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FGF/MAPK/Ets signaling renders pigment cell precursors competent to respond to Wnt signal by directly controlling *Ci-Tcf* transcription

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SUMMARY

FGF and Wnt pathways constitute two fundamental signaling cascades, which appear to crosstalk in cooperative or antagonistic fashions in several developmental processes. In vertebrates, both cascades are involved in pigment cell development, but the possible interplay between FGF and Wnt remains to be elucidated. In this study, we have investigated the role of FGF and Wnt signaling in development of the pigment cells in the sensory organs of *C. intestinalis*. This species possesses the basic features of an ancestral chordate, thus sharing conserved molecular developmental mechanisms with vertebrates. Chemical and targeted perturbation approaches revealed that a FGF signal, spreading in time from early gastrulation to neural tube closure, is responsible for pigment cell precursor induction. This signal is transmitted via the MAPK pathway, which activates the *Ci*-Ets1/2 transcription factor. Targeted perturbation of *Ci*-TCF, a downstream factor of the canonical Wnt pathway, indicated its contribution to pigment cell differentiation Furthermore, analyses of the *Ci-Tcf* regulatory region revealed the involvement of the FGF effector, *Ci*-Ets1/2, in *Ci-Tcf* transcriptional regulation in pigment cell precursors. Our results indicate that both FGF and the canonical Wnt pathways are involved in *C. intestinalis* pigment cell induction and differentiation. Moreover, we present a case of direct transcriptional regulation exerted by the FGF signaling cascade, via the MAPK-ERK-Ets1/2, on the Wnt downstream gene *Ci-Tcf*. Several examples of FGF/Wnt signaling crosstalk have been described in different developmental processes; however, to our knowledge, FGF-Wnt cross-interaction at the transcriptional level has never been previously reported. These findings further contribute to clarifying the multitude of FGF-Wnt pathway interactions.

KEY WORDS: *Ciona intestinalis*, Ascidian, Sensory organs, Pigment cells, FGF signaling, Ets, Tcf, Canonical Wnt signaling, FGF/Wnt crosstalk

INTRODUCTION

FGF and Wnt signaling cascades perform fundamental roles in a variety of processes, both in vertebrates and invertebrates. The extracellular FGF ligand-dependent/FGF receptor activation starts the intracellular signal transduction, which is conveyed in the cell mainly by the more common MAPK signaling cascade (Bottcher and Niehrs, 2005; Thisse and Thisse, 2005; Chen et al., 2007). The canonical Wnt signaling activation leads to stabilization and nuclear import of β-catenin. The association of β-catenin with TCF/LEF (T-cell/lymphocyte enhancer) transcription factors regulates the expression of Wnt downstream genes (Clevers, 2006; MacDonald et al., 2009).

FGF and Wnt signaling pathways cross talk, in either cooperative or antagonistic fashions, in several developmental and cellular processes, such as embryonic morphogenesis and patterning, cell proliferation, cell fate determination and tissue induction (Gunhaga et al., 2003; Katoh, 2006; Takemoto et al., 2006; Canning et al., 2008; Hong et al., 2008). Cross-interaction between these pathways can further modulate individual fluctuations of each signal, generating specific intracellular environments in different tissues and/or developmental stages. The two signaling pathways can intermingle with each other, either acting on the same target genes (Takemoto et al., 2006), or intervening on signaling cascade protein activity. In pathological conditions, such as mouse carcinogenesis, FGF-dependent GSK3β downregulation indirectly provokes β-catenin stabilization, reinforcing β-catenin/TCF signal transduction, which leads to more dramatic malignant phenotypes (Katoh, 2006).

Pigment cells comprise a broad category of highly specialized cells present across the animal kingdom, including melanocytes of the skin, pigment cells of the pineal organ and retinal pigment epithelium (RPE) of the eye (King et al., 1995). Despite their different functional properties and different embryonic origin, pigment cells share the common feature of producing melanin (Schraermeyer, 1996). Melanin-producing cells cover disparate functional roles, spreading from immune response to photo-protection (Steel and Barkway, 1989; Marks and Seabra, 2001; Sulaimon and Kitchell, 2003; Nappi and Christensen, 2005). Several inheritable pathologies affecting pigment cell development and function are linked to skin, eye and ear disorders, including more severe diseases such as various carcinomas (Goding, 2007).

Both the FGF and Wnt signaling cascades take part in pigment cell development (Stocker et al., 1991; Sauka-Spengler and Bronner-Fraser, 2008; Fujimura et al., 2009). Although a clear function of FGF factors in promoting pigment cell formation has not yet been demonstrated, it is worth mentioning that five different forms of bFGF are expressed at the onset of melanocyte precursor differentiation in avian embryos (Sherman et al., 1991).

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Furthermore exogenous bFGF treatments are able to induce pigmentation in embryonic quail neural crest-derived cells (Stocker et al., 1991). The canonical Wnt signaling pathway has been associated with neural crest-pigment cell differentiation in zebrafish and mouse, as well as RPE differentiation in the murine optic cup (Dorsky et al., 1998; Dorsky et al., 1999; Dorsky et al., 2000; Schmidt and Patel, 2005). Altogether, these findings point to the involvement of FGF and Wnt signaling cascades in pigment cell development; however, any possible crosstalk between these two pathways has yet to be described.

The swimming larva of the urochordate ascidian *C. intestinalis* (L., 1767) possesses distinctive chordate body plan features, including a notochord and hollow dorsal nerve cord. In the *C. intestinalis* larval anterior sensory vesicle, there are two pigment cell sensory organs: the anterior geotactic otolith and the posterior photoreceptive ocellus (Dilly, 1969; Eakin and Kuda, 1971; Torrence, 1986; Ohtsuki, 1991; Tsuda et al., 2003), which directs larval swimming behavior before metamorphosis (Svane, 1989; Tsuda et al., 2003). The otolith is a single cell, containing a melanin granule and is connected by a narrow stalk to the sensory vesicle ventral floor (Dilly, 1962).

The ocellus is a multicellular organ composed of 30 photoreceptor cells, three lens cells and one cup-shaped pigment cell (Horie et al., 2005). Functional similarities and conservation of basic gene expression patterns led to infer that ascidian sensory organs, vertebrate eye and pineal organ could be derived from a common archetypal 'visual organ' (Kusakabe et al., 2001; Sato and Yamamoto, 2001; Lamb et al., 2007). Molecules belonging to arrestin and opsin classes are expressed in ocellus photoreceptors as in vertebrate retina photoreceptor cells and pineal organ (Kusakabe et al., 2001; Nakagawa et al., 2002; Nakashima et al., 2003). Ascidian sensory organ pigment cells, analogously to the vertebrate RPE, pineal gland and melanocytes (Tief et al., 1996), express the melanogenic genes tyrosinase (*tyr*) (Caracciolo et al., 1997) and tyrosinase related protein (*tyrp*) (Toyoda et al., 2004). In both ascidians and vertebrates, these genes can be considered as early developmental pigment cell markers, as their transcript production is not restricted to differentiated pigment cells, but appears in the early pigment cell precursor lineage (Palumbo et al., 1991; del Marmol and Beermann, 1996; Tief et al., 1996; Caracciolo et al., 1997; Sato et al., 1997). Cell-manipulation studies have indicated that, at least for pigment cell precursor specification, an inductive signal from the adjacent A-line nerve cord precursors is required (Nishida and Satoh, 1989; Nishida, 1991). Nevertheless, the nature of the signaling factor, as well as the molecular events that it triggers, remains to be elucidated. During the early steps of *C. intestinalis* embryogenesis, FGF signaling activity via the MAPK pathway is necessary for neural induction (Hudson et al., 2003) and patterning of the caudal neural plate along the anterior posterior axis (Hudson et al., 2007; Imai et al., 2009). Given these data, and considering the roles that FGF and Wnt signaling perform in vertebrate melanocytes and RPE development, we investigated the functional role of FGF and Wnt signaling cascades in *C. intestinalis* larval sensory organ pigment cell development. By performing chemical and transgene-mediated inhibition, we demonstrated an inductive role for FGF-MAPK signaling pathway in pigment cell precursors through the activation of *Ci*-Ets1/2, a well known FGF downstream effector (Wasylyk et al., 1998). Transgene-mediated inhibition also revealed a functional role of the direct Wnt-downstream effector *Ci*-TCF in pigment cell development (Molenaar et al., 1996). We found *Ci-Tcf* transcript expressed in pigment cell precursors shortly after the onset of FGF signaling cascade. Our data demonstrated that the Wnt/β-catenin pathway contributes to pigment cell differentiation process through *Ci*-TCF activity. Functional analyses of *Ci-Tcf* cis-regulatory region revealed a novel crosstalk between the FGF and Wnt/β-catenin signaling cascades, in which the FGF downstream effector, *Ci*-Ets1/2, is involved in the transcriptional regulation of the main downstream effector of the Wnt signaling pathway, *Ci-Tcf*.

MATERIALS AND METHODS

Animals and embryos

Animal handling and embryo treatments and transgenesis via electroporation were carried out as previously described (Corbo et al., 1997; Ristoratore et al., 1999). Embryo imaging capture was performed with a Zeiss Axio Imager M1 and a Zeiss LSM 510 META confocal microscope.

Chemical inhibitor treatments

Embryos were obtained by in vitro fertilization, then chemically dechorionated and grown in Millipore-filtered sea water (MFSW) at room temperature, until the desired stage. Experimental embryos were treated with 4 μM MEK inhibitor compound U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) in MFSW (U0126 stock solution: 2 mM in DMSO) (Sigma) for 10 or 20 minutes and then washed with MFSW to remove the compound. Treated embryos were allowed to develop to the desired stage and then fixed for whole-mount in situ hybridization or immunohistochemistry, or directly observed. For lithium treatment, *ptyrp1a*>Ets:VP16 electroporated embryos and control embryos were incubated in MFSW containing 40 mM LiCl up to the larval stage.

Histochemistry and in situ hybridization

RNA probes were synthesized from the following gene clones: *Ci-ets1/2* (N. Satoh Gene Collection 1 ID: ciad08k16), *Ci-Tcf*, *Ci-tyr* (N. Satoh Gene Collection 1 ID: citb41l04) and *gfp*.

Single-gene whole-mount in situ hybridizations were performed as previously described (Ristoratore et al., 1999). Double fluorescent whole-mount in situ hybridization were performed as described previously (Dufour et al., 2006). Whole-mount immunohistochemistry procedures have been performed as previously described (Shimeld et al., 2005). Rabbit anti-*Ciona* βγ crystallin antibody was kindly provided by Dr Sebastian M. Shimeld (Department of Zoology, University of Oxford, UK).

Characterization of *Ci-Tcf* cDNA

A search of the *C. intestinalis* cDNA database (Ghost Database, http://ghost. zool.kyoto-u.ac.jp/indexr1.html) with the entire JGI2 *Ci-Tcf* gene model (gw1.06q.3.1) led to the identification of two cDNA clones: cicl049a12 (N. Satoh Gene Collection 1 ID: CiGC13p03), sequence 1-1086 bp; and ciad081m17 (N. Satoh Gene Collection 1 ID: CiGC08g07), sequence 1070 bp-end. By exploiting the *Ssp*I restriction enzyme site, located in position 1006, the cicl049a12 and ciad081m17 clones have been ligated to obtain a bona fide full-length *Ci-Tcf*.

Construct preparation

The oligonucleotides carrying suitable restriction sites on the 5′ ends that were used to prepare the constructs are listed in Table S1 in the supplementary material.

The *Ci-tyrp1a* and *Ci-tyr* 5′flanking regions were PCR-amplified from genomic DNA using the primers: *Ci-ptyrpF*/*Ci-ptyrpR*, and *Ci-ptyrF*/*Ci-ptyrR*, respectively.

For *ptyr*>FGFR^{DN}, the MESP promoter was replaced with the *ptyr* 5′ fragment in a MES*P*>FGFR^{DN} vector (a generous gift from L. Christiaen, NYU School of Medicine, New York, USA) using the PCR-incorporated *Xba*I and *Not*I sites.

For, *ptyr*>mCherry, mCherry sequence was amplified by PCR from a pmCherry vector (Clontech) with the oligonucleotides *mCheF* and *mCheR*, and inserted into the *ptyr*>FGFR^{DN} vector, previously digested with *Not*I and *Eco*RI to eliminate FGFR^{DN}.

For *ptyr*>FGFR^{DN}mChe, FGFR^{DN}, which lacks the STOP codon, was amplified by PCR from MES*P*>FGFR^{DN} template using the primers *FGFRDNFw* and *FGFRDNRev*, and double digested with *Not*I and *Bam*HI

DEVELOPMENT

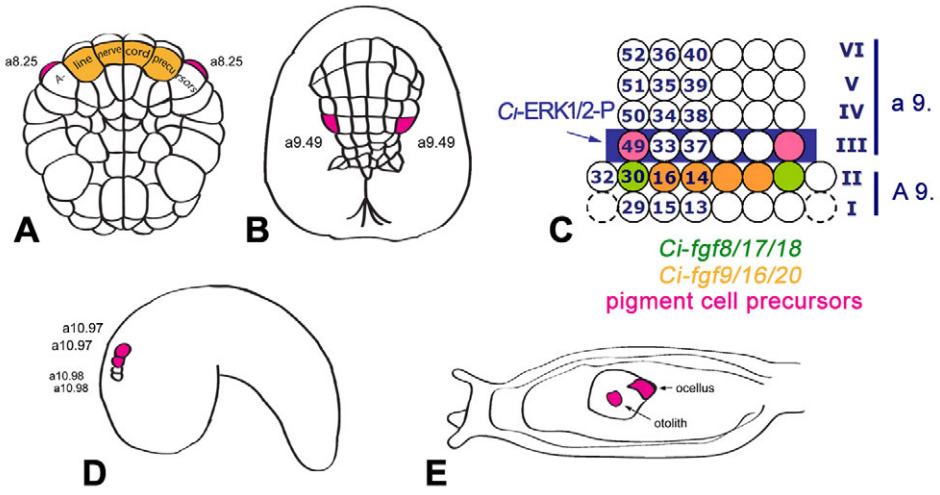

Fig. 1. Pigment cell lineage during *C. intestinalis* embryogenesis. During gastrulation (**A-C**), pigment cell precursor (pink) induction occurs. In this period, A-line nerve cord cells express *Ci-fgf9/16/20* (from 110-cell stage, yellow) and *Ci-fgf8/17/18* (from the late gastrula, green) and *Ci*-ERK1/2 activation appears in row III of the neural plate (C, blue rectangle). (A) 110-cell stage; (B) late gastrula stage; (C) neural plate scheme at the late gastrula stage [adapted, with permission, from Hudson et al. (Hudson et al., 2007)]. Vegetal view, anterior is upwards. (**D**,**E**) During neural tube closure, pigment cell precursors divide and differentiate into sensory organ pigment cells. D, tailbud stage; E, larval stage. Dorsal is upwards, anterior is towards the left.

restriction enzymes. mCherry, lacking both the Kozak signal and the first methionine, was amplified by PCR using the primers *mCheFw* and *mCheR*, and then double digested with *Bam*HI and *Eco*RI restriction enzymes. The fragments *Not*I-FGFR^{DN}-*Bam*HI and *Bam*HI-mCherry-*Eco*RI were then fused in-frame downstream from *ptyr* promoter, replacing FGFR^{DN} in the *ptyr*>FGFR^{DN} vector, previously digested with *Not*I/*Eco*RI to eliminate FGFR^{DN}.

For *ptyrp1a*>mChe, *ptyrp1a*>FGFR^{DN}, *ptyrp1a*>FGFR^{DN}mChe, *ptypr1a*>Ets:VP16, *ptyrp1a*>Ets:WRPW and *ptyrp1a*>ΔN1/*Ci*-Tcf:mChe, the 5′ *ptyrp1a* fragment replaced the *ptyr* fragment (in the constructs *ptyr*>mChe, *ptyr*>FGFR^{DN}, *ptyr*>FGFR^{DN}mChe and *ptyr*>ΔN1/*Ci*-Tcf:mChe) or the MESP promoter (in the constructs MES*P*>Ets:VP16 and MES*P*>Ets:WRPW, kindly provided by Brad Davidson, University of Arizona, Tucson, AZ, USA), previously digested with *Hin*dIII-*Not*I to eliminate *ptyr* or MESP enhancers.

For *ptyrp1a*>Sβcat-mChe, stabilized β-catenin (Sβcat) lacking the N-terminal (1-47 amino acid residues) that includes putative phosphorylation sites, was amplified using the oligonucleotides *SβcatFw* and *SβcatRev*. Sβcat, digested with *Not*I/*Bam*HI was inserted in place of FGFR^{DN} in the construct *ptyrp1a*>FGFR^{DN}mChe.

For *ptyr*>ΔN1/*Ci*-Tcf:mChe, ΔN1/*Ci*-Tcf, which lacks the N-terminal β-catenin-binding domain, was prepared by PCR from a construct containing the full coding sequence of *Ci-Tcf*, using two oligonucleotides *N1F* and *N1R*. ΔN1/*Ci*-Tcf, digested with *Not*I/*Bam*HI was inserted in place of FGFR^{DN} in the construct *ptyr*>FGFR^{DN}mChe.

pTcf 2.0>GFP: 2.0 kb of the 5′ flanking *Ci-Tcf* region was PCR-amplified from genomic DNA using the oligonucleotides *ptcf1* and *ptcf9* (see Table S2 in the supplementary material) and cloned into a pBS>GFP vector upstream from *gfp*. *ptcf* 0.4, *ptcf* 0.21 and *ptcf* 0.22 fragments were all generated by PCR amplification, using appropriate primers (*cf0.4*/*cr0.4*, *cf0.4*/*c2r* and *c2*/*cr0.4*, respectively; see Table S2 in the supplementary material) and cloned into a pBS plasmid containing the Epstein Barr virus TATA (E1bTATA) upstream from GFP (Leong et al., 1988; Parks et al., 1988; Poleev et al., 1995). This construct is transcriptionally inactive and functions in *C. intestinalis* as minimal promoter (A.S., unpublished). The *ptcf* 0.4 regulatory sequence was searched for transcription factor binding sites by using Genomatix software (http://www.genomatix.de). Point mutations were introduced in *pTcf* 0.4 regulatory sequence, using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and suitable oligonucleotides (see Table S3 in the supplementary material).

Electro mobility shift assay (EMSA)

Ci-Ets1/2 DNA-binding domain (DBD) in vitro transcription and translation was carried out using the TNT Quick Coupled Transcription/Translation System (Promega), according to manufacturer's instructions. EMSA assay has been performed as previously reported (Fanelli et al., 2003), using 5.0 μl of in vitro synthesized *Ci*-Ets1/2 DBD protein, and labeled and competitor DNAs prepared by annealing the wild-type (*Wt2*) or mutant (*Mut2*) oligonucleotides (see Table S3 in the supplementary material).

RESULTS

MAPK signaling is required for sensory organ pigment cell formation in *Ciona intestinalis*

C. intestinalis pigment cell precursors arise at the early gastrula stage (the 110-cell stage) as two symmetric progeny of the a4.2 brain/sensory vesicle lineage (Nishida, 1987). In the course of gastrulation, these cells and their descendants (the a9.49 cell pair) require an inductive signal from the sub-adjacent A-line nerve cord precursors to be directed towards pigment cell fate (Nishida, 1991). During neurulation, the a.9.49 pair divides into two pairs of cells: the a10.98 blastomeres, which form part of the right-dorsal sensory vesicle epithelium; and the a.10.97 blastomeres, which become pigment cells (Nishida and Satoh, 1989) (Fig. 1). At gastrulation, expression of two FGF genes, *Ci-fgf8/17/18* and *Ci-fgf9/16/20*, in the six A-line nerve cord precursors of row II is accompanied by the activation of *Ci*-ERK1/2 (a known downstream FGF-MAP kinase cascade factor) in the adjacent six a-line cells of the neural plate row III, which includes the two pigment cell precursors (Fig. 1C) (Hudson et al., 2007) (ANISEED; http://www.aniseed.cnrs.fr/). As a first approach to test the role of the MAPK cascade on pigment cell precursor induction, the pharmacological agent U0126 was used to block the ERK1/2 kinase, MEK1/2. Wild-type in vitro fertilized embryos were treated with U0126 at late gastrula and neurula stages for 20 minutes at room temperature. The resulting larvae showed normal morphology and tail motility, with the exception of the sensory vesicle that appeared totally deprived of pigment cells (Fig. 2B,C). U0126 treatment at progressively later developmental intervals, starting from the early tailbud stage, did not affect larval pigment cell formation. No alterations were observed in control DMSO-treated embryos (Fig. 2A,D,D′).

Melanogenic genes, *Ci-tyrp1a* and *Ci-tyr*, are expressed specifically in pigment cell lineage up to the larval stage. The *Ci-tyrp1a* gene starts to be expressed in pigment cell lineage from the late gastrula stage (aniseedV3_7490 on the ANISEED website: http://www.aniseed.cnrs.fr/), whereas the *Ci-tyr* gene is activated in the same territory starting from the neurula stage (Caracciolo et al., 1997) and aniseedV3_7634). We have identified enhancers of both genes (*ptyrp1a* and *ptyr*) that are able to recapitulate endogenous *Ci-tyrp1a* and *Ci-tyr* expression patterns (F.R. and

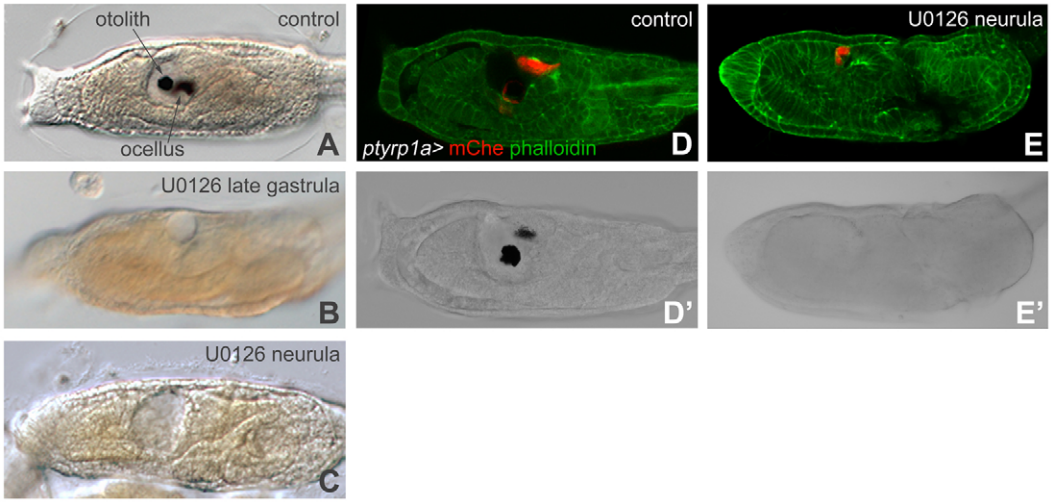

Fig. 2. MAPK signaling is required for pigment cell induction. Control larva (**A**) and larvae treated with U0126 at the midgastrula stage (**B**) and neurula stage (**C**). The sensory vesicle is smaller in B compared with C and pigment cells are absent. (**D**,**D′**) *ptyrp1a*>mCherry larvae show mCherry expression in pigment cell lineage. (**E**,**E′**) *ptyrp1a*>mCherry larvae treated with U0126 at the neurula stage show expression in grouped cells in the sensory vesicle; mCherry (red), phalloidin (green). Lateral view, anterior towards the left.

A.S., unpublished; see Fig. S1 in the supplementary material). *ptyrp1a*>mCherry embryos were used to verify any pigment cell lineage perturbation following U0126 treatment at the neurula stage. The resulting larvae were deprived of pigment cells; however, they showed fluorescent cells within the sensory vesicle (Fig. 2E,E′). These data indicate that the a10.97s and a10.98s pigment cell precursors are still present, although they lack the phenotypic characteristics of the pigment cell lineage. Interestingly, U0126 treatment of *ptyrp1a*>mCherry embryos at earlier developmental time points also blocked transgene expression (data not shown). In summary, MAPK activity is necessary, from gastrula to neurula stage, to direct pigment cell precursors through their final fate.

FGF signaling pathway operates upstream of the MAPK cascade in pigment cell precursor induction

To test whether MAPK cascade activation in pigment cell precursors is FGF dependent, both the *ptyrp1a* and *ptyr* cis-regulatory sequences were used to conditionally express a dominant-negative form of the sole *Ci*-FGFR (FGFR^{DN}) in the pigment cell precursors (Davidson et al., 2006). Targeted expression of FGFR^{DN} resulted in larvae lacking one or both pigment cells, showing normal morphology of the palps, tail and sensory vesicle.

Perturbed larvae were assayed for the presence of *Ci*-βγ crystallin protein, which specifically marks the otolith sensory organ and the sensory adhesive palps (Fig. 3A). In all larvae *Ci*-βγ crystallin was detected in the anterior palps (Fig. 3B-D). Among the larvae possessing only one pigment cell, ~50% showed *Ci*-βγ crystallin in the single pigment cell, confirming its identity as the otolith (Fig. 3D). Once assessed that the use of both promoters resulted in a similar range of phenotypes, we have chosen the *ptyrp1a* promoter for subsequent analyses, as its activity begins at late gastrula stage (see Fig. S1 in the supplementary material), almost contemporaneously with the requirement of the MAPK cascade to direct pigment cell fate (see Fig. 1). Transgenic *ptyrp1a* >FGFR^{DN} embryos resulted in a high percentage of larvae showing one (33%) or no (53%) pigment cell (Fig. 3G).

A construct coding for the *Ci*-FGFR^{DN}mCherry fusion protein under the control of the *ptyrp1a* regulatory region (*ptyrp1a*>FGFR^{DN}mChe), was used to identify cells in which the endogenous FGFR function was perturbed. *ptyrp1a*>FGFR^{DN}mChe larvae, which lack both pigment cells, showed two to four fluorescent cells grouped in the dorsal sensory vesicle epithelium (Fig. 3F,F′). Thus, it appears that FGF signaling is necessary for pigment cell induction in *C. intestinalis*.

The role of the FGF-MAPK downstream factor *Ci*-Ets1/2 in pigment cell precursor induction

Ci-Ets1/2 belongs to the ETS family of transcription factors, characterized by a DNA-binding domain that recognizes the consensus sequence GGA(A/T) (Graves and Petersen, 1998). Subgroups of this family, including *Ci*-Ets1/2, possess a MAPK phosphorylation site on a threonine residue, and their activity is therefore driven by MAPK phosphorylation (Graves and Petersen, 1998; Wasylyk et al., 1998). In the distantly related ascidian *Halocynthia roretzi*, *Hr*-Ets, the *Ci*-Ets1/2 ortholog, is involved in brain induction and sensory pigment cell formation, as a downstream effector of the FGF-MAPK signaling cascade (Miya and Nishida, 2003). We examined the expression of *Ci-Ets1/2* at the late gastrula, early and late neurula stages. During this developmental time period, the transcript appears in a-line neural territories, including pigment cell precursor blastomeres (Fig. 4A-C). To test the role of *Ci*-Ets1/2 in pigment cell precursor induction, constitutively inactive (Ets:WRPW) and active (Ets:VP16) forms of the *Ci*-Ets1/2 protein have been selectively expressed in pigment cell lineage driven by the *ptyrp1a* regulatory region. The Ets:WRPW repressor form (Fisher et al., 1996; Kang et al., 2005; Davidson et al., 2006) caused the same phenotype alterations as FGFR^{DN}, showing a slight increase in the percentage of larvae totally deprived of pigment cells (Fig. 4D,D′,F). Conversely, the constitutive active form, Ets:VP16 (Cress and Triezenberg, 1991; Hall and Struhl, 2002), led to the formation of extranumerary pigment cells (up to four) in the larval sensory vesicle (Fig. 4E,E′,F).

Remarkably, we noted that the contemporary block of *Ci*-FGFR and *Ci*-Ets1/2 activity (*ptyrp1a*>Ets:WRPW plus *ptyrp1a*>FGFR^{DN}mChe) resulted in a higher percentage of specimens lacking pigment cells, in comparison with single perturbations (Fig. 4F). To determine whether *Ci*-Ets1/2 acted as FGF signaling downstream effector in pigment cell induction, we simultaneously expressed Ets:VP16 and FGFR^{DN} in pigment cell precursors. The constitutively active form of *Ci*-Ets1/2 (Ets:VP16) was able to induce pigmentation in the FGFR^{DN} background, rescuing to a large extent the FGFR^{DN} perturbation (Fig. 4F). This result indicates that these two molecules belong to the same pathway and cooperate in pigment cell induction.

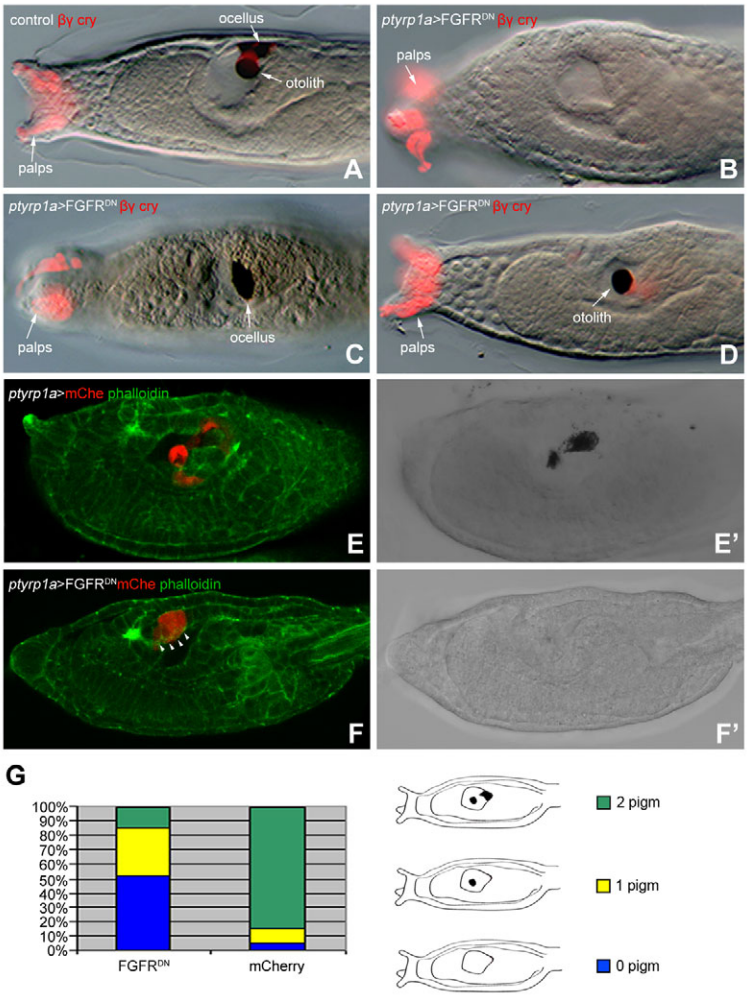

Fig. 3. FGF signaling is required for pigment cell induction. (**A**) Control larvae showing *Ci*-βγ-crystallin expression in the palps and in the otolith. (**B-D**) All *ptyrp1a*>FGFR^{DN}-transgenic larvae show *Ci*-βγ-crystallin expression in the anterior palps. Among larvae possessing one pigment cell (C,D), around 50% show *Ci*-βγ crystallin in the single otolith-like pigment cell (D). Lateral view, anterior towards the left. (**E**,**E′**) Transgenic *ptyrp1a*>mCherry larvae showing mCherry expression in the pigment cell lineage. (**F**,**F′**) *ptyrp1a*>FGFR^{DN}mChe larvae without pigment cells show mCherry expression in grouped cells in the sensory vesicle (arrowheads). Lateral view, anterior towards the left. (**G**) Percentage of *ptyrp1a*>FGFR^{DN}mChe transgenic larvae showing no pigment cells (blue), one pigment cell, either otolith or ocellus (yellow), or two pigment cells (green), as illustrated schematically on the right. Within each column is the combined number of larvae counted during at least three trials; *n*>150 embryos scored for transgene expression.

Role of the canonical Wnt downstream effector *Ci*-TCF in *C. intestinalis* pigment cell differentiation

The canonical Wnt signaling pathway promotes pigment cell differentiation in zebrafish neural crest through the action of TCF/LEF family of transcription factors (Dorsky et al., 1998; Dorsky et al., 1999). To investigate a possible involvement of the canonical Wnt signaling pathway in *C. intestinalis* sensory organ pigment cell development, we have focused our attention on *Ci-Tcf*, the single *Tcf/Lef* ortholog gene identified in *C. intestinalis* genome (Yamada et al., 2003).

Ci-TCF protein prediction revealed the presence of a N-terminal β-catenin-binding domain (amino acids 1-50), showing 60% of sequence similarity with the other TCF/LEF orthologs, and of a HMG DNA-binding domain (amino acids 277-350), possessing up to 87% of sequence identity with the HMG DNA-binding domains of zebrafish and human TCF-3.

Ci-Tcf transcripts are maternally provided and ubiquitously localized until gastrulation (Imai et al., 2004) (this work). From the mid-neurula stage, the gene is expressed amongst different territories, including the two blastomeres corresponding to pigment cell precursors (a10.97s) (Fig. 5A). Expression persists in these two blastomeres until neural tube closure (Fig. 5B). Co-localization analysis of *Ci-Tcf* with *Ci-tyr* transcripts, which are present in all four a9.49 progeny (Caracciolo et al., 1997), confirmed *Ci-Tcf* expression to the most posterior a10.97 blastomeres (Fig. 5C-C″).

To test *Ci*-TCF function in pigment cell development, a dominant-negative form of Ci-TCF (ΔN1/*Ci*-Tcf:mChe) was selectively expressed in pigment cell precursors, driven by the *Ci-tyr* enhancer (*ptyr*), the activity of which starts at the neurula stage, almost concurrently with *Ci-Tcf* appearance in the pigment cell lineage (compare Fig. 5A with Fig. S1 in the supplementary material). Targeted expression of ΔN1/*Ci*-Tcf:mChe resulted in larvae showing defective pigment cell melanization phenotypes compared with the control (Fig. 5D-G). In 69% of specimens, one pigment cell appeared normally melanized, whereas the other was poorly or completely lacking melanization (Fig. 5E-E′,G). In 17% of the observed larvae both pigment cells appeared partially melanized (Fig. 5F,F′,G). Anticipating the timing of ΔN1/*Ci*-Tcf expression, by using, as further control, the *ptyrp1a* enhancer resulted in a similar range of pigment cell phenotype (data not shown). These findings suggest that *Ci*-TCF perturbation does not affect pigment cell specification, but rather pigment cell differentiation, contributing to the regulation of melanogenesis.

Functional cis-acting ETS-binding sites are necessary for *Ci-Tcf* transcriptional regulation in pigment cell precursors

Expression within the pigment cell precursors, coupled with its detected role in the melanization process during pigment cell differentiation, prompted us to explore *Ci-Tcf* transcriptional regulation in order to identify upstream factors. We found that 2.0

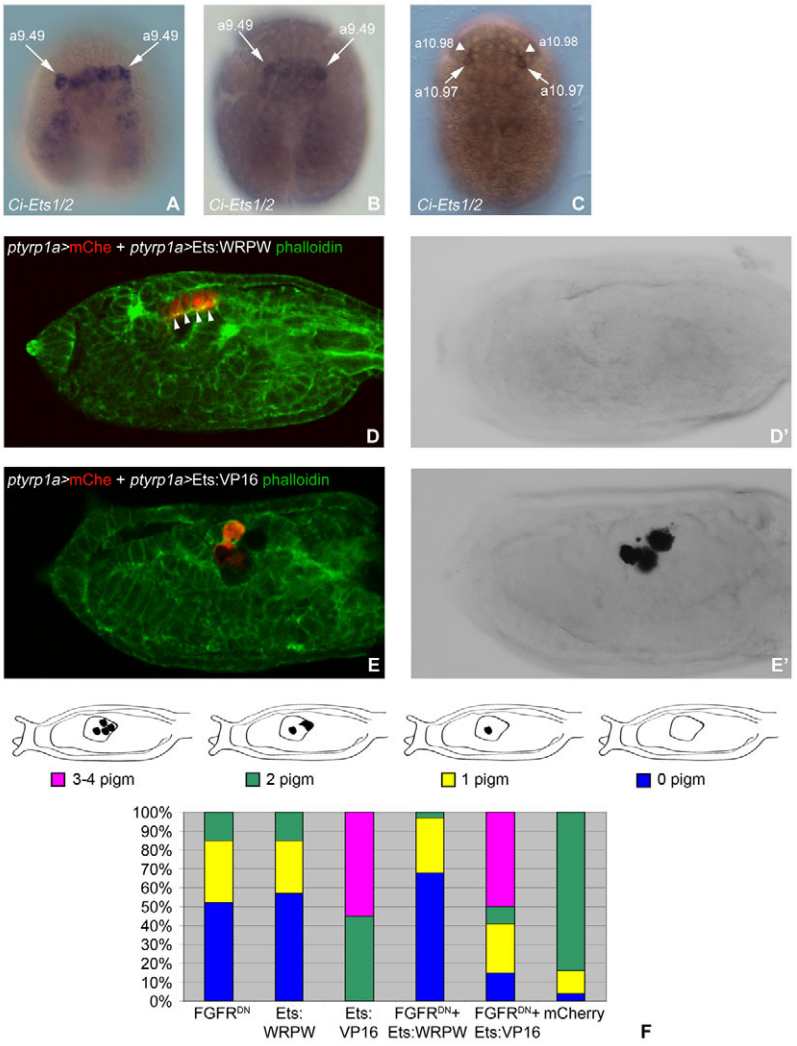

Fig. 4. *Ci-Ets 1/2* expression and function in pigment cell precursors. (**A-C**) During gastrulation, *Ci-Ets1/2* is broadly expressed in the neural plate region, including pigment cell lineage (a9.49 in A,B; a10.97 and a10.98 in C). (A) Late gastrula, (B) early neurula and (C) late neurula stages. Vegetal view, anterior is towards the top. (**D-E′**) Double transgenic *ptyrp1a*>mChe plus *ptyrp1a*>Ets:WRPW (D,D′) and *ptyrp1a*>mChe plus *ptyrp1a*>Ets:VP16 (E,E′) larvae observed with confocal and transmitted light, respectively. Pigment cells are absent (D′), there are four grouped cells in the sensory vesicle (D, arrowheads) and accessory pigment cells are present in the sensory vesicle (E′). (**F**) Percentage of transgenic larvae (different transgenic conditions are indicated on the bottom) showing three or more pigment cells (pink), two pigment cells (green), one pigment cell [either otolith or ocellus (yellow)] and no pigment cells (blue), as illustrated schematically above.

kb of the 5′ flanking region (*ptcf* 2.0, –1937 from the 5′ of the EST predicted transcript) was sufficient to recapitulate endogenous *Ci-Tcf* expression in nervous system and mesenchyme territories, starting from the neurula developmental stage (see Fig. S2 in the supplementary material). Phylogenetic footprinting between *C. intestinalis* and *C. savignyi* genomes led to the identification of a 0.25 kb conserved block (–1113/-867 bp) in the *ptcf* 2.0 region. The fragment *ptcf* 0.4 (–1165/–780 bp: containing the 0.25 kb conserved sequence) was able to mimic endogenous gene expression into pigment cell precursors from the neurula stage (50-60% of the observed specimens) (Fig. 6A-C). Two complementary and partially overlapping deletions of *ptcf* 0.4 region, *ptcf* 0.21 (–1165/–958 bp) and *ptcf* 0.22 (–996/–780 bp), showed reduced activity in larval pigment cells (20-25%) in comparison with *ptcf* 0.4 (50-60%) (data not shown). These results revealed that Ci-*Tcf* expression in pigment cell precursors depends on elements present on both sub-regions. The entire *ptcf* 0.4 region was thus subjected to bioinformatic analyses in order to identify putative conserved cis-acting modules. These analyses revealed the presence of four consensus ETS-binding sites (GGAA) (Fig. 6D). Three of these ETS elements (2, 3 and 4) fell within the 0.25 kb *C. savignyi* shared region, with element 2 and 4 conserved in their respective positions. The functionality of *ptcf* 0.4 ETS-binding sites has been tested in vivo. Single point mutations (Bertrand et al., 2003) performed separately on each putative ETS element, revealed that whereas mutated site 1 showed no change in transgene expression (Fig. 6E), a consistent reduction in transgene expression was observed upon mutating sites 2, 3 and 4, in comparison with unaltered *ptcf* 0.4. Combined mutation on sites 2, 3 and 4 completely abolished *ptcf* 0.4 enhancer activity (Fig. 6E), thus indicating that the putative ETS-binding sites 2, 3 and 4 are necessary and collectively cooperate to drive full *Ci-Tcf* expression in the pigment cell lineage.

The FGF downstream effector *Ci*-Ets1/2 is involved in *Ci-Tcf* transcriptional regulation

The FGF and the Wnt signaling downstream effectors *Ci-Ets1/2* and *Ci-Tcf* are contemporarily expressed in *C. intestinalis* pigment cell precursors at the neurula stage. Moreover, both signaling cascades are involved in pigment cell development, intervening in both induction and differentiation processes. (l) The identification of functional ETS binding sites on *Ci-Tcf* regulatory region strongly suggests a direct control of FGF signaling on *Ci-Tcf* expression at the transcriptional level. In a first attempt to verify this interaction, we evaluated *Ci*-Ets1/2 affinity for ETS binding sites located on the *Ci-Tcf* regulatory region. Single point mutation of *ptcf* 0.4 indicated site 2 as the stronger ETS element for *Ci-Tcf* expression in pigment cell territory; therefore, we focused on examining *Ci*-Ets1/2 DNA-binding domain (DBD) affinity for site 2. Gel shift assay confirmed specific interaction between *Ci*-Ets1/2 DBD and

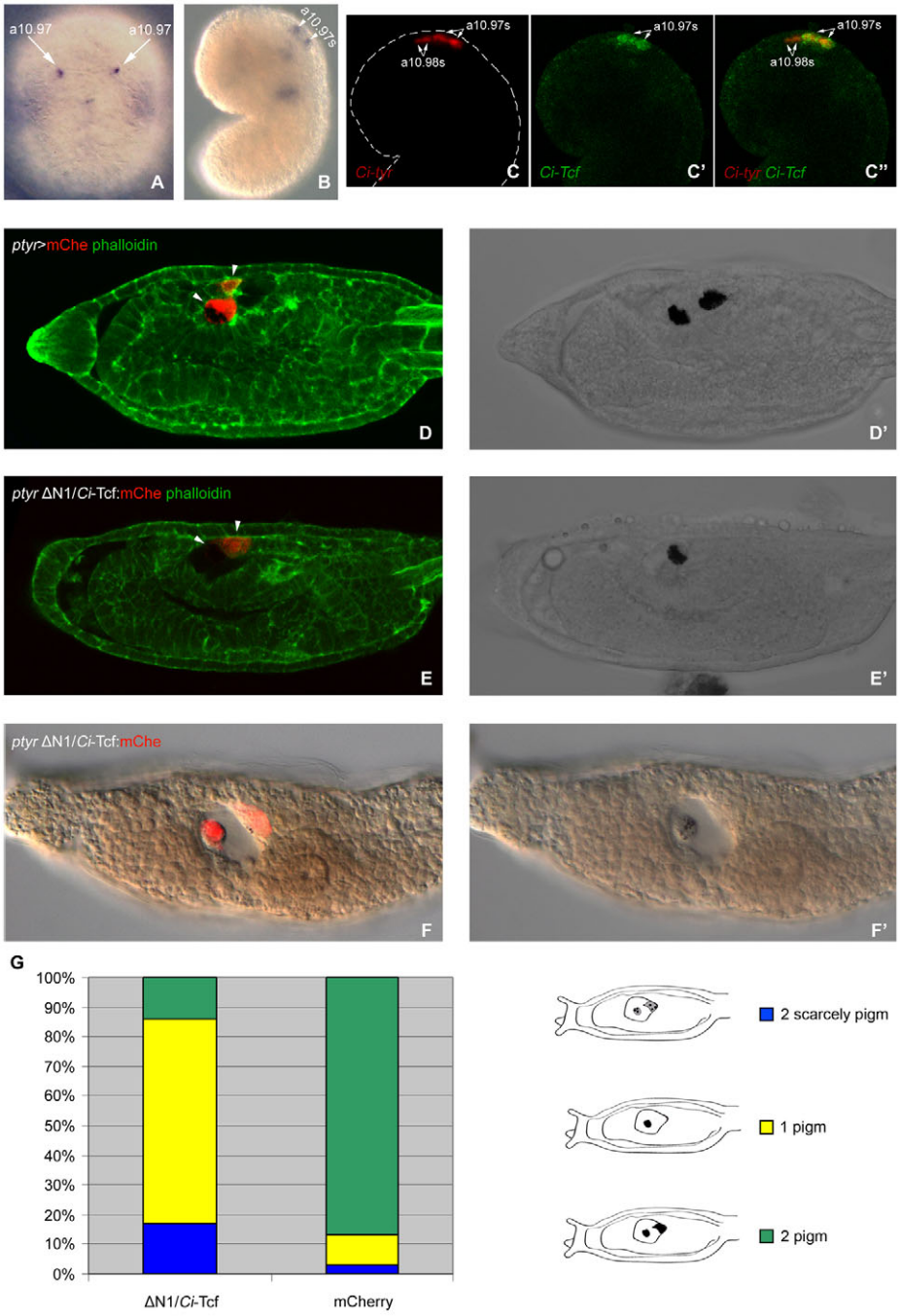

Fig. 5. *Ci*-TCF expression and function in pigment cell precursors. (**A**,**B**) *Ci-Tcf* expression in pigment cell precursors at the mid-neurula and early tailbud stages (A,B, white arrows). (**C-C‴**) Confocal images of a tailbud embryo (lateral view, anterior is towards the left) hybridized with both *Ci-tyrosinase* (C, red) and *Ci-tfc* (C′, green) antisense probes. The merged image (C″) shows *Ci-Tcf* and *Ci-tyrosinase* co-expression of in the posterior a10.97 pair. (**D**,**D′**) Transgenic *ptyr*>mCherry and (**E**,**E′**) *ptyr*> ΔN1/*Ci*-Tcf:mChe larvae. Lateral view, anterior is towards the left. mCherry is localized in pigment cell lineage in D,D′, and in a non-pigmented cell in E,E′. (**F**,**F′**) Merged bright-field/fluorescent and bright-field images of a *ptyr*>ΔN1/*Ci*-Tcf:mChe transgenic larva showing mCherry expression (F) in two poorly melanized cells (F′). (**G**) Percentage of *ptyr*>ΔN1/*Ci*-Tcf:mChe transgenic larvae showing two scarcely pigmented cells (blue), one pigment cell, either otolith or ocellus (yellow), or two pigment cells (green), as illustrated schematically on the right.

ptcf 0.4 ETS binding site 2 (Fig. 7, lanes 2-5). Furthermore, the same single nucleotide substitution in the ETS-binding site 2, which disrupted reporter gene expression, also inhibited competition by unlabeled oligonucleotide (Fig. 7, lanes 6-8). To evaluate this interaction in vivo, we treated transgenic *ptcf*0.4>GFP embryos with U0126, from the late gastrula stage, and compared the results with *ptcf*0.4>GFP transgenic embryos in terms of GFP expression. The results revealed that U0126 treatment almost completely abolishes pigment cell formation and blocks GFP transcription under the *ptcf*0.4 enhancer, indicating FGF/MEK1/2 involvement in *Ci-Tcf* transcriptional regulation (Fig. 8A). Additionally, *ptcf*0.4>GFP/*ptyrp1a*>Ets:WRPW transgenic embryos showed that targeted interference with *Ci*-Ets1/2 function in pigment cell precursors also strongly reduced the activity of the *ptcf*0.4 enhancer (marked by expression of GFP), compared with the control larvae (Fig. 8A), confirming the participation of *Ci*-Ets1/2 factor in *Ci-Tcf* transcription. As further evidence, targeted expression of the constitutive repressor form of *Ci*-Ets1/2 (Ets:WRPW) caused a reduction or absence of endogenous *Ci-Tcf* expression (Fig. 8B-D).

FGF-Wnt crosstalk in pigment cell formation

In the canonical Wnt signaling pathway, the inactivation of GSK3 leads to the nuclear accumulation of β-catenin, which promotes LEF/TCF-dependent transcription and functions as a transcriptional co-activator. Studies of other Wnt pathway components include the use of lithium chloride (LiCl) as a GSK3 inhibitor, with consequent stabilization of free cytosolic β-catenin (Klein and Melton, 1996;

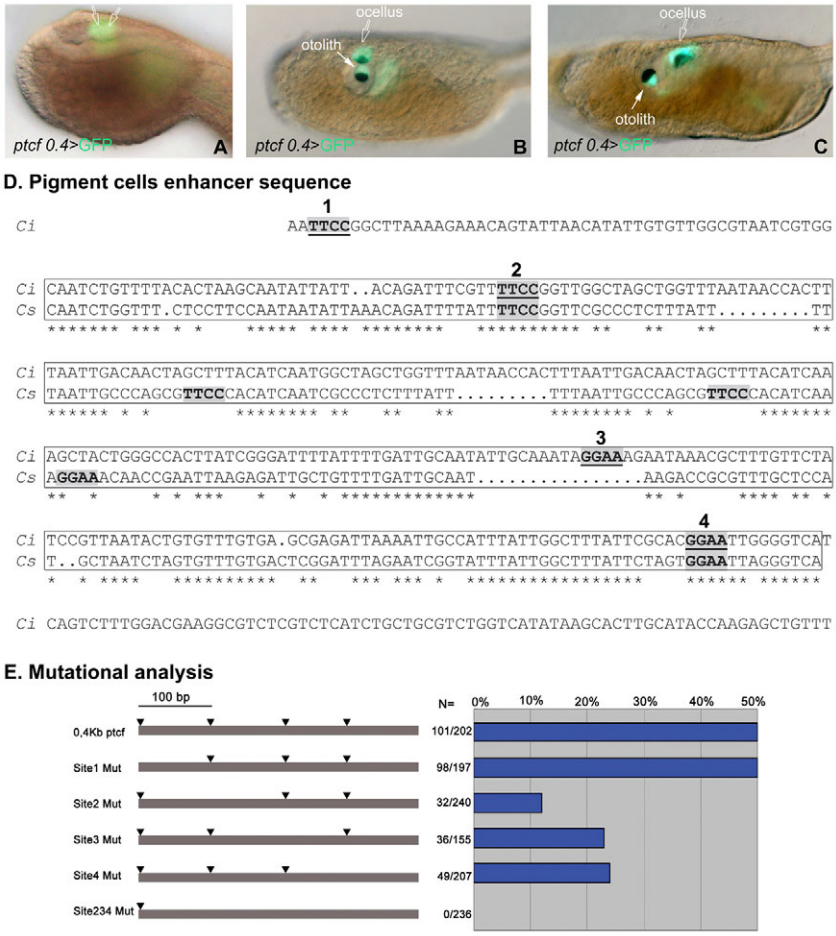

Fig. 6. Identification of pigment cell *Ci-Tcf* enhancer. (**A-C**) Merged bright-field/fluorescent images of GFP expression driven by the *ptcf0.4* enhancer at the tailbud (A), early (B) and middle (C) larval stages. Lateral view, anterior is towards the left. Transgene expression occurs in the pigment cell lineage (arrows). (**D**) Phylogenetic footprinting of the ETS-element (gray boxes) between *C. intestinalis* (Ci) and *C. savignyi* (Cs). Stars indicate conserved nucleotides. (**E**) Mutational analysis. In the left-hand diagram, gray bars indicate the *ptcf* 0.4 fragment and the arrowheads indicate ETS-binding sites (numbered 1-4). An absence of arrowheads means mutation in the corresponding ETS-binding site [GGAA to AGAA (Bertrand et al., 2003)]. The histogram on the right displays the proportions of embryos showing transgene expression (N=total embryos).

Stambolic et al., 1996), and the creation of stabilized β-catenin that lacks phosphorylation sites and thus is much more resistant to degradation (Yost et al., 1996). LiCl and stabilized β-catenin were therefore tested in *C. intestinalis* embryos to determine whether the correlation between FGF signaling and *Ci*-TCF activation can be framed into the more general mechanism of a FGF/Wnt signaling crosstalk for pigment cell differentiation.

LiCl treatment or targeted expression of stabilized β-catenin (*ptyrp1a*>Sβcat) in transgenic *ptyrp1a*>Ets:VP16 embryos resulted, in both cases, in the presence of extranumerary pigment cells in a much higher percentage compared with the single *ptyrp1a*>Ets:VP16 transgenic embryos (Fig. 9A-D). Notably, LiCl treatment or targeted expression of stabilized β-catenin (*ptyrp1a*>Sβcat) alone (without *ptyrp1a*>Ets:VP16) both result in normal larvae having two pigment cells that frequently appear highly melanized (data not shown). Collectively, these results reveal that FGF signaling activates the transcription of *Ci-Tcf,* which in turn acts as a downstream effector of the canonical Wnt signaling cascade in pigment cell formation.

DISCUSSION

Here, we describe the functional roles of FGF and the canonical Wnt signaling pathways in *C. intestinalis* sensory organ pigment cell development. Previous studies have demonstrated the temporal events in which ascidian pigment cell precursor specification and differentiation take place; however, they have not given many clues on the molecular events controlling these processes (Darras and Nishida, 2001). Our results show that the FGF signaling pathway, via the MAPK cascade, is the inductive signal responsible for conditional pigment cell precursor specification, whereas canonical Wnt signaling plays a role in pigment cell differentiation. Moreover, we show that during these processes, the two signaling pathways cross talk in a novel, previously unknown, manner that involves FGF cascade-dependent transcriptional control of the Wnt downstream effector *Ci-Tcf*.

Time requirement for induction of pigment cell precursors

During a time window spanning from the onset of gastrulation until the end of neurulation, the FGF signaling cascade appears widely activated in *C. intestinalis* neural plate cells. Previous cell-dissociation and recombination studies, using other ascidian species, suggest that specification of pigment cell precursors is accomplished between the early and the middle gastrula developmental stages (Nishida and Satoh, 1989). Our data derived from studies of *C. intestinalis* whole embryos, demonstrate that specification of pigment cell precursors, in vivo, extends for a longer time period. Inhibition of the inducing signal at the neurula developmental stage causes failure of pigment cell precursor specification. The discrepancy between the data presented here and previous studies could reside in the diverse experimental conditions, with the whole embryo making the difference with respect to dissociated cells. Embryonic integrity is most probably fundamental to supply the proper cellular context needed for the appropriate pigment cell precursor specification. However, species-specific differences between the two model systems cannot be excluded.

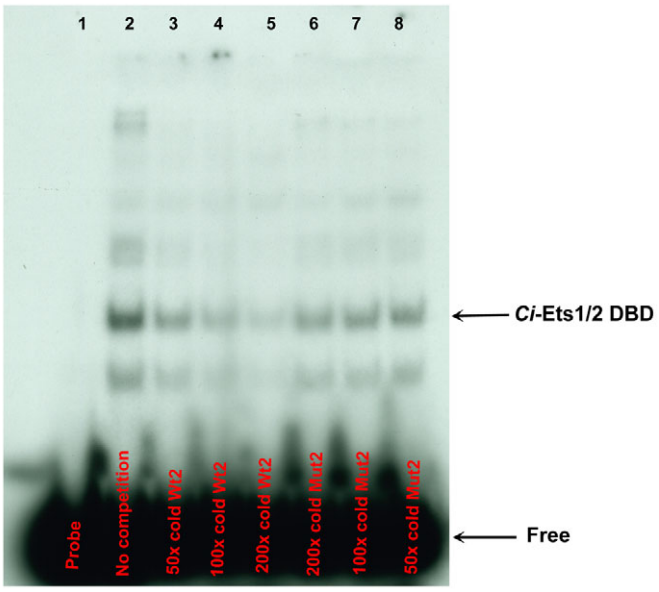

Fig. 7. Interaction between *Ci*-Ets1/2 (DBD) and *ptcf 0.4* ETS-binding site 2. A shifted band (indicated by the *Ci*-Ets1/2DBD, arrow) is observed in lane 2 where *Ci*-Ets1/2 (DBD) protein was incubated with the labeled Wt2 oligonucleotide. DNA-protein complex formation was inhibited by incubation with 50, 100 and 200-fold molar excess of Wt2 unlabeled oligonucleotide (lanes 3-5). Competition by 50, 100 and 200-fold molar excess of unlabeled mutated oligonucleotide Mut2 fails to inhibit binding (lanes 6-8). Free indicates unbound labeled oligonucleotide.

The source of inducing signal

At the late gastrula stage, the *C. intestinalis* neural plate is well organized in a grid-like fashion, in which cells are located in invariant positions. In this context, the pigment cell precursors (a9.49 cell pair of the neural plate) are in close proximity to two sources of FGF diffusible signaling molecules, *Ci*-FGF8/17/18 and *Ci*-FGF9/16/20. These molecules are released from the lateral and medio-central nerve cord precursor cells of the underneath row, respectively (Hudson et al., 2007). At the mid-neurula stage, the last division of the a9.49 pairs gives rise to the a10.97 and a10.98 pairs, of which only the a10.97 cell pair becomes pigment cells. FGF molecules act at a very short range from the target cells (Tassy et al., 2006); thus, the contact distance between the source and the target cell is a fundamental requirement for the induction mediated by FGF signaling. Intriguingly, morphogenetic rearrangements that build the *C. intestinalis* embryo morphology push the a10.98 blastomeres in a more rostral position, outdistancing them from FGF sources. In contrast, the a10.97 cell pair remains within the FGF signaling range. The fact that only the a10.97 cells, and not their posterior sister cells a10.98, develop into *C. intestinalis* pigment cells could be framed in this context. Moreover, during the last a9.49 cell pair division, FGF responsiveness could be differentially inherited by the a10.97 and a10.98 cell pairs, contributing to cell-specific fate diversification.

The mechanism of induction

Starting from early gastrulation, the inductive FGF signal is released from the A-nerve cord precursors to the future pigment cells. The signal is transmitted in the cells through the MAPK cascade, which in turn activates the transcription factor *Ci*-Ets1/2. A first important finding, related to *Ci*-Ets1/2, concerns its presence in the a9.49 pair at the neural plate stage at the right time and place to behave as FGF signaling effector, serving to instruct the fate of the a9.49 pair down the pigment cell precursor lineage. With next cellular division, *Ci*-Ets1/2 is partitioned between the two a9.49 cell-progeny, of which the a10.97 pair remains physically close to FGF-emitting sources. This strict contact would retain *Ci*-Ets1/2 activation in only the a10.97 blastomeres, leading to initiation of the pigment cell-specific program. Our findings support this interpretation, highlighting the importance of FGF signaling through *Ci*-Ets1/2 in determining pigment cell fate. Indeed, FGF signal inhibition, both by drug treatment and targeted interference with *Ci*-FGF receptor or *Ci*-Ets1/2 function, results in embryos in which the pigment cell program is blocked. Remarkably, alteration of pigment cell fate is not accompanied by apoptosis. Perturbed cells survive; however, they assume an incorrect position inside the larval brain vesicle, aligning on the dorsal side of sensory vesicle wall. These data indicate that FGF signal induction is indispensable for both pigment cell fate determination and to settle on their final

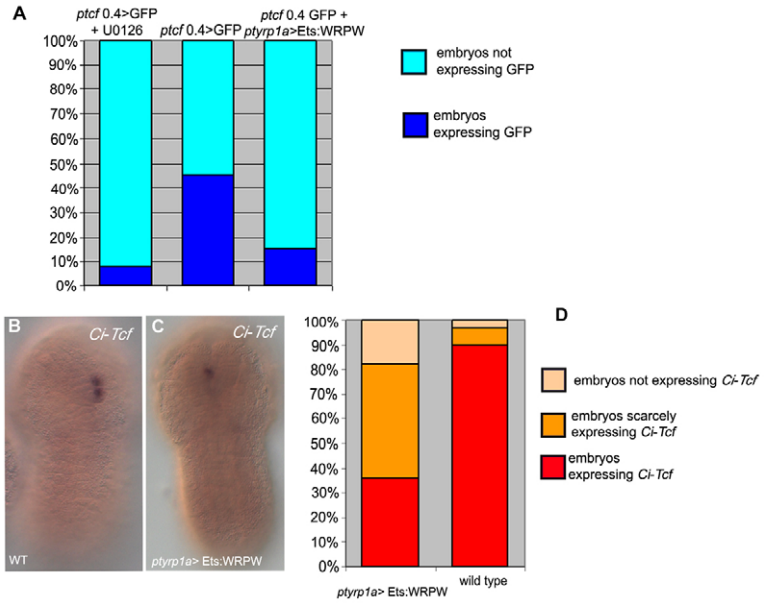

Fig. 8. *Ci*-Ets1/2 involvement in *Ci-Tcf* transcriptional regulation. (**A**) Percentage of *ptcf* 0.4>GFP expressing larvae treated with U0126 or co-expressing *ptyrp1a*>Ets:WRPW. GFP expression driven by the *ptcf* 0.4 enhancer is blocked by U0126 treatment and in double *ptcf* 0.4>GFP/*ptyrp1a*>Ets:WRPW transgenic embryos. (**B**,**C**) Ets:WRPW transgenic embryos show also a reduction (C) or the absence of endogenous *Ci-Tcf* expression compared with control (B). (**D**) Percentage of embryos expressing *Ci-Tcf* in wild type and in *ptyrp1a*>Ets:WRPW transgenic condition.

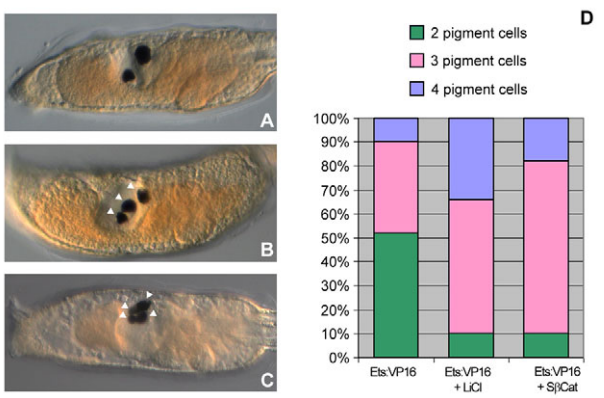

Fig. 9. FGF-Wnt signaling interaction in pigment cell formation. (**A-D**) LiCl treatment or targeted expression of stabilized β-catenin (*ptyrp1a*>Sβcat), in transgenic *ptyrp1a*>Ets:VP16 embryos, results in a much higher percentage of larvae with three or four pigment cells, compared with the *ptyrp1a*>Ets:VP16 transgenic larvae, as indicated in D. A representative of control larva (A) with two pigment cells, or of transgenic larvae with three (B) or four (C) pigment cells (lateral view, anterior towards the left). Arrowheads indicate the pigment cells.

location in the sensory vesicle. Ectopic activation of *Ci*-Ets1/2, in a10.98s and a10.97 sibling pair, results in supernumerary pigment cell formation, further supporting this hypothesis. Studies on *C. intestinalis* cardiac development showed a similar dynamic action of FGF signaling, through *Ci*-Ets1/2 asymmetrical activation. The B7.5 lineage gives rise to both heart and anterior tail muscle precursors and this cell fate decision depends on *Ci*-Ets1/2 activation in future heart, but not in tail muscle precursors. Symmetrical *Ci*-Ets1/2 activation in B7.5 lineage is sufficient to transform proximal tail muscles into supernumerary heart cells, and eventually causes the formation of additional functional heart tissue in juveniles. On the other hand, FGF signal inhibition, both at the level of the *Ci*-FGF receptor and of *Ci*-Ets1/2 activity, blocks heart field specification and cardiac precursor migration (Davidson et al., 2006). Analogous cell migrating defects have been observed in our system. It has been further demonstrated that FGF signaling also presides over mechanisms of heart cell migration, contributing to the formation of protrusions on migrating cell membranes (Christiaen et al., 2008). The aforementioned correlations encourage further investigations into whether or not FGF-mitigated migration can be extended to pigment cell formation.

Canonical Wnt signaling is involved in sensory organ pigment cell differentiation through the action of *Ci*-TCF

In the *C. intestinalis* genome, a single member of the TCF/LEF family of transcription factors, *Ci-Tcf*, has been identified. *Ci-Tcf* is probably a direct ortholog of the single primordial *tcf* gene that gave rise to the four genes present in mammals, after the two genome-duplication events that occurred in the evolution of vertebrates (Holland et al., 1994). In the *C. intestinalis* pigment cell lineage, *Ci-Tcf* expression appears exclusively in the a10.97 blastomeres, where it remains confined up to the late tailbud developmental stage. Interestingly, all pigment cell lineage-specific genes identified so far, such as *Ci-tyr* and *Ci-tyrp1a*, are expressed in both a10.97 and a10.98 cell pairs. *Ci-Tcf* is the only gene so-far identified, specifically localized in only the a10.97 pair, thus offering insights on the molecular events involved in pigment cell differentiation in *C. intestinalis*. In zebrafish, experimental perturbation of TCF/LEF functions, through injection of β-catenin domain-truncated *tcf3* mRNA into single premigratory neural crest cells, promotes neuronal fate at the expense of pigment cells (Dorsky et al., 1998). This evidence parallels our finding in *C. intestinalis* where targeted perturbation of *Ci*-TCF activity disrupts the pigment cell program. *C. intestinalis* TCF-perturbed cells retain their respective positions in the larval brain but present a range of defective pigmentation phenotypes, strongly suggesting a role for *Ci*-TCF in pigment cell terminal differentiation. Variability of the observed phenotypes coherently supports the proposition that the pigmentation process may require threshold levels of *Ci*-TCF protein. In addition our data point to *Ci*-TCF acting in conjunction with β-catenin as downstream effector of Wnt signaling in pigment cell differentiation. Indeed, LiCl treatment or targeted expression of stabilized β-catenin (*ptyrp1a*>Sβcat) in transgenic *ptyrp1a*>Ets:VP16 embryos resulted in a higher percentage of larvae showing extranumerary pigment cells compared with the single *ptyrp1a*>Ets:VP16 transgenic embryos. It is easy to suppose that this finding is related to the increased availability of a stable β-catenin, to act as a transcriptional co-activator, compared with the pool of unstable β-catenin usually present in the cells.

FGF and Wnt: a new mode of interaction

FGF and Wnt signaling cascades can elicit many biological effects in different cell types through the transcriptional regulation of specific subsets of genes. These cascades work through signal transduction pathways that ultimately result in the activation of specific downstream effectors. The key point therefore is the availability in the responding cells, at the right time, of these target effectors whose action determines the appropriate response to the input. Here, we have clarified the mechanism by which *Ci*-TCF appears ready, in pigment cell lineage, to function as context-dependent regulator of Wnt signaling. In classical terminology this phenomenon can be referred to as ‘competence’, i.e. FGF signal makes a10.97 cells able to respond to Wnt signal, by directly controlling *Ci*-Tcf transcription through *Ci*-Ets1/2 that represents the first *Ci*-TCF upstream regulator identified so far. This is a novel finding in the light of FGF-Wnt crosstalk and opens new perspectives for studies centered on the mechanisms by which different inputs are intertwined to control the specificity of cellular responses.

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Competing interests statement
The authors declare no competing financial interests.

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