levine, and Zeller 1997; Christiaen et al. 2009a;
Stolfi and Christiaen 2012). Putative enhancers are cloned upstream of neutral reporters or
active genetic elements for tissue-specific transgene expression, generally recapitulating
endogenous patterns of activity (Wang and Christiaen 2012). Pleiotropic cis-regulatory regions can be dissected to isolate tissue-specific enhancers, but this process is not guaranteed to generate a transgene with specific enough enhancer activity.

Heterologous transgene activation systems provide an attractive addition to the molecular toolbox for studies using *Ciona*. Gal4/UAS-based systems can be inhibited by expression of Gal80, generating a logic by which tissue specificity is achieved by activation of the response element within the domain of gene A, “but not” within the domain of partially overlapping gene B (Brand and Perrimon 1993; Suster et al. 2004).

We chose the proximal enhancer of *Hand-related*, which is expressed in A7.6 and anterior trunk endoderm (Davidson and Levine 2003; Woznica et al. 2012; and this paper) to test the specificity, efficiency, and toxicity of the heterologous, repressible transgenic systems Gal4/UAS (Brand and Perrimon 1993), LexA/LexAop (R. Yagi, Mayer, and Basler 2010; Lai and Lee 2006), TrpR/tUAS (Suli et al. 2014), and QS/QF (Potter et al. 2010) (Fig. S1). Because of its rapid activation, efficiency, specificity, lack of obvious toxicity, and ability to be inhibited by Gal80, we used the Gal80-repressible form of a transcription activator consisting of the DNA binding domain from the bacterial LexA protein fused to the trans-activation domain of the yeast Gal4 protein, LexA::Gal4AD (“LHG”) (R. Yagi, Mayer, and Basler 2010) downstream of the *Hand-related* proximal enhancer (Hand-r>LHG). We electroporated zygotes using a combination of *Hand-r>H2B::mCherry;Hand-r>LHG;LexAop>GFP* constructs in order to determine the extent to which the binary LexA/LexAop system recapitulated the original *Hand-r*-driven expression pattern (Fig. 1A,B). We observed that 83.1% (± 17.3%; n=107) of larvae expressing *Hand-r>H2B::mCherry* also expressed *Hand-r>LHG;LexAop>GFP* in patterns that overlapped almost completely, meaning that the LexA/LexAop binary system faithfully recapitulates the
activity of unary transgenes, albeit with a ~3 hours delay compared to reach levels similar
to those of unary Hand-r-driven transgenes (Fig. S1C).

We then used the Nkx2-1/Ttf1 enhancer to drive Gal80 (Nkx2-1>Gal80) in order to
inhibit LHG trans-activity specifically in the endoderm and restrict LexAop activation to the
A7.6 lineage (Figure 1C, D; Ristoratore et al., 1999). We marked the endoderm membranes
with Nkx2-1>hCD4::GFP (Gline et al. 2015), and used either Hand-
r>LHG;LexAop>H2B::mCherry or Hand-r>LHG;Nkx2-1>Gal80;LexAop>H2B::mCherry. We
observed a 96% reduction in the proportion of hCD4::GFP+ larvae that also had endodermal
cells marked with H2B::mCherry (Fig. 1C,D). These results demonstrate the efficacy and
specificity of the LHG/Gal80/LexAop system to achieve refined transgene expression using
a tertiary "but not" logic. 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.

The detailed A7.6 origins of oral siphon muscles (OSM)

Although previous work has characterized gene expression patterns attributed to
the A7.6-derived TLCs in tailbud embryos (Imai, Satoh, and Satou 2003; Jeffery et al. 2008;
Shi and Levine 2008; Tokuoka et al. 2004; Tokuoka, Satoh, and Satou 2005), the detailed
development and morphogenesis of the A7.6-derived cells remained uncharacterized.
Therefore, we undertook a comprehensive description of the entire A7.6 lineage through
8.5 hours post-fertilization (hpf; stage 17, initial tailbud I;(Hotta et al. 2007)), and of the
cells that give rise to the OSM through 24hpf (stage 26+).

Because mCherry proteins generated by the LexA/LexAop constructs are not easily
detectable in embryos until approximately 8hpf (Fig. S1C), we marked the A7.6 lineage by
the overlap of Hand-r>H2B:mCherry and MyT>unc76::GFP transgenes, (The MyT driver is
active in A7.6 and a-line neural cells (Imai et al. 2004; Shi and Levine 2008); Fig. 2B-F). We visualized the A7.6 blastomeres in samples fixed every fifteen minutes starting at 5hpf (approx. stage 10), and applied Conklin’s nomenclature (Conklin 1905) to identify and name every cell within the A7.6 lineage up to 8.5hpf (approx. stage 17; Fig. 2). In parallel, we tested whether early divisions coincided with specific gene activities, as is often the case in early ascidian embryos (Satoh 2013, ch.12): we performed in situ hybridization on early stage embryos to detect expression of Hand-related and MyT immediately following each one of the earliest divisions, while marking A7.6-derived cells with MyT-driven nuclear H2B::mCherry (MyT>H2B::mCherry) (Fig. 2G-P).

At 5hpf, newborn A7.6 blastomeres express both Hand-r and MyT (Fig. 2G,L); and by 5.5hpf, they have divided along the antero-posterior axis (Fig. 2C, H, M). Although there is no obvious morphological asymmetry between the two daughter cells, the anterior A8.12 preferentially maintains Hand-r expression, while the posterior A8.11 maintains MyT expression (Fig 2H,M). By 6hpf, both A8.12 and A8.11 have divided; A8.12 along the antero-posterior axis and A8.11 dorsoventrally, again without obvious morphological asymmetry between their daughter cells, A9.21/A9.22 and A9.23/A9.24, respectively (Fig. 2A,D). A9.24/A9.23 both maintained roughly equal levels of Hand-r expression, which was no longer detected in the posterior A9.22/A9.21-pair, which instead expressed sustained levels of MyT (Fig. 2I,N).

MyT expression was rapidly and strongly re-activated in A9.23, the sister of the anteriormost Hand-r+ A9.24 (Fig. 20), prior to dividing at ~7.5hpf. Therefore, A9.23 expresses both MyT and Hand-r when it divides at ~7.5hpf. This division occurs along the dorsoventral axis of the embryo, giving rise to the ventral A10.45 and dorsal A10.46 cells, both of which maintained Hand-r and MyT expressions until about 8.5hpf, at which point expression of Hand-r was decreased substantially (arrows in 2H and M point to A8.12,
which maintains Hand-r, but not MyT). By 8.5 hpf, the anteriormost TLC, A9.24, had divided along the anteroposterior axis and its daughters A10.48/47 maintained expression of Hand-r (Fig. 2K). At that time, which is during the late neurula stage, the anteriormost TLC, A10.48 activates Ebf (Fig. 2K,P,R). In summary, A7.6 blastomeres and their anterior-most progeny always divided somewhat antero-posteriorly such that A10.48, the anterior-most Hand-r+/Ebf+ great-granddaughter of A7.6, always stood out among the TLCs. Given the established roles of Hand-r and Ebf in ASM specification within the cardiogenic B7.5 lineage (Stolfi et al., 2010; Razy-Krajka et al., 2014), we regarded A10.48 as the most likely OSM progenitor in tailbud embryos.

Between 8.5hpf and 16hpf, although the derivatives of A9.23 and A9.22/21 can still be distinguished from each other by position and gene expression patterns, more detailed clonal relationships could not be inferred from time series of fixed embryos. Therefore, we refer to all A9.23/22/21 derivatives as “posterior TLC”. By contrast, the A9.24 derivatives, A10.48 and A10.47, which we will call the “anterior TLC”, continue to follow stereotyped division patterns and gene expression dynamics until 16hpf (stage 24; LTBI; Fig. 3).

In order to test whether the OSM derive from the anterior Hand-r+/Ebf+ A10.48 progenitors, we sought to further characterize the division, migration and gene expression patterns of the latter throughout tailbud and larval stages (Figs. 3, 4). For time-points after 9hpf, we used our LexO(A7.6)>>H2B::mCherry binary reporter system to label the A7.6 lineage, and applied the Conklin nomenclature only to the A10.48 cells and their descendants. Finally, It has been shown that Hand-r, Tbx1/10 and Ebf are expressed sequentially and are necessary to activate the ASM program in B7.5-derived progenitors (Wang et al. 2013; Razy-Krajka et al. 2014; Stolfi et al. 2014). Therefore, we added Tbx1/10
to Hand-r and Ebf in our analysis of candidate markers of early fate-restricted OSM precursors.

The Hand-r+/Ebf+ A10.48 did not divide until 11hpf (mid-tailbud II), at which point its daughter cells, the anterior A11.96 and posterior A11.95, exhibit distinct and stereotyped differences in nuclear volumes and positions. Live imaging showed that this size asymmetry resulted from an inflation of A11.95 during interphase rather than an initially asymmetric division (movie S1). Meanwhile, A10.47 divides dorsoventrally giving rise to A11.94/93. During this time, both A11.96 and A11.95 continue to express Hand-r and Ebf, while A11.94/93 lost the Hand-r mRNAs that were still detectable in their A10.47 mother (Fig. 2K; Fig. 3B-E,L). By 13hpf, A11.95 has divided dorsoventrally and both daughters A12.190 and A12.189 continue to express Hand-r and Ebf so that all three of A11.96, and A12.190/A12.189 co-express Hand-r and Ebf. However, A11.96 expresses higher levels of Ebf mRNAs than the A12.190/189 pair (Fig. 3H). Moreover, by 13hpf, only A11.96 activates Tbx1/10 expression (Fig. 3J). By 16hpf, A11.96 has divided along the anteroposterior axis, giving rise to anterior A12.192 and posterior A12.191. The remaining anterior TLC have also divided, such that there were always 8 anterior TLCs by 16hpf, with only the anterior-most A12.192 and A12.191 cells co-expressing Hand-r, Ebf and Tbx1/10 (Fig. 3E,I,K). Our observation that A11.96 is the first and only A7.6-derived cell to co-express the siphon muscle determinants Hand-r, Tbx1/10 and Ebf opened the possibility that A11.96 is the fate-restricted OSM precursor.

In order to determine whether the A11.96-derived A12.191/192 cells are the sole fate-restricted OSM founder cells, we characterized the expression dynamics of Mrf mRNA and the Isl>unc76::Venus reporter construct - hereafter referred to as Isl>YFP, which marks all siphon muscle cells (Fig. 1; (Stolfi et al. 2010)). Within the A7.6 lineage, we found that Isl>YFP was first expressed in A12.191/192 at 16hpf (Fig. 4A,A'). Subsequently, A12.192
and .191 cells each divide once between 18hpf and 22hpf, giving rise to four Isl>YFP+/LexO(A7.6)>>H2B:mCherry+ cells on either side of the larva (A13.382 to A13.386; Fig. 4A-D, with close-ups of the same data shown in A’-D’). These Isl>YFP+/LexO(A7.6)>>mCherry+ cells will migrate to form an 8-cell ring underneath the oral ectoderm (a.k.a. stomodeum; Christiaen et al, 2005), where at 24hpf the complete OSM rudiment will contain 8 cells, all of which are mCherry+/YFP+ (Fig.4D-D’).

Since Mrf and Bhlh-tun1 were previously shown to be expressed in the 28hpf OSM ring (Razy-Krajka et al., 2014), we reasoned that the earliest expression of these genes would also point toward the OSMP. Therefore, we sought to describe their expression dynamics in the labeled A7.6 lineage. We found that Mrf first turned on at 18hpf in the most anterior pair of marked cells, which we inferred to be A12.191 and A12.192 (Fig. 4E).

Between 18 and 22hpf, one of these cells divides first, so that at around 19hpf the OSMP are comprised of three cells—one Mrf+/bHLH-tun1--; one Mrf-/Bhlh-tun1++; and one expressing both genes (Fig. 4F). At later time points, we found that each set of OSMP divided into two Mrf-expressing and two Bhlh-tun1-expressing cells (Fig. 4G, H). The existence of Bhlh-tun1++;Mrf- OSMPs suggests that, by analogy with the ASMPs (Razy-Krajka et al. 2014), the OSM anlage contains stem-cell-like precursors that may contribute to siphon growth and/or regeneration later in life (e.g. Hamada et al., 2015). Taken together, these observations indicate that A7.6-derived fate-restricted OSM founder cells are A12.192 and A12.191, which express Hand-r, Ebf, Tbx1/10, Isl>unc76::Venus and Mrf before producing Mrf+/Bhlh-tun1- and Mrf-/Bhlh-tun1+ OSMPs in a manner analogous to ASMPs (Fig. 4I, J).

Novel regulatory relationships between Tbx1/10 and Ebf

Because early determinants of ASM specification are expressed in the progenitors of the OSM, but follow different spatiotemporal dynamics, we 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, we ruled out a role for Tbx1/10 in the onset of Ebf expression but reasoned that Tbx1/10 could instead contribute to Ebf maintenance in fate-restricted OSMPs.

We first tested whether Tbx1/10 misexpression would maintain Ebf expression in A11.95-derived anterior TLCs that transiently express Ebf but fail to maintain it and never activate Tbx1/10 (see Figs. 3L, 4I for the clonal context of this discussion). We used the LexO(A7.6)>>Tbx1/10 to overexpress Tbx1/10 throughout the A7.6 lineage, and analyzed the effect on Ebf expression at 16hpf (Fig. 5A-D). At 16hpf, Ebf is normally expressed in the nervous system, the ASMFs and the OSMs (A12.192/191; Fig 3I; Fig. 5B; Wang et al. 2013). Upon misexpression of Tbx1/10, we did not observe substantial ectopic Ebf expression in either the A11.95-derived anterior TLC, nor anywhere in the posterior TLCs (n=62; Fig. 5C). Thus Tbx1/10 misexpression does not appear to be sufficient to cause ectopic Ebf expression in the posterior TLC, or maintain Ebf expression in the anterior TLC. 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).

We next sought to test the effects of loss of Tbx1/10 on Ebf expression in the A7.6 lineage. In the B7.5 lineage, RNAi-mediated loss of Tbx1/10 function inhibited Ebf expression in the ASMF (Wang et al. 2013). To test whether Tbx1/10 is required for the maintenance Ebf expression in the OSM, we used CRISPR/Cas9-based tissue-specific mutagenesis to inhibit Tbx1/10 function. Here, we adapted methods developed by Gandhi, Stolfi, et al (Stolfi et al. 2014; Gandhi, Stolfi and Christiaen, in preparation) to direct editing of the Tbx1/10 locus by CRISPR/Cas9 (see Materials and Methods and Fig. S2), and used a
pair of sgRNA constructs targeting bp303-322 and bp558-577 in exon 1 of Tbx1/10 in all TBX1/10 loss-of-function experiments.

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. Using Ebf>GFP as a proxy for Ebf activation (Wang et al. 2013), we found that Tbx1/10 mutagenesis caused an 87% reduction in the proportion of electroporated larvae showing Ebf>GFP+ ASMs relative to control larvae, thus mimicking the published RNAi-mediated phenotype and demonstrating the efficacy Tbx1/10-targeting sgRNA constructs (Fig. S3B).

We then targeted Cas9 expression to the A7.6 lineage with our LexA/LexAop system and found that, in contrast to what has been demonstrated in the B7.5 lineage, loss of Tbx1/10 function in the A7.6 lineage did not alter Ebf expression in the OSMPs at 16hpf (n=66; Fig. 5E). Taken together, these results indicate that, by 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, we sought to test the role of Ebf in activation of Tbx1/10 in the A7.6 lineage. It seems unlikely that Ebf alone activates Tbx1/10 in the anterior TLC, since Ebf is expressed more broadly than Tbx1/10, and Ebf misexpression throughout the A7.6 lineage using a Lex0(A7.6)>>Ebf strategy was not sufficient to cause ectopic Tbx1/10 expression in any part of the TLC (Fig. 5F). However, Ebf may still be required together with (an) unknown co-factor(s) to activate Tbx1/10 in the A11.96 OSM founder cells. To test this, we used published sgRNA constructs and Lex0(A7.6)>>Cas9 for A7.6-lineage-specific CRISPR/Cas9-mediated targeted mutagenesis of the Ebf coding sequence (Stolfi et al. 2014). In control conditions (i.e. either Lex0(A7.6)>>LacZ or Lex0(A7.6)>>Cas9;U6>sgControl), Tbx1/10 was
expressed in the OSMP, sensory vesicle, STVC, and endoderm (Fig. S4). We found that Ebf-targeted mutagenesis inhibited Tbx1/10 expression specifically in the OSMP at 16hpf in
86% of embryos (n=14; Figure 5I). We therefore conclude that the OSM precursors
uniquely require Ebf to express Tbx1/10, while Ebf expression is independent of Tbx1/10
expression. This is in stark contrast with 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 within the cardiopharyngeal mesoderm
(Wang et al. 2013).

Tbx1/10 and Ebf are required in parallel to specify the OSM fate
We have shown that, although ASM and OSM both express Mrf in the siphon muscle
founder cells, the upstream regulators of Mrf expression in B7.5-derived ASMP are deployed
differently in the A7.6 lineage. The regulatory relationships that we have demonstrated
between Ebf and Tbx1/10 open the possibility that the function of each in Mrf activation
may also be unique in OSM vs. ASM. We first tested whether loss of either Ebf or Tbx1/10
function throughout the A7.6 lineage inhibited OSM fate specification by assaying Mrf
expression in 24hpf larvae (Fig. 6). In samples electroporated with
LexO(A7.6)>>nls:Cas9:nls and control sgRNA (U6>sgControlF+E; Stolfi et al, 2014), Mrf was
co-expressed with LexO(A7.6)>>H2B:mCherry in the OSM of 91.9% of larvae (n=37; Fig. 6A,
D). Co-electroporating the sgTbx1.303 and sgTbx1.558 constructs targeting Tbx1/10
reduced the proportion of transfected larvae expressing Mrf in the vicinity of the oral
ectoderm to 35.1% (n=77; Fig. 6B, D). Similarly, targeted mutagenesis of Ebf reduced the
proportion of larvae with Mrf+/LexO(A7.6)>>mCherry+ OSM to 23.3% (n=43; Fig. 6C, D).
Because Tbx1/10 does not appear to be involved in Ebf activation (Fig. 5), we interpret
these results to indicate that Tbx1/10 has a direct, Ebf-independent role upstream of Mrf in
OSM fate. On the other hand, since Ebf is necessary for Tbx1/10 expression in the OSM (Fig. 5), loss of Mrf upon Ebf mutagenesis could be due to loss of Tbx1/10 in cis. Therefore, these data indicate that although the role of Ebf and Tbx1/10 as siphon muscle regulators is conserved between OSM and ASM, these functions may result from different regulatory logic in the two contexts.

We sought to further test this possibility using gain-of-function assays by misexpression. We used LexO(A7.6)>>Ebf or LexO(A7.6)>>Tbx1/10 to over-express Ebf and/or Tbx1/10 throughout the A7.6 lineage and assayed Mrf expression in 24hpf larvae (Fig. 7). Using LexO(A7.6)>>LacZ as a control, we observed that 4.9% of larvae express low levels of Mrf among scattered mesenchymal cells (n=41; Fig. 7A, E). Similarly, misexpression of Tbx1/10 resulted in only 2.1% of larvae showing any ectopic Mrf expression (n=47; Fig. 7B, E). Meanwhile, misexpression of Ebf increased the proportion of larvae with ectopic Mrf to 32.8% (n=64; Fig. 7C, E). In these larvae, A7.6-derived cells that showed ectopic Mrf expression clustered towards the dorsal midline. We observed increased proportions of ectopic Mrf+ A7.6-derived cells when we co-expressed LexO(A7.6)>>Ebf and LexO(A7.6)>>Tbx1/10 together: 60.7% of larvae showed ectopic Mrf expression (n=84; Fig. 7D, E). Remarkably, A7.6-lineage cells that ectopically expressed Mrf upon Ebf and Tbx1/10 co-expression tended to cluster near the atrial siphon primordium, whereas the anterior-born OSMP normally migrate towards the oral siphon primordium (a.k.a. stomodeum; e.g. Fig. 7D). This observation suggests that when combined misexpression of Ebf and Tbx1/10 induced ectopic Mrf expression and siphon muscle fate in the posterior TLcs, siphon muscle precursor cells home towards the closest siphon primordium during larval development. Taken together, these data demonstrate that Ebf and Tbx1/10 functions are both required and act in parallel to activate Mrf and promote siphon muscle specification in the A7.6 lineage.
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, where it must be activated by Tbx1/10. 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 (Racioppi et al., unpublished construct) to restore Ebf expression specifically in the STVCs and their progeny. Importantly, the enhancer activity of this Tbx1/10 construct was not affected by expression of Cas9 and sgTbx1.303;558 (Fig. S3A, B; Tbx1/10(-7333/-2896)::bpTbx1>Ebf).

In control larvae, there are four ASM precursors (ASMP) at 22hpf, with the outer ASMP expressing Mrf and the inner ASMP expressing Bhlh-tun1 ((Razy-Krajka et al. 2014) and Fig. 8A; SHP marked with “*”). In the B7.5 lineage, loss of Tbx1/10 results in loss of Ebf in the ASMF (Fig. S3C; Wang et al., 2013) and Ebf activates ASM-predictive expression of Mrf and Bhlh-tun1 (Razy-Krajka et al., 2014). Consistent with this, we found that loss of Tbx1/10 abolished expression of Mrf and Bhlh-tun1 in 100% of larvae (n=10), likely due to loss of Ebf expression (Fig. 8C). Because the Tbx1/10 enhancer drives transgenic expression in the ASM founder cells (ASMF) as well as the second heart precursors (SHP; see Fig. 4J), we expected to see the effects of Ebf overexpression expanded to the SHP. Indeed, electroporation of Tbx1/10>Ebf with control guide RNA caused an expansion of Mrf and Bhlh-tun1 expression to the SHP in 9 out of 11 larvae (Fig. 8B). Having also confirmed that mutation of Tbx1/10 did not interfere with expression of Tbx1>GFP (Fig. S3B), we introduced Tbx1>Ebf and found that Ebf alone was able rescue, and cause ectopic,
expression of Mrf and Bhlh-tun1 (Fig. 8D). These data indicate Tbx1/10 function is
dispensable for ASM specification downstream of Ebf. Therefore, whereas Ebf can regulate
siphon muscle fate without Tbx1/10 in the ASM, its function as an activator of siphon
muscle fate in the OSM depends on co-expression with Tbx1/10 (Fig. 8E).
Discussion

In this paper, we use two clonally distinct but molecularly similar muscle groups to examine context-dependent control of muscle specification. Though the regulatory mechanisms upstream of Mrf expression in the atrial siphon muscle (ASM) founder cells of the basal Chordate Ciona intestinalis have been described (Stolfi et al. 2010; Wang et al. 2013; Razy-Krajka et al. 2014), very little was known about the origins of the oral siphon muscles (OSM) or the mechanisms of OSM specification. We present a detailed description of the developmental origins of the oral siphon muscles in the ascidian Ciona. We have traced the clonal origins of the OSM from a single multipotent mesodermal progenitor in the gastrula, and explored the mechanisms that activate myogenesis in this lineage. In order to identify and characterize the descendants of the A7.6 lineage, we adapted the heterologous Gal80-repressible binary transgenic system from E. coli, LexA/LexAop (R. Yagi, Mayer, and Basler 2010), which is poised to permit refined transgenic strategies for studies using Ciona.

Conserved synergy between Ebf and Tbx1/10

Muscle differentiation across bilaterians depends on the activity of muscle regulatory factor (MRF) family of bHLH DNA binding transcription factors (Braun et al. 1994; Summerbell, Halai, and Rigby 2002; Kassar-Duchossoy et al. 2004; Rudnicki et al. 1993; Wei, Rong, and Paterson 2007; Fukushige and Krause 2005; Andrikou et al. 2015). These MRFs are regulated by, and work in concert with, transcription factors that can differ between muscle groups, but appear to be conserved across species. Pax3/7 homologs have long been recognized as regulators of somitic muscle specification and regeneration in vertebrates, and have recently been shown to have a role in limb muscle specification and regeneration in arthropods (Buckingham and Relaix 2007; Sambasivan et al. 2013; von Maltzahn et al. 2013; Konstantinides and Averof 2014). Tbx1 homologs are crucial...
regulators of pharyngeal muscle development in vertebrates (Aggarwal et al. 2010; Z. Zhang, Huynh, and Baldini 2006; Sambasivan et al. 2009; Diogo et al. 2015). In mammals, \textit{Tbx1} regulates \textit{Myf5} and \textit{MyoD} specifically in the mandibular arches, but is not involved in specification of other head muscles, or in the somitic muscles (Sambasivan et al. 2009). The \textit{Drosophila} ortholog of \textit{Tbx1}, \textit{org-1}, is required for expression of \textit{ladybird} and \textit{slouch}, both muscle identity genes, all of which occurs downstream of \textit{nautilus} expression (Crozatier and Vincent 1999; Wei, Rong, and Paterson 2007; Schaub et al. 2012).

Transcription factors of the \textit{Collier/Olf1/Ebf} (COE) family are emerging as important upstream regulators of \textit{Mrf} expression and myogenesis. COE orthologs have documented roles in neurogenesis, cellular immunity, and hematopoiesis (Crozatier and Vincent 1999; Pang, Matus, and Martindale 2004; Kratsios et al. 2011; Benmimoun et al. 2015). Earliest evidence for COE's myogenic properties implicated it in specification of a hemisegmentally-repeated abdominal muscle subtype by interactions with \textit{nautilus}, though in a domain distinct from the action of \textit{org-1} (Crozatier and Vincent 1999; Schaub et al. 2012; Wei, Rong, and Paterson 2007) (Crozatier and Vincent 1999). The COE homologs, \textit{Ebf2} and \textit{Ebf3}, have been found to regulate \textit{Myf5} and \textit{MyoD} expressions in \textit{Xenopus} (Green and Vetter 2011). In the mouse, \textit{Ebf1} and \textit{Ebf3} interact with \textit{MyoD} in the developing diaphragm to activate muscle-specific gene transcription (Jin et al. 2014). \textit{Ciona} has only one copy of \textit{Ebf}, which has been found to be necessary for both neurogenesis and myogenesis (Kratsios et al. 2011; Razy-Krajka et al. 2014), in keeping with an ancient role for COE homologs in both of those functions (Jackson et al. 2010).

Future studies in other systems will be needed to determine whether, as shown to be the case in \textit{Ciona} (this study and Wang et al. 2013; Razy-Krajka et al. 2014), regulatory interactions between COE and \textit{Tbx1} contribute to muscle specification as could be predicted by their overlapping expressions in developing pharyngeal muscles.
Comparison of ASM and OSM

We have shown that, while Tbx1/10 and Ebf are both expressed in the ASM and OSM precursors and required for Mrf expression, their regulatory relationships differ between the B7.5/ASM and A7.6/OSM lineages (Fig. 8). In the B7.5 lineage, Ebf is necessary and sufficient to activate Mrf, even in the absence of Tbx1/10 (Fig. 8; Wang et al. 2013; Razy-Krajka et al. 2014). In the A7.6 lineage, on the other hand, Mrf requires the combined inputs of Ebf and Tbx1/10 (Fig. 6 and 7). When misexpression of Ebf leads to ectopic Mrf expression (Fig. 7), it was likely due to the ability of Ebf to activate Tbx1/10 in the A7.6 lineage (Fig. 5), so that Tbx1/10 was provided in trans, and both together activated Mrf.

Ectopic activation of Mrf in the A7.6 lineage was nearly twice as efficient when Tbx1/10 and Ebf were provided together, supporting the notion that they act in combination to activate Mrf in the OSM.

Why would the temporal deployment and regulatory logic of two such deeply conserved muscle-regulatory genes differ so greatly in each context? Specification of the siphon muscle founder cells in both the A7.6 lineage and the B7.5 lineage is an instance of binary fate choice, the outcome of which is expression of Mrf and the terminal differentiation gene battery specific to siphon muscles. However, the alternative fate and embryonic context in each case is different. Therefore, the rewiring of the Ebf-Tbx1/10 interactions that we have demonstrated upstream of Mrf may reflect larger network constraints specific to the A7.6 and B7.5 lineage, respectively. Meanwhile, the shared need for either Tbx1/10 or Ebf in muscle specification likely reflects the fact that Ebf and Tbx1/10 regulate not only Mrf, but is also involved in 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). COE in
Drosophila has also been shown to bind directly to enhancers of muscle-specific identity genes (de Taaffin et al. 2015), as has org-1 (Schaub et al. 2012). Our results indicate that although Ebf has been largely overlooked as a muscle regulator in vertebrates (perhaps because of genetic redundancies between Ebf1, -2 and -3), its function in concert with MyoD homologues may be deeply conserved, and medically relevant. Further work investigating genes directly activated by Ebf in ASM and OSM in Ciona, will provide important insights into the conservation and evolution of the complex regulatory interactions involved in muscle development.

Materials and Methods

Animals and Electroporation

Gravid C. intestinalis type A, also known as Ciona robusta (Brunetti et al. 2015) adults were obtained from M-REP, Santa Barbara, 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.

Cloning of unary transgenic enhancers

Enhancers were amplified from larval gDNA using specific primers shown in Table 1 and cloned into backbones containing reporters using standard molecular cloning procedures.
<table>
<thead>
<tr>
<th><strong>Cloning of Gal4/UAS, LexA/LexAop, TrpR/tUAS and QF/QS constructs</strong></th>
</tr>
</thead>
</table>
| Gal4 was amplified from Tubp-Gal4 (Addgene #17747; (Lee and Luo 1999)) using specific primers and adding NotI and EcoRI restriction sites on the 3’ and 5’ ends, respectively (NotI-Gal4-F: 3’- ATGAAGCTACTGTCTTCTATC -5’; EcoRI-Gal4-R: 3’- TTACTCTTTTTTGTTTGG -5’). Gal80 was amplified from Tubp-Gal80 (Addgene #17748; (Lee and Luo 1999)) using specific primers and adding NotI and EcoRI restriction sites (NotI-Gal80-F 5’- AAAGCGGCGCAGATTGCGTGGTGTGGAATG -3’; EcoRI-Gal80-R 5’- AAAGAATTCCTTATAAATGTCGAGATTG -3’). Plasmids containing a 5x concatamer of

<table>
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<th><strong>FGF8/17/18</strong></th>
<th><strong>NotIR</strong></th>
<th><strong>5’- TTTTCGCGCGCAACGTAGATATCTTTCGCAAAT</strong></th>
<th><strong>3’-</strong></th>
</tr>
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<tr>
<td><strong>KH2012:KH.C</strong></td>
<td><strong>1.116</strong></td>
<td><strong>Hand-related (-622) AsclF</strong></td>
<td><strong>5’- AAAGGGGGCAAAACAGTTGTCTCCCCTGAG</strong></td>
</tr>
<tr>
<td><strong>KH2012:KH.C</strong></td>
<td><strong>1.143</strong></td>
<td><strong>LBP (-3198) AsclF</strong></td>
<td><strong>5’- TTTGGCGCGCTCAACACAGGAGACATCCA</strong></td>
</tr>
<tr>
<td><strong>KH2012:KH.C</strong></td>
<td><strong>1.274</strong></td>
<td><strong>MyT (-3271) AsclF</strong></td>
<td><strong>5’- AAAGGCGGCTTTTTCGTCGCAAGAGCTG-3’</strong></td>
</tr>
<tr>
<td><strong>KH2012:KH.I</strong></td>
<td><strong>1.084</strong></td>
<td><strong>OASIS (-3345) AsclF</strong></td>
<td><strong>5’- TTTGGCGCGCAGCATAGCGTTCCTACAGTCG</strong></td>
</tr>
<tr>
<td><strong>KH2012:KH.S3</strong></td>
<td><strong>90.2</strong></td>
<td><strong>TLC2 (-1804) AsclF</strong></td>
<td><strong>5’- TTTGGCGGCGCCAGTGGGAGTGGGTAT</strong></td>
</tr>
<tr>
<td><strong>KH2012:KH.I</strong></td>
<td><strong>1.084</strong></td>
<td><strong>OASIS (+31) NotIR</strong></td>
<td><strong>5’- AAAGCGGCAACAGCGGTGTGCACTACAGATG</strong></td>
</tr>
<tr>
<td><strong>KH2012:KH.S3</strong></td>
<td><strong>90.2</strong></td>
<td><strong>TLC2 (+54) NotIR</strong></td>
<td><strong>5’- TTTGGCGCGCCAGGGAACATGFGGTAGAATG</strong></td>
</tr>
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</table>
the UAS response element fused to the HSP70 basal enhancer were created by excising CD8:GFP from the pUAS-mCD8:GFP backbone (Addgene #17746; (Lee and Luo 1999)) using NotI/XbaI restriction digest, and then using an XbaI/EcoRI linker fragment to clone all reporters flanked by NotI/EcoRI sites.

The LHG coding sequence and LexAop enhancer sequences were taken from pDPPattB-LHG and p28-pJFRC19-13xLexAop2-IVS-myr--GFP (R. Yagi, Mayer, and Basler 2010). The LHG coding sequence was amplified using specific primers and adding restriction sites NotI/EcoRI (NotI-LHG-F: 5’-AAAGCG GCC GCAAC CATG AAAGC GTTA ACGGC CAG-3’; EcoRI-LHG-R 5’-TTTGAAT TCTTA CTC TTTTT TTG GGTT TG-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 Ascl/NotI restriction enzymes and cloned into a backbone containing H2B::mCherry and subsequently subcloned using standard molecular cloning methods.

TrpR coding sequence was amplified from pCMV:nlsTrpR-Gal4AD using specific primers and adding NotI and EcoRI restriction sites at the 3’ and 5’ ends, respectively (NotI-TrpR-F: 3’-AAAGCGGCCGCAACCATGAAAGCCTTAAGGCAG-3’; EcoRI-TrpR-R: 3’-GCCCAAATCACCCTATTC-5’)

The QF coding sequence was amplified from pAC-QF (Addgene #24338; (Potter et al. 2010)) using specific primers and adding NotI and EcoRI restriction sites at the 3’ and 5’ ends, respectively (NotI-QF-F: 3’-AAAGCGGCCGCAACCATGAAAGCCTTAAGGCAG-5’; EcoRI-QF-R: 3’-AAAGAATTC CTAT TGC TCATA CGT G TTGAT-5’). The QUAS response element and D. melanogaster HSP70 basal promoter was amplified from p5E-QUAS (Addgene #61374)
using specific primers and adding NotI and EcoRI restriction sites at the 3' and 5' ends, respectively (NotI-QUAS-F: 3' - TTT GG CGCG CC  GG G TTA TCT ATC CT C G G – 5'; EcoRI-QUAS-R: 3' - TTT G CGG CCG CC AA T TC C CT AT T CA G A GT TCT – 5').

Genome editing using CRISPR

Single Guide RNAs against Ebf were used according to the methods described in (Stolfi et al. 2014). Single guide RNAs against Tbx1/10 were designed using CRISPRdirect (Naito et al. 2014), 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 cds of the first base-pair targeted): sgTbx1.303(+); sgTbx1.422(–); sgTbx1.558(+); sgTbx1.673(+) ; sgTbx1.783(+) ; sgTbx1.835(+); sgTbx1.982(+) ; sgTbx1.971(–); sgTbx1.1067(+) (Fig. S2A). We verified that sgRNAs directed cutting of genomic DNA by electroporating embryos with the ubiquitously-expressed Ef1α >nls:Cas9:nls and 25 μg 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 pCR-II 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 (Fig. S2B). Therefore, for all tissue-specific manipulations of Tbx1/10 function, we used LexO(A7.6)>>nls:cas9:nls;U6>sgTbx1.303;U6>sgTbx1.558, with 25ug 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.

Acknowledgements

We are grateful to Anthony Filipovic for help characterizing the efficacy and toxicity of various candidate binary systems. We are indebted to Claudia Racioppi and Alberto Stolfi for the Tbx1/10 STVC-specific enhancer. We give special thanks to Maximilien Courgeon and Claude Desplan for the LexA/LexAop plasmids. This work was supported by National Institutes of Health/National Heart, Lung and Blood Institutes R01 HL108643 award to L.C. and 2T32HD007520-16 award to TRT as part of the New York University Developmental Genetics Training Grant program (P.I.: Jessica Treisman). The authors declare no competing interests.

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Figure Legends

Figure 1. The Gal80-repressible LexA/LexAop binary transgenic system efficiently and specifically labels the A7.6 blastomeric lineage of *Ciona robusta*. (A) Confocal image of 24hpf larva showing expression of Handr>H2B:mCh and Isl>unc76:GFP. OSM are mCherry+/GFP+ while ASM are mCherry-/GFP+. Endoderm is marked with "*". (B) 24hpf larva expressing Hand-r>LHG;LexAop>H2B:mCherry and Isl>unc76:GFP, showing that Handr>LHG;LexAop>unc76:GFP fully recapitulates expression of Handr>H2B:mCherry. (C) Single confocal slice of 24hpf larva expressing Hand-r>LHG;LexAop>H2B:mCherry and the endodermal marker Nkx2-1>CD4:GFP, showing that Hand-r>LHG;LexAop>H2B:mCherry is expressed throughout the endoderm. (D) Single confocal slice of 24hpf larva showing that expression of Nkx2-1>Gal80 abolishes expression of Hand-r>LHG;LexAop>H2B:mCherry in the endoderm only.

Figure 2. 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 at each time-point in panels G-R. (B-F) Embryos electroporated with Hand-r>H2B:mCherry and MyT>unc76:YFP, which overlap exclusively in the A7.6 lineage. 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. Expression of MyT>GFP or of *MyT* mRNA in the a-line neural cells is marked with an “*”. (G-R) Expression dynamics of Hand-related (G-K), MyT (L-P), or Ebf (Q,R) mRNA, with A7.6 lineage marked by MyT>H2B:mCherry. (S) 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.

Figure 3. 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 Fig. 2K and R, to emphasize continuity of A10.48 cell and to show the anterior TLC in the whole-embryo context. Panels B-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; small white arrows mark the daughters of A11.96, named A12.192/191.

Scale bars = 25 μm. (E) 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.

Figure 4. 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 OSM at 16hpf (A), followed by cell divisions, anterior migration of the OSM, 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 OSM in 18-24hpf larvae labeled with LexO(A7.6)>>H2B:mCh and with Mrf (blue) and Orphan-bHLH-1 (green) mRNA revealed by in situ hybridization. (I) Complete schematic diagram of the development of the A7.6 lineage, from the time of its birth at 5hpf, until differentiation of the OSM around 24hpf, with gene expression dynamics labeled and mapped on clonally. (J) For comparison, a simplified schematic diagram of key steps in ASM development within the B7.5 lineage based on (Davidson and Levine 2003; Stolfi et al. 2010; Wang et al. 2013).

Figure 5. Effects of Tbx1/10 gain-of-function and loss-of-function on expression of Ebf(F-E) and effect of Ebf gain-of-function and loss-of-function on Tbx1/10 at 16hpf (F-I). (A) Cartoon showing whole-embryonic context of the anterior TLC at 16hpf. (B-E) Close-up of anterior TLC population at 16hpf, electroporated with LexO(A7.6)>>H2B:mCherry and LexAop>LacZ (B); LexAop>Tbx1/10 (C); LexAop>nls:Cas9:nls;U6>sgControlF+E (D); or LexAop>nls:Cas9:nls;U6>sgTbx1.303;U6>sgTbx1.558 (E). (F-I) Close-up of anterior TLC in 16hpf embryos electroporated with LexO(A7.6)>>H2B:mCherry and LexAop>LacZ (F); LexAop>Ebf (G); LexAop>nls:Cas9:nls;U6>sgControlF+E (H); or LexAop>nls:Cas9:nls;U6>sgTbx1.303;U6>sgTbx1.558 (I). Scale bars = 10μm. Total n are pooled from two biological replicates of batch-electroporation of zygotes. n=61 for (B); n=62 for (C); n=77 for (D); n=66 for (E).
Figure 6. Tissue-specific knockdown of Ebf or Tbx1/10 in the A7.6 lineage leads to loss of OSM. (A-C) 26hpf larvae electroporated with LexO(A7.6)>H2B:mCherry;LexAop>nls>Cas9:nls and U6>sgControlF+E (A), U6>sgTbx1.303;U6>sgTbx1.558 (B), or U6>sgEbf.774 (C). (D) Boxplot showing the proportion of larvae in which LexO(A7.6)>H2B:mCherry and Mrf mRNA were both expressed in the OSM, with sample sizes indicated. The total n are pooled from two biological replicates of batch-electroporation of zygotes: n=37 for sgControl; n=77 for sgEbf; n=43 for sgTbx1/10. Scale bars = 25μm.

Figure 7. Effect of Ebf, or Tbx1/10 gain-of-function on expression of Mrf (A-D) 24hpf larvae electroporated with LexO(A7.6)>>H2B:mCherry and LexAop>LacZ (A); LexAop>Ebf (B); LexAop>Tbx1/10 (C); or LexAop>Ebf; LexAop>Tbx1/10 (D). (E) Boxplot showing proportion of larvae in each condition in which we observed ectopic Mrf expression, with sample sizes indicated. n= 41 for LacZ; n=47 for Ebf; n=64 for Tbx1/10; n=84 for Ebf + Tbx1/10.

Figure 8. Ebf is an independent master regulator of siphon muscle fate in the B7.5 lineage. (A-D) Close-up of ASM and first and second heart precursors (SHP; marked with "*"), derived from the B7.5 lineage, revealing Mrf mRNA (blue) and Blh-tun-1 mRNA (green). (A,B) Larvae electroporated with Mesp>H2B:mCherry;Mesp>nls:Cas9:nls;U6>sgControlF+E and Tbx1>LacZ (A) or Tbx1>Ebf (B). In (B), note that expression of Mrf and Blh-tun-1 has expanded to the SHP, due to earlier Tbx1>Ebf expression in the secondary TVCs. (C, D) Larvae electroporated with Mesp>H2B:mCherry;Mesp>nls:Cas9:nls;U6>sgTbx1.303;U6>sgTbx1.558 and Tbx1>LacZ (C) or Tbx1>Ebf (D). Note that in (C) there is a complete loss of Mrf or Blh-tun-1 expression, whereas in (D), Mrf and Blh-tun-1 show wild-type expression patterns in the ASM, and are also expressed in the SHP. Scale bars = 25μM. n=. All data was collected from a single technical replicate. (E) Schematic diagram comparing core regulatory interactions upstream of Mrf-driven differentiation and Notch-driven stemness in ASM and OSM. In black, documented shared expression of Hand-r in cells that give rise to, among other tissues, siphon muscles may be involved in activation of the core common regulators Tbx1/10 and Ebf, as indicated by green dashed arrows. Although both Ebf and Tbx1/10 impinge on Mrf expression, the distinct regulatory relationships in place in ASM vs. OSM are indicated by...
purple and brown arrows, respectively. The shared direct input from *Ebf* to *Mrf* in both ASM and OSM is indicated by the solid green arrow. In grey, Notch signaling downstream of *Mrf* activation has been established in the ASM (Razy-Krajka et al. 2014) as the mechanism for cells to choose between stemness and differentiation, but has not yet been tested in the OSM.
Figure 2 (Christiaen)
Figure 4 (Christiaen)
Ebf mRNA \textit{LexO(A7.6)} \textgreater H2B:mCherry 16hpf

Tbx1/10 mRNA; \textit{LexO(A7.6)} \textgreater H2B:mCherry; 16hpf
**Figure 6 (Christiaen)**

**LexO(A7.6) > H2B:mCherry; Mrf mRNA**

(A) sgControl

(B) sgTbx1.303

(C) sgTbx1.558

(D) sgEbf.774

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*Figure showing images of larvae expressing Mrf in OSM under different conditions.*

- **Notes:**
  - The images illustrate the expression of Mrf mRNA in OSM under various conditions.
  - The graph below the images depicts the percentage of larvae expressing Mrf in OSM, with different conditions and corresponding n-values.
Figure 7 (Christiaen)
Figure 8 (Christiaen)