A central question in chordate evolution is the origin of sessility in adult ascidians, and whether the appendicularian complete free-living style represents a primitive or derived condition among tunicates. According to the ‘a new heart for a new head’ hypothesis, the evolution of the cardiopharyngeal gene regulatory network appears as a pivotal aspect to understand the evolution of the lifestyles of chordates. Here we show that appendicularians experienced massive ancestral losses of cardiopharyngeal genes and subfunctions, leading to the ‘deconstruction’ of two ancestral modules of the tunicate cardiopharyngeal gene regulatory network. In ascidians, these modules are related to early and late multipotency, which is involved in lineage cell-fate determination towards the first and second heart fields and siphon muscles. Our work shows that the deconstruction of the cardiopharyngeal gene regulatory network involved the regressive loss of the siphon muscle, supporting an evolutionary scenario in which ancestral tunicates had a sessile ascidian-like adult lifestyle. In agreement with this scenario, our findings also suggest that this deconstruction contributed to the acceleration of cardiogenesis and the redesign of the heart into an open-wide laminar structure in appendicularians as evolutionary adaptations during their transition to a complete pelagic free-living style upon the innovation of the food-filtering house.

Cardiac developmental atlas and ontogeny

The open-wide laminar heart of appendicularians is considered the simplest chordate heart, consisting just of two layers, the myocardium and the pericardium, the former of which pumps against the stomach (Fig. 1a). Here we provide a developmental atlas of the appendicularian heart and show that cardiogenesis in Oikopleura dioica is fast, spanning only 3.5 h from the early hatching stage (5 h post-fertilization (hpf)), at which no morphological evidence of the cardiac primordium could yet be distinguished, until the late hatching stage (8.5 hpf), when the heart began to beat (Supplementary Video 1).

Analysis of muscular Actin 1 (Actn1) expression (in lieu of Mesp, which is the preferred precardiac marker in ascidians, but is absent in appendicularians (Fig. 1b)) integrated with data from 4D microscopy nuclear tracing (Fig. 1c, Extended Data Fig. 1) identified B8.9 blastomere at the incipient tailbud stage as the first cardiac progenitor cell (CPC). Our analysis revealed that cardiac cells shared lineage with the first three anterior tail muscle cells, following the same ontogenetic origin as in ascidians and therefore provided evidence that ascidian and appendicularian hearts are homologous.

Loss of cardiopharyngeal GRN early module

In vertebrates, the cardiopharyngeal field is the developmental domain that gives rise to the heart and branchiomeric muscles from a common pool of early cardiopharyngeal multipotent progenitors. After a binary-stepwise process of fate choices, early cardiopharyngeal progenitors give rise to the first and second heart fields and to branchiomeric muscles in the head and neck. In ascidians, pharyngeal muscles (that is, siphon and longitudinal muscles) are considered homologous to vertebrate branchiomeric muscles, and their cardiopharyngeal GRN is...
highly conserved with vertebrates using a homologous binary-stepwise model. Consistent with this model, the precardiac master regulator *Mesp* is expressed in multipotent pre-CPCs in both vertebrates and ascidians, followed by FGF–MAPK signalling mediated by ETS1/2 phosphorylation, and finally the activation of the cardiogenic kernel (that is, *Gata4/5/6, Ftx, Nk4* and *Hand1/2*) and BMP signalling.

Our genomic survey of seven appendicularian species and eleven ascidians revealed the absence of *Mesp, Ets1/2, Gata4/5/6, Mek1/2* and *Hand-r* homologues in all analysed appendicularians, whereas they were present in all ascidians (Fig. 1b, Extended Data Figs. 2–8). Phylogenetic analyses suggested that the absence of these genes was probably due to ancestral gene losses that occurred at the base of the appendicularian lineage after its split from ascidians. Following the surprising absence of a homologue of *Mesp*, considering its precardiac master role in ascidians and vertebrates, we tested for the possibility of ‘function shuffling’ among *Mesp*-related basic helix–loop–helix...
**Fig. 2 | Comparison of the cardiopharyngeal cell lineage and GRN in ascidians and appendicularians.** The determination of the cardiopharyngeal lineage (blue) and its split from the germline (brown) occurs one cleavage earlier in *O. dioica* than in *C. robusta*. In contrast to *O. dioica*, in ascidians, the precardiac *Mesp*-positive cell B7.5 divides before the split of the anterior tail muscle lineage (ATM; purple) and the cardiopharyngeal lineage (blue). In *O. dioica*, the daughter cell of B7.5 rapidly activates the expression of the cardiogenic kernel (*Nk4* and *Hand1/2*) and becomes a CPC (red), whereas in ascidians, their counterpart TVCs (green) maintain a multipotent state, which will give rise to the first heart precursors (FHPs; red), second heart precursors (SHPs; pink) and atrial siphon muscle field (ASMF; yellow), the last two through intermediate secondary multipotent cells (STVCs; orange). The lack of *Dach* expression in the heart of *O. dioica* suggests the absence of a homologue of the ascidian second heart field. The numerous losses of cardiopharyngeal genes (grey strikethrough) and subfunctions (grey) highlight the deconstruction of the ‘early’ and ‘late’ ancestral multipotent GRN modules related with the early precardiac multipotency (EM module) of the TVCs in ascidians, and the late multipotency (LM module) of the STVCs and their derivatives such as pharyngeal muscles and second heart field, respectively. The developmental timelines depict the acceleration of cardiogenesis in *O. dioica* compared with ascidians, with the differentiation of the first CPC (red) as soon as 2.5 hpf in *O. dioica*.

(bHLH) genes. However, we did not observe any expression domain of *Math or Neurogenin* (the closest bHLH genes according to blast analysis) that were compatible with precardiac progenitors. We also tested for the possibility of function shuffling among appendiculardian-specific duplications of paralogues closely related to the lost genes (that is, two *Ets1/2a* and four *Gata1/2/3*) as well as *MEK7* and *MEK3/6*, but again no tissue-specific expression domains compatible with CPCs were observed. We found homologues of *Nk4*, *Hand1/2* and *FoxF*. While *Nk4*, first, and then *Hand1/2* were sequentially expressed in the CPCs at incipient tailbud and mid-tailbud stages, respectively (Fig. 1d, e), no expression of *FoxF* was observed in the CPCs. These results showed that the first CPC (B8.9) resulting from the split of the anterior tail muscle lineage (B8.10) did not maintain a multipotent state (as their counterpart trunk ventral cells (TVCs) do in ascidians), but rapidly activated the expression of the cardiogenic kernel (*Nk4* and *Hand1/2*). In contrast to ascidians and vertebrates, this activation had become independent of *Mesp, Ets1/2*-mediated FGF signalling, *FoxF* and *Gata4/5/6* (ref.2). The co-elimination of *Mesp, Ets1/2b, MEK1/2, Gata4/5/6* and the loss of the cardiac subfunction of *FoxF* highlighted the deconstruction of what can be considered an ancestral ‘early multipotent’ module that in ascidians and vertebrates is related to the early maintenance of the multipotent state of the precardiac progenitors3, and consequently result in accelerated cardiogenesis in *O. dioica* (Fig. 2).

The deconstruction of this early multipotent module suggested that the conserved cardiogenic roles of FGF–MAPK and BMP signalling in ascidians and vertebrates could have also been altered in appendicularians. To test this hypothesis, we performed inhibitory treatments against the FGF receptor, the surviving paralogues *MEK3/6* and *MEK7*, and BMP receptors, at different concentrations and time windows, and treated embryos were analysed by whole-mount in situ hybridization (Extended Data Fig. 9, Supplementary Data 2). Results revealed that the formation of the CPCs and the onset of *Nk4* expression were not altered in treated embryos in which FGF–MAPK or BMP signalling pathways had been inhibited (Fig. 1f). These results suggested that the determination and differentiation of the CPCs and the onset of the cardiogenic kernel had become independent of these two signalling pathways during the evolution of appendicularians.

**Loss of cardiopharyngeal GRN late module**
In ascidians, TVCs undertake a series of asymmetric cell divisions and regulatory transient secondary multipotent states (that is, STVCs) that...
not only give rise to the first and second heart precursors but also to the atrial siphon muscle founder cells. We surveyed the appendicularian homologous genes encoding cardiopharyngeal transcription factors such as \textit{Hand}-r, \textit{Tbx}1/10, \textit{Islet}1 and \textit{Ebf} (also known as \textit{Coe}), which in ascidians become activated in an FGF–MAPK-dependent manner to determine the trajectory towards the atrial siphon muscles, including the activation of \textit{MyoD} (also known as \textit{Mrf}) \cite{4,19,28} (Extended Data Fig. 10). In addition to the aforementioned absence of \textit{Hand}-r, \textit{Tbx}1/10 was present in any appendicularian, suggesting again an ancestral gene loss in the appendicularian lineage. We found single homologues for \textit{Islet}1, \textit{Ebf} and \textit{MyoD}; the two former genes were expressed in the nervous system, and the latter in the oikoplastic epithelium, but no expression was found for any of these homologues in the trunk that could suggest the presence of a homologous tissue to the atrial or any other pharyngeal muscle. To test for the presence of a presumptive second heart field in \textit{O. dioica}, we analysed the expression of the homologue of \textit{Dach}, which in ascidians is activated by \textit{Tbx}1/10 in the absence of FGF–MAPK signalling, and is necessary to determine the identity of the second heart precursors \cite{39}. Whole-mount in situ hybridization revealed that, while the single homologue of \textit{Dach} was expressed in the nervous system, the endoderm and the trunk epidermis, no expression was detected in the heart, suggesting the absence of a second heart field homologue in \textit{O. dioica} (Extended Data Fig. 10). In addition to \textit{Dach}, our genome survey in \textit{O. dioica} revealed the absence of 12 out of 25 genes that a recent single-cell transcriptomic analysis had revealed to be specific for the first or second heart field precursors in ascidians \cite{Supplementary Data 3}. In summary, our results highlight that during the deconstruction of the cardiopharyngeal GRN, the loss of \textit{Tbx}1/10 and the loss of cardiopharyngeal subfunctions for \textit{Dach}, \textit{Islet}1, \textit{Ebf} and \textit{MyoD} might represent the loss of an ancestral ‘late multipotent’ module that is required for regulating secondary multipotency and differentiation of the second heart field and atrial muscle in ascidians, structures that appear to have been lost during the evolution of ascidians \cite{Fig. 2}.

**Discussion**

**Homology and one cleavage earlier trend**

Understanding the evolution of the cardiopharyngeal GRN in appendicularians and ascidians is key to infer the ancestral lifestyle of tunicates. Our work provides strong evidence supporting the homology between the hearts of ascidians and appendicularians despite their remarkable differences of morphology, developmental pace and physiology. A key difference is that the specification of the cardiopharyngeal cell lineage and its split from the tail muscle cell lineage occurs one cleavage earlier in appendicularians than in ascidians (Fig. 2). This ‘one cleavage earlier’ trend can be considered a general aspect of the evolution of the development of appendicularians, consistent with the observation already made by Delsman \cite{29} that gastrulation in \textit{O. dioica} occurred one cleavage earlier than in ascidians, and later corroborated by modern studies \cite{30}. This trend has probably contributed to developmental acceleration, morphological simplification and reduction in cell number in this group of tunicates.

**Deconstruction and evolutionary impact**

One of the most striking findings of our work is the numerous losses of genes and subfunctions that highlight a process of deconstruction of the cardiopharyngeal GRN in appendicularians. The term ‘deconstruction’, originally coined in philosophy and later applied in literature, architecture, fashion and cookery, or even developmental biology \cite{31}, is not synonymous with destruction or homogeneously distributed erosion, but instead it refers to the process of dismantling or breaking apart elements that traditionally are combined, and whose analysis facilitates the recognition of structural modules. Our EvoDevo work here, in agreement with the modular model for the control of heart cell identity proposed by Wang et al. \cite{32}, unveils the deconstruction of ‘evolvable modules’ of the cardiopharyngeal GRN, accompanied by developmental system drift and GRN rewiring during the evolution of the heart and pharyngeal muscle in appendicularians (Fig. 2). The loss of the ‘early’ and ‘late’ multipotent ancestral modules correlates with the loss of the multipotent states that in ascidians are maintained in the TVC and STVCs, respectively. The losses of these two ancestral modules can be connected to three evolutionary innovations that accompanied, and plausibly facilitated, the evolution from an ancestral sessile ascidian-like adult lifestyle to the pelagic fully free lifestyle of appendicularians: (1) an accelerated cardiogenesis, (2) the formation of an open-wide laminar heart, and (3) the loss of the siphon muscle (Fig. 3).

First, the accelerated cardiogenesis driven by the ‘one-cleavage earlier’ CPC specification and by the deconstruction of the GRN is probably the result of a primary adaptation to the faster development in appendicularians than in other tunicates. Moreover, accelerated cardiogenesis also enabled the heart to adaptively begin beating as soon as 8.5 hpf in \textit{O. dioica}—in contrast to a few days post-metamorphosis in ascidians—driving haemolymph circulation to be ready when juveniles inflate the first house (10 hpf) and begin pelagic filter feeding.

Second, the low number of cardiac cells (the myocardiun is made of only six cells) \cite{14} together with the apparent loss of the second heart field homologue in appendicularians is compatible with the transformation of an ascidian-like tubular heart into an open-wide laminar heart that beats against the stomach. Considering that haemolymph circulation in appendicularians is not only powered by the heart but also by tail movements \cite{33}, the adaptive innovation of a laminar cardiac structure plausibly offered a more efficient system to pump haemolymph waves propelled by the tail movements through an open-wide structure than through the less accessible space of a tubular ascidian-like heart.

Third, the loss of the ‘body wall’ and pharyngeal/siphon muscles in the trunk of \textit{O. dioica} \cite{21} can be considered the result of regressive evolution during the transition from a sessile ascidian-like to the pelagic style of appendicularians, in which their functions in sessile ascidians (siphon opening/closing and water squirming as a response to large...
debris, predators, low tide, or the ejection of faeces or gametes) became useless upon the innovation of the house in appendicularians.

**Future perspective**

This work exemplifies how the study of gene loss and the application of the concept of deconstruction in evolutionary biology facilitates the recognition of modules and rewiring of the GRN to better understand the evolution of species. Our study, for instance, supports an evolutionary scenario in which the deconstruction of the cardiopharyngeal GRN was linked to regressive loss of features that characterize the ascidian-like sessile lifestyle such as the siphon muscles, and to the evolution of the accelerated cardiogenesis and the transformation to a laminar heart that could have been adaptively selected during the transition of appendicularians to a pelagic complete free-living active style connected to the innovation of the house (Fig. 3). Our evidence, supporting the view that the last common tunicate ancestor had a biphasic lifestyle alternating motile larva and sessile adults, is compatible with the commonly accepted assumption that appendicularian branching is basal among tunicates, but it is also compatible with the possibility that appendicularians are phylogenetically related to some groups of ascidians (that is, Aplousobranchia). Thus, our work provides a useful framework for future comparative studies of the cardiopharyngeal GRN among different tunicates, as well as for future efforts to clarify the potential neotenic origin of appendicularians and their phylogenetic relationship with other tunicates.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-021-04041-w.

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**Methods**

**Biological material**

O. dioica specimens were obtained from the Mediterranean coast of Barcelona (Catalonia, Spain). Culturing of O. dioica and embryo collections were performed as previously described. This project did not involve any ethical issues related to informed consent, data protection issues, or humans. Experimentation on aquatic invertebrate animals such as the planktonic Oikopleura dioica is not subject to regulations regarding animal experimentation, because this applies only to vertebrate organisms (Real Decreto 223 14-3-1998, in Catalonia Ley 5/1995, DOGC 2073, 5172). Nevertheless, experimental procedures followed EU animal care guidelines and were approved by the Ethical Animal Experimentation Committee (CEEA 2009) of the University of Barcelona.

**Genome database searches and phylogenetic analysis**

Protein sequences from the tunicate Ciona robusta and the vertebrate Homo sapiens were used as queries in BLASTp and tBLASTn searches in genome databases of selected species: https://blast.ncbi.nlm.nih.gov/Blast.cgi for Branchiostoma floridae in genome databases of selected species: https://blast.ncbi.nlm.nih.gov/Blast.cgi were used as queries in BLASTp and tBLASTn searches. Genome database searches and phylogenetic analysis were performed as previously described. This project did not involve any ethical issues related to informed consent, data protection issues, or humans. Experimentation on aquatic invertebrate animals such as the planktonic Oikopleura dioica is not subject to regulations regarding animal experimentation, because this applies only to vertebrate organisms (Real Decreto 223 14-3-1998, in Catalonia Ley 5/1995, DOGC 2073, 5172). Nevertheless, experimental procedures followed EU animal care guidelines and were approved by the Ethical Animal Experimentation Committee (CEEA 2009) of the University of Barcelona.

**Cardiac lineage tracing using 4D microscopy**

Cardiac lineage tracing was performed using Supplementary Video 2 from Stach et al. We followed cell divisions starting from B5.2 blastomere at the 16-cell stage until the late tailbud stage when the CPC divides from the first anterior tail muscle cell. Blastomere nomenclature follows that of Conklin for ascidians (vegetal blastomeres in capital letters, animal blastomeres in lower case).

**Cloning and expression analysis**

O. dioica genes were PCR amplified from cDNA obtained as previously described. Then, they were cloned using the Topo TA Cloning Kit (K4530-20, Invitrogen) to synthesize antisense digoxigenin (DIG) and fluorescein (FITC) riboprobes for whole-mount in situ hybridization (FWMISH).

**Pharmacological treatments**

For FGF receptor inhibition, animals were treated with 50 µM and 100 µM of SU5402 (SML0443, Merck) and AZD4547 (9403, BioVision) for 3 h. For FGF2 inhibition, animals were treated with 50 µM and 100 µM of SU5402 (SML0443, Merck) and AZD4547 (9403, BioVision) for 3 h. For FGF receptor inhibition, animals were treated with 50 µM and 100 µM of SU5402 (SML0443, Merck) and AZD4547 (9403, BioVision) for 3 h. For FGF receptor inhibition, animals were treated with 50 µM and 100 µM of SU5402 (SML0443, Merck) and AZD4547 (9403, BioVision) for 3 h.

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from the two-cell stage (30 min post-fertilization (mpf)) and from 32-cell stage (70 mpf), respectively, to hatching stage (4 hpf) in darkness. For MEK3/6 inhibition, animals were treated with gossypetin (1176, Extrasyntese) 100 µM from the 2-cell to the 32-cell stage in darkness. For MEK7 inhibition, animals were treated with 1 µM and 25 µM of 5Z-7-oxozeaenol (O9890, Merck) from the 2-cell stage to the 32-cell stage, respectively. For BMP inhibition, animals were treated with 10 µM of LDN (SML1119, Merck) and dorsomorphin (P5499, Merck) from the 2-cell stage and from the 32-cell stage until hatching stage. To perform these treatments, eggs were pooled in 4 ml of SSW and fertilized with 200 µl of sperm dilution (the sperm of three males in 5 ml of SSW). At the desired time, embryos were transferred to a 3-mm Petri dish plate with 4 ml of treatment solution at 19 °C. Control embryos were incubated in DMSO 0.2% or 0.3% (v/v) depending on the concentration of the treatment. The effects of the treatments were scored by in situ hybridization. For tailbud embryos, we used cross-hybridizing ActnM1, Nk4 and Brachyury probes34,35, whereas for hatching embryos, we used the specific ActnM1 probe33.

Statistics and reproducibility
No statistical methods were used to predetermined sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment. Descriptions of morphological features in live animals or expression domains obtained by WMISH or FWMISH were performed in at least five specimens (usually from 10 to 20) in each analysed developmental stage. WMISH and FWMISH were performed at least twice for each probed gene. Inhibitory treatments were performed at least twice for each condition, and numbers of phenotype counts out of the total number of analysed embryos are indicated in Fig. 1f, Extended Data Fig. 9 and Supplementary Data 2. The sex condition of embryos does not influence experimental design.

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

Data availability
Accession numbers and URLs of databases from publicly available sources are provided in the Methods, Supplementary Information and Supplementary Data 1.

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Author contributions
A.F.-R. carried out the cardiac developmental atlas, genome surveys, phylogenetic analyses, WMISH experiments, cell lineage mapping and BMP inhibitory treatments. M.F.-T. contributed to the Fgf/Mapk genome survey and FGF inhibitory treatments. E.D.-B. contributed to the Tbx genome survey and WMISH. M.J.-L. contributed to the Ets and Tbx phylogenies. M.P.-C. contributed to Islet characterization. A.F.-R. interpreted the data and made the figures. C.C. conceptualized the project. J.G.-F. and R.A. provided resources. R.A. and C.C. supervised the experiments. C.C. and A.F.-R. wrote the manuscript. All authors commented on the manuscript and agreed to its final version.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | 4D-reconstruction of a virtual cardiac cell tracing, based on nuclear position from the 30-cell stage to tailbud stages of *O. dioica* embryos (modified from Stach 2008)

B8.9 appears as the first CPC. Blastomere nomenclature follows that of Conklin for ascidians (vegetal blastomeres in capital letters, animal blastomeres in small letters, and blastomeres from the right underlined), and their fate are indicated in different colors: muscle + heart (purple), posterior tail muscle cells (yellow), anterior tail muscle cells (ATM, green), heart (red), germ-line (blue). Circles and hexagons represent blastomeres derived from right and left sides of the embryo, respectively. Dashes encircle sister cells resulting from a cell division.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Mesp ML phylogenetic tree and Math and Neurogenin expression. a, Unrooted phylogenetic tree, represented in a rectangular layout for the sake of clarity, showing the presence of bHLH homologs of Neurogenin and Math in appendicularians, but the absence of Mesp. The presence of Mesp in cephalochordates, vertebrates and all analyzed ascidians suggests an ancestral loss of Mesp at the base of the appendicularian lineage after its split from the lineage leading to ascidians. Bootstrap values are shown in the nodes. Scale bar indicates amino acid substitutions. Vertebrates (black): Gallus gallus (Gga), Homo sapiens (Hsa), Latimeria chalumnae (Lch), Lepisosteus oculatus (Loc); Ascidian tunicates (blue): Botrylloides leachii (Ble), Botrylloides schlosseri (Bsc), Ciona robusta (Cro), Ciona savignyi (Csa), Halocynthia aurantium (Hau), Halocynthia roretzi (Hro), Molgula occidentalis (Mocci), Molgula occulta (Mocc), Molgula ocularata (Mocu), Phallusia fumigata (Pfu), Phallusia mammillata (Pma); Appendicularian tunicates (red): Bathochordaeus sp. (Bsp), Fritillaria borealis (Fbo), Mesochordaeus erythrocephalus (Mer), Oikopleura albicans (Oal), Oikopleura dioica (Odi), Oikopleura longicauda (Olo), Oikopleura vanhoeffeni (Ova); Cephalochordates (green): Branchiostoma belcheri (Bbe), Branchiostoma floridae (Bfl), Branchiostoma lanceolatum (Bla). b–f, Developmental expression pattern of O. dioica Math homolog. Whole mount in situ hybridization in different stages of O. dioica development showing expression in the notochord in tailbud and early-hatchling embryos (red arrowheads) (c, d), in epidermis (blue arrowheads) (c–f), in the rectum domain in hatchling stages (yellow arrowheads) (d–f), in later stages of neural system development (pink arrowheads) (e, f), and in later stages of digestive system development (green arrowheads) (e, f). g–k, Developmental expression pattern of O. dioica Neurogenin homolog. Whole mount in situ hybridization in different stages of O. dioica development shows that Neurogenin expression was restricted to nervous system in tailbud and early-hatchling stages (pink arrowheads) (h, i) but no expression was detected in any region compatible with cardiac function. Images from tailbud in advance correspond to left lateral views orientated anterior towards the left and dorsal towards the top.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Ets ML phylogenetic tree and expression. a, Unrooted phylogenetic tree of the Ets and Erg protein families showed a high bootstrap value separating both protein families what corroborated the existence of two Ets1/2 genes in appendicularians. Scale bar indicates amino acid substitutions. Vertebrates (black): Gallus gallus (Gga), Homo sapiens (Hsa), Latimeria chalumnae (Lch), Lepisosteus oculatus (Loc); Ascidian tunicates (blue): Botryllodes leachi (Ble), Botryllodes schlosseri (Bsc), Ciona robusta (Cro), Ciona savignyi (Csa), Halocynthia aurantium (Hau), Halocynthia roretzi (Hro), Molgula occidentalis (Mocci), Molgula occulta (Moccu), Molgula oculata (Mocul), Phallusia fumigata (Pfu), Phallusia mammillata (Pma); Appendicularian tunicates (red): Bathochordaeus sp. (Bsp), Fritillaria borealis (Fbo), Mesochordaeas erythrocephalus (Mer), Oikopleura albicans (Oal), Oikopleura dioica (Odi), Oikopleura longicauda (Olo), Oikopleura vanhoeffeni (Ova); Cephalochordates (green): Branchiostoma belcheri (Bbe), Branchiostoma floridae (Bfl), Branchiostoma lanceolatum (Bla). b, Phylogenetic analysis of chordate Ets/2, using cephalochordate sequences as outgroup, suggested that the two Ets1/2 genes of appendicularians were co-orthologs to the ascidian Ets1/2a. c–h, Whole mount in situ hybridization of O. dioica Ets1/2a1 did not show any clear expression before hatching stages (c–f). In early-hatching stage Ets1/2a1 revealed expression in the migratory endodermal strand cells (pink arrowheads) (g). In late-hatching the expression signal was restricted to the buccal gland (green arrowheads) (h). i, j, Ets1/2a2 did not show expression until tailbud stage. k, l, In tailbud embryos, expression signal was detected in tail muscle cells (orange arrowheads), the notochord (red arrowheads) and the epidermis of the trunk (blue arrowheads). m, In early-hatching expression signal continued in the tail muscle and the notochord and increased in the anal domain (yellow arrowhead). n, In late-hatching stage, the Ets1/2a2 expression covered the entire oikoplasic epithelium, and continued in the muscle cells of the tail. Large images from tailbud in advance correspond to left lateral views oriented anterior towards the left and dorsal towards the top. Inset images are dorsal views of optical cross sections at the levels of dashed lines.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | FGF/MAPK ML phylogenetic tree and expression.

a, ML phylogenetic tree of the MEK subfamilies in chordates revealing the loss of the MEK4, MEK5 and MEK1/2 subfamilies in appendicularians, but the surviving of MEK3/6 and MEK7 subfamilies. Scale bar indicates amino acid substitutions. Bootstrap values are shown in the nodes. Vertebrates (black): Gallus gallus (Gga), Homo sapiens (Hsa), Latimeria chalumnae (Lch), Lepisosteus oculatus (Loc); Ascidian tunicates (blue): Botrylloides leachi (Ble), Botrylloides schlosseri (Bsc), Ciona robusta (Cro), Ciona savignyi (Csa), Halocynthia aurantium (Hau), Halocynthia roretzi (Hro), Molgula occidentalis (Mocci), Molgula occulta (Moccu), Molgula oculata (Mocul), Phallusia fumigata (Pfu), Phallusia mammillata (Pma); Appendicularian tunicates (red): Bathochordaeus sp. (Bsp), Fritillaria borealis (Fbo), Mesochordaeus erythrocephalus (Mer), Oikopleura albicans (Oal), Oikopleura dioica (Odi), Oikopleura longicauda (Olo), Oikopleura vanhoeffeni (Ova); Cephalochordates (green): Branchiostoma belcheri (Bbe), Branchiostoma floridae (Bfl), Branchiostoma lanceolatum (Bla).

b–g, Whole mount in situ hybridization of ERK homolog in different stages of O. dioica development did not detect expression in any studied stage (b–f) until late-hatchling when expression was detected in an specific central domain in the oikoplastic epithelium (blue arrowheads) (g).

h–m, Whole mount in situ hybridization of MEK7 homolog in O. dioica revealed expression in the developing neural tissue in tailbud stages (pink arrowheads) (i,j), and in the esophagus (green arrowhead) and the oikoplastic epithelium (blue arrowheads) in the late-hatchling stage (m).

n–s, Whole mount in situ hybridization of MEK3/6 homolog in different stages of O. dioica development did not show any obvious tissue specific expression domain in the trunk, but the signal was generalized, with the exception of muscle cells in the tail at late-hatchling stages. Images from tailbud in advanced correspond to left lateral views orientated anterior towards the left and dorsal towards the top.
Extended Data Fig. 5 | Gata and FoxF ML phylogenetic trees in chordates. a, Gata ML phylogenetic tree reveals the loss of the Gata4/5/6 in appendicularians, but the surviving and lineage specific duplications of Gata1/2/3 in appendicularians. b, FoxF ML phylogenetic tree reveals the presence of an ortholog of FoxF in appendicularians. The sister FoxQ subfamily was used as outgroup to root the tree. Scale bar indicates amino acid substitutions. Bootstrap values are shown in the nodes. Vertebrates (black): Gallus gallus (Gga), Homo sapiens (Hsa), Latimeria chalumnae (Lch). Lepisosteus oculatus (Loc); Ascidian tunicates (blue): Botrylloides leachi (Ble), Botryllus schlosseri (Bsc), Ciona robusta (Cro), Ciona savignyi (Csa). Halocynthia aurantium (Hau), Halocynthia roretzi (Hro), Molgula occidentalis (Mo), Molgula oculata (Moc), Phallusia mammillata (Pfu), Phallusia fumigata (Pfa), Phallusia chlorophana (Pch); Appendicularian tunicates (red): Bathochordaeus sp. (Bsp), Fritillaria borealis (Fbo), Mesochordaeus erythrocephalus (Mer), Oikopleura albicans (Oal), Oikopleura dioica (Odi), Oikopleura longicauda (Olo), Oikopleura vanhoeffeni (Ova); Cephalochordates (green): Branchiostoma belcheri (Bbe), Branchiostoma floridae (Bfl), Branchiostoma lanceolatum (Bla).
Extended Data Fig. 6 | NK ML phylogenetic tree in chordates reveals the presence of an ortholog of Nk4 in appendicularians and two orthologs of the Nk2 subfamily. Scale bar indicates amino acid substitutions. Bootstrap values are shown in the nodes. Vertebrates (black): Gallus gallus (Gga), Homo sapiens (Hsa), Latimeria chalumnae (Lch), Lepisosteus oculatus (Loc); Ascidian tunicates (blue): Botrylloides leachii (Ble), Botrylloides schlosseri (Bsc), Ciona robusta (Cro), Ciona savignyi (Csa), Halocynthia aurantium (Hau), Halocynthia roretzi (Hro), Molgula occidentalis (Mocci), Molgula occulta (Moccu), Molgula oculata (Mocul), Phallusia fumigata (Pfu), Phallusia mammillata (Pma); Appendicularian tunicates (red): Bathochordaeus sp. (Bsp), Fritillaria borealis (Fbo), Mesochordaeus erythrocephalus (Mer), Oikopleura albicans (Oal), Oikopleura dioica (Odi), Oikopleura longicauda (Olo), Oikopleura vanhoeffeni (Ova); Cephalochordates (green): Branchiostoma belcheri (Bbe), Branchiostoma floridae (Bfl), Branchiostoma lanceolatum (Bla).
Extended Data Fig. 7 | Hand ML phylogenetic tree suggests that member of this family in *O. dioica* is homologous to ascidian Hand1/2. Despite the tree suggests that the second paralog of ascidian (Hand-r) arose by a duplication at the base of the tunicate clade, and therefore subsequently lost in appendicularians. The low node support –bootstrap and approximate likelihood-ratio test (aLRT)– and the presence of shared long amino acid domain rich in K between the Hand1/2 and Hand-r in ascidians, but absent in appendicularians, do not allow us to discard the possibility that Hand-r was originated by a duplication within the ascidian lineage, and its basal branching in the tunicate clade is due to a long branch attraction phenomenon. Scale bar indicates amino acid substitutions. Bootstrap values are shown in the nodes.

**Vertebrates (black):** *Gallus gallus* (Gga), *Homo sapiens* (Hsa), *Latimeria chalumnae* (Lch), *Lepisosteus oculatus* (Loc); **Ascidian tunicates (blue):** *Botryllodes leachi* (Ble), *Botryllodes schlosseri* (Bsc), *Ciona savignyi* (Csa), *Halocynthia aurantium* (Hau), *Halocynthia roretzi* (Hro), *Molgula occidentalis* (Mocci), *Molgula oculata* (Mocul), *Phallusia fumigata* (Pfu), *Phallusia mammillata* (Pma); **Appendicularian tunicates (red):** *Bathochordaeus sp.* (Bsp), *Fritillaria borealis* (Fbo), *Mesochordaeus erythrocephalus* (Mer), *Oikopleura albicans* (Oal), *Oikopleura dioica* (Odi), *Oikopleura longicauda* (Olo), *Oikopleura vanhoeffeni* (Ova); **Cephalochordates (green):** *Branchiostoma belcheri* (Bbe), *Branchiostoma floridanae* (Bfl), *Branchiostoma lanceolatum* (Bla).
Extended Data Fig. 8 | Developmental coexpression patterns of ActnM1 and potential cardiac transcription factors. Double fluorescent in situ hybridization of ActnM1 with Nk4, Hand1/2, FoxF, Gata1/2/3b and Gata1/2/3d. Nk4 expression signal was detected in ventral epidermis and the CPC (B8.9) from the incipient-tailbud stage (a) until the early-tailbud (a'). In later stages, we only detected expression in the epidermis, but not in the cardiac precursors (a''–a'''). Hand1/2 was specifically expressed in the cardiac progenitors from late-tailbud to hatchling stages (b''–b'''). We did not detect expression of FoxF, Gata1/2/3b nor Gata1/2/3d in cardiac precursors, but they were expressed in different epidermal domains (c–e'''). The images correspond to the overlay of a stack of confocal sections with expression of the different genes. The small overlapping color in e''' is due to the overlay of the stack, and not to actual co-expression. White arrowheads indicate co-expression of ActnM1 with the corresponding gene in cardiac progenitors. Incipient- and early-tailbud stages correspond to ventral views oriented anterior towards the top. Late-tailbud and early-hatchling stages correspond to lateral views oriented anterior towards the left and dorsal towards the top.
### From 2-cell stage

| Compound  | DMSO 0.2% | SU5402 50 µM | AZD4547 50 µM | Gossypol 100 µM | 5Z7O 1 µM | LDN 10 µM | DORSO 10 µM |
|-----------|------------|---------------|----------------|-----------------|-----------|-----------|-------------|
| a         |            |               |                |                 |           |           |             |
| 30 µm     | 20/20      |               |                |                 |           |           |             |
| b         |            |               |                |                 |           |           |             |
| b'        |            |               |                |                 |           |           |             |
| c         |            |               |                |                 |           |           |             |
| c'        |            |               |                |                 |           |           |             |
| d         |            |               |                |                 |           |           |             |
| d'        |            |               |                |                 |           |           |             |
| e         |            |               |                |                 |           |           |             |
| e'        |            |               |                |                 |           |           |             |
| f         |            |               |                |                 |           |           |             |
| f'        |            |               |                |                 |           |           |             |
| g         |            |               |                |                 |           |           |             |
| g'        |            |               |                |                 |           |           |             |

**ActnM1**

### From 32-cell stage

| Compound  | DMSO 0.2% | SU5402 100 µM | AZD4547 100 µM | Gossypol 100 µM | 5Z7O 2.5 µM | LDN 10 µM | DORSO 10 µM |
|-----------|------------|---------------|----------------|-----------------|-------------|-----------|-------------|
| h         |            |               |                |                 |             |           |             |
| i         |            |               |                |                 |             |           |             |
| i'        |            |               |                |                 |             |           |             |
| j         |            |               |                |                 |             |           |             |
| j'        |            |               |                |                 |             |           |             |
| k         |            |               |                |                 |             |           |             |
| k'        |            |               |                |                 |             |           |             |
| l         |            |               |                |                 |             |           |             |
| l'        |            |               |                |                 |             |           |             |
| m         |            |               |                |                 |             |           |             |
| m'        |            |               |                |                 |             |           |             |
| n         |            |               |                |                 |             |           |             |
| n'        |            |               |                |                 |             |           |             |

**Nk4 + Brachyury**

### From 32-cell stage

| Compound  | DMSO 0.2% | SU5405 100 µM | AZD4547 100 µM | Gossypol 100 µM | 5Z7O 2.5 µM | LDN 10 µM |
|-----------|------------|---------------|----------------|-----------------|-------------|-----------|
| o         |            |               |                |                 |             |           |
| o'        |            |               |                |                 |             |           |
| p         |            |               |                |                 |             |           |
| p'        |            |               |                |                 |             |           |
| q         |            |               |                |                 |             |           |
| q'        |            |               |                |                 |             |           |
| r         |            |               |                |                 |             |           |
| r'        |            |               |                |                 |             |           |
| s         |            |               |                |                 |             |           |
| s'        |            |               |                |                 |             |           |
| t         |            |               |                |                 |             |           |
| t'        |            |               |                |                 |             |           |

**ActnM1**

*Extended Data Fig. 9* See next page for caption.
Extended Data Fig. 9 | FGF, MEK and BMP inhibition during heart development of O. dioica. a–g’, Whole mount in situ hybridization of ActnM1 in DMSO-control (a) and treated embryos with inhibitors of FGFR (SU5402 and AZD4547), MEK3/6 (Gossypetin), MEK7 (SZ7-Oxozeaenol) and BMP inhibitors (LDN and Dorsomorphin) from 2-cell stage up to early-tailbud stage (b–g’). Embryos treated with FGFR and MEK inhibitors affected gastrulation and caused abnormal phenotypes in which mesodermal derivatives showed either abnormal domains (b’–e’) or complete absence (b’’–e’’). However, those treated embryos that reached fairly normal incipient morphologies (b–g), showed the presence of CPCs (red arrowheads). h–n’, Whole mount in situ hybridization of NK4+Brachyury in DMSO-control (h) and treated embryos with FGFR, MEK and BMP inhibitors (i–n’) from 32-cell stage to early-tailbud stage. A majority of the treated embryos showed the Nk4 expression in the CPCs (i–n), even in some with obvious abnormalities in the notochord (l). Only in embryos with severe abnormal morphologies or arrested, we could not distinguish the CPCs from other Nk4 expression domains (l’–n’). o–t’, Whole mount in situ hybridization of ActnM1 in DMSO-control (o) and treated embryos with FGFR, MEK and BMP inhibitors from 32-cell stage to early-hatchling stage (p–t’). Most of the treated embryos showed abnormal tails (p’–t’), in which the elongation and rotation had been affected. Moreover, while the CPCs had converged near the midline into a single cardiac field, we observed that in many embryos with tail malformations, the CPCs had not converged and were still bilaterally separated at the right and left sides of the trunk (red numbers in brackets). These results suggest that FGF/MEK/MAPK and BMP signaling pathways may be involved in tail elongation/rotation and late cardiac organogenesis. Tailbud embryos images correspond to dorsal views with anterior to the left. Hatchling images represent dorsal views with anterior to the top.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Tbx ML phylogenetic tree and Islet, Ebf, MyoD and Dach expression. a, ML phylogenetic tree of the Tbx subfamilies in chordates reveals the loss of Tbx1/10 and Tbx21/Eomes/Tbr1 subfamilies in appendicularians and the ancestral loss of Tbx4/5 subfamily in tunicates. Scale bar indicates amino acid substitutions. Bootstrap values are shown in the nodes. Vertebrates (black): Gallus gallus (Gga), Homo sapiens (Hsa), Latimeria chalumnae (Lch), Lepisosteus oculatus (Loc); Ascidian tunicates (blue): Botrylloides leachi (Ble), Botrylloides schlosseri (Bsc), Ciona robusta (Cro), Ciona savignyi (Csa), Halocynthia aurantium (Hau), Halocynthia roretzi (Hro), Molgula occidentalis (Mooc), Molgula occulta (Moocu), Molgula oculata (Moocu), Phallusia fumigata (Pfu), Phallusia mammillata (Pma); Ascidian appendicularians (red): Bathochordaeus sp. (Bsp), Fritillaria borealis (Fbo), Mesochordaeas erythrocephalus (Mer), Okipleura albicans (Oal), Okipleura dioica (Odi), Okipleura longicauda (Olo), Okipleura vanhoeffeni (Ova); Cephalochordates (green): Branchiostoma floridae (Bfl), Branchiostoma lanceolatum (Bla). b–w, Whole mount in situ hybridization of O. dioica Islet, Ebf, MyoD and Dach homologs. 64-cell embryos did not showed expression of Islet (b) which was only detected in the developing nervous system from tailbud to hatching embryos (c–f). Ebf (COE) did not show expression in early stages (g, h) but we detected expression in the nervous system from tailbud to mid-hatchling stage (i–k) and in the oikoplastic epithelium of late-hatchling embryos (l). We did not detect expression of MyoD from 32-cell to hatching embryos (m−p). In late-hatchling embryos MyoD was expressed in the oikoplastic epithelium (q). Dach expression started at the 64-cell stage in the developing nervous system (pink arrowheads) and continued until late-tailbud stage (r–t). In tailbud stages, Dach started expressing in the trunk epidermis (blue arrowheads) which was maintained until late-hatchling stages when it was expressed in the whole oikoplastic epithelium (blue arrowheads) (u–v). In mid-hatchling stage, beside the epidermis, Dach expression was also detected in the endostyle (green arrowheads) (w). Large images from tailbud in advance correspond to left lateral views oriented anterior towards the left and dorsal towards the top. Inset images are dorsal views of optical cross sections at the levels of dashed lines. Pink arrowheads indicate the developing nervous system. Blue arrowheads indicate the oikoplastic epithelium.
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Our web collection on statistics for biologists contains articles on many of the points above.

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- Data collection: no software was used for data collection
- Data analysis: AliView v1.17.1, PhyML 3.0 (http://atgc.lirmm.fr/phyml/), FIJI (ImageJ v2.0.0-rc-69/1.52n), BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

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Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description
The study implies surveys of public genome databases, phylogenetic analyses, gene expression analyses by embryo whole-mount in situ hybridization, and inhibitory chemical treatments.

Research sample
The Oikopleura dioica embryos were obtained by in vitro fertilization from gametes obtained from pools of animals of our animal facility.

Sampling strategy
No statistical methods were used to predetermine sample size.

Data collection
All embryos and material samples were generated in our facilities, and therefore, no field collection was needed.

Timing and spatial scale
Timing and scaling do not apply since samples were obtained from our facility, in which the conditions are constant throughout the year.

Data exclusions
No data were excluded from the analyses.

Reproducibility
Gene expression analyses and treatments were performed at least two times, using embryos from pools produced from multiple females and males. Normal expression patterns were observed in a majority of normal developed embryos, and the number of abnormal patterns are indicated. All attempts at technical replication were successful.

Randomization
Embryos for experiments were obtained by in vitro fertilization from pooling hundreds of oocites and sperm from multiple parents to increase genetic diversity and avoid potential random biases. Treatments and control conditions were always performed in embryos from the same pool to avoid potential random biases among different pools.

Blinding
The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment.

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Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | We have used the marine zooplanktonic animal Oikopleura dioica, which belong to the subphylum tunicata |
|--------------------|--------------------------------------------------------------------------------------------------|
| Wild animals       | All animals are inbred lines cultured in our animal facility for generations during at least three years. No wild animals were used in this study. |
| Field-collected samples | The study did not involve samples collected from the field. |
| Ethics oversight   | This project does not involve any ethical issues related to informed consent, data protection issues, or Humans. The experimentation of aquatic invertebrate animals such as the planktonic Oikopleura dioica is not subjected to the regulation of animal experimentation, because this only applies to vertebrates organisms (Real Decreto 223 de 14 Marzo de 1998, en Cataluna Ley 5/1995, DOGC2073,5172). In any case, experimental procedures followed the EU animal care guidelines, and have been approved by the Ethical Animal Experimentation Committee (CEEA-2009) of the University of Barcelona. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.