**HES and Mox genes are expressed during early mesoderm formation in a mollusk with putative ancestral features**

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The mesoderm is considered the youngest of the three germ layers. Although its morphogenesis has been studied in some metazoans, the molecular components underlying this process remain obscure for numerous phyla including the highly diverse Mollusca. Here, expression of *Hairy and enhancer of split* (HES), *Mox*, and *myosin heavy chain* (MHC) was investigated in *Acanthochitona fascicularis*, a representative of Polyplacophora with putative ancestral molluscan features. While *AfaMHC* is expressed throughout myogenesis, *AfaMox1* is only expressed during early stages of mesodermal band formation and in the ventrolateral muscle, an autapomorphy of the polyplacophoran trochophore. Comparing our findings to previously published data across Metazoa reveals *Mox* expression in the mesoderm in numerous bilaterians including gastropods, polychaetes, and brachiopods. It is also involved in myogenesis in molluscs, annelids, tunicates, and craniates, suggesting a dual role of *Mox* in mesoderm and muscle formation in the last common bilaterian ancestor. *AfaHESC2* is expressed in the ectoderm of the polyplacophoran gastrula and later in the mesodermal bands and in putative neural tissue, whereas *AfaHESC7* is expressed in the trochoblasts of the gastrula and during foregut formation. This confirms the high developmental variability of HES gene expression and demonstrates that *Mox* and HES genes are pleiotropic.

Germ layers form early in animal development and give rise to the various adult tissues and cell types. The most ancient germ layers, the ectoderm and endoderm, are established during gastrulation, while the third germ layer, the mesoderm, is argued to be the youngest and probably evolved in the bilaterian lineage1–4, but see5,6 for alternative view. The mesoderm is considered a key innovation, since numerous bilaterian organ systems such as muscles, bone, and connective tissue derive from this germ layer7. In a number of protostomes, the mesoderm is formed by cells that immigrate from the blastopore margin into the blastocoel. These sometimes form a pair of mesodermal bands as, for example, in animals that exhibit spiral cleavage (the Spiralia; e.g., Platyhelminthes, Annelida, and Mollusca8–12). In several other protostomes, as well as in deuterostomes, the mesoderm-forming cells typically detach from the archenteron wall1,13,14. While the mesodermal cell lineage has been investigated in a number of lophotrochozoan representatives including the flatworm *Hoploplana*8, the polychaete annelids *Podarke, Polygordius*, and *Scoloplos*15, the gastropods *Planorbis*16 and *Crepidula*17, and the polyplacophoran mollusk *Acanthochitona*, the molecular mechanisms underlying mesoderm specification remain largely unclear12.

*MHC* or *myosin class II* is a member of the myosin superfamily. It is, together with *myosin class I*, often assumed to constitute the most ancient myosin class, having evolved at the bikont-unikont split22. The protein products of MHC build the myosin fibres of cnidarian, ctenophore, and bilaterian muscle cells23–26. In the annelid *Platynereis dumerilii*, MHC is expressed in both, striated and smooth muscles of the early nectochaete larva20. In *Drosophila melanogaster*, MHC is expressed in somatic and visceral muscles as well as in cardioblasts18, and...
in the cephalochordate Branchiostoma belcheri, MHC expression is found during somite formation and in the notochord. In vertebrates, MHC is involved in the development of skeletal, cardiac, and smooth muscles. In the non-bilaterian cnidarian Nematostella, MHC transcripts are present in the tentacle muscles and in retractor muscles of primary polyps. They are also found in muscle progenitor cells in the tentacle root of the ctenophore Pleurobrachia pileus.

Max genes possess a conserved helix-turn-helix DNA-binding homeodomain. Previous studies have suggested a sister group relationship to the homeotic gene even-skipped (Eve). In chordates, Max expression was reported during formation and differentiation of the main mesodermal derivatives, the somites, that give rise to muscles, bones, and connective tissue. Expression of the Drosophila Max ortholog buttonless is restricted to dorsal median cells which play a crucial role in axon guidance. Importantly, buttonless expression was not detected in Drosophila muscle progenitor cells or muscle tissue, suggesting a loss of Max in myogenesis in this lineage.

HES genes are members of the basic helix-loop-helix superfamily and direct downstream targets of the Delta-Notch signalling pathway. They possess an additional HES-specific hairy orange domain and a WWPW motif at the C-terminal end. HES genes are involved in a variety of developmental processes such as mesoderm formation, maintaining stem cell potential, or partitioning of morphological territories (e.g., segmentation in annelids, arthropods, chordates, as well as budding in Hydra). HES genes in mollusks have so far only been studied in the gastropod Crepidula fornicate, where one HES gene was found to be expressed around the mouth as well as in neurosensory cells in the early larva, while the other one shows more dynamic expression domains in the lateral ectoderm around the mouth.

In order to test whether MHC, Max, and HES are expressed during mesoderm formation in mollusks, we investigated tempo-spatial expression of MHC, Max, and HES genes in Acanthochitona fascicularis, a member of Polyplacophora that displays several morphological characteristics thought to be ancestral for one of the two major molluscan lineages, the Aculifera. In addition, we provide a metazoan-wide comparative survey on the tempo-spatial expression domains of these genes. By plotting these data on current phylogenies and by applying a ground pattern reconstruction approach using parsimony, we discuss scenarios concerning the emergence and loss of involvement of these genes in mesoderm formation and myogenesis across major lineages of the metazoan tree of life.

Material and methods

Animals and fixation. Adult Acanthochitona fascicularis specimens were collected in the intertidal region between the Station Biologique de Roscoff and the Île Verte in Roscoff, France (48° 43′ 44.70″ N 3° 59′ 13.53″ W). Adults and all developmental stages were maintained in glass dishes with filtered seawater at 18–21 °C. Spontaneous spawning of mature males and females generally occurred 1 to 3 days after collection. Gametes were inseminated by adding drops of sperm to the eggs. Upon the first observation of 2-cell stages (~80 min after fertilization), the embryos were washed multiple times with filtered sea water to prevent polyspermy and bacterial or fungal infection.

The gastrula stage was reached at around 8 h post fertilization (hpf). Trochophore larvae hatched from 18 hpf onwards. At 48–60 hpf, larvae reached the metamorphic competent stage (referred to as “late trochophore larva” herein). Early juveniles that had completed metamorphosis appeared between 60 and 90 hpf.

In order to fix samples for RNA extraction, specimens were centrifuged, the seawater was removed, and liquid nitrogen was added. Specimens were stored at −80 °C until RNA extraction. For in situ hybridization experiments, specimens were fixed for 1–2 h in 4% paraformaldehyde (PFA Sigma-Aldrich #158127; St. Louis, USA) in MOPS-EGTA (0.1 M MOPS Sigma-Aldrich #69947; 2 mM MgSO₄, Thermo Fisher Scientific #52044; Waltham, USA; 1 mM EGTA, Sigma-Aldrich #E4378; 0.5 M NaCl, Roth #HN00.1; Karlsruhe, Germany) and washed twice or thrice in ice-cold 100% methanol. Fixed specimens were stored at −20 °C.

RNA probe design. Total RNA extraction from pooled developmental stages spanning early cleavage stages to juveniles was performed using the Qiagen RNeasy mini kit 50 (#74104; Venlo, Netherlands). Reverse transcription into cDNA was performed with the Roche 1st strand cDNA synthesis kit for RT-PCR (Roche #11483188001; Rotkreuz, Switzerland). Specific primers for each gene of interest were designed manually and purchased from Microsynth AG (Zürich, Switzerland) (Supplementary Table 1). Reading frames and orientation of the transcripomomic templates were assessed with the ExPaSy translate tool (https://web.expasy.org/translate/). Melting temperatures of designed primers were assessed with the Promega Oligo Calculator tool (https://biotools.nubic.northwestern.edu/OligoCalc.html). The genes of interest were amplified by PCR (Promega protocol #9PIM829; 5× Go-Taq Flexi Buffer Promega #M890A, Fitchburg, USA; magnesium chloride, Promega #A351; dNTP Mix, Promega #1141; Go Taq Flexi DNA Polymerase, Promega #M780B) and the gene-specific primers. The amplified genes were ligated into a pGEM-T easy vector (Promega #A1380). The plasmid was amplified using E. coli competent cells (Promega #L2001). Plasmid DNA was purified using the QiAprep spin miniprep kit 250 (Qiagen #27106). Inserts were sequenced by Microsynth AG (Vienna) using sp6 primers. Amplification of the insert was done by PCR (Promega protocol #9PIM829; M13 Primer, Microsynth AG). In vitro transcription was done using the DIG RNA Labeling Mix, 10× conc. (Roche #11277003910) with either T7 RNA polymerase (Roche #10881767001) or sp6 RNA polymerase (Roche #10810274001). Additionally, 1 µl of DTT (Sigma-Aldrich #D6032) was added to each sample and incubation was performed for three instead of two hours to increase the RNA probe yield. The RNA probes were sephadex-purified using the Illustra ProbeQuant G-50 Micro Columns (GE Healthcare Life sciences #28903408; Pittsburgh, USA) and precipitated overnight at −20 °C (4 M LiCl, Sigma-Aldrich #L7026; 96–100% ethanol). Precipitated probes were washed...
twice for 15 min each in 70% ethanol, air-dried at room temperature, and dissolved in 20 µl nuclease-free water (Thermo Fisher Scientific #R0581). The probes were stored at –80 °C.

**In situ hybridisation.** Fixed and stored *Acanthochitona fascicularis* specimens were incubated in EGTA in methanol (90% methanol; 0.05 M EGTA pH 8). Subsequently, the EGTA solution was stepwise exchanged by an ascending (20%, 50%, 50%, 80%, 100%) phosphate buffered saline series (Roth #10581; 0.9546, 47% PFA for decalcification and were subsequently washed thrice for 10 min each in PBT. Specimens were incubated in a solution of 50 µg/ml proteinase-K in PBT (Roche #03115879001) for 10 min at 37 °C and then washed twice for 5 min each and twice for 10 min each in PBT at room temperature. In order to reduce charged probe binding, specimens were subsequently incubated for 10 min each in 1% triethanolamine (PBT with 1% TEA added; Sigma-Aldrich #90279), for 5 min each in 1% TEA with 0.15% acetic anhydride (Prolabo #21390293; Bern, Switzerland), and for 5 min each in 1% TEA with 0.3% acetic anhydride added. Specimens were then washed twice for 5 min each and twice for 10 min each in PBT and post-fixed in 4% PFA for 45 min. Afterwards, the specimens were washed twice for 5 min each and twice for 10 min each in PBT and were incubated in hybridization buffer (50% formamide, Roth #P040; 5× saline sodium citrate SSC, Roth #10541; 100 µg/ml heparin, Sigma-Aldrich #H3149; 5 mM EDTA, Roth #80401; Denhardt's block reagent, Sigma-Aldrich #D2531; 100 µg/ml yeast tRNA, Sigma-Aldrich #R675; 0.1% Tween20; 5% dextran sulfate, Sigma-Aldrich #D8906) for 10 min at room temperature and additionally for approximately 24 h at 60–62 °C in a water bath. Complementary antisense probes and sense probes (0.5–2 ng/µl) were preheated in 300 µl 100% hybridization buffer for 10 min at 85 °C. One RNA probe per specimen patch was added and hybridization was performed at 60–62 °C for approximately 24 h. Next, the specimens were washed thrice for 20 min each in 4× Wash (50% formamide; 4× SSC; 0.1% Tween20), twice for 20 min each in 2× Wash (50% formamide; 2× SSC; 0.1% Tween20), and thrice for 15 min each in 1× Wash (50% formamide; 1× SSC; 0.1% Tween20). Subsequently, specimens were washed thrice for 10 min each in SSCT (1× SSC; 0.1% Tween20) and then washed four times for 10 min each in 0.1 M maleic acid buffer (MAB) (0.1 M MAB pH 7.5 Sigma-Aldrich #M0375; 0.15 M NaCl, 0.1% Tween20). To prevent non-specific anti-digoxigenin antibody binding, specimens were incubated for two hours in 2% MAB block solution (0.08 M MAB, pH 7.5; 2% block reagent #11096176001). Afterwards, specimens were incubated in an anti-digoxigenin antibody conjugated to an alkaline phosphatase enzyme (1:5000; Roche #11093274910) in 2% MAB block solution overnight at 4–7 °C. Alkaline phosphatase enzyme requires a pH of 9.5 to function, thus a respective alkaline phosphatase buffer (AP) was used (0.5 M Tris pH 9.5, Roth #48551; 0.5 M NaCl). Next, the specimens were washed four times for 10 min each in PBT and then thrice for 10 min each in alkaline phosphatase buffer (AP; 0.1% Tween20). Signal was developed with a staining buffer (1× AP-buffer; 3.75 µl/ml BCIP, Roche #1138321001; NBT 5 µl/ml, Roche #11383213001) or, alternatively, with a staining buffer that contained 7.5% polyvinyl alcohol (1× AP-buffer without Tween20 but with 75 mg/ml polyvinyl alcohol, Sigma-Aldrich #P1763; 3.75 µl/ml BCIP; NBT 5 µl/ml). Staining time ranged from 20 to 30 min in case of MHC and from 3 to 4 h in case of *Mox*, *HESC1*, and *HESC3-C6*. In case of *HESC1* and *HESC3-C6*, staining was additionally performed over a longer time period, ranging from 16 to 23 h, but yielded no signal. Negative controls were performed by following the same in situ hybridization protocol but replacing the antisense probe with its corresponding sense probe. These experiments yielded no signal (for gene phylogenies, see Suppl. Figs. 1–3, for negative controls, see Suppl. Fig. 4).

Signal development was stopped by washing the specimens twice for 5 min each in AP buffer and thrice for 10 min each in PBT. Then, the specimens were post-fixed in 4% PFA for 30 min each and subsequently washed twice for 5 min each and twice for 10 min each in PBT. Specimens were stored in 50% glycerol (Roth #37831.3) diluted in PBT. Prior to clearing, specimens were washed twice for 10 min each in an ascending DEPC series in PBT (20%, 40%, 60%, 80%, 100%) and afterwards twice for 10 min each in an ascending ethanol series in DEPC (20%, 40%, 60%, 80%, 100%). Specimens were mounted on glass slides and cleared in 2:1 benzyl benzoate:benzyl alcohol (Sigma-Aldrich #B9550 and #402834). Specimens were studied with an Olympus BX53 light microscope (Olympus, Tokyo, Japan) and images were taken with a DP73 camera (Olympus). Images were edited with Fiji44. Expression pattern schemes were designed with Inkscape (version 0.92.4; https://inkscape.org) and Gimp 2 (Version 2.8.22; https://www.gimp.org).

Between 15 and 40 specimens per gene and developmental stage were investigated in detail for precise location of their expression domains. In almost all cases, 100% of the specimens showed identical expression patterns. Exceptions to this are *HES2* expression in the gastrula (consistent expression in 20 out of 25 specimens) and in the early larva (25 consistent patterns out of 35 specimens) as well as *HES7* in the early larva (20 consistent expression domains out of 25 specimens). For *HES7* expression experiments in the mid-trochophore stage only five specimens were available, all of which showed identical expression patterns.

**Screening for genes of interest.** The publicly available *Acanthochitona fascicularis* translated transcriptome45 (erroneously assigned to as *Acanthochitona crinita* therein) was downloaded (https://zoology. unive.ac.at/open-data/) and de-duplicated using cd-hit (Version 4.7), setting the sequence identity threshold to 0.9546. *Mox* and *MHC* sequences from other mollusks and lophotrochozoans were obtained from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) (Supplementary Tables 2, 3, 4) and were used for reciprocal similarity-based searches of the *A. fascicularis* database using the blastp tool (Version 2.8.1+)48 with the e-value set to 1e−6. Protein domain architecture of the resulting *A. fascicularis* candidate sequences was determined using the hmmscan algorithm against the Pfam A database (https://pfam.xfm.org/). In the case of the *HES* genes, a hmm search (Version 3.1b2)49 was performed with the *HES* family-specific hairy orange domain as a query (Pfam code: PF07527.13). The hairy orange hmm file (Pfam code: PF07527.13) was downloaded from the
enrolling muscle

dorsoventral muscle

ventrolateral muscle

transversal muscle

apical grid

ventromedian muscle

rectus muscle

prototroch muscle ring
Figure 1. Expression of AfaMHC during Acanthochitona fascicularis development. (B, D, F, H, J, L) are schematic representations of gene expression signatures of the respective developmental stages. Colour code indicates respective muscle systems. (A–D) AfaMHC expression in the early trochophore larva (A) AfaMHC expression in the developing rectus, enrolling, and ventrolateral muscles. (B) Ventral view of the developing muscles. (C) Lateral right view of the AfaMHC expression in developing muscles. (D) Lateral right view. (E–H) AfaMHC expression in the late trochophore larva. (E) AfaMHC expression is found in all muscles. Dorsally located muscles such as the rectus muscle and the transversal muscles are partially masked by the intense staining of the more ventrally positioned muscles. (F) Ventral view. Rectus and transversal muscles are not shown. (G) Lateral view showing weak expression in the transversal muscles. (H) Lateral right view. (I–L) AfaMHC expression in the early juvenile. (I) AfaMHC expression is retained in the enrolling muscle, the ventrolateral muscle, the dorsoventral muscles, and the transversal muscles. (J) Ventral view. (K) Lateral right view of AfaMHC expression. (L) Lateral right view. Asterisks mark the mouth. Roman numbers correspond to the future juvenile shells. a anterior, d dorsal, l left, p posterior, r right, v ventral. Scale bars equal 20 μm. Expression pattern schemes were designed with Inkscape (version 0.92.4; https://inkscape.org) and Gimp 2 (Version 2.8.22; https://www.gimp.org).

Pfam database. Seven HES gene candidates turned out to possess a complete basic helix–loop–helix domain, a WRPW motif, and these were used for further analysis.

Gene annotation trees. To obtain additional HES sequences, the Crassostrea gigas Ensembl peptide file49 (https://metazoa.ensembl.org/index.html) was queried with hmmsearch (Version 3.1b2) from the HMMER package48 using the Pfam HES hidden markov model (Pfamcode: PF07527.13). Using this approach, we identified six HES gene candidates that met the threshold e-value of 1e – 3 and also possessed the two complete HES Identity of genes of interest.

Myosin heavy chain (MHC). One AfaMHC ortholog was found in the Acanthochitona fascicularis transcriptome50 (see Suppl. Fig. 1A). The annotated AfaMHC sequence contains one myosin head domain and one myosin tail domain. A MHC-specific glycine (peptide sequence: idfGxdl) insertion within the myosin head domain51 provides further confirmation of gene identity (Suppl. Fig. 1B). Phylogenetic analysis was performed with the Akaike information criterion (–sort A)52. Selected amino acid substitution models were LG58 for MHC and HES, and WAG59 for Mox. Maximum likelihood trees and Bootstrap analyses (100 bootstraps, -b 100) were performed using phylml (Version 20120412)50. Tree topology (t), branch length (l), and rate parameters (r) were optimized (-o lr). Visualisation and annotation of alignments was performed using allview (Version 1.0.0.0; https://ormbunkar.se/allview/54), Jalview (version 2.11.0.; https://www.jalview.org/)55, Gimp 2 (Version 2.8.22; https://www.gimp.org), and Inkscape (version 0.92.4; https://inkscape.org). Visualisation and annotation of phylogenetic trees was performed with FigTree (Version 1.4.4; http://tree.bio.ed.ac.uk/software/figtree/56).

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Data availability

The raw data and source code of the manuscript is available at: https://www.nature.com/sr/srep-data/doi/10.1038/s41586-021-05678-9.
The third group of genes related to the HES family are the Helt genes (Hairy and enhancer of split-related protein Helt), which only possess the bHLH domain and the Hairy orange domain but lack the specific tetrapeptide at the C-terminal end. The most distantly related gene group, Clockwork orange, was used as an outgroup. Similar to Helt, Clockwork orange only possesses the bHLH and the Hairy orange domain.

**MHC is expressed in all larval and most adult muscle systems.** Expression of AfaMHC was first detected in early trochophore stages during muscle formation (Fig. 1A–D). AfaMHC is expressed in three small, paired regions which give rise to the rectus muscle that spans the region below the future shell plates in anterior–posterior direction. In addition, expression is in the enrolling muscle that laterally engulfs the larva and the ventrolateral muscle that lies ventrally and consists of two longitudinal muscle strands (see5,65 for detailed description of polyplyacophoran larval myoanatomy) (Fig. 1A–D). In the late trochophore larva, all larval muscle systems (i.e., muscles that do not persist until adulthood) are labelled (Fig. 1E–H), including the prototroch muscle ring that underlies the prototroch, the paired ventrolateral muscle, the single ventromedian muscle, and the apical muscle grid59,65. Muscles that are maintained and elaborated after metamorphosis are the enrolling muscle, the dorsal longitudinal rectus muscle, seven sets of paired dorsoventral muscles (with the eighth being formed only considerably later during post-metamorphic development), and a set of dorsal transversal muscles that underlie the shell plates59,65. Of these, expression of AfaMHC is in the enrolling muscle, the rectus muscle, and the dorsoventral muscles (Fig. 1E–H). Relatively weak expression domains are found in the region of the developing dorsal transversal muscles (Fig. 1G,H). In the juvenile polyplyacophoran, the larval muscles disappear and the muscles of the future adult body plan become elaborated. Accordingly, individual myocytes become concentrated into distinct sets of dorsoventral and transversal muscles. Adult-specific muscles, such as the buccal musculature that forms several strands around the mouth, and the paired radula retractors develop59,65. The ventrolateral muscle is still partly visible at this point and is reduced during further growth. Of these juvenile muscle systems, AfaMHC expression is found in the ventrolateral muscle, the enrolling muscle, the dorsoventral muscles, and in the transversal muscles (Fig. 1I–L).

**Mox is expressed in the mesodermal bands and in a subset of the musculature.** Of the two Mox sequences identified we were only able to produce expression data by in situ hybridisation for AfaMox1. Expression of this gene was first detected in the early trochophore larva (Fig. 2A,B), where it is prominently expressed in the developing paired mesodermal band (Fig. 2C–F). In the late trochophore larva, AfaMox1 expression is confined to the ventrolateral muscle (Fig. 2G–J). No Mox expression was detected in later stages of development.

**HES genes are expressed in ectodermal and mesodermal domains.** Two of the seven HES family genes identified (AfaHESC2 and AfaHESC7) yielded expression signals. Both genes start to be expressed in the late gastrula stage. Their expression is maintained in early larval stages but only AfaHESC2 is expressed in the late trochophore larva. In the gastrula, AfaHESC2 is expressed in ectodermal cells (Fig. 3A,B). In the early trochophore larva, AfaHESC2 is expressed in the mesodermal bands. A weaker expression domain extends from the anterior pole of the mesodermal bands into the apical region of the larva where it closes in an inverted U-shaped manner (Fig. 3C–F). In the late trochophore larva, AfaHESC2 expression is limited to the region of the adult buccal ganglion close to the dorsal ectoderm, where two spot-like expression domains are located (Fig. 3G–J). Expression of AfaHESC7 first occurs in the prospective trochtoblasts in the equatorial region of the gastrula (Fig. 4A,B). In the early larval stage, AfaHESC7 expression is restricted to a domain around the mouth (Fig. 4C,D). Throughout larval development, AfaHESC7 expression continues to be expressed around the mouth and in the region of the presumptive foregut. AfaHESC7 expression ceases in the late trochophore larva (Fig. 4E–H).

**Discussion**

**Myosin heavy chain: a conserved marker of metazoan myogenesis.** Results from the cnidarian *Nematostella* suggest that myosin heavy chain (MHC) was already a key component of contractile cells in the last common ancestor of cnidarians and bilaterians. MHC has been used as a marker to study early muscle differentiation during formation of the paired coelomic cavities of the metasome14. Data are inconclusive as to whether or not MHC is expressed during the early formation of several muscle systems in *Acanthochitona* larvae, including the ventrolateral muscle, the enrolling muscle, and the rectus muscle. These results confirm the utility of MHC as a marker of early myogenesis in Mollusca, although further studies are needed to allow for a more detailed comparison of the initial stages and domains of muscle differentiation in this phylum.

**Conserved Mox expression in nephrozoan mesoderm and muscle formation.** Most metazoans possess only one Mox gene5,7,14,13,32–24 with exception of the vertebrates that have two31,32 and the anthozoan *Nematostella vectensis* that has four Mox genes that evolved by tandem duplications35. Cnidarian Mox genes are expressed exclusively in the endoderm7,13,24, whereas in most bilaterians, Mox expression initially coincides with mesoderm formation and is later restricted to the developing muscle72.

In deuterostomes, Mox expression typically begins around the time of gastrulation in early mesodermal precursors. In the hemichordate *Saccoglossus kowalevskii*, *Mox* (*SkoMox*) is expressed in the ventral mesoderm during formation of the paired coelomic cavities of the metasome14. Data are inconclusive as to whether or not *SkoMox* expression continues during subsequent development14. In the ascidian *Ciona intestinalis*, the Mox ortholog *Mox* (*CimMox*) is specifically expressed in muscle precursor cells in the early gastrula77 and in the cephalochordate *Branchiostoma floridae*, *BbeMox* is expressed in the paraxial mesoderm during somite formation14. In the mouse, two *Mox* genes, *MmuMox1* and *MmuMox2*, were identified. These show slightly different expression...
dynamics and have been implicated in the early anterior–posterior patterning of the embryonic mesoderm as well as in somite specification and differentiation. A reduction of limb muscle tissue in *MmuMox2* null mice revealed the importance for muscle development. A *Mox* mutation in zebrafish causes defects in bone development such as vertebral fusion, congenital scoliosis, and asymmetry of the pectoral girdle, providing evidence for the involvement of *Mox* in establishing mesodermal derivatives. These data imply a conserved involvement of *Mox* in the initial specification of the deuterostome mesoderm and in the development of its derivatives.

In the diverse Lophotrochozoa, *Mox* expression has only been studied in three species, namely the gastropod *Haliotis asinina*, the brachiopod *Terebratalia transversa*, and the polychaete *Alitta virens*. For each of these, only one *Mox* gene has been described, while we found a second *Mox* sequence in the polyplacophoran *Acanthochitona fascicularis*. All four species start to express *Mox* shortly after gastrulation in lateral mesodermal bands that flank the endoderm. Accordingly, an early role for *Mox* in mesodermal band specification appears to be an ancestral feature of lophotrochozoans. During later stages, *Mox* continues to be expressed in the developing foot musculature in *Haliotis*, in precursor cells of the future body wall and pharyngeal muscles in *Alitta*, and in the ventrolateral muscle of late *Acanthochitona* trochophore larvae. Since we were not able to produce consistent expression results for *AfaMox2*, a putative role of this gene remains speculative. However, taken together, these data support a dual role of *Mox* in early mesoderm specification and in myogenesis, that is conserved among lophotrochozoans and deuterostomes. Notably, however, several lineage-specific evolutionary events have resulted in the loss of conserved roles and in co-option of *Mox* into novel ones. The *Mox* ortholog of the sea urchin *Strongylocentrotus purpuratus*, for example, is not expressed during mesoderm formation but in ectodermal neurons in the region of the larval apical organ. This expression disappears in later stages, indicating that *SpuMox* plays a role in early neurogenesis rather than in mesoderm or muscle formation. A similar situation is found in the fruit fly *Drosophila melanogaster*, where the *Mox* ortholog *buttonless (DmeMox)* is expressed in the ventral median cells which derive from the ventral mesoderm and play a crucial role in axon guidance. Importantly, however, *DmeMox* is not expressed in muscle progenitors or muscular tissue. In the second major

**Figure 2.** Expression of *AfaMox1* during early mesoderm formation in *Acanthochitona fascicularis*. (B, D, F, H, J) are schematic representations of gene expression patterns of the respective developmental stages with gene expression domains indicated in purple and the ventrolateral muscle in green. (A) The gastrula is devoid of *AfaMox1* expression. (B) Lateral right view. (C–F) *AfaMox1* expression in the early trochophore larva. (C) *AfaMox1* is expressed in the mesodermal bands. (D) Ventral view. (E) Lateral right view of *AfaMox1* expression in the mesodermal bands. (F) Lateral right view. (G–J) *AfaMox1* expression in the late trochophore larva. (G) *AfaMox1* expression in the ventrolateral muscle. (H) Ventral view. (I) Lateral right view of *AfaMox1* expression in the ventrolateral muscle. (J) Lateral right view. Asterisks mark the blastopore and the mouth, respectively. a anterior, d dorsal, l left, p posterior, r right, v ventral. Scale bar equals 20 µm. Expression pattern schemes were designed with Inkscape (version 0.92.4; https://inkscape.org) and Gimp 2 (Version 2.8.22; https://www.gimp.org).
ecdysozoan lineage, Nematoda, Mox was very likely lost altogether since other ecdysozoans and echinoderms are yet to be tested for Mox expression, a potential association between the loss of mesodermal Mox expression and the evolution of a neurogenesis-related role remains uncertain.

In summary, the data currently available suggest that Mox was recruited into mesoderm formation in the last common bilaterian ancestor (LCBA) and may thus have played an important role in mesoderm evolution (Fig. 5). In addition, it appears that Mox was simultaneously recruited into myogenesis in the LCBA with loss of this role at least in Drosophila and putatively in both, myogenesis and mesoderm formation, in echinoderms (Fig. 5).

Variability of HES gene expression in metazoan development. HES genes are fast evolving genes that have undergone repeated species-specific, independent gene duplications. The actual number of HES copies varies from one single sequence in the cnidarian Hydra to up to 22 copies in the zebrafish Danio. In Acanthochiton fasicularis, seven HES genes were identified, and two (AfaHESC2 and AfaHESC7) were further investigated here by in situ hybridization.

HES genes have been implicated in a wide range of developmental processes including neurogenesis as well as digestive tract and mesoderm formation. Thus, HES expression domains vary considerably between taxa. A comparative overview of the identified Mox, HES, and MHC genes and their respective expression domains across Metazoa is provided in Supplementary Table 5. In the sea anemone Nematostella, two HES genes, NveHES2 and NveHES3, are expressed in ectodermal cells of the gastrula, while NveHES3 expression expands to oral ecto- and endoderm in the planula larva. In contrast, the single Hydra HES gene (HvuHES) is expressed during budding at the bud base shortly before separation from the mother animal, but was not detected in earlier stages. In early embryos of the acoelomorph Symsagittifera roscophensis, the only HES gene, SroHES, is expressed in the
anterior-median region. In juveniles, it is expressed posterior to the statoblast, dorsally in the nerve cords, and mid-ventrally in the brain, but not in the mesoderm. These data indicate that *HES* genes were initially involved in neurogenesis and in development of anterior ecto- and endodermal tissues and that their mesodermal expression might be a nephrozoan (or even bilaterian) novelty.

Deuterostomes, such as the cephalochordate *Branchiostoma*, and vertebrates possess multiple *HES* genes that are broadly expressed across all germ layers. In *Branchiostoma*, four out of eight *HES* genes (*BbeHESA-D*) are expressed in the anterior endoderm, in the presumptive neural plate, and in the presomitic mesoderm of the mid-gastrula. In neurula stages, expression is further found in the endoderm, in the neural tube, in the somites, as well as in the paraxial mesoderm, the foregut, the neural plate, and in the notochord. In vertebrates (mouse, chicken, and *Xenopus*), *HES* genes also play a crucial role during somitogenesis, gut formation, neurogenesis, as well as in the maintenance of stem cell potential and separation of different brain areas from each other. A functional study employing *HES* gene knockdown in *Xenopus laevis* resulted in a decrease of cell proliferation. This indicates anti-apoptotic functions and highlights the ability for transcriptional repression of *HES* genes. In the sea urchin *Strongylocentrotus*, no mesodermal expression of *HES* was observed. Instead, *HES* is expressed from blastula to gastrula stages in the oral ectoderm and (weakly) in the archenteron. This is consistent with data on *SpuMox* that, in contrast to *Mox* genes of other deuterostomes, is also absent from the mesoderm and is exclusively expressed in ectodermal neurons in the sea urchin.

Interestingly, *Mox* and *HES* genes also seem to be of relatively little importance for mesoderm development and myogenesis in ecdysozoans. In the nematode *Caenorhabditis elegans*, ref-1 (*CelHES*) is only expressed

**Figure 4.** Expression of *AfaHESC7* during development of *Acanthochitona fascicularis*. (B, D, F, H) are schematic representations of gene expression signatures (in purple) of the respective developmental stages. (A, B) Expression of *AfaHESC7* in the gastrula. (A) *AfaHESC7* is expressed in the trophoblasts. (B) Lateral view. (C, D) *AfaHESC7* expression in the early trochophore larva. (C) Expression of *AfaHES7* is found in ectodermal cells around the mouth. (D) Ventral view. (E, F) Expression of *AfaHESC7* in the mid-trochophore larva. (E) The expression domain remains around the posterior margin of the mouth and extends anteriorly into the region of the foregut. (F) Ventral view. (G) Loss of *AfaHESC7* expression in the late trochophore larva. (H) Lateral right view. Asterisks mark the blastopore and the mouth, respectively. a anterior, d dorsal, l left, p posterior, r right, v ventral. Scale bar equals 20 µm. Expression pattern schemes were designed with Inkscape (version 0.92.4; https://inkscape.org) and Gimp 2 (Version 2.8.22; https://www.gimp.org).
in descendants of the AB blastomere, which contribute to the nervous system. The single Drosophila HES gene, hairy (DmeHES), is mainly expressed ectodermally during segmentation, where it acts as a pair-rule gene. In later stages, DmeHES is also expressed in the nervous system, the foregut, and the developing muscles not investigated. X = no Mox ortholog present. Lophotrochozoa: Mox is expressed in the mesodermal bands of early lophotrochozoan larvae and additionally in a small pre-oral ectomesodermal domain in Alitta virens. Mox is also expressed in muscle progenitor cells and/or muscle tissue in later-stage mollusk and annelid larvae. Data on brachiopods are inconclusive. Ecdysozoa: the Mox ortholog buttonless is expressed in dorsal median cells in Drosophila which originate from the mesoderm and play a role in axon guidance but are not associated with myogenesis. Nematodes have no Mox ortholog. Deuterostomia: Mox expression in the mesoderm in hemichordates and chordates. In the sea urchin, Mox is only expressed in neural cells of the larva. Mox expression in myogenesis in hemichordates is unknown. In chordates, Mox is expressed during somitogenesis in amphioxus and vertebrates. In amphioxus, no Mox expression was observed after somitogenesis. In vertebrates, both Mox genes are expressed in myogenesis. Xenacoelomorpha: No unambiguous Mox ortholog described. Cnidaria: Mox expression is restricted to the endoderm. Parsimony analysis suggests recruitment of Mox in mesoderm formation and myogenesis at the base of bilaterians with a loss in myogenesis in Drosophila and a loss in mesoderm formation in echinoderms. Asterisks mark the mouth. Data from previous investigations and present study. Expression pattern schemes were designed with Inkscape (version 0.92.4; https://inkscape.org) and Gimp (Version 2.8.22; https://www.gimp.org).
A potential reason for this is that only two out of seven HES genes were detectable by in situ hybridization during Acanthochitona ontogeny. While in-situ hybridization sensitivity is high, it is possible that one or more of the remaining five HES genes are indeed involved in myogenesis but did not meet the minimum expression threshold required for detection. Although we were unable to unequivocally assign AfaHESC2 expression to distinct morphological features, it is briefly expressed in the mesodermal bands and later appears to overlap with the region of the developing buccal ganglia. AfaHESC7 expression was observed in the oral ectoderm, around the foregut, and, surprisingly, in the trochoblasts. The latter are specialized founder cells that give rise to the ciliated cells of the prototroch and have so far not been reported to express a HES gene in any other lophotrochozoan.

Taken together, these data show that mesodermal and muscular HES gene expression is likely an ancestral feature of bilaterians that was lost in multiple lineages including echinoderms, nematodes, planarians, and possibly also acoelomorphs and polyplacophoran mollusks. Involvement in endoderm specification, on the other hand, likely emerged in the last common ancestor of Metazoa and was also lost in several lineages, such as acoelomorphs, nematodes, planarians, and brachiopods. Altogether, ectodermal and/or neural HES gene expression appears to be particularly well conserved across metazoans. Since HES genes chiefly act in separating tissues from each other that are destined to undergo fate determination ("territorialisation"), they have been co-opted into various additional developmental processes, such as the formation of the chaete in annelids and brachiopods, segmentation in annelids and arthropods, somitogenesis in chordates, and budding in cnidarians. This enormous variability highlights their importance for the evolution of distinct ontogenetic pathways throughout the animal kingdom (Fig. 6, Supplementary Table 5).
Conclusion

The present study shows that Mox and HES genes are expressed during mesoderm formation in the mollusk Acanthochiton fasicularis. Expression of Mox in the mesodermal bands and in their major derivatives, the muscles, is congruent with the situation in other lophotrochozoans, suggesting a dual role of this gene in the last common bilaterian ancestor. Mox experienced loss in myogenesis in ecdysozoans and loss in both myogenesis and mesoderm formation in echinoderms, where it is instead expressed in the ectoderm. Expression of HES occurs during early mesoderm development, neurogenesis, and digestive tract formation in a number of bilaterians as well as in ectodermal and endodermal domains in cnidarians, implying either a wide variety of roles already at the dawn of bilaterian evolution or a particularly high degree of variability (co-option) of HES genes with various independent gain-of-function events along individual bilaterian lineages.

Received: 16 February 2021; Accepted: 13 August 2021
Published online: 09 September 2021

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Acknowledgements
We thank Thomas Rattei (University of Vienna) for providing access to the Life Science Computer Cluster and Christian Baranyi (University of Vienna) for help with animal rearing and fixation. We thank Andreas Hejnol (University of Bergen) and Pedro Martinez (University of Barcelona) for comments on the presence of putative Max orthologs in acoelomorphs. We thank the staff of the Station Biologique de Roscoff (France) for providing lab space. This study was supported by a grant of the Austrian Science Fund (FWF) to Andreas Wanninger (Grant number: P29455-B29). The research leading to these results has also received funding from the European Union’s Horizon 2020 research and innovation programme under Grant agreement No 730984 to Elisabeth Zieger.

Author contributions
A.W. designed and supervised the project. A.S. performed research with contributions from E.Z. and A.C. A.S. drafted the manuscript. E.Z., A.C., and A.W. contributed to writing of the manuscript. All authors contributed to, read, and approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-96711-y.

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