Muscle-based movement is a hallmark of animal biology, but the evolutionary origins of myocytes are unknown. Although believed to lack muscles, sponges (Porifera) are capable of coordinated whole-body contractions that purge debris from internal water canals. This behavior has been observed for decades, but their contractile tissues remain uncharacterized with respect to their ultrastructure, regulation, and development. We examine the sponge *Ephydatia muelleri* and find tissue-wide organization of a contractile module composed of actin, striated-muscle myosin II, and transgelin, and that contractions are regulated by the release of internal Ca$^{2+}$ stores upstream of the myosin-light-chain-kinase (MLCK) pathway. The development of this contractile module appears to involve myocardin-related transcription factor (MRTF) as part of an environmentally inducible transcriptional complex that also functions in muscle development, plasticity, and regeneration. As an actin-regulated force-sensor, MRTF-activity offers a mechanism for how the contractile tissues that line water canals can dynamically remodel in response to flow and can re-form normally from stem-cells in the absence of the intrinsic spatial cues typical of animal embryogenesis. We conclude that the contractile module of sponge tissues shares elements of homology with contractile tissues in other animals, including muscles, indicating descent from a common, multifunctional tissue in the animal stem-lineage.
Animal movement is predominantly enabled by the coordinated activity of two cell types: myocytes (contractile cells of the muscular system) and neurons (signaling cells of the nervous system). But not all animals have these cell types—sponges lack both yet are still capable of coordinated tissue movements during whole-body contractions. Understanding how sponges contract has key relevance for understanding the evolutionary origins of animal movement.

Strictly sessile, sponges must actively pump water through an internal canal system for feeding, gas exchange, waste removal, and sexual reproduction. The canal system is partitioned into incurrent cavities (atria) and canals, feeding chambers lined by flagellated cells (choanocytes) that generate direction flow and phagocytose bacteria, and incurrent canals that channel wastewater to exhalant openings (oscula) (Fig. 1A). Contractions serve to clear canal blockages and maintain water internal water pressure increases to dislodge debris1 (Fig. 1A). After a contraction, incurrent pores close and canals narrow, and internal water pressure increases to dislodge debris3 (Fig. 1A).

Lab-grown freshwater sponges are useful models for studying contractions because they are small, transparent, and amenable to microscopy. In *Ephydatia muelleri*, contraction cycles can be described as biphasic. During phase I, atrial volume decreases, and incurrent canals narrow as water is displaced into incurrent canals which expand in diameter. During phase II, atrial volume and incurrent canal diameter increase as incurrent canals narrow (Fig. 1B). This is a progressive sequence, leading from the incurrent tissues of the system to the incurrent tissues, and propagates towards oscula3.

From a signaling perspective, it is known that canal blockage (natural or induced by the addition of Sumi ink) activates ciliated sensory cells, which release nitric oxide (NO). Nitric oxide diffuses across tissues and is modulated by glutamate and gammaminobutric acid (GAGA), initiating contraction2–4. A major gap in this model is that the cellular mechanisms of the contractile response remain essentially uncharacterized. How is the contractile force generated structurally, and how are contractions regulated at the level of cellular physiology? Answers to these questions are needed to clarify whether sponge contractile tissues are similar to non-muscle contractile tissues in other animals such as epithelia that undergo apical constriction, or to muscles?

In well-studied bilaterian animal models with muscle tissues, fast-contracting somatic myocytes are involved in voluntary or reactive movements, and slow-contracting visceral myocytes are involved in organ movements and tissue tension5. In both, contractions require interactions between actin filaments and type-II myosin, composed of two myosin heavy chains (MyHCs), two regulatory light chains (RLCs), and two essential light chains. The contraction speed of different cell and tissue types reflects the kinetics of the MyHC expressed. For example, striated-muscle myosin heavy chain (stMyHC) is found in fast-contracting skeletal and cardiac muscles of vertebrates, and smooth and striated invertebrate muscles6–8 (Fig. 2B). Non-muscle myosin heavy chain (nmMyHC) is found in slow-contracting vertebrate smooth muscle, and some invertebrate visceral muscles, but also in non-muscle contractile contexts such as cell motility, cytokinesis, and apical constriction9.

Bilaterian myocytes can be further distinguished by their regulatory mechanisms (see below). In fast contracting myocytes, the tropomysosin/troponin C complex hinders stMyHC from binding to actin but is released by Ca2+10. Tropomysin C-regulation is unique to bilaterian muscle. A potentially more ancient mechanism is the MLCK pathway, in which cytoplasmic Ca2+ binds to calmodulin, activating MLCK to phosphorylate the RLC of myosin II11,12. The MLCK pathway functions in muscle and non-muscle contractile contexts, and appears to regulate non-bilaterian muscles7,13.

During development, unique transcription factor combinations specify the identity of either cardiac and smooth muscles, or striated muscles in vertebrates (see below)3. These are well-conserved between species and indicate cell type homology between lineages14. An interaction central to the developmental specification of all muscle types is between a myocardin-related transcription factor (MRTF) and a MADS-box transcription factor—either serum response factor (SRF) or myocyte enhancer factor 2 (Mef2). Vertebrate smooth/cardiac muscle development also involves transcription factors from GATA, NK homeobox, and Fox families, and vertebrate striated/skeletal muscle development also involves E12 and MyoD transcription factors (restricted to bilaterians)7.

Here, we examine tissues of the sponge *Ephydatia muelleri* and find evidence of a contractile module with evolutionary links to muscle. Specifically, contractions depend upon the motor-activity of striated-muscle myosin II and are regulated by MLCK pathway, and the development of the contractile module appears to

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**Fig. 1 Sponge body plan and contractions.** A The tent-like outer layer is supported by spicules (light blue) and is composed of two epithelia, which house a thin extracellular matrix (pink) containing migratory cells. The incurrent system (Inc) comprises the atrium and incurrent canals. Water is drawn into the atrium through incurrent pores (ostia; Ost) and into choanocyte chambers (orange), then into incurrent system (Exc) and through the osculum where it exits the sponge. B Still images from a mechanically induced contraction show that a 40 min contraction cycle involves at least two phases. During phase I (t = 15 min), incurrent canals (red) narrow as incurrent canals (blue) widen. During phase II (t = 36 min), incurrent canals widen as incurrent canals narrow.
Results

Contractile actin-bundles in pinacocytes contain stMyHC. The tissues lining the atrial cavity and canals are believed to play a primary role in the contraction in sponges. These tissues are regionally differentiated but are all composed of endothelial-like cells (pinacocytes) that form a watertight barrier, express genes involved in contraction,15,16, and decrease in area during contraction.17 Moreover, in *E. muelleri*, pinacocytes contain linear actin-bundles that align between adjacent cells, exhibiting tissue-wide organization (Fig. 2A).

Searching the transcriptome,18 we identified two type II myosin heavy chains, which clearly fall into stMyHC and nmMyHC groups (Fig. 2C). We generated and validated a custom antibody targeting a divergent region in the motor head for stMyHC (Fig. 2D, Supplementary Fig. 9). To test whether these actin-bundles are associated with myosin II, we immunostained for stMyHC (Fig. 2E). Staining was initially diffuse but increased throughout development. In fully differentiated tissues, stMyHC was organized into linear structures that resemble actin-bundle orientation (Fig. 2F), but co-staining was incompatible with fixation conditions. To further test for association with actin-bundles we treated sponges with latrunculin B, an inhibitor of actin polymerization, and observed that loss of stMyHC staining mirrored the dynamics of actin-bundle disassembly (Supplementary Fig. 1).

Contractions are regulated by the MLCK pathway. Previous studies have shown that sponge contractions abate in Ca^{2+}/Mg^{2+}-free medium (CMFM).1,19 To test the inverse of this, whether elevated cytoplasmic Ca^{2+} can induce contractions, we treated *E. muelleri* in CMFM with the calcium ionophore, ionomycin, and with the SERCA pump inhibitor, thapsigargin. Both treatments induced a strong biphasic contraction cycle (Fig. 3A). In ionomycin-treated sponges, contractions appeared to be more drawn-out and less intense compared with thapsigargin-treated sponges.
sponges, which closely mirrored mechanically induced contractions at the tested concentrations. We interpret this as a difference in the kinetics of the molecules, as well as the mechanism of action; thapsigargin likely requires sufficient intracellular stores to elicit a response.

Since treatments were administered globally, the observed response could be a secondary effect downstream of NO signaling from activated sensory cells. To decouple sensation and contraction, we treated sponges with the NO-synthase inhibitor L-NAME (Fig. 3B). Sponges deficient for NO signaling did not contract in response to treatment with Sumi ink (which activates sensory cells) but exhibited a strong response to thapsigargin (Fig. 3C). This suggests that thapsigargin is acting on cells downstream of the sensory cells, which is consistent with the presence of endoplasmic reticulum-associated Ca\(^{2+}\) stores in contractile tissues.

With respect to the regulation of Ca\(^{2+}\)-dependent contraction (Fig. 4A), *E. muelleri* has homologs of calmodulin, MLCK, and an RLC ortholog with conserved functional residues (Fig. 4B). If the release of internal Ca\(^{2+}\) is upstream of MLCK signaling, then contractions should be disrupted by the MLCK inhibitor ML-7. To test this, we first established a concentration of Sumi ink that permanently blocked water flow in L-NAME treated sponges (i.e., sponges deficient for NO synthesis), but could be cleared by contraction of untreated sponges. Ink clearance and the reestablishment of flow was determined by post-treating sponges with Dyl to see if it entered canals. We found that ML-7-treated sponges were unable to clear ink and restore flow, nor could they be induced to contract with thapsigargin (Fig. 4C, D). Whereas ML-7 targets MLCK and is predicted to affect only a subset of myosin II activity, treatment with more general myosin ATPase inhibitors is expected to have global effects on myosin activity. We found that blebbistatin and para-amino blebbistatin caused a complex phenotype similar to phase I of the contraction cycle, but sponges never entered phase II and could not be induced to do so with thapsigargin (Supplementary Fig. 2).

In vertebrates, the RLC is the substrate for MLCK during smooth-muscle contraction\(^{20}\). To test for RLC phosphorylation during contraction, we immunostained sponges for phosphorylated RLC (pRLC). In thapsigargin-treated sponges, there was increased phosphorylation of the RLC (Fig. 4A E). If the RLC was not phosphorylated, it would not be expected to elicit a contraction.

Collectively, these results support that contractions depend upon the release of ER-associated Ca\(^{2+}\) stores, downstream of NO signaling. Elevated Ca\(^{2+}\) then activates MLCK, leading to increased phosphorylation levels of RLC associated with contractile actin-bundles in pinacoderm tissues.

**MRTF drives development of the contractile module.** To test for conserved developmental mechanisms involved in specifying contractile cell-fate in sponges and bilaterians, we focused on the transcriptional cofactor, MRTF (Fig. 5A). In bilaterians, MRTF homologs are broadly expressed, but inhibited through the interaction between G-actin with N-terminal RPEL (RPxxEL) repeats\(^{21}\). Actin polymerization disrupts this interaction, exposing a nuclear localization signal and leading to nuclear translocation\(^{22,23}\). In the nucleus, MRTF interacts with SRF or...
Mef2 to drive the expression of contractile genes and can induce the differentiation of myocytes\textsuperscript{24–27} and myofibroblasts\textsuperscript{28}, and is involved in the regulation of epithelial-mesenchyme transitions\textsuperscript{29,30}. Loss of function studies in mice have shown that the loss of myocardin results in early embryonic lethality due to improper formation of vascular smooth muscle\textsuperscript{31}. Loss of MRTF-B also results in early embryonic lethality due to cardiac defects\textsuperscript{32,33}. Though most ubiquitously expressed, loss of MRTF-A is not lethal but results in deficiencies in lactation, likely resulting from loss of myoepithelial tissue\textsuperscript{34}.

\textit{E. muelleri} has a single, broadly expressed (Supplementary Fig. 3) MRTF ortholog that is predicted to have conserved RPEL mechanisms.\textsuperscript{35} Loss of function studies in sponges have shown that it may act primarily on MRTF in sponges. To test this, we monitored \textit{EmTAGLN2} (a predicted MRTF-target) expression in control, MRTF-activator ISX, and MRTF-inhibitor CCG-207319 treated \textit{E. muelleri} sponges. ISX treatment caused increased \textit{EmTAGLN2} expression, whereas CCG-207319 treatment caused a decrease in \textit{EmTAGLN2} expression, whereas ISX treatment caused an increase in \textit{EmTAGLN2} expression (Fig. 5D). Since ISX is less specific than CCG-207319, we also corroborated this result by treatment with cytochalasin D—a potent MRTF-activator through competitive binding of G-actin\textsuperscript{44}—and similarly observed an increase in \textit{EmTAGLN2} expression. From a functional perspective, MRTF-inhibited sponges had a reduced ability to clear Sumi ink—an indicator of diminished contractile activity (Fig. 5E).

Because MRTF activity is regulated by G-actin, it is possible to pharmacologically manipulate this interaction. Highly specific MRTF inhibitors such as CCG-203971\textsuperscript{36,37} have been identified, as well as activators that act through less specific mechanisms. One of these, \textit{N}-cyclopropyl-5-((thiophen-2-yl)isoxazole-3-carboxamide (ISX) is an MRTF activator\textsuperscript{38–40} that drives cardiac-myoﬁbocyte differentiation in vivo\textsuperscript{48}. ISX also drives the expression of secretory programs and neuronal differentiation through the transcription factor NeuroD1 in a Mef-2-dependent manner\textsuperscript{41–43}. However, as sponges lack neurons and NeuroD1, we reasoned that ISX may act primarily on MRTF in sponges. To test this, we monitored \textit{EmTAGLN2} (a predicted MRTF-target) expression in response to CCG-207319 and ISX treatment and observed corollary changes; CCG-207319 treatment caused a decrease in \textit{EmTAGLN2} expression, whereas ISX treatment caused an increase in \textit{EmTAGLN2} expression (Fig. 5D). Since ISX is less specific than CCG-207319, we also corroborated this result by treatment with cytochalasin D—a potent MRTF-activator through competitive binding of G-actin\textsuperscript{44}—and similarly observed an increase in \textit{EmTAGLN2} expression. From a functional perspective, MRTF-inhibited sponges had a reduced ability to clear Sumi ink—an indicator of diminished contractile activity (Fig. 5E).
Fig. 5 Development of contractile structures depends on MRTF-activity. A Myocytes are developmentally specified by a transcriptional complex that includes MRTF interactions with SRF or MeF2. B Predicted domain organization of E. muelleri MRTF compared with vertebrate homologs (NLS; nuclear localization signal, B1, basic rich domain; SAP, SAP domain; LZ, leucine zipper; TAD, transcription activation domain). C Confocal images of pinacocytes (top) and archocytes (bottom) immunostained for MRTF (grayscale and magenta), phalloidin (yellow), and Hoechst (cyan). Immunostainings for MRTF were performed on multiple sponges over five independent experiments with consistent results. D Top: confocal images of an actin-bundle immunostained for TAGLN2 (grayscale and magenta), phalloidin (yellow) and Hoechst (cyan). Bottom: qPCR of TAGLN2 in response to treatment with CCG-203971 with consistent results.

To test the effects of MRTF-activation on contractile tissue differentiation, we dissociated juvenile sponges and treated archocyte-enriched cell fractions with either DMSO or ISX and placed them in an attachment-free environment (this allows for the formation of primary aggregates—primmorphs—but delays differentiation). After three days, control primmorphs lacked contractile-bundles, whereas primmorphs treated with ISX developed linear actin-bundles that aligned at adhesion plaques (Fig. 6A). These stained positive for pRLC (Supplementary Fig. 6) and treated primmorphs contained elevated levels of stMyHC (LogFC = 1.03, P = 1.0E−3), and components of the Ca2+-dependent MLCK pathway including MLCK-like serine/threonine kinase (LogFC = 1.58, P = 6.29E-5), calmodulin (LogFC = 1.55, P = 0.002), sodium/calcium exchanger 1 (SLC8A1) (LogFC = 1.05, P = 0.029), and Phospholipase C gamma (PLCγ) (LogFC = 1.01, P = 0.005) (Fig. 6D). Upregulated signaling genes included metabotropic glutamate receptors and GABA receptor subunits (Fig. 6D), consistent with a role for glutamatergic and GABAergic signaling in contractile behavior.23 Uregulated developmental factors included the myogenic transcription factor SRF (LogFC = 1.18, P = 0.001). Four Forkhead transcription factors showed increased expression, including FoxL2 (LogFC = 1.07, P = 0.008), FoxG (LogFC = 1.31, P = 4.16E−5), which is expressed in myocytes of invertebrates,45 FoxO, and Fox1 (LogFC = 0.60, P = 0.016 and LogFC = 1.86, P = 0.001 respectively). The phylogenetically broad muscle marker Crip/Csrp46 was also upregulated (LogFC = 1.10, P = 0.001).

MRTF activates myogenic factors, signaling, contractile, and adhesion genes. To understand the transcriptional response of primmorphs to MRTF activation, we sequenced mRNA from ISX or DMSO treated primmorphs. Differential expression analysis of 16,712 mapped transcripts revealed that 1390 were upregulated and 1091 were downregulated (Supplementary Figs. 7–8). We interpret upregulated genes as candidate targets of MRTF regulation, and downregulated genes as archocyte-enriched. The ISX-treated samples had elevated expression of genes involved in contraction, signaling, development, and adhesion (Fig. 6D).

To understand the evolutionary origin of bilaterian smooth and striated myocytes, we analyzed a phylogenetically broad set of MRTF sequence alignments, highlighting central interactions, expressing relationships based on our phylogenetic analysis of MRTF family proteins,44 Creative Commons Attribution 4.0 International—CC BY 4.0. Scale bars 500 μm (E), 5 μm in C, and 2 μm in D. Contraction assays were performed three independent times with consistent results.
Contraction dynamics of ISX-treated primmorphs in response to thapsigargin. Data are presented as mean values ± SEM. Scale bars 10 μm. Heatmap of select transcripts that were differentially expressed in ISX-treated samples. Primmorphs treated with ISX and vehicle controls were maintained in an attachment-free environment in nine independent experiments. Phalloidin stainings were performed twice, total protein extraction was performed on one sample, and total RNA was extracted from three independent experiments with consistent results. Scale bars 10 μm.

**Discussion**

A challenge for tracing the ancestry of myocytes is that muscles are very diverse in modern animals. For example, like vertebrates, the ascidian *Ciona robusta* and the annelid *Platyneris dumerilii* have striated myocytes that express stMyHC, are regulated by the troponin C/tropomyosin complex, and are developmentally specified by a skeletal muscle-like transcriptional complex. They also have smooth muscles that express nmMyHC and are patterned by a smooth/cardiac muscle-like transcriptional complex.

Some of the most highly upregulated genes belonged to the collagen family, suggesting a role for pinacocytes in the secretion of extracellular matrix (Fig. 6D). As many as fourteen collagens had elevated expression in ISX-treated samples. Adhesion molecules, including cadherins, integrins, and down syndrome cell adhesion molecule, had increased expression levels as well (Fig. 6D). Though many upregulated genes correspond to the transcriptional profile of pinacocytes based on scRNA-seq data, upregulation of silicatein—a sclerocyte marker—suggests that ISX treatment caused differentiation of other cell types as well, directly or indirectly.

Here, we establish that *E. muelleri* also contains a contractile module with elements of homology to contractile tissues in other animals, including myocytes. Actomyosin bundles containing stMyHC and transgelin exhibit tissue-wide organization in pinacoderm tissues that line internal body cavities and canals. Contraction depends upon the release of ER-associated Ca\(^{2+}\) stores and MLCK regulation of stMyHC, and the development of the module appears to depend upon MRTF-activity. Our interpretation of this contractile module as muscle-related is corroborated by single-cell sequencing data from the related species, *Spongilla lacustris*, which indicate that pinacocytes, and another cell type—myopeptidocytes—cluster with myocytes from other animals (myopeptidocytes are solitary cells found between tissues) that express contractile genes including nmMyHC.

Although sponge contractile tissues and muscles have similarities that can only be explained by common ancestry, we do not assert that this reflects their one-to-one homology. This is partly due to the similarity of muscle and non-muscle contractile mechanisms, but also because invertebrate muscles are often multifunctional. For example, the epitheliomuscles of *Hydra* function in contraction, the formation of an epithelial barrier, innate immunity, and regeneration. In the planarian *Schmidtea mediterranea*, muscle also acts as a connective tissue that secretes ECM proteins (including 19 collagens) and signaling molecules that provide spatial cues for regeneration from neoblasts. High expression of numerous collagens has also been found in the epitheliomuscles of *Nematostella vectensis* (30 in the circular body muscles), which is interpreted as a sign of multifunctionality. Similarly, sponge pinacoderm tissues form an endothelial-like barrier to the environment, are capable of phagocytosis, and express genes involved in sensation, metabolism, and defense. Also, in an intriguing parallel with *S. mediterranea*...
and N. vectensis, MRTF-induction of contractile tissue development was associated with the upregulation of as many as 14 collagen genes. Our findings can inform hypotheses about the organization of contractile tissues in the first animals, as well as the sequence of events that gave rise to early muscles. Specifically, the epithelial-like nature of contractile tissues in both sponges and cnidarians suggests that this represents the ancestral state, and that narrowly specialized myocytes emerged later. This fits well with the view of epithelial tissues as the “building blocks” of animal body plans, which may have been among the first tissues to evolve. It has been hypothesized that myoepithelia represent a retained feature of an ancient muscle precursor. The hypothesis that myoepithelia represent a retained feature of an ancient muscle precursor, the long-held view that they lack muscles has been interpreted as evidence that myocytes evolved after sponges diverged from other animals, or that myocytes were lost in the evolution of animal body plans. Even during sexual reproduction, there is limited correspondence between the epithelial- and muscular tissues predates modern animals. Consistent with this interpretation, the stMyHC, and nmMyHC paralogs diverged in the holozoan stem lineage and the choanoflagellate Choanoeca flexa forms colonies that resemble a polarized epithelium with coordinated contractile behaviors.

Finally, the possible role of MRTF in specifying contractile tissues in E. muelleri helps to explain the plasticity and regenerative capacity of sponges. Evidently, without the need for intrinsic spatial cues characteristic of embryogenesis, sponges can develop from archecyte-enriched aggregates and gemmules, and adult tissues can remodel in response to flow dynamics. In vertebrates, MRTF is an actin-regulated force-sensor involved in muscle plasticity and regeneration, and our data indicate that similar mechanisms are operating in E. muelleri. This supports a model in which environmental-feedback mechanisms drive contractile tissue development, and we speculate that this could be common in sponges. Even during sexual reproduction, there is limited correlation between embryonic patterning and adult tissue identity. It is conceptually plausible that the evolution of animal developmental mechanisms involved a transition in which ancient environmental feedback mechanisms were later harnessed by genetically encoded, intrinsic patterning mechanisms.

Methods

Sponge collection and cultivation. Gemmules of E. muelleri were collected from an unnamed lake in the Brainard Lake Recreation Area in the Colorado Rocky Mountains (40°04′48.0″N 105°32′34.6″W) and stored in autoclaved lake water (LW) at 4 °C. Before use, gemmules were treated with 1% hydrogen peroxide for 5 min, washed thoroughly with autoclaved lake water, and plated in either six-well plates (CellTreat #229106) or coverslip-bottom dishes (MatTek Corporation #P35G-1.5-10-C) in LW containing 100 µg/mL ampicillin. Plates/dishes were then placed in a dark cabinet at room temperature until hatching (~3 days). After hatching, water was renewed daily without the addition of ampicillin.

Induction of contraction by mechanical agitation, Sumi ink, and elevated Ca2+ - Sponges were induced to contract using mechanical agitation by placing the plate or dish on a vigorous rocking platform for 3 min before transfer to an inverted microscope for imaging. Alternatively, contractions were induced with Sumi ink (Yasutoma #K6Y) by incubating sponges in 1:1000 solution for 10 min, followed by LW washes. To test for Ca2+ dependence of contractions, sponges were placed in 2 mL LW and left on the microscope stage for 1 h to confirm they were not contracting prior to treatment. After 5 min of imaging, treatments were applied by gently removing 1 mL of LW and replacing it with 1 mL of either thapsigargin or external Ca2+ inactivating solutions. To induce contractions in the absence of NO signaling or external Ca2+ ions, sponges were pretreated with either 50 µM L-NAME (Cayman Chemical #80210), 0.1 mM EDTA, or deionized water (Milli-Q, Milipore). Time lapse images were collected, generally, at a rate of one image every 20 s and stacks were compiled into videos using ImageJ software. Major excurrent canals were manually annotated from still images. For changes in canal diameter, the excurrent and excurrent areas were chosen with ImageJ and the diameter across the same plane was measured every 6th frame over the course of the video. The ratio of diameter at time t to the initial diameter at t = 0 was then taken to represent change in diameter over the course of treatment. Graphs of canal dynamics were generated using Microsoft Excel (version 16).

Antibody production and validation. Polyclonal antibodies against stMyHC and MRTF were produced in rabbits (Syd Labs), and polyclonal antibodies against L-NAME (Cayman Chemical #80210), TGFβ, and TGFβ receptor 2 were produced in chickens (Pacific Immunology). The coding region of each antigen (Supplemental Sequences) was cloned into the PET His6 GST TEV LIC cloning vector (Addgene, plasmid #29655) from synthesized gBlocks (Integrated DNA technologies) (stMyHC) or from the E. muelleri cDNA library. Recombinant proteins were expressed in Escherichia coli (Rosetta strain, Novagen, Madison, WI) and purified with Pierce Glutathione Sepharose 4B (ThermoFisher Scientific #16101) following the manufacturer’s protocol. Antibodies were affinity purified on a column made by coupling each recombinant protein to AminoLink Resin (ThermoFisher Scientific #20381) and validated by Western blotting and by Peptide Competition Assay (Supplementary Figs. 9–12).

Immunostaining. Sponges were grown in glass-bottom dishes for 3–4 days post hatching and then fixed using one of two methods: (1) to prepare samples for multiplexed hybridization, sponges were fixed in Carnoy’s solution (60% EtOH, 30% chloroform, 10% glacial acetic acid) for 3 min at RT, followed by a gentle 10 min wash in 100% EtOH at RT. All samples were then incubated in PBS and blocked with PBS containing 0.5% Triton X-100 for 1 h at RT. Primary antibodies in blocking solution were applied for 1 h at RT, or overnight at 4 °C (1:100 for anti-PRLC, 1:200 for anti-MRTF, 1:250 for anti-TAGLN2, 1:500 for anti-TAGLN3, 1:1000 for anti-EmVIn1 and 1:500 for anti-stMyHC). Secondary antibodies, either anti-rabbit AF488 (Life Technologies) or anti-Chicken AF555 (Invitrogen #A32935), were applied at a 1:120 phallolidin AF 568 (ThermoFisher Scientific #A12380), and 1:100 Hoechst dye in blocking solution. Samples were incubated for 45 min at RT in the dark, washed in PBS, and mounted in mounting medium (0.1% Propyl gallate, PBS pH 7.6, 90% glycerol). Samples were imaged on an Olympus Fluoview FV3000 confocal microscope.

In order to understand the developmental dynamics of stMyHC, sponges were fixed in 8 h time-intervals after attachment to the dish. The only change to the fixation protocol observed was that the glacial acetic acid component of Carnoy’s solution was added dropwise during the first minute to help preserve the delicate structure of the newly formed tissues. Since actin filaments were not preserved using this fixation method, we instead performed a timed sequence of latrunculin B treatments to test whether stMyHC staining was disrupted in conjunction with actin-bundle dissolution. Specifically, gemmules were plated in glass-bottom dishes and grown until canals and choanoocytes were clearly visible. Sponges were then treated with 20 µM latrunculin B in LW for either 1, 5, 10, or 30 min, then fixed and stained using appropriate methods for either stMyHC or F-actin.

To test for elevated pRLC staining in contracted tissues, 10 z-stacks were taken through comparable regions of the pinacoderm using the same confocal settings for relaxed and contracted samples. Projections of the images were made in Fiji and the F-actin and pRLC images were merged. pRLC staining intensity was measured as pixel intensity and expressed as a ratio to actin-bundle length (nine biological and three technical replicates). Measurements were obtained from nine biological and three technical replicates, and a simple regression analysis was performed for pixel intensity and actin-bundle length in RStudio.

HCR RNA fluorescent in situ hybridization of MRTF and SRF. Multiplexed HCR RNA-FISH was performed with custom split initiator probes ordered from Molecular Instruments®. Sponges were grown in coverslip-bottom dishes (Mattek) for 6 days. Cellbrite-fix 640 (Biotium) was added at 1:1000 dilution for 15 min, and sponges were then washed once in lakewater. Immediately after washing, sponges were fixed in ice-cold 4% (v/v) paraformaldehyde in 1% Holfreter’s solution overnight at 4 °C. Sponges were then washed with Holfreter’s a dehydration series was then performed to transfer the samples to 100% MeOH and then transferred to 100% ETOH before rehydration into 1x PBS. Samples were digested with 5 µg/mL protease K for 90 s and digestion was halted with 2 µg/mL glycine. Samples were then postfixed in 4% (v/v) paraformaldehyde in 1x PBS at 4 °C and then washed twice with PBS/0.1% Tween 20. A dehydration series was then performed using buffers supplied by Molecular Instruments® and following the general protocol with minor modifications. Samples were prehybridized for 30 min in hybridization buffer at 37 °C. Samples were then hybridized with 0.5, 2, 5, 10, and 10 nmol probes for each target for ~16 h at 37 °C in humidified chambers, on a rocking platform. Sponges were washed in 2x SSC and then transferred to 1x SSC and then brought to room temperature. Samples were pre-amplified in provided amplification buffer for 30 min at room temperature and 24 pmol of amplification hairpins for each target
were prepared by snap cooling, separately, in amplification buffer. These were combined and added to the sample for 16 h at room temperature in the dark. Samples were then washed into 1× PRST and stained with 1:100 Hoechst dye before mounting.

Testing the role of MLCK and MRTF in physiological contractions. Gemmules were plated in 24-well dishes and treated with the 1 μM ML-7 (MLCK inhibitor66,67), or 50 μg/mL L-NAME for 2 h, or 20 μM or 50 μM CCG-203791 (MRTF inhibitor68,69) for 3 days before testing, with treatment refreshed daily. To disrupt flow, sponges were each treated with 1:20 (v/v) Sumi ink for 10 min, carefully washed with LW, and allowed to sit, undisturbed, for 3 h (long enough to allow ink blockages to be cleared in sponges capable of contraction). To test if water flow was restored by contraction, samples were then treated with 1:1000 (v/v) DiI (ThermoFisicrScientific #PD282) in LW for 10 min in the dark and washed with LW, which under normal flow conditions strongly stains choanocyte chambers. Samples were manually screened and imaged via epifluorescence microscopy for DiI staining within water canals. Laser-level and exposure time was set based on control sponges and DiI staining was judged based on overlap with Sumi ink-stained choanocyte chambers. Washout experiments and extended treatments were performed to confirm sponges remain viable under treatment conditions.

gRT-PCR. Gemmules were plated in six-well format on 22 mm coverslips with ~10 sponges per well. For MRTF inhibition, sponges were treated with 20 μM CCG-203791 in LW, beginning at ‘tent-stage’ (i.e., before choanocyte chambers formed) and with treatment refreshed daily. For MRTF activation, sponges were treated with 50 μM ISX58,9 beginning at tent-stage and with treatment refreshed daily, or with 10 μM cytochalasin D (ThermoFisicrScientific #PHZ1063) for 30 min prior to harvesting (cytochalasin D disrupts the MRTF/g-actin association through competitive binding of g-actin90). RNA was extracted by washing sponges off of coverslips with Trizol Reagent (ThermoFisicrScientific #1596-018) in a 50 mL tube, followed by ethanol precipitation and rehydration in nuclease-free water. The concentration of purified total RNA was measured by spectrophotometry and an equal mass from each extraction was used to generate cDNA.

Activation of MRTF in archeocytes. Approximately 50 gemmules were grown to 3 days post-hatching, then dissociated in LW containing 1 mM EDTA and passed through a 70 μm cell strainer. Cell suspensions were centrifuged at 1000 × g for 5 min to enrich for archeocytes. The supernatant was removed by pipetting and the cell pellet was resuspended in LW containing 50 μM iSx or DMSO. Using a wide-bore pipette tip, 25 μL of the cell suspension was plated as a hanging drop and maintained for 2–3 days.

Contraction assays were performed by transferring aggregates to the center of a coverslip-bottom dish and adding thapsigargin to a final concentration of 1 nM. Aggregates were photographed every 30 s for 60 min to create time-lapse videos. Time-lapse series were analyzed in Fiji to measure the changes in aggregate size in response to thapsigargin. Immunosstaining of aggregates was conducted as described above, except aggregates were stained in 1.5 mL tubes instead of in dishes.

RNAseq and differential gene expression analysis. Approximately 100 gemmules were grown till 3 days post-hatching, then dissociated as described above. Cells were resuspended in treatment solution (LW, 50 μM iSx) or in control solution (LW, DMSO), and were maintained in 10 mL of solution in a 60 mm petri dish on an orbital rocker to prevent attachment. After 24 h, primorphs were collected and total RNA was extracted using Triol Reagent. Experiments were performed in triplicate. Generation of a poly-A-selected library and paired-end sequencing on Illumina HiSeq 4000 platform was performed by Novogene (Sacramento California). Adapter sequences were removed from the raw reads and were quality filtered using fastq91. Cleaned reads were mapped to the E. muelleri genome using HISAT292 and transcripts were assembled using StringTie93 and htseq-count94. Differential expression analysis was performed using EdgeR95,96 and was visualized from normalized expression using HeatMap97. Gene ontology analysis was performed using BLAST2GO Pro98. The identity of proteins of interest was assessed by performing reciprocal BLAST99, searching Pfam100, and by phylogenetic analysis using phylogeny.40 Phylogenetic trees and analysis of proteins of interest in this study are available in the supplement (Supplementary Figs. 13–15).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Sequences and accession numbers for all genes and proteins analyzed in this study are made available in the supplement. Raw RNAseq reads have been deposited at the NCBI SRA (accession number/BioProject PRJNA178521, BioSamples SAMN18537458, SAMN18537459, SAMN18537460, SAMN18537461, SAMN18537462, SAMN18537463). Raw confocal image files are available at figshare (https://doi.org/10.6084/m9. figshare. 8003945). Source data are provided with this paper.

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Correspondence and requests for materials should be addressed to S. A. Nichols.

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