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FGF signalling controls formation of the apical sensory organ in the cnidarian *Nematostella vectensis*

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Fibroblast growth factor (FGF) signalling regulates essential developmental processes in vertebrates and invertebrates, but its role during early metazoan evolution remains obscure. Here, we analyse the function of FGF signalling in a non-bilaterian animal, the sea anemone *Nematostella vectensis*. We identified the complete set of FGF ligands and FGF receptors, of which two paralogous FGFs (*NvFGFa1* and *NvFGFa2*) and one FGF receptor (*NvFGFRa*) are specifically coexpressed in the developing apical organ, a sensory structure located at the aboral pole of ciliated larvae from various phyla. Morpholino-mediated knockdown experiments reveal that NvFGFa1 and NvFGFRa are required for the formation of the apical organ, whereas NvFGFa2 counteracts NvFGFRa signalling to prevent precocious and ectopic apical organ development. Marker gene expression analysis shows that FGF signalling regulates local patterning in the aboral region. Furthermore, NvFGFa1 activates its own expression and that of the antagonistic *NvFGFa2*, thereby establishing positive- and negative-feedback loops. Finally, we show that loss of the apical organ upon *NvFGFa1* knockdown blocks metamorphosis into polyps. We propose that the control of the development of sensory structures at the apical pole of ciliated larvae is an ancestral function of FGF signalling.

KEY WORDS: Cnidaria, *Nematostella*, Apical organ, FGF signalling

INTRODUCTION

All key features of animal body plans are established during embryonic development, and thus an understanding of the early steps in body plan evolution requires an understanding of the embryonic development of phylogenetically basal metazoans. One such metazoan phylum is the Cnidaria, which represents the sister group to the Bilateria. It is estimated that Bilateria and Cnidaria split about 600 million years ago (Fig. 1A). The cnidarian model organism *Nematostella vectensis*, a sea anemone, is a member of the Anthozoa and is considered to be a representative of the basal group within the Cnidaria (Bridge et al., 1995; Bridge et al., 1992; Collins, 2002; Medina et al., 2001) that has retained much of the ancestral genetic complexity of the cnidarian-bilaterian ancestor (Chourrout et al., 2006; Putnam et al., 2007; Ryan et al., 2006; Technau et al., 2005). In contrast to the triploblastic Bilateria, Cnidaria are composed only of ectodermal and endodermal epithelia separated by an acellular extracellular matrix, the mesogloea. Juvenile and adult *Nematostella* polyps consist of a tube-shaped body column with longitudinal endodermal lamellae, the mesenteries, reaching into the gastric cavity. The pharynx is an ectodermal invagination, the only opening at the oral end is surrounded by a ring of tentacles (Fig. 1E). *Nematostella* gastrulation (Kraus and Technau, 2006; Magie et al., 2007) is followed by a free-swimming, ciliated planula larva and gradual metamorphosis into a primary polyp (Fig. 1B-E) (Hand and Uhlinger, 1992). During the planula stage, a sensory ciliary tuft develops at the aboral pole (Chia and Koss, 1979). Larvae swim with this apical ciliary organ facing forward and also settle on this pole. Ciliary tuft-bearing apical organs are also located at the aboral pole of marine ciliated larvae of both protostomes (e.g. molluscs and annelids) and deuterostomes (e.g. echinoderms and hemichordates, see Fig. 1A), but their function is not well characterised. Because they are only present in the free-swimming larvae and disappear after metamorphosis, they are thought to be required for the detection of suitable conditions for metamorphosis and/or for directed swimming. However, apical organs are absent in major model organisms, such as *Caenorhabditis elegans*, *Drosophila* and vertebrates, and therefore the molecular basis of its development, despite its evolutionary significance, is completely unclear.

In order to identify signalling molecules that are involved in the embryonic patterning of *Nematostella*, we identified and isolated the complete set of 15 homologous transcripts of Fibroblast growth factors (FGFs) and the only two Fibroblast growth factor receptors (FGFRs) present in the genome. This extends the recent predictive identification and selective cloning of 13 FGF ligands from the *Nematostella* genome (Matus et al., 2007). FGF signalling is involved in a wide variety of developmental processes in vertebrates and invertebrates (Borland et al., 2001; Bottcher and Niehrs, 2005; Huang and Stern, 2005; Thisse and Thisse, 2005). It regulates the migratory behaviour of cells during gastrulation in vertebrates, sea urchin and *Drosophila* (Keller, 2005; Leptin, 2005; Rottinger et al., 2008; Wilson and Leptin, 2000), mesoderm formation in vertebrates (Kimelman, 2006), and neural induction in vertebrates and urochordates (Bertrand et al., 2003; Stern, 2005; Wilson and Edlund, 2001). At later developmental stages, it is involved in anteroposterior patterning of the neuroectoderm and the mesoderm in vertebrates (Altmann and Brivanlou, 2001), branching morphogenesis in the *Drosophila* and mouse respiratory systems (Ghabrial et al., 2003; Metzger and Krasnow, 1999; Warburton et al., 2000), limb development in vertebrates (Capdevila and Izpisua Belmonte, 2001; Niswander, 2002; Tickle, 1999), and notochord and heart formation in urochordates (Davidson et al., 2006; Imai et al., 2002; Yasuo and Hudson, 2007). In planarians, a role for FGF signalling in brain development has been proposed (Cebria et al., 2002; Ogawa et al., 2002), and, in the hydrozoan *Hydra*, a FGF receptor is expressed both in the tip and the foot region of

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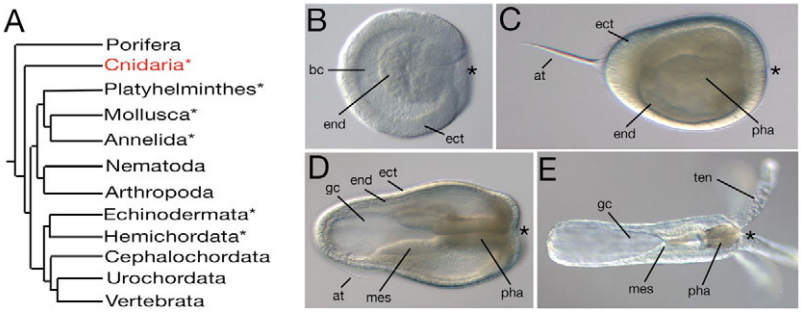

Fig. 1. Phylogenetic position and embryonic development of the cnidarian *Nematostella*. (**A**) Simplified phylogenetic tree according to published data (Bourlat et al., 2006; Delsuc et al., 2006), showing that Cnidaria is a sister phylum to Bilateria. The presence of apical organs is indicated by asterisks. (**B-E**) Overview of *Nematostella* embryonic development: (B) gastrula, (C) planula, (D) metamorphosis and (E) primary polyp. The blastopore/oral pole is marked by asterisks. *at*, apical tuft; *bc*, blastocoel; *ect*, ectoderm; *end*, endoderm; *gc*, gastric cavity; *mes*, mesentery; *pha*, pharynx; *ten*, tentacle. Note the short cilia covering the ectoderm in C.

developing buds (Sudhop et al., 2004). However, to date, no functional data about the role of individual FGFs in lower metazoans have been reported.

We show here that two paralogous FGF genes (*NvFGFa1* and *NvFGFa2*) and one FGF receptor gene (*NvFGFRa*) are expressed at the aboral pole and, later, in the apical organ of the *Nematostella* embryo. We employ morpholino-mediated knockdown to show that signalling of NvFGFa1 via NvFGFRa is required for specification of the ciliary tuft of the apical organ. By surprising contrast, NvFGFa2 is required to prevent the precocious and ectopic formation of ciliary tuft cells by antagonising NvFGFRa signalling. Furthermore, NvFGFa1 maintains its own expression and that of *NvFGFRa* and *NvFGFa2*, indicating that both positive- and negative-feedback loops are involved in development and maintenance of the apical organ. Finally, we show that, whereas absence of the ciliary tuft upon *NvFGFa1* knockdown does not affect the swimming behaviour of the planula larvae, it does completely block metamorphosis.

MATERIALS AND METHODS

Animal culture

Nematostella culture and the induction of spawning have been described previously (Fritzenwanker and Technau, 2002; Hand and Uhlinger, 1992). Embryos were raised in one-third filtered seawater (Nematostella medium) at 23°C.

Identification of *Nematostella* FGFs and FGF receptors, phylogenetic analysis and RT-PCR

All described genes were identified by searching a *Nematostella* EST database (http://genome.jgi-psf.org/Nemve1/Nemve1.home.html) with corresponding vertebrate sequences and tBLASTN. Incomplete 5′ ends of *NvFGFRb* and *NvFGFRa* were obtained by 5′ RACE using GeneRacer (Invitrogen). GenBank Accession numbers are: *NvFGFa2*, DQ882654; *NvFGFa1*, DQ882655; *NvFGFRb*, EF173462; *NvFGFRa*, EF173463.

Oligo dT-primed cDNAs for developmental RT-PCR were generated by standard procedures. Primer sequences and PCR conditions are available from the authors upon request.

Morpholino tests with coupled transcription-translation assays

For the rabbit reticulocyte lysate-based assay, the open reading frame and morpholino target site of *NvFGFa1*, *NvFGFa2* and *NvFGFRa* were cloned into the pCS2+ vector. In each case, 200 ng of plasmid, 0.5 nmol morpholino and 4 μCi ^{35}S-labeled methionine (Amersham, UK) were added to 10 μl of SP6 TnT-Quick coupled transcription/translation reaction mix (Promega, USA) and incubated for 90 minutes at 30°C. Reactions were separated by SDS-PAGE and synthesised proteins were visualised by autoradiography.

In situ hybridisation and immunocytochemistry

Embryos were fixed for 1 hour in cold 4% paraformaldehyde in PBS or 3.7% formaldehyde in *Nematostella* medium and stored in methanol until use. Hybridisations were carried out as described (Rentzsch et al., 2006). Probes were synthesised from full-length cDNA clones with Megascript Kits (Ambion, USA) and digoxigenin- or FITC-labeled UTP (Roche, Switzerland).

For staining with anti-acetylated tubulin antibody (Sigma T6793), embryos were fixed for 1 hour in cold 4% paraformaldehyde/PBS, followed by 20 minutes in 10% DMSO in PBS and 15 minutes in 2% H_2O_2 in PBS. The antibody was diluted 1:400 in 10% lamb serum, 1% DMSO, 0.1% Triton X-100 in PBS.

Microscopy

Scanning electron microscopy was carried out at the Molecular Imaging Centre (MIC) of the University of Bergen (FUGE, Norwegian Research Council) using a Jeol JSM7400-F microscope. Samples were treated as described previously (Kraus and Technau, 2006); the blastopore was used for orientation of the larvae.

Injection of morpholinos and inhibitor treatments

For microinjections, fertilised eggs were dejellied with 2.5% cysteine in *Nematostella* medium (Fritzenwanker and Technau, 2002). Injections were done with a Femtojet microinjector (Eppendorf, Germany) on a Nikon TE2000-S inverted microscope.

Morpholinos (MOs) were purchased from Gene Tools, USA. Sequences are: *NvFGFa2*, CGTTAGCATGGTGATCGTCATGTTG; *NvFGFa1*, ATAAGGTGGACGCATGACTTTGTAG; *NvFGFRa*, TCCACCAAG-CTCGAAGAGCCGTCAT; and control MO, CATGGAGAAATCG-GACTTCATATTT. Nucleotides complementary to the start ATG are underlined. The sequence of the control MO does not yield any hits in the available *Nematostella* genome assembly or EST database (http://genome.jgi-psf.org/Nemve1/Nemve1.home.html). MOs were diluted in water and injected at 0.25 nmol/μl (*NvFGFa2*) or 0.5 nmol/μl (*NvFGFa1* and *NvFGFRa*) with 0.5 μg/μl rhodamine-dextran (M_r 10,000, Molecular Probes, USA) as a tracer.

SU5402 (Calbiochem, USA) was applied at a final concentration of 20 μM, UO126 (Promega, USA) at 10 μM, each in 0.1% DMSO. Control animals were incubated in 0.1% DMSO only. Solutions were changed after 8 hours.

Double phosphorylated ERK was detected with monoclonal anti-phospho p42/44 antibody E10 (Cell Signaling Technologies, USA).

RESULTS

Identification of FGFs and FGFRs in *Nematostella*

Based on genomic and EST resources, we identified 15 FGF-domain containing transcripts (see Fig. S1 in the supplementary material) (J.H.F. and U.T., unpublished) (Matus et al., 2007), two of which will be described here in detail. They encode putative proteins of 202 amino acids (aa; NvFGFa1) (Matus et al., 2007) and 197 aa (NvFGFa2, this study). The N terminus of each protein contains a predicted signal peptide [SignalP3.0 (Bendtsen et al., 2004)], indicating that they can act as secreted factors. Phylogenetic analyses based on the FGF domain shows that NvFGFa1 and NvFGFa2 belong to a eight-membered paralogous group that cannot be assigned with certainty to a particular subfamily (see Fig. S1 in the supplementary material). Within this paralogous group, *NvFGFa1* and *NvFGFa2* are distantly related. *NvFGFa1* has been identified in parallel and named *NvFGF1A* (Matus et al., 2007), whereas *NvFGFa2* has not yet been described. To avoid the

impression that *NvFGFa1* and *NvFGFa2* belong to the FGF1 subfamily, we prefer to use the names *NvFGFa1* and *NvFGFa2* in the following sections.

We also isolated the complete open reading frame of two paralogous FGF receptors, which we term *NvFGFRa* and *NvFGFRb* in accordance with Matus et al. (Matus et al., 2007) (see Fig. S2 in the supplementary material). No other FGF receptors could be identified in the genome. Both contain three extracellular immunoglobulin domains, a single transmembrane domain and an intracellular split tyrosine kinase domain (for domain organisation, see Fig. S3 in the supplementary material). From the hydrozoan *Hydra*, a single FGFR, termed Kringelchen, has been reported so far (Sudhop et al., 2005), which clusters with the *Nematostella* FGFRs (see Fig. S2 in the supplementary material). As the duplication leading to FGFRa and FGFRb appears to be lineage specific, we conclude that the common ancestor of Cnidaria and Bilateria had one FGFR, provided that no other FGFR has been lost in the cnidarian lineage.

FGF ligands and receptors are expressed at the apical pole

To determine whether FGF signalling might be involved in embryonic development of *Nematostella*, we analysed the temporal and spatial expression pattern of *NvFGFa1*, *NvFGFa2*, *NvFGFRa* and *NvFGFRb* by RT-PCR and in situ hybridisation, respectively. RT-PCR on cDNA from different developmental stages shows that both *NvFGF* receptors and *NvFGFa2* are expressed maternally and zygotically, whereas *NvFGFa1* is expressed only zygotically (Fig. 2A). By in situ hybridisation, the earliest localised expression detected for *NvFGFa1* and *NvFGFa2* was in early gastrula stages, as a broad domain encompassing almost the complete aboral half of the embryo (Fig. 2B,F). During gastrulation, this broad domain becomes gradually restricted to the small patch at the aboral pole that marks the site where the apical organ will develop in the early planula larva (Fig. 2C,G). Expression of both genes remains confined to this site throughout planula stages and the first 2 to 3 days after metamorphosis (Fig. 2D,E,H,I), and becomes undetectable afterwards. Double in situ hybridisation did not reveal a difference in the width of the expression domains of *NvFGFa1* and *NvFGFa2* during midgastrulation, when the expression becomes restricted to the aboral pole (Fig. 2N-P).

The expression of *NvFGFRa* is very similar to that of *NvFGFa1* and *NvFGFa2*: it commences broadly in the aboral half and then becomes restricted to the aboral pole (Fig. 2J-M). However, fluorescent double in situ hybridisation experiments show that, in contrast to *NvFGFa1*, the expression domain of *NvFGFRa* is slightly wider than that of *NvFGFa2* during the narrowing of the aboral expression domain (Fig. 2Q-S). In addition, *NvFGFRa* is expressed in the whole endoderm during planula stages, with

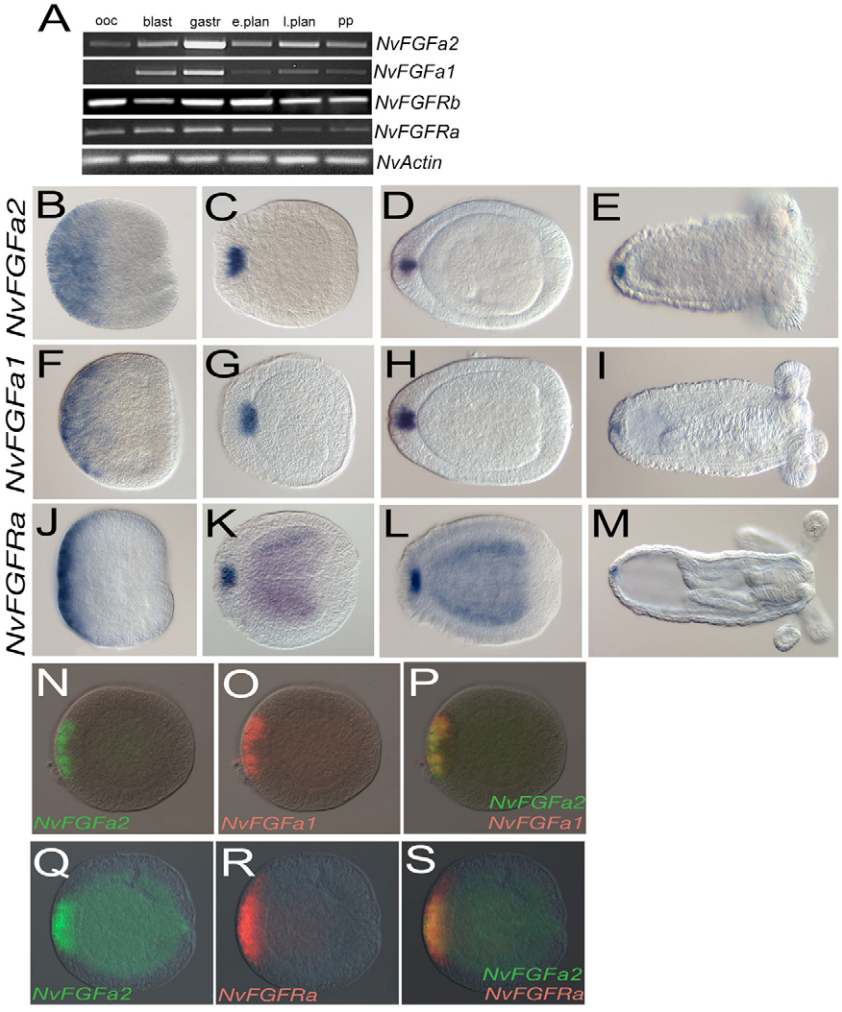

Fig. 2. Expression pattern of *Nematostella FGFa*, *FGFb* and *FGFRa*. (**A**) Temporal expression profile determined by RT-PCR; *NvFGFa1* is not expressed maternally. ooc, unfertilised eggs; blast, blastula (12 hpf); gastr, gastrulation (24 hpf); e.plan, early planula (48 hpf); l.plan, late planula (96 hpf); pp, primary polyp (7 dpf). (**B-S**) Spatial expression pattern determined by in situ hybridisation. Lateral views, blastoporus/mouth to the right, apical pole and swimming direction of planula larvae to the left. (B-E) *NvFGFa2*, (F-I) *NvFGFa1*, (J-M) *NvFGFRa*. (B,F,J) Gastrula, (C,G,K) early planula, (D,H,L) late planula, (E,I,M) primary polyp. (N-S) Fluorescent double in situ hybridisation at late gastrula stage with indicated probes. The *NvFGFa1* and *NvFGFa2* expression domains are identical; the *NvFGFRa* expression domain is wider than that of *NvFGFa2* (and *NvFGFa1*).

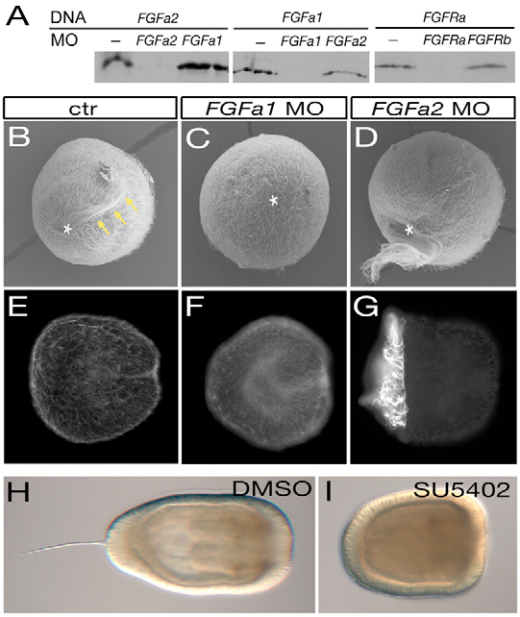

Fig. 3. Opposite effects of *NvFGFa1* and *NvFGFa2* morpholinos on apical organ formation. (**A**) Autoradiograph of transcription-translation reactions in the presence of ^{35}S-labeled methionine. Plasmids and MOs added to the reaction are indicated above the lanes. Tested MOs only inhibit translation of the corresponding transcripts. (**B-D**) Scanning electron microscopy of 4-day-old planulae injected with the MOs indicated. The aboral pole is marked by asterisks; arrows in B point to the apical tuft. *NvFGFa1* MO leads to loss, *NvFGFa2* MO to expansion of the apical organ. (**E-G**) Visualisation of the ciliary tuft by anti-acetylated tubulin antibody staining of 48-hpf planulae injected with MOs indicated. Lateral views, aboral pole to the left. The *NvFGFa2* MO causes premature formation of an expanded apical tuft. (**H**,**I**) Animals with a differentiated apical organ treated from 72 hpf to 120 hpf with 0.1% DMSO or 20 μM SU5402/0.1% DMSO. SU5402 causes loss of the apical organ.

pronounced signals detectable in the mesenteries (Fig. 2K,L). The second FGF receptor, *NvFGFRb*, is expressed uniformly throughout the endoderm during gastrulation and planula stages (data not shown) (see Matus et al., 2007).

Taken together, *NvFGFa1*, *NvFGFa2* and *NvFGFRa* share a common expression pattern at the aboral pole, compatible with a function in the development of the apical organ. We therefore focused subsequent functional analyses on these genes.

Opposing activities of NvFGFa1 and NvFGFa2 control the development of the ciliary tuft

We used morpholino antisense oligonucleotides (MO) (Summerton, 1999) to analyse the function of NvFGFa1, NvFGFa2 and NvFGFRa during *Nematostella* development. To test whether the morpholinos can suppress translation of the targeted transcripts, we employed a reticulocyte lysate-based transcription-translation system (see Material and methods). Synthesis of NvFGFa1 protein from a NvFGFa1 encoding plasmid was readily suppressed by addition of the *NvFGFa1* MO to the reaction, but not by the *NvFGFa2* MO. Similarly, the *NvFGFa2* MO, but not the *NvFGFa1* MO, suppressed synthesis of NvFGFa2, and translation of *NvFGFRa* was suppressed by the *NvFGFRa* MO, but not by the *NvFGFRb* MO (Fig. 3A).

Embryos injected with a control morpholino or with dextran developed normally into planula larvae, although with a slight developmental delay compared with uninjected embryos. Scanning electron microscopy of mid-planula (96 hpf) embryos revealed that *NvFGFa1* MO- or *NvFGFRa* MO-injected embryos lack the ciliary tuft (Fig. 3B,C; see also Fig. S4 in the supplementary material). By striking contrast, the injection of morpholinos against the co-expressed paralog *NvFGFa2* resulted in a pronounced expansion of the ciliary tuft (Fig. 3D). Furthermore, staining of cilia with an antibody against acetylated tubulin showed that, in embryos injected with the *NvFGFa2* MO, the long apical cilia develop significantly earlier than in control embryos. At early planula stage (48 hpf), before the apical tuft is visible in control embryos, a vastly oversized tuft of apical cilia is detectable in *NvFGFa2* morphants (Fig. 3E-G).

The observation that both FGFs and the FGF receptor are expressed in the apical organ cells throughout planula stages and in the young primary polyp suggested that FGF signalling might still be required after the initial formation of the apical organ. To test this possibility, we used a chemical FGF receptor inhibitor, SU5402, which binds to a region of the tyrosine kinase domain of FGF receptors that is highly conserved in both *Nematostella* FGF receptors (Mohammadi et al., 1997). Incubation of *Nematostella* embryos with SU5402 leads to a clear reduction in phosphorylation of the MAP kinase ERK, which is phosphorylated and thereby activated by FGF signalling in various higher metazoans (Fig. 6A). We selected planula larvae after formation of a visible apical tuft (72 hpf), and incubated them in 20 μM SU5402. Within 48 hours, 67% of the SU5402-treated planulae (n=33) completely lost the apical cilia (Fig. 3I) and 24% had a clearly thinner apical tuft; control incubation in 0.1% DMSO had no effect (Fig. 3H; n=38).

We conclude that opposing activities of NvFGFa1 and NvFGFa2 regulate proper development of the apical organ in *Nematostella*, and that FGFR signalling is required to maintain the apical cilia throughout planula stages.

FGF signalling controls patterning within the aboral region

To obtain a better understanding of the patterning defects that underlie the observed phenotypes, we used a panel of marker genes that demarcate distinct regions along the oral-aboral axis. Expression analysis was carried out at 48 hpf and, thus, about 24 hours before differentiation of the apical organ becomes apparent by the emergence of the apical cilia.

NvCOE is a homolog of the Collier/Olf/EBF family of transcription factors that are implicated in neuronal development in various organisms (Dubois and Vincent, 2001). *NvCOE* is expressed in the apical organ of the *Nematostella* early planula larvae (Fig. 4A) (Pang et al., 2004). This expression is lost in embryos injected with the *NvFGFa1* or *NvFGFRa* MO (Fig. 4E,I), whereas it is strongly expanded upon *NvFGFa2* MO injection (Fig. 4M). *NvFoxD1* is a winged helix transcription factor that is expressed in a broad aboral domain of the *Nematostella* planula larvae and thus includes, but goes beyond the expression domain of *NvCOE* at this stage (Fig. 4B) (Magie et al., 2005). The expression of *NvFoxD1* is unaffected in *NvFGFa1* and *NvFGFRa* MO-injected embryos (Fig. 4F,J), whereas its expression in *NvFGFa2* morphants includes the expanded apical organ, but does not exceed the expression domain of *NvCOE* (Fig. 4N). We also analysed the expression of *NvWnt2*, which is expressed in a belt-like domain in the central part of the planula (Fig. 4C) (Kusserow et al., 2005), and that of *NvFkh*, which at this stage is expressed around the blastopore, marking the oral end of the planula

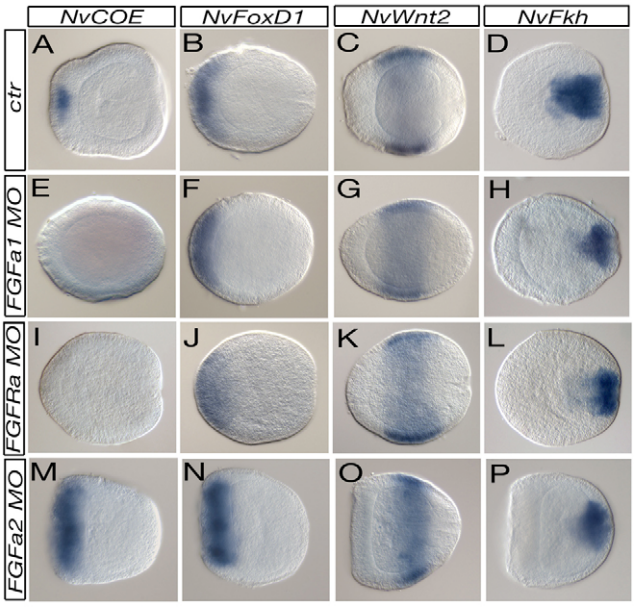

Fig. 4. NvFGF signalling regulates patterning within the aboral region. (**A-P**) In situ hybridisation of 48 hpf planulae. Lateral views, aboral pole to the left; probes are indicated above the panels, injected morpholinos to the left. Knockdown of *NvFGFa1* and *NvFGFRa* causes specific loss of the apical organ marker, knockdown of *NvFGFa2* results in expansion of the apical tuft. Displayed expression patterns were obtained in: (A) 38/42, (B) 31/38, (C) 21/23, (D) 36/36, (E) 48/53, (F) 58/65, (G) 14/18, (H) 29/31, (I) 39/42, (J) 24/33, (K) 22/24, (L) 46/52, (M) 29/34, (N) 27/29, (O) 26/36 and (P) 40/42 embryos.

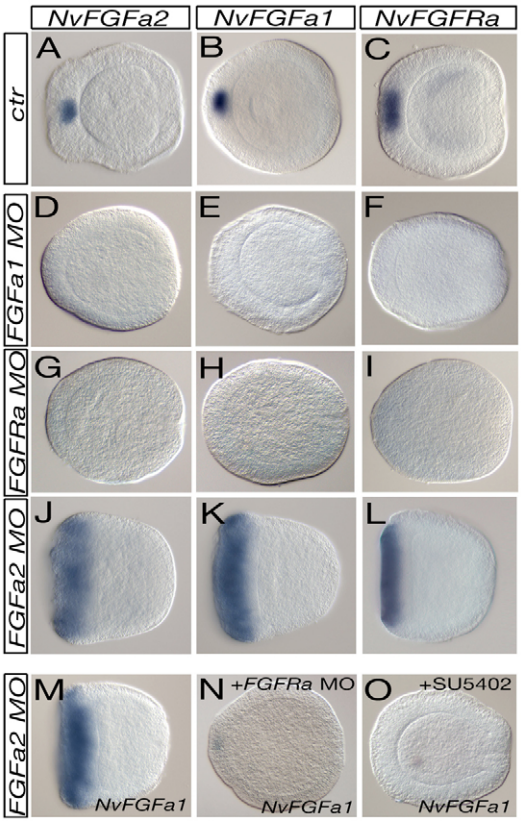

Fig. 5. NvFGFRa signalling is required for the expression of FGF pathway components and expansion of the apical organ in *NvFGFa2* morphants. (**A-O**) In situ hybridisation of 48-hpf planulae. (A-L) Lateral views, aboral pole to the left; injected morpholinos are indicated to the left of the panels, probes above. NvFGFa1 and NvFGFRa are required for their own transcription. (M-O) In situ hybridisation of 48-hpf planulae probed with *NvFGFa1*. (M) *NvFGFa2* MO-injected planula; (N) planula co-injected with *NvFGFa2* and *NvFGFRa* MOs; (O) planula injected with the *NvFGFa2* MO and treated with 20 μM SU5402 from 20 hpf on.

(Fig. 4D) (Fritzenwanker et al., 2004; Martindale et al., 2004). Both markers were unaffected by injection of the *NvFGFa1*, *NvFGFa2* or *NvFGFRa* MOs (Fig. 4G,H,K,L,O,P).

Taken together, knockdown of *NvFGFa1* or *NvFGFRa* leads to a specific loss of the apical organ marker *NvCOE*, without affecting other markers along the oral-aboral axis, whereas knockdown of *NvFGFa2* results in expansion of the apical organ territory into the *NvFoxD1*-positive aboral region. This suggests that NvFoxD1 specifies a broad aboral identity rather than the apical organ itself, but possibly defines the domain where an apical organ can be formed. As *NvFoxD1* expression is not affected in knockdown experiments, it appears to act upstream or in parallel to the FGF signalling pathway.

Expression of FGF pathway components is maintained by FGFRa signalling

The above results indicate that proper development of the apical organ requires a balance between the opposing activities of NvFGFa1 and NvFGFa2. One possible way to achieve such a balance would be the use of feedback mechanisms. We therefore analysed whether the expression of *NvFGFa1*, *NvFGFa2* and *NvFGFRa* is regulated by FGF signalling. Indeed, we found that knockdown of *NvFGFa1* or *NvFGFRa* leads to a loss of transcription of *NvFGFa1* and *NvFGFa2*, and to a nearly complete loss of *NvFGFRa* expression (Fig. 5A-I). By contrast, knockdown of *NvFGFa2* resulted in an expansion of the expression domains of *NvFGFa1* and *NvFGFRa*, and of *NvFGFa2* itself (Fig. 5J-L). The expanded expression domains of *NvFGFa1* and *NvFGFRa* in *NvFGFa2* morphants suggest that the expansion of the apical organ in these embryos might be caused by excessive signalling of NvFGFa1 via NvFGFRa. To test this possibility, we co-injected *NvFGFa2* and *NvFGFRa* MOs, and found that co-injection suppressed the expansion of the apical organ territory caused by injection of *NvFGFa2* MO alone (Fig. 5M,N). Similarly, incubation of *NvFGFa2* MO-injected embryos with the chemical FGF receptor inhibitor SU5402 completely blocked formation of the apical cilia (Fig. 5O).

We conclude that NvFGFa1 signalling via NvFGFRa maintains its own expression, as well as that of *NvFGFRa* and the antagonistic *NvFGFa2* (Fig. 7H), and that the expansion of the apical organ caused by knockdown of *NvFGFa2* is mediated by NvFGFRa signalling.

Nematostella FGF signalling is transduced by the MAP kinase pathway

Activation of FGF receptors can trigger several intracellular transduction pathways. One of the most prominent among these is a conserved Ras/Raf/MEK/MAP kinase pathway, which mediates FGF signalling in many developmental processes in other animals. Manipulation of this pathway can be achieved by the application of UO126, a chemical compound that specifically blocks the activity

of MEK, which is a specific activator of the MAP kinase ERK (Favata et al., 1998). Western blot analysis with an antibody against double-phosphorylated (i.e. activated) ERK shows that UO126 almost completely abolishes ERK activation in *Nematostella* (Fig. 6A). As ERK is also involved in FGF-independent processes, we applied UO126 only after blastula stage (20 hpf) to minimise the risk of non-FGF related effects that might secondarily affect apical organ formation. Treatment with UO126 blocked apical organ formation and expression of the apical organ markers *NvCOE* and *NvFGFa1*, but did not affect the expression of *NvFoxD1* (Fig. 6B-D; data not shown). Moreover, UO126 blocked the expansion of apical organ markers caused by the injection of *NvFGFa2* MOs (Fig. 6E-G). These data suggest a major role for a Ras-MEK-ERK cascade in FGF-dependent apical organ formation.

Knockdown of *NvFGFa1* and SU5402 treatment block metamorphosis

The analysis of marker gene expression has shown that knockdown of *NvFGFa1* and *NvFGFa2* specifically affects formation of the apical organ. This allowed us to examine the possible consequences of loss or expansion of the apical organ for the behavior and later development of the larvae. After gastrulation, *Nematostella* larvae begin to swim freely, first mainly in circles, later in a more directional manner (see Movies 1, 2 in the supplementary material). In both cases they swim with the apical organ pointing forward. We observed that, despite the absence of the apical organ, larvae injected with the *NvFGFa1* MO start to swim freely with the aboral pole forward (see Movie 3 in the supplementary material). Although a minority of them is also capable of directional swimming (Fig. 7A,B; see also Movie 4 in the supplementary material), they predominantly swim in circles. *NvFGFa2* morphants with an oversized apical organ are also able to swim with their apical pole forward; however, they swim slowly and only upon agitation (see Movie 5 in the supplementary material). Hence, the apical organ is not essential for swimming, which appears to be solely accomplished by the cilia that cover the whole ectoderm.

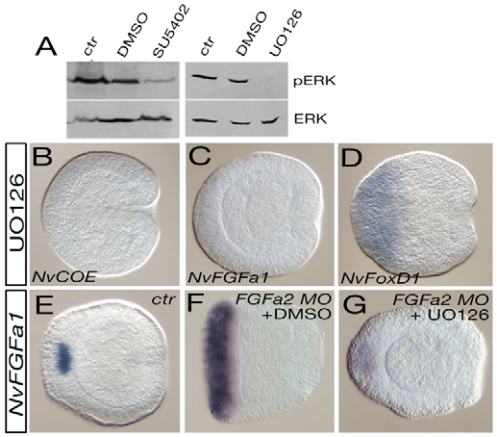

Fig. 6. MAP kinase ERK acts downstream of NvFGF signalling in apical organ formation. (**A**) Western blot to detect levels of phosporylated and total ERK. (**B-G**) In situ hybridisation of 48-hpf planulae. Lateral views, aboral pole to the left. (B-D) Embryos treated with 10 μM UO126 from 20-48 hpf; probes are indicated in the lower left corner. (E-G) In situ hybridisation with a *NvFGFa1* probe; treatments are indicated in the upper right corner, inhibitor treatment was carried out from 20-48 hpf.

As the apical organ of bilaterian larvae has been suggested to be involved in metamorphosis, we next examined whether *NvFGFa1* or *NvFGFa2* morphants undergo normal metamorphosis into primary polyps. After 12 days, 71% of uninjected (n=156), 67% of control MO-injected (n=133), 64% of *NvFGFa2* MO-injected (n=85), but only 2% of *NvFGFa1* MO-injected (n=98) embryos had become primary polyps (Fig. 7C-F).

The experiments with the FGF receptor inhibitor SU5402 had shown that continuous FGF receptor activity is required for the maintenance of the apical organ. To support the idea that the apical organ is essential for the induction of metamorphosis, we incubated *Nematostella* larvae from late planula stages (120 hpf) in SU5402. Whereas 93% (n=159) of the control embryos had become primary polyps at 9 dpf, only 10% (n=165) of the SU5402-treated planula had begun metamorphosis, and none had become primary polyps (Fig. 7G). Although inhibition of FGF receptor signalling by SU5402 is not restricted to the apical organ, these results suggest that loss of the apical organ in *NvFGFa1* morphants and upon SU5402 treatment impairs the ability to undergo metamorphosis.

DISCUSSION

Formation of the apical organ is controlled by FGF signalling

Our results show that development of the apical ciliary organ in *Nematostella* is controlled by the antagonistic interplay of NvFGFa1 and NvFGFa2. However, our finding that the expression domains of *NvFoxD1* and *NvWnt2* are essentially unaltered in *NvFGFa1* and *NvFGFa2* morphants indicates that, despite the early broad expression of *NvFGFa1* and *NvFGFa2*, they are not involved in global patterning of the embryo. Rather, their function is specifically required within the aboral *NvFoxD1*-positive territory for the proper development of the most aboral cells, which form the apical organ. NvFoxD1 thus probably acts upstream or in parallel to the FGF signalling pathway.

We propose that in the wild-type situation, the apical organ-promoting activity of NvFGFa1 is antagonised by NvFGFa2 until the initially broad expression domain of *NvFGFa1* is restricted to a small patch at the aboral pole in the early planula larvae. Because NvFGFa1 activates transcription of both *NvFGFa2* and *NvFGFa1*, it maintains a balance between promoting and suppressing signals, which in turn prevents precocious apical organ formation. Upon suppression of *NvFGFa2* translation by injection of the *NvFGFa2* MO, NvFGFa1 activity is enhanced by the lack of the antagonistic NvFGFa2 protein and by elevated autoregulation of its own transcription (Fig. 7H), resulting in earlier differentiation of the long apical cilia from a broader, i.e. not fully restricted, domain. As in wild-type larvae, expression of *NvFGFa1* and *NvFGFa2* is then maintained in the differentiated apical organ cells, and this late expression is required for the maintenance of the apical cilia, as even late inhibition of the FGF receptor by the specific inhibitor SU5402 leads to loss of the apical organ. These data are consistent with a reaction-diffusion type of patterning mechanism. It would predict that the inhibitor (NvFGFa2) diffuses faster and thus has a longer diffusion range than the activator (NvFGFa1) in order to delimit the range of signalling and, thereby, the formation of the apical organ to a small area. Although theoretical models have stressed for a long time the power of reaction-diffusion mechanisms to generate spot- or stripe-like patterns (Meinhardt and Gierer, 2000), molecular evidence has remained relatively scarce. The best-studied example is the pair of antagonistically acting TGFβ ligands, Nodal and Lefty, in vertebrate development (for reviews, see Juan and Hamada, 2001; Solnica-Krezel, 2003). Although attracting and repulsive FGF

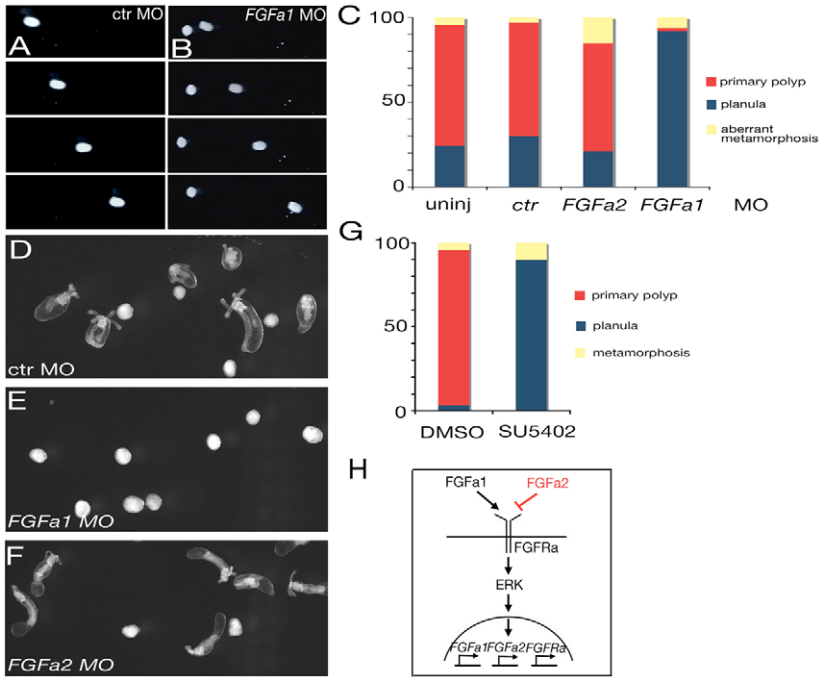

Fig. 7. Metamorphosis, but not swimming, is affected by the manipulation of FGF signalling. (**A**,**B**) Frames from movies of individual 4-dpf planula larvae injected with the morpholino indicated. Total width of the frame is ~3 mm; time interval between first and last frame was ~1.2 seconds in A and ~1.7 seconds in B. (**C**) Illustration of the effect of the indicated morpholinos on metamorphosis at 12 dpf. Uninjected, *n*=156; control (ctr) MO, *n*=133; *NvFGFa1* MO, *n*=98; *NvFGFa2* MO, *n*=85. (**D-F**) Micrographs showing live animals at 12 dpf; injected morpholinos are indicated. All animals injected with the *NvFGFa1* MO are still planula larvae, most of the *NvFGFa2* MO-injected animals are primary polyps. (**G**) Effect of SU5402 treatment on metamorphosis. Late planula stage embryos (5 dpf) were continuously incubated with DMSO or 20 μM SU5402 until 9 dpf. DMSO, *n*=159; SU5402 treatment, *n*=165. (**H**) Schematic model of the autoregulation of FGF pathway components in *Nematostella* apical organ development.

signals have been invoked to act in mesoderm migration during chick gastrulation (Yang et al., 2002), our data are, to our knowledge, the first example of two FGF ligands that are co-expressed, are auto- and crosscatalytic, and have activating and inhibiting effects consistent with a reaction-diffusion type mechanism.

NvFGFa1 and *NvFGFa2* are co-expressed from early gastrula stages on, yet only after restriction of their expression domains in the early planula does differentiation of the apical cilia occur. The premature differentiation of the apical cilia upon knockdown of *NvFGFa2* suggests that it is a quantitative change in NvFGFa1 activity that triggers this differentiation. An excess of NvFGFa1 protein might accumulate over time by a slightly higher rate of synthesis, or a longer half-life of the transcripts or the protein, and finally reach a threshold level for cilia differentiation. Alternatively, a subtle change in expression levels of *NvFGFa1* and/or *NvFGFa2* at early planula stage might lead to an elevated net NvFGFa1 signalling activity.

The mechanism that drives the restriction of *NvFGFa1*, *NvFGFa2* and *NvFGFRa* expression into the small aboral domain during gastrulation remains unclear. The activity of NvFGFa2 itself might be required for this process, as an expansion of apical pole markers in NvFGFa2 morphants was already visible at mid-gastrulation (data not shown). However, expression of apical organ markers is maintained in differentiated apical organ cells, and it is therefore difficult to distinguish whether the early expansion of the expression domains is caused by a direct role of NvFGFa2 in their restriction, or indirectly by the premature onset of differentiation of the apical organ cells. Interestingly, all described apical organ markers in *Nematostella* either display the same expression dynamics as the FGFs, or their expression commences only at later stages. So far, no gene has been described that is expressed in a spot-like domain at the apical pole during gastrulation, and that would thereby provide a localised source for initiation of the restriction. Dissection experiments have shown that only the oral half of gastrula-stage embryos is able to regenerate a complete planula larva, including a correctly patterned apical tuft (Fritzenwanker et al., 2007; Lee et al., 2007), and it therefore appears possible that the restriction is not triggered by an 'attractive' signal from the aboral pole, but rather by a 'repulsive' signal from the oral pole. Wnt factors would be a candidate group of molecules for such a function, as several of them are expressed in distinct ring-like domains located exclusively in the oral half of the embryo (Kusserow et al., 2005). A similar mechanism operates in the sea urchin embryo, where the size of the apical organ is restricted by β-catenin signalling from the opposite, vegetal pole (Yaguchi et al., 2006).

The *Nematostella* genome encodes two FGF receptors, but only *NvFGFRa* is co-expressed with *NvFGFa1* and *NvFGFa2* in the apical organ. This co-expression suggests that both FGFs use NvFGFRa as their receptor. As knockdown of *NvFGFa1* and *NvFGFRa* have identical effects on apical organ formation and marker gene expression, one obvious possibility to account for the opposing activities of the two ligands would be that only NvFGFa1 is able to activate the receptor, whereas NvFGFa2 binds to NvFGFRa without activating it and thus acts as a dominant-negative ligand. Alternatively, binding of NvFGFa1 and NvFGFa2 could activate different transduction pathways that antagonise each other intracellularly. To our knowledge, an antagonism of two co-expressed FGFs that signal via the same receptor would represent a novel mechanism for the fine-tuning of FGF activity. Interestingly, a similar mechanism has been described for a member of the TGFβ family of growth factors, whereby BMP3 can bind and block ActRIIB, a receptor that is activated by the binding of BMP4 or Activin (Gamer et al., 2005).

Our results suggest that signalling of NvFGFa1 via NvFGFRa might be mediated by conserved intracellular pathway components. The ability of the MEK inhibitor UO126 to block apical organ formation in wild-type embryos and in *NvFGFa2* morphants is compatible with an involvement of a Ras/MEK/ERK signalling cascade in NvFGFa1 signalling. This pathway mediates the response to FGFs in many developmental processes in higher metazoans (Eswarakumar et al., 2005; Thisse and Thisse, 2005). However, the MAP kinase pathway can also be activated by other signals, and indeed blocking of FGF receptor signalling only partially abolishes

the phosphorylation of ERK/MAP kinase (Fig. 6A). This indicates that ERK is also activated by another pathway besides the FGF signalling pathway. This other signalling input is apparently not essential for apical organ formation, as expansion and abolishment can all be achieved by manipulation of the corresponding FGF signalling pathway.

Evolution of apical sensory organs

Apical organs with a tuft of long cilia have been described in marine ciliated larvae from the two major bilaterian groups, protostomes and deuterostomes, but the evolutionary relationship of cnidarian, protostomian and deuterostomian apical organs is not yet clear.

Strikingly, FGFs or FGF receptors are expressed in the region of apical organ formation in sea urchin and hemichordate embryos [two deuterostomians (Gerhart et al., 2005; Lapraz et al., 2006)], and in the polychaete *Platynereis* (a protostomian; P. Steinmetz and D. Arendt, personal communication). Thus, although functional data are lacking for these organisms, it is tempting to speculate that regulation of apical organ formation by FGF signalling is common to cnidarian, protostomian and deuterostomian larvae, and might thus represent an ancestral function of FGF signalling that was present in the common ancestor of all eumetazoans. However, broader species sampling and more marker genes are necessary to conclude on the homology of apical organs in cnidarian and bilaterian larvae.

In bilaterian larvae, morphological observations, and pharmacological and cell ablation experiments, have suggested that apical organs are chemo- and/or mechanosensory structures with neuroendocrine functions that might be involved in the induction of metamorphosis (Hadfield et al., 2000; Kempf et al., 1997; Voronezhskaya and Khabarova, 2003). Our results strongly indicate that FGF signalling is required for metamorphosis, most likely through the formation of the apical organ. Because metamorphosis of larvae into adults is of pivotal importance for all pelago-benthic directly and indirectly developing organisms, future work will attempt to identify the external cues and their internal processing.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/10/1761/DC1

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