Figures and figure supplements

Logics and properties of a genetic regulatory program that drives embryonic muscle development in an echinoderm

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Figure 1. Expression analysis of genes encoding sea urchin FGF-signaling components and FoxC by double FISH. FGFR1 and FGF were stained in green and FoxC in red at the very early gastrula stage (30–32 hr). Nuclei were labeled blue with DAPI. Yellow circles indicated by yellow arrowheads show cells co-expressing the analyzed genes. Panels A and B are stacks of merged confocal Z sections of all three channels, while separate channels over DAPI are presented in the other panels. Insets in panels A–A″ show representative single confocal sections to confirm that the two genes are indeed expressed in the same cell. Embryos in A–A″ are seen in a lateral view along the animal-top/vegetal-down axis. Embryos in B–B″ are displayed in a vegetal view. fv, frontal view; vv, vegetal view; o, oral, ab, aboral. The position of the putative unspecified myoblast precursors is indicated in Figure 1—figure supplement 1. Phylogenetic analyses of sea urchin fibroblast growth factor (FGF) and FGFR protein sequences are reported in Figure 1—figure supplements 2, 3, respectively. A co-expression analysis of FGFR1 and FoxC at late gastrula stage (48 hr) is reported in Figure 1—figure supplement 4.

DOI: 10.7554/eLife.07343.003
Figure 1—figure supplement 1. Three-color FISH of Gcm, Ese, and FoxA. Co-staining of Gcm as a marker for aboral pigment cell precursors, Ese for oral blastocoelar cell precursors, and FoxA for endoderm at 26 hr. Gcm transcript was stained in light green, Ese in red, and FoxA in cyan. Nuclei were labeled blue with DAPI. Yellow circles indicate the SMs, and white circles indicated by white arrows show single cells that do not express any of the analyzed genes. In 85% of the embryos analyzed, approximately two triple-negative cells were observed that might represent myoblast precursors. All embryos are in a vegetal view. Panels A, B, and C are merged confocal stacks, while panels A'–A‴ depict separate channels over DAPI. Panel D is a schematic representation of the vegetal surface of a sea urchin mesenchyme blastula orientated along the oral right/aboral left (O/Ab) axis, seen from the vegetal pole. The different NSM domains identified by distinct regulatory signatures at the vegetal plate are shown in different colors as indicated in the legend. Primary mesenchyme cells are not visible as they have already ingressed into the blastocoel at this stage.

DOI: 10.7554/eLife.07343.004
Figure 1—figure supplement 2. Phylogenetic analysis of the sea urchin FGFA protein sequence. (A) The protein domain structure of the SpFGFA contains a signal peptide and a FGF core domain. (B) Phylogenetic analysis of FGF proteins. The tree was constructed by the neighbor-joining method based on the multiple alignments of the FGF core domains from various organisms. Bootstrap values over 50% are shown at the branch points. Sea urchin FGFs (Sp-FgfA and Pl-FgfA) do not group with the seven vertebrate FGF families from A to G. The scale indicates the % amino acid difference with Poisson correction. GenBank accession numbers for FGFs are: acorn worm SkFgf20 like, NM_001171225; amphioxus AmphiFgf1/2, EU606032; AmphiFgf8/17/18, EU606035; AmphiFgf9/16/20, EU606036; ascidian CiFgf8/17/18, NM_001032476; CiFgf11/12/13/14, NM_001032561; Caenorhabditis elegans CeEGL17, NM_075706; Drosophila melanogaster DmPYR, AY55396; DmTHS, NM_136857; human HsFgf19, NM_005117; mouse MmFgf1, NM_010197; MmFgf2, NM_008006; MmFgf3, Y00848; MmFgf4, NM_010202; MmFgf5, NM_010203; MmFgf6, NM_010204; MmFgf7, NM_008008; MmFgf8, NM_010205; MmFgf9, U33535; MmFgf10, NM_008002; MmFgf11, NM_010198; MmFgf12, NM_183064; MmFgf13, NM_010200; MmFgf14, NM_010201; MmFgf15, NM_008003; MmFgf16, AB049219; MmFgf17, NM_008004; MmFgf18, NM_008005; MmFgf20, AB049218; MmFgf21, NM_020013; MmFgf22, NM_023304; MmFgf23, NM_022657; sea urchin Sp-FgfA, HQ107979; PI-FgfA, EF157978. DOI: 10.7554/eLife.07343.005
Figure 1—figure supplement 3. Phylogenetic analysis of the sea urchin FGFR protein sequences. (A) The protein domain structures of SpFGFR1 and SpFGFR2. The Fibronectin III domain (FN3), three Ig domains (Ig1-3), acid box (AB), transmembrane region (TM), and Tyrosine kinase domain are indicated. (B) For the phylogenetic analysis, the tyrosine kinase domains of FGFRs were aligned, and the tree was built in the same manner as the FGF tree. SpFGFR1 and SpFGFR2 are not orthologous to the human FGFR1 and FGFR2, respectively. GenBank accession numbers for FGFRs are: human HsFgfR1, AB208919; HsFgfR2, NM_000141; HsFgfR3, NM_000142; HsFgfR4, AY892920; ascidian CiFgfR, NM_001044355; C. elegans CeEGL-15, NM_077441; D. melanogaster DmBreathless, NM_168577; DmHeartless, NM_169784; sea urchin SpFgfR1, NM_214537; SpFgfR2, JF499690.
DOI: 10.7554/eLife.07343.006
Figure 1—figure supplement 4. Coexpression analysis of FGFR1 and FoxC by double FISH. Relative spatial expression domains of FGFR1 and FoxC at late gastrula stage (48 hr). Image is a stack of merged confocal Z sections in all channels. Inset shows representative single confocal section of the tip of the archenteron. Color code of channel association to each gene is shown in each panel. Nuclei are stained blue with DAPI. Embryo is seen in a frontal view.
DOI: 10.7554/eLife.07343.007
Figure 2. Perturbation of the FGF pathway. To analyze the phenotype of FGF perturbation, bright-field images were taken with differential interference contrast (DIC). Effects on muscle formation were also tested by detection of MHC expression by fluorescent in situ hybridization (FISH) or of myosin heavy chain (MHC) protein localization by immunostaining on pluteus larvae (72 hr). The ciliary band and gut internal cilia were stained with an anti-acetylated tubulin antibody (AcT). Panels (A–D) show the effect of SU5402 in the formation of the coelomic pouches (B) and MHC expression (D). Panels (E–H) show the effect of anti-FGFR1 translation morpholino oligonucleotide (MO) in the formation of the coelomic pouches, MHC protein localization, and gut morphology. Two representative phenotype embryos, both with impaired muscles while differing for gut sphincter formation, are reported in (F) (normal gut, 70% of cases) and (H) (reduced sphincters, 30% of cases). Panels (E, G, I, and J) show the effect in MHC protein localization caused by injection of FGFR1 dominant negative RNA (DnRNA) (J). Panels (A, C) show control embryos treated with DMSO. Panel (G) shows a larva injected with a fluo-control MO and panels (E, I) show control uninjected larvae (for MO injection controls see also ‘Materials and methods’ and Figure 2—figure supplement 2). The inset in panel H is a magnified view of the cilia at the apical organ. Pictures in C, D, and G–J are stacks of merged confocal Z sections. MHC was stained in red and acetylated tubulin in green. Nuclei were labeled blue with DAPI. Spicules are seen in DIC analysis as reflecting polarized light objects. All embryos are seen in frontal view except the ones in panels E, F, and I that are seen in lateral view with the oral side on the right (fv, frontal view; lv, lateral view). White arrows indicate the position of cardiac sphincters, whilst yellow and red arrows show, where present, the pyloric and anal sphincters, respectively. Black lines indicate pigment cells (pc). White lines indicate muscle fibers (mf). The asterisks indicate the absence of coelomic pouches (cp). A summary of SU5402 and U0126 treatments as well as MHC protein expression analysis after MEK pathway perturbation is reported in Figure 2—figure supplement 1. Control MO experiments are reported in Figure 2—figure supplement 2. Co-expression analysis of genes encoding putative MAPK effectors and FoxC as well as P-Elk protein detection is reported in Figure 2—figure supplement 3.

DOI: 10.7554/eLife.07343.008
Figure 2—figure supplement 1. Summary of SU5402 and U0126 treatments and MHC protein detection by immunostaining after MEK pathway perturbation. (A) Scheme summarizing the drug treatments performed and the morphological phenotypes observed. (B, C) MHC protein localization was tested by immunostaining in (B) control pluteus larva and (C) pluteus larva treated with the MEK inhibitor U0126 as indicated in (A) by the green line. The ciliary band and gut internal cilia were stained with an anti-acetylated tubulin antibody. MHC was stained in red and acetylated tubulin in green. Nuclei were labeled blue with DAPI. All embryos are seen in lateral view with the oral side on the right. DOI: 10.7554/eLife.07343.009
Figure 2—figure supplement 2. Control experiments for MOs. Panels A–D show control uninjected embryos at very early gastrula (A), late gastrula (B), and pluteus larva (C, D). Panels (E–G) show embryos injected with fluo-control MO at very early gastrula (E), late gastrula (F, H), and pluteus larva (G, I). Panels J and K show the effect in the translation of FGFR1-GFP fusion protein expression after FGFR1 MO injection (K–K′) compared to controls injected.
Figure 2—figure supplement 2. Continued

with FGFR1-GFP mRNA only (J–J'). A–C, E–G, J and K are bright-field images taken with DIC, E'–G', J' and K' are fluorescent images, whilst D, H, and I are stacks of merged confocal Z sections. In panels D, H, and I, MHC was immunostained in red, acetylated tubulin in green, and nuclei were labeled blue with DAPI. Embryos in panels D and I are the same shown in Figure 2I and Figure 4A, respectively. Embryos in panels A–E, G, and I–K are seen in lateral view while those in panels B, F, and H are in frontal view. fv, frontal view; lv, lateral view; cp, coelomic pouches; pc, pigment cells; sp, spicules; mo, mouth; st, stomach; in, intestine.

DOI: 10.7554/eLife.07343.010
Figure 2—figure supplement 3. Immunostaining of P-Elk and expression analysis of genes encoding putative MAPK effectors and FoxC by double FISH. (A, B) Localization of P-Elk protein by immunostaining. (C–F) Spatial expression domains of (C, D) Erg and (E, F) Ets1/2 with respect to FoxC by double FISH. Panels A, B are DIC images, and panels C–F are stacks of merged confocal Z sections. Insets in panels E, F show representative single confocal sections. FoxC is red, Erg is green, and Ets1/2 is cyan. Nuclei were stained blue with DAPI. Embryos in A and C panels are viewed from the vegetal pole while all the others are seen in frontal view.

DOI: 10.7554/eLife.07343.011
Figure 3. Spatial analysis of gene expression after FGF pathway perturbation by FISH. FoxY (A–D), FoxC (E–H), FoxF (I–L), MHC (I, J), and Ese (A, B) transcript localization tested by FISH in control embryos (A, C, E, G, I, K) and in embryos treated with SU5402 (B, F, J) or injected with FGFR1 MO (D, H, L) (for MO injection controls see also ‘Materials and methods’ and Figure 2—figure supplement 2). Panels A, B, I, and J show double FISH. FoxY was stained in green, FoxC and FoxF in red, MHC in cyan, and Ese in magenta. Nuclei were labeled blue with DAPI. Each picture is a stack of merged confocal Z sections. Yellow circles indicated by yellow arrowheads show cells co-expressing the analyzed genes. The orientation of the larvae is reported for each panel: fv, frontal view; av, animal view; lv, lateral view.
DOI: 10.7554/eLife.07343.012
Figure 4. MHC protein detected by immunostaining after perturbation of putative myogenic regulators. MHC protein localization was tested by immunostaining in fluo-control MO-injected pluteus larvae (72 hr) (A) and in embryos of the same age injected with MOs against FoxY (B), FoxC (C), FoxF (D), FoxL1 (E), MyoD2 (F), Six1/2N (G), and Tbx6 (H) (for MO injection controls see also Materials and methods and Figure 2—figure supplement 2). The ciliary band and gut internal cilia were stained by immunohistochemistry with an anti-acetylated tubulin antibody. Each picture is a stack of merged confocal Z sections with MHC in red and acetylated tubulin in green. Nuclei were labeled blue with DAPI. All embryos are seen in lateral view with the oral side on the right. White arrows indicate the position of cardiac sphincters. White lines indicate muscle fibers (mf). Below each panel, statistics of muscle fiber phenotype observed are reported as normal (6–7 mf), mild (4–5 mf), or strong (0–2 mf). A co-expression analysis of Six1/2 and FoxC is reported in Figure 4—figure supplement 1. Analysis of the temporal expression profile of two distinct Six1/2 isoforms and visualization of pigmentation after perturbing Six1/2N isoform are reported in Figure 4—figure supplement 2.

DOI: 10.7554/eLife.07343.013
Control  | FoxC MO2 400 μM  | FoxF MO2 300 μM  | FoxL1 MO2 200 μM
--- | --- | --- | ---
A  | B  | C  | D
A'  | B'  | C'  | D'

Figure 4—figure supplement 1. Control experiments for MOs. Circumesophageal muscles were tested by phalloidin staining in fluo-control MO-injected pluteus larvae (72 hr) (A, A') and in embryos of the same age injected with MOs against FoxC (B, B'), FoxF (C, C'), and FoxL1 (D, D') at different concentrations. Each picture is a stack of merged confocal Z sections. Phalloidin is seen in green and nuclei are labeled blue with Hoechst. All embryos are seen in lateral view with the oral side on the left. Below each panel, statistics of muscle fiber phenotype observed are reported.

DOI: 10.7554/eLife.07343.014
**Figure 4—figure supplement 2.** Co-expression analysis of *Six1/2* and *FoxC* by double FISH. Relative spatial expression domains of *Six1/2* and *FoxC* at the mid gastrula stage (42 hr). Image is a stack of merged confocal Z sections in all channels. Inset shows representative single confocal section of the tip of the archenteron. Color code of channel association to each gene is shown in each panel. Nuclei are stained blue with DAPI. Embryo is seen in a frontal view.

DOI: 10.7554/eLife.07343.015
Figure 4—figure supplement 3. The two Six1/2 isoforms. (A) Upstream sequence of the of the Six1/2 gene. Two ATGs are shown in red. The upstream one, highlighted in bold, corresponds to the first ATG in the long isoform (Six1/2N) that is probably generated by an alternative transcription start. The downstream ATG indeed corresponds to the first ATG of the short isoform in which transcription starts a few nucleotides upstream of it (Andrew Ransick, personal communication). Highlighted in different colors show the regions where the different set of qPCR primers were designed: the ones used to amplify the upstream sequence belonging to Six1/2N isoform only, are in yellow, while the ones used to amplify part of the homeobox domain (in bold), common to both isoforms, are highlighted in olive green. The target sequence used to design the MO against the long isoform Six1/2N is highlighted in cyan.

(B) Temporal expression profiles of Six1/2 distinct isoforms during sea urchin embryogenesis. Graph shows the number of transcripts per embryo during embryogenesis revealed by qPCR. Six1/2HD represents the sum of the number of transcripts of the two isoforms, while Six1/2N shows only the number of transcripts for Six1/2N. The columns.
Figure 4—figure supplement 3. Continued

represent average of various measurements, and the error bars are standard deviations. (C, D) Bright-field images were taken with DIC of (C) control uninjected larva and (D) Six1/2N morphant (72 hr) for visualizing the effect on pigmentation.

DOI: 10.7554/eLife.07343.016

Figure 4—figure supplement 4. Control experiments for MOs. Circumesophageal muscles were tested by phalloidin staining in flou-control MO-injected pluteus larvae (72 hr) (A, A’ in Figure 4—figure supplement 1) and in embryos of the same age injected with MOs against Six1/2 (A, A’) and Tbx6 (B, B’) at a concentration of 100 μM. Each picture is a stack of merged confocal Z sections. Phalloidin is seen in green and nuclei are labeled blue with Hoechst. All embryos are seen in lateral view with the oral side on the left. Below each panel, statistics of muscle fiber phenotype observed are reported.

DOI: 10.7554/eLife.07343.017
Figure 5. Spatial analysis of gene expression after MO perturbation of selected putative myogenic regulators by FISH. FoxC, FoxY, FoxF, MHC, and nanos transcripts were detected by FISH in fluo-control MO injected embryos (A, C, E, G, I) and in embryos injected with MOs against FoxY (B, D, F), FoxC (H), and FoxF (J). All images are obtained as stacks of merged confocal Z sections. Panels G, H show double FISH. In panel G, single channels over DAPI are shown as insets. FoxY was stained in green, FoxC and FoxF in red, MHC in cyan, and Nanos in magenta. Nuclei were labeled blue with DAPI. Yellow circles indicated by yellow arrowheads show cells co-expressing the analyzed genes. The orientation of the embryos is indicated in each panel: fv, frontal view; lv, lateral view.
DOI: 10.7554/eLife.07343.018
Figure 6. Effects of FoxY, FoxC, FoxF, FoxL1, MyoD2, Six1/2, and Tbx6 perturbations on transcript levels of selected mesodermal genes at 44 hr and 48 hr. Each diamond represents a single measurement of three independent biological experiments. Fold differences were calculated between experiments and control counts using the quantitative data obtained from the NanoString nCounter. Onefold change represents no change; ≥ 2 indicates increased expression level significantly (blue labels); ≤ 0.5 indicates decreased expression level significantly (red labels). Asterisks indicate perturbation effects as measured in independent biological experiments by qPCR. NanoString and qPCR perturbation data normalized against controls are provided in Figure 6—source data 1, and raw NanoString data are provided in Figure 6—source data 2.
DOI: 10.7554/eLife.07343.019
Figure 7. Schematic representation and view from all nuclei of the NSM regulatory interactions in early, mid, and late sea urchin gastrulae. On the left side, three developmental stages of the sea urchin embryo are schematized: (A) early, (B) mid, and (C) late gastrula stage. On the right side, the genetic interactions found within this study are summarized. Different colors are used for each domain showing exclusive regulatory states: oral animal non-skeletogenic mesodermal (NSM) (OR AN NSM), salmon pink; NSM, blue; aboral NSM (AB NSM), lavender; small micromere derivatives (SM), green; myogenic domain (M), light red; endoderm (ENDO), yellow-green; oral ectoderm (OR ECTO), light gray. Genes are presented as horizontal thick lines and their names are reported below the thick lines. The wiring among the genes is shown with solid lines, although none of them has been demonstrated to be direct. Arrows represent positive regulation, bars represent repression, and white bullets, together with the dashed lines, indicate signaling events. Genes that are expressed in more than one domain, for which the putative inputs were revealed by NanoString but not validated by spatial expression analysis, are shown on a shaded background. The asterisk in A relates to the fact that Figure 7. continued on next page.
that we did not demonstrate which FGF factor signals to FGFR1. A co-expression analysis of several genes included in the gene regulatory network (GRN) diagrams is reported in Figure 7—figure supplement 1. Numbers associated to inputs indicate the evidence for all interactions reported and are listed in Figure 7—source data 1.

DOI: 10.7554/eLife.07343.022

Figure 7—figure supplement 1. Co-expression analysis of genes encoding mesodermal factors by double FISH. Relative spatial expression domains of (A) SoxE and FoxC at the mid gastrula stage (40 hr), (B) Not and FoxC at the mid gastrula stage (44 hr), and (C) Pitx2 and FoxC at the late gastrula stage (48 hr). Each picture is a stack of merged confocal Z sections in all channels. Inset in panel A shows a representative single confocal section of the tip of the archenteron. Color code of channel association to each gene is shown in each panel. Nuclei are stained blue with DAPI. All embryos are in a frontal view.

DOI: 10.7554/eLife.07343.024