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

Paola Squarzoni*,†, Fatema Parveen‡, Laura Zanetti, Filomena Ristoratore‡,§ and Antonietta Spagnuolo‡,§

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 (Schaeremeyer, 1996). Melanin-producing cells cover disparate functional roles, spreading from immune response to photoprotection (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).
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, pigment gland and melanocytes (Tief et al., 1996), express the melanogenic genes tyrosinase (*tyr*) (Caraciolo 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; Caraciolo 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 (Husdon et al., 2003) and patterning of the caudal neural plate along the anterior posterior axis (Husdon 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 (Waslyky 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-diamo-no-2,3-dicyano-1,4-bis[2-amino-phenylthio]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, *ptyrpla* 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-tyr*, *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- *Ci-Ona* β 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: 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- *Ci-Ona* β crystallin antibody was kindly provided by Dr Sebastian M. Shimeld (Department of Zoology, University of Oxford, UK).

**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-tyr5a and Ci-tyr 5′ flanking regions were PCR-amplified from genomic DNA using the primers: *Ci-ptyrpF/Ci-ptyrpR*, and *Ci-ptyrp/Ci-ptyrR*, respectively.

For *ptyr>*FGFRDN, the MESP promoter was replaced with the *ptyr* 5′ fragment in a MESP>*FGFRDN* vector (a generous gift from L. Christianen, NYU School of Medicine, New York, USA) using the PCR-incorporated XbaI and NotI 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>*FGFRDN vector, previously digested with *NotI* and EcoRI to eliminate *FGFRDN*.

For *ptyr>*FGFRDN*mChe, *FGFRDN*, which lacks the STOP codon, was amplified by PCR from MESP>*FGFRDN* template using the primers *FGFRDNFw* and *FGFRDNRev*, and double digested with *NotI* and BamHI.
Restoration 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 BamHI and EcoRI restriction enzymes. The fragments Notl-FGFRDN-BamHI and BamHI-mCherry-EcoRI were then fused in-frame downstream from ptyr promoter, replacing FGFRDN in the ptyr-FGFRDN vector, previously digested with Notl/EcoRI to eliminate FGFRDN.

For ptyr1a>mChe, ptyr1a>FGFRDN, ptyr1a>FGFRDN>mChe, ptyr1a>Ets:VP16, ptyr1a>Ets:WRPWP and ptyr1a>ΔN1/Ci-Tcf>mChe, the 5’ ptyr1a fragment replaced the ptyr fragment (in the constructs ptyr>mChe, ptyr>FGFRDN, ptyr>FGFRDN>mChe and ptyr>ΔN1/Ci-Tcf>mChe) or the MESP promoter (in the constructs MESP>Ets:VP16 and MESP>Ets:WRPWP, kindly provided by Brad Davidson, University of Arizona, Tucson, AZ, USA), previously digested with HindIII-NotI to eliminate ptyr or MESP enhancers.

For ptyr1a>Sßcat-mChe, stabilized β-catenin (Sßcat) lacking the N-terminal (1-47 amino acid residues) that includes putative phosphorylation sites, was amplified using the oligonucleotides SßcatFw and SßcatRev. Sßcat, digested with Notl/BamHI was inserted in place of FGFRDN in the construct ptyr1a>FGFRDN>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 Notl/BamHI was inserted in place of FGFRDN in the construct ptyr>FGFRDN>mChe. pTEF 2.0-GFP. 2.0 kb of the 5’ flanking Ci-Tcf region was PCR-amplified from genomic DNA using the oligonucleotides ptef1 and ptef9 (see Table S2 in the supplementary material) and cloned into a pBS-GFP vector upstream from gfp. pTEF 0.4, pTEF 0.21 and pTEF 0.22 fragments were all generated by PCR amplification, using appropriate primers (cf0.4/0.4c, cf0.4/0.4c2r and c2r/c2r0.4, respectively; see Table S2 in the supplementary material) and cloned into a pBS plasmid containing the Epstein Barr virus TATA (E1TATA) upstream from GFP (Leong et al., 1988; Parks et al., 1988; Polickey et al., 1995). This construct is transcriptionally inactive and functions in C. intestinalis as minimal promoter (A.S., unpublished). The pTEF 0.4 regulatory sequence was searched for transcription factor binding sites by using Genomatix software (http://www.genomatix.de). Point mutations were introduced in pTEF 0.4 regulatory sequence, using the QuickChange 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 (W2) or mutant (M2) 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 cell precursors (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-tyrpl and Ci-tyr, are expressed specifically in pigment cell lineage up to the larval stage. The Ci-tyrpl 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 (ptyrpl and ptyr) that are able to recapitulate endogenous Ci-tyrpl and Ci-tyr expression patterns (F.R. and
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\textsuperscript{DN}) in the pigment cell precursors (Davidson et al., 2006). Targeted expression of FGFR\textsuperscript{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\textsuperscript{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\textsuperscript{DN}mCherry* fusion protein under the control of the *ptyrp1a* regulatory region (*ptyrp1a>FGFR\textsuperscript{DN}mChe*), was used to identify cells in which the endogenous FGFR function was perturbed. *ptyrp1a>FGFR\textsuperscript{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; Waslylk 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\textsuperscript{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’). Remarkably, we noted that the contemporary block of *Ci-FGFR* and *Ci-Ets1/2* activity (*ptyrp1a>Ets:WRPW plus ptyrp1a>FGFR\textsuperscript{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\textsuperscript{DN} in pigment cell precursors. The constitutively active form of *Ci-Ets1/2* (Ets:VP16) was able to induce pigmentation in the FGFR\textsuperscript{DN} background, rescuing to a large extent the FGFR\textsuperscript{DN} perturbation (Fig. 4F). This result indicates that these two molecules belong to the same pathway and cooperate in pigment cell induction.
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 (∆N1/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 ∆N1/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 ∆N1/Ci-Tcf expression, by using, as further control, the ptyr>p1a 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
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 precursors; 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
ptcf0.4 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 ptcf0.4>GFP embryos with U0126, from the late gastrula stage, and compared the results with ptcf0.4>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 ptcf0.4 enhancer, indicating FGF/MEK1/2 involvement in Ci-Tcf transcriptional regulation (Fig. 8A).

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;
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.
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 position.
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 C-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’s 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>β-catenin) 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.

**Supplementary material**

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