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Evolution of Developmental Control Mechanisms

Ephrin-mediated restriction of ERK1/2 activity delimits the number of pigment cells in the *Ciona* CNS

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ABSTRACT

Recent evidence suggests that ascidian pigment cells are related to neural crest-derived melanocytes of vertebrates. Using live-imaging, we determine a revised cell lineage of the pigment cells in *Ciona intestinalis* embryos. The neural precursors undergo successive rounds of anterior–posterior (A–P) oriented cell divisions, starting at the blastula 64-cell stage. A previously unrecognized fourth A–P oriented cell division in the pigment cell lineage leads to the generation of the post-mitotic pigment cell precursors. We provide evidence that MEK/ERK signals are required for pigment cell specification until approximately 30 min after the final cell division has taken place. Following each of the four A–P oriented cell divisions, ERK1/2 is differentially activated in the posterior sister cells, into which the pigment cell lineage segregates. Eph/ephrin signals are critical during the third A–P oriented cell division to spatially restrict ERK1/2 activation to the posterior daughter cell. Targeted inhibition of Eph/ephrin signals results in, at neurula stages, anterior expansion of both ERK1/2 activation and a pigment cell lineage marker and subsequently, at larval stages, supernumerary pigment cells. We discuss the implications of these findings with respect to the evolution of the vertebrate neural crest.

Introduction

The ascidian pigment cells form part of the ocellus and the otolith, light and gravity sensing organs, respectively (Jiang et al., 2005; Tsuda et al., 2003). Previously, the ascidian pigment cells have been proposed as the evolutionary precursors of the vertebrate retinal pigment epithelium or pineal organ, based on expression of gene markers and the probable function of the ocellus pigment cell in shielding the photoreceptors (Lamb et al., 2007; Sato and Yamamoto, 2001). However, recent evidence using a larger panel of genes supports a closer evolutionary relationship to the cephalic neural crest of vertebrates (Abitua et al., 2012; Ivashkin and Adameyko, 2013). This hypothesis is strengthened by the acquisition of migratory properties upon the misexpression of Twist, a cell behavior exhibited by neural crest.

Of the four founder embryonic lineages of *Ciona* (A- and B- of the vegetal hemisphere and a- and b- of the animal hemisphere), the central nervous system (CNS) derives from three lineages, A-, a- and b- (Nishida, 1987). This study is concerned with the a-line neural lineages, from which the pigment cells arise. At the 64- to 76-cell stage, the a-line neural lineages form one row of six cells (Fig. 1) (Lemaire et al., 2002). These cells then divide twice along the A-P axis to generate four rows of six cells at the 6-row neural plate stage (Fig. 1). The 6-row neural plate is arranged with row I posterior-most and row VI anterior-most (Nicol and Meinertzhagen, 1988). The a-line cells comprise rows III–VI (pink cells in Fig. 1) and the A-line cells, rows I and II. Of the a-line cells, only rows III and IV will undergo neurulation and contribute to the CNS. These two rows generate the anterior part of the sensory vesicle, which is the ascidian larval brain, and also contribute to the oral siphon primordium (Christiaen et al., 2007; Cole and Meinertzhagen, 2004; Nishida, 1987; Taniguchi and Nishida, 2004; Veeman et al., 2010). The anterior two rows, V and VI, generate the palps, an adhesive organ, as well as non-neural ectoderm. The pigment cell lineage is situated in the lateral-most cell of row III. Between the 6-row neural plate and the 12-row neurula stages,

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each row of cells divides in a specific sequence, with row II cells dividing first, followed by row I, then row III and V and lastly row IV and VI (Nicol and Meinertzhagen, 1988). In each row, cells in the medial four columns divide prior to those in the lateral columns. At the 12-row neural plate stage, the pigment cell lineage is segregated into the posterior row III (IIIp) cell, a10.97. As we will describe in this manuscript, the a10.97 pigment cell precursors undergo a further and final round of cell division along the A-P axis, whereby the posterior-positioned daughters, a11.193, intercalate at the midline to become the pigment cells of the otolith and ocellus.

FGFs are secreted ligands that bind to a class of receptor tyrosine kinases, the FGF receptors (FGFRs), and predominantly activate the Ras/MEK/ERK cascade. FGF/ERK signaling is implicated in a vast range of processes during vertebrate neural development including the induction of neural crest (Guillemot and Zimmer, 2011; Hébert, 2011; Stuhlmiller and Garcia-Castro, 2012). During ascidian neural development, FGF/ERK signaling is responsible for the specification of different cell types. It is involved in the process of neural induction of a-line neural lineages at the 32-cell stage (Bertrand et al., 2003) and later during patterning of the developing neural plate and neural tube (Abitua et al., 2012; Hudson et al., 2003, 2007; Squarzoni et al., 2011; Stolfi et al., 2011; Wagner and Levine, 2012). Recently, Eph/ephrin signaling via RasGAP has been shown to limit the spatial extent of activation of ERK1/2 during several developmental cell fate choices in *Ciona* embryos including during neural development (Haupaix et al., 2013; Ohta and Satou, 2013; Picco et al., 2007; Shi and Levine, 2008; Stolfi et al., 2011). Here we investigated the role of Eph/ephrin signaling and the MEK/ERK pathway during pigment cell specification at neural plate stages of *Ciona intestinalis* development.

Materials and methods

Embryo experiments and constructs

Adult *C.intestinalis* were purchased from the Station Biologique de Roscoff (France) or M-Rep (San Diego, CA). Cell nomenclature, lineage and the fate maps are previously described (Cole and Meinertzhagen, 2004; Conklin, 1905; Nishida, 1987). Ascidian embryo culture has been described (Sardet et al., 2011). Electroporation was carried out as described (Christiaen et al., 2009). U0126 (Calbiochem) was added to artificial seawater at 2 μM. All data was pooled from at least two independent experiments.

For the electroporation constructs, the upstream sequences *ZicL*, *Tyr*, *Mitf*, *Dmrt*, *Msx* and *Fog* are previously described (Abitua et al., 2012; Haupaix et al., 2013; Rothbächer et al., 2007; Wagner and Levine, 2012). These were used to drive expression of *LacZ*, *mCherry*, *Eph1ΔC*, *Histone 2B* (*H2B*), *unc76GFP*, *Eph3ΔC*, *RG*(*R818E*) (Abitua et al., 2012; Haupaix et al., 2013; Hudson et al., 2007; Stolfi et al., 2011; Wagner and Levine, 2012). Following electroporation, embryos were selected at gastrula stages for correct morphology. In Fig. 3, *Dmrt::Eph3ΔC* electroporated embryos were co-electroporated with *Trp::mCherry*. Only fluorescent (i.e. electroporated) embryos were counted for this experiment, in all other experiments, all embryos were counted. In order to assess ocellus and otolith pigment cell type, embryos were mounted in 80% glycerol and slightly compressed. Ocellus type or otolith type pigment cells were defined based on pigment granule morphology; such that tiny dispersed granules were defined as ocellus type and larger, round or flower shaped granules were defined as otolith. The number of ocellus type pigment cells was likely underestimated since it was more difficult to distinguish individual ocelli from one another. In both control and electroporated embryos, even very large ocelli were counted as one.

In situ hybridization and immunofluorescence

In situ hybridization was carried out as previously described (Hudson et al., 2013). Dig-labeled probes were synthesized from the following *Ciona* cDNA clones: *Trp* (Hudson et al., 2003), *FoxC* (cilv050a24) (Imai et al., 2004), *ZicL* (cicl002e04) (Imai et al., 2002b), *Mitf* (cilv41b12) (Abitua et al., 2012). *Six3/6* (cicl021e08), *Dll-B* (cicl022f04), *ephrinAc* (ciad074h16), *ephrinAb* (cieg037l08), *ephrinAd* (ciad008n17), *Eph3* (cieg009e01), *FgfR* (citb040h06), *Fgf9/16/20* (citb007k01) and *Fgf8/17/18* (citb002j04) are all described (Imai et al., 2004). Clone numbers refer to clones from the 'Release 1 Gene Collection Plates' (Satou et al., 2002). For *Dll-B* in situ hybridization, DAPI staining was used to verify cell identity. In some batches of embryos, *Dll-B* was also detected in row IV. These batches of embryos were removed from the analysis. For *Trp* in situ hybridization in Fig. 5, it should be noted that the intensity of the *Trp* signal increases with developmental time. At the 6-row neural plate stage, the color reaction was developed for approximately 12 h, whereas at the early tailbud stage, the color reaction time was 1 to 3 h.

dpERK1/2 immunofluorescence is described previously (Haupaix et al., 2013). LacZ immunofluorescence is described previously (Stolfi et al., 2011) except that anti-β-galactosidase (Molecular Probes, A11132) was used at 1/500 and the secondary antibody used was goat anti-rabbit Alexa Fluor 488 (Molecular Probes, A11008) at 1/1000.

In our analysis, we refer to embryos as the 6-row neural plate stage to indicate that no row of a-line cells has divided, though cells in row II have sometimes divided. We refer to the 12-row neurula stage to indicate that row III cells have divided. Embryos in Figs. 2 and 6 and Supplementary Fig. 1 were mounted in VECTASHIELD/DAPI (Vector Laboratories). Embryos in Figs. 2, 5–7 and Supplementary figures were photographed on an Olympus BX51 using Leica DFC310FX camera and those in Figs. 3 and 4G on a Zeiss Axio Imager AZ using a SPOT RT3 camera.

Time-lapse movie

The time-lapse sequence was obtained using a Zeiss LSM 700 microscope using a plan-apochromat 20 × objective. Confocal stacks contained 20 optical slices at a thickness of approximately 1 μm each and were taken every 3 min for 2 h. Images were rendered in 3D using Volocity 6 with the 3D opacity visualization tool.

Results

Differential activation of ERK1/2 in the neural plate is required for patterning along the A–P axis

It has previously been shown that differential ERK1/2 activity between rows III/IV (CNS) and V/VI (palp) at the 3-row neural plate stage (Fig. 1) accounts for acquisition of their distinct genetic programs (Wagner and Levine, 2012). ERK1/2 is subsequently differentially activated between rows III and IV at the 6-row neural plate stage with ERK1/2 active in row III cells (Fig. 2A) (Hudson et al., 2007). This pattern of ERK1/2 activation is required for the differential fate specification of these two sister rows (Fig. 2C). We inhibited MEK/ERK signal transduction by applying U0126, an inhibitor of the MAP kinase kinase, MEK1/2, at precise developmental time points. The following markers were used to assess neural plate patterning: *FoxC* (rows V and VI), *Dll-B* (epidermis and rows V and VI), *ZicL* (rows III and IV), *Six3/6* (row IV), *Mitf* and *Trp* (row III) (Abitua et al., 2012; Irvine et al., 2007; Wagner and Levine, 2012). As reported previously, application of U0126 at the 76-cell stage disrupts the choice between row III/IV and row V/VI fates

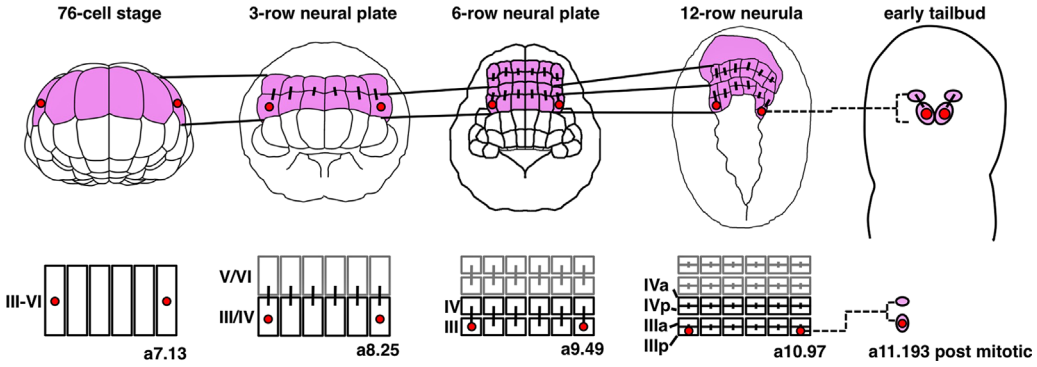

Fig. 1. The a-line neural lineages of *Ciona*. Throughout the indicated stages, the a-line derived neural plate cells are colored pink in the illustrated embryos. Below the embryo illustrations are simplified schematics of the a-line derived neural plate, with the row III-IV CNS lineages in black and the row V–VI palp lineages in gray. Bars indicate sister cell relationships. A red dot indicates the pigment cell lineage at each stage with the corresponding cell nomenclature indicated. After segregation from the non-neural ectoderm, the a-line neural lineage is a single row of six cells at the 76-cell stage. During gastrulation, the six a-line neural precursors divide along the A-P axis, making two rows of six cells. Together with the single row of A-line neural precursors, this makes a 3-row neural plate. All cells of the 3-row neural plate divide in a specific order to generate the 6-row neural plate (Nicol and Meinertzhagen, 1988). The 6-row neural plate then divide in an asynchronous but predictable order (see text for details) to produce 12-rows of neural plate cells in neurula embryos. At this stage the pigment cell lineage is positioned in the lateral column in row IIIp. This cell undergoes one final division to produce a11.194 and a11.193 prior to their intercalation at the midline (see also Supplementary movie 1 and Fig. 4).

(Wagner and Levine, 2012). Genes expressed in rows III and IV (*ZicL, Six3/6, Trp* and *Mitf*) were lost and genes normally restricted to rows V and VI (*FoxC* and *Dll-B*) were ectopically expressed in rows III and IV (Fig. 2B and C). In contrast, application of U0126 after the separation of rows III/IV and V/VI, at the early gastrula 3-row neural plate stage (approximate stage 11+ (Hotta et al., 2007)), resulted in loss of row III gene expression (*Trp, Mitf*) and ectopic expression of the row IV gene, *Six3/6,* in row III. Thus, inhibition of MEK1/2 signals at these two successive time points results, in each case, in a distinct posterior to anterior fate transformation of sister cells. In the first instance, differential activation of ERK1/2 specifies row III/IV over V/VI fate and then, following division of row III/IV cells, it promotes row III over row IV fates.

Eph/ephrin signals are not required for neural plate patterning at the 3- and 6-row neural plate stages

There are several examples during *Ciona* embryogenesis whereby Eph/ephrin signals control the spatial extent of ERK1/2 activation (Haupaix et al., 2013; Ohta and Satou, 2013; Picco et al., 2007; Shi and Levine, 2008; Stolfi et al., 2011). Three ephrin ligands are expressed in the neural lineages during neural plate and neurula stages and the receptor *Eph3* is expressed ubiquitously, becoming upregulated in the pigment cell lineage soon after the 6-row neural plate stage (Supplementary Fig. 1) (Imai et al., 2004; Tassy et al., 2010). These gene expression profiles suggest that Eph/ephrin signals could also be involved in the modification of ERK1/2 activity during neural plate patterning. In order to address this possibility, we expressed a dominant negative form of the Eph receptor Eph3, *Eph3ΔC* (Picco et al., 2007), in the a-line neural lineages. For this, we used the *Dmrt* promoter which drives expression throughout the a-line neural lineages from the 64-cell stage and has previously been used to disrupt gene function at the 3-row neural plate stage (Ikeda et al., 2013; Wagner and Levine, 2012). Following electroporation of *Dmrt::Eph3ΔC,* we observed that expression of *FoxC, Dll-B, ZicL, Six3/6, Mitf* and *Trp* were unaffected at the 6-row stage neural plate stage (Fig. 2B and C). Consistently, the spatial pattern of ERK1/2 activation at the 6-row neural plate stage was not perturbed in these embryos (Fig. 2A). We conclude that Eph/ephrin signals are not required to generate the spatial pattern of ERK1/2 activation in the neural plate during the 3-row or 6-row neural plate stages. A similar conclusion was reached analyzing embryos electroporated with an early-active promoter, *Fog,* to drive *Eph3ΔC* in all ectodermal cells from the 16-cell stage (Rothbächer et al., 2007) (Supplementary Fig. 2).

An extra cell division in the pigment cell lineage

Despite little effect on the neural plate genes analyzed, we consistently observed ectopic formation of pigment cells in larvae resulting from embryos electroporated with *Dmrt::Eph3ΔC* (Fig. 3). In some cases, five or more pigment cells could be observed. This result suggests that Eph/ephrin signals play a role in neural patterning. We thus set out to understand how Eph/ephrin signals are involved in this process.

In order to study the role of Eph/ephrin signals in pigment cell formation, we first observed the pigment cell lineage in detail, revealing a previously unrecognized cell division (Fig. 4, Supplementary movie 1). We analyzed the time-lapse sequence of embryos electroporated with *Dmrt::H2BYFP* (Histone 2B fused with YFP, magenta in images) to label rows III through VI and *ZicL::H2BCFP* (green in images) to label rows I through IV. These reporters are thus co-expressed in rows III and IV allowing for the delineation of cell lineages. Analyzing the time-lapse sequence revealed a further cell division of the a10.97 cell, which was previously described as a postmitotic pigment cell precursor (Cole and Meinertzhagen, 2004; Whittaker, 1979). The pigment cell lineage and cell positions in the neural tube are thus modified to account for this additional division (Fig. 4G). We also recognized this extra division by analyzing *Trp* expression every 15 min from the 6-row neural plate stage until the early tailbud stage (Fig. 5A-H). *Trp* expression begins at the 6-row neural plate stage in a9.49 (Fig. 5A). Following cell division of a9.49, *Trp* expression is observed in both daughter cells, but rapidly becomes stronger in the posterior-positioned a10.97 compared to its anterior sister cell, a10.98 (Fig. 5B–D). Next, a10.97 divides into a11.194 and a11.193, both cells retain *Trp* expression and converge towards the midline (Figs. 4D and 5E), touch (Figs. 4E and 5F) and begin to intercalate (Fig. 5G). Finally these cells are positioned with the two smaller a11.194 cells side by side anterior to the two larger a11.193 pigment cell precursors intercalated at the midline (Fig. 4F and G; Supplementary movie 1).

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2014.07.010.

MEK1/2 activity is required until approximately 30 min after the final division in the pigment cell lineage

Since patterning of the 6-row neural plate was not affected by inhibition of Eph/ephrin signaling (Fig. 2; Supplementary Fig. 2), we hypothesized that the supernumerary pigment cells observed following inhibition of Eph/ephrin signals resulted from a failure to

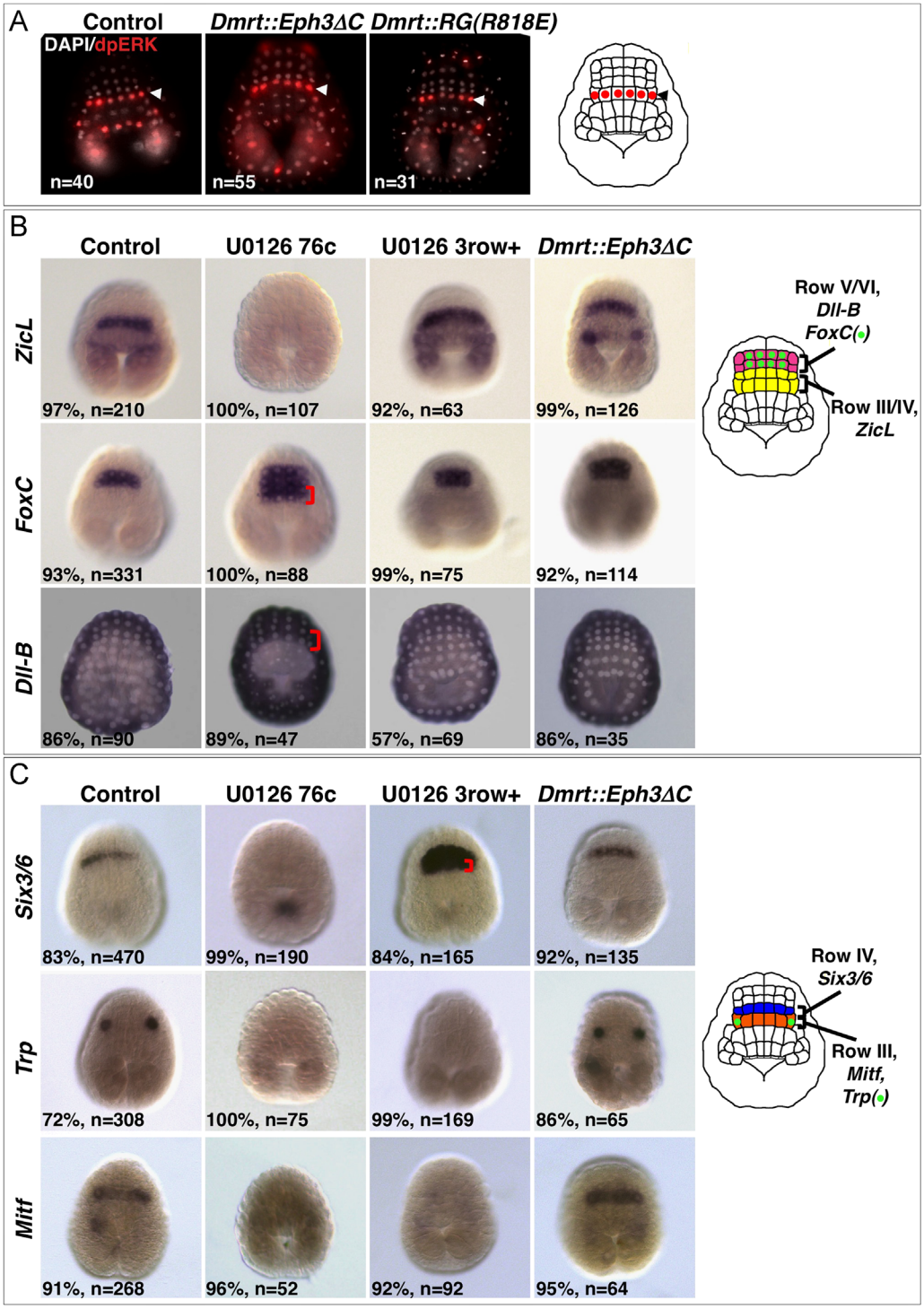

Fig. 2. Role of MEK1/2 and Eph/ephrin signals at the 3-row and 6-row neural plate stage. Experimental conditions are indicated above each column of panels. (A) dpERK1/2 immunofluorescence at the 6-row neural plate stage showing ERK1/2 activation in row III (arrowhead). (B, C) Analysis of gene expression following various treatments. U0126 was added to embryos at the 76-cell stage (76c), or at the late 3-row neural plate stage when endoderm invagination is well underway (3row+). An embryo illustration on the right shows the control expression domains. Percentages indicate the proportion of embryos that the panel represents. For the *Dll-B* U0126 3row+panel, the remaining embryos showed *Dll-B* expression in rows III and IV, although this was often weaker than in rows V and VI, and/or patchy. The red brackets indicate ectopic expression.

control the spatial extent of ERK1/2 activation after the 6-row neural plate stage. We first analyzed precisely when pigment cell specification is dependent on MEK/ERK signals. We applied U0126 to embryos every 15 min from the 6-row neural plate stage to the early tailbud stage, when pigment cell formation has been shown to occur independently of MEK1/2 signaling (Squarzoni et al., 2011). We monitored the precise developmental stage at which embryos were placed in U0126 by analyzing a sample of embryos at each time point for *Trp* expression (Fig. 5A–I). We then assessed the presence of pigmented cells in resultant larvae (Fig. 5J). This analysis shows that pigment cell formation requires MEK1/2 signaling until approximately 30 min after the pigment cell lineage has undergone its final cell

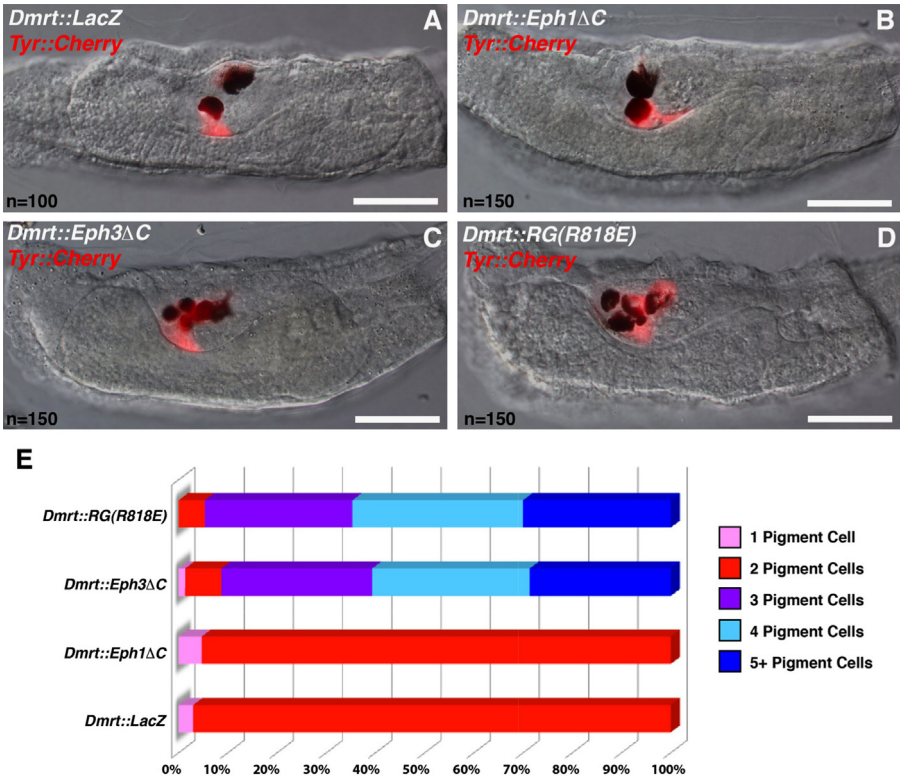

Fig. 3. Inhibition of Eph/ephrin signaling results in excess pigment cells. Embryos were electroporated with the constructs indicated on the panels. *Dmrt::Eph1ΔC* and *Dmrt::LacZ* were used as controls for electroporation, showing that the results were specific to disruption of Eph3 and RasGAP. Co-electroporation with *Tyr::mCherry* (red) shows that the pigment cells derive from *Tyrosinase* positive cells. The graph (E) shows the proportion of embryos with the number of pigment cells indicated in the key, following electroporation. In embryos exhibiting five or more pigment cells, it was difficult to distinguish each pigment cell, so these embryos were scored as 5+. Scale bars, 50 μm.

division (corresponding approximately to the embryonic stage shown in Fig. 5G). Following this time point, U0126-treatment had little effect on specification of pigment cells, albeit their pigmentation was somewhat reduced.

ERK1/2 is activated in the pigment cell lineage during neurulation

We showed that pigment cell specification requires an intact MEK1/2 signaling pathway until around 30 min after the final cell division of this lineage (Fig. 5). Consistent with a direct requirement for the pathway during pigment cell specification, we found that ERK1/2 remains active in the pigment cell lineage following the 6-row neural plate stage. When the neural plate consists of 12-rows of cells (in this analysis all row III cells and medial row IV cells had completed division), ERK1/2 activation was preferentially detected in the posterior daughters of rows III and IV (row IIIp and IVp) (Fig. 6A). At this stage, the pigment cell lineage is segregated into a10.97, the lateral-most cells of row IIIp (Figs. 1 and 6A). At the early tailbud stage (corresponding to the stage in Fig. 5F), we observed higher ERK1/2 activity in a11.193, the posterior daughter of a10.97, compared to the anterior daughter a11.194 (Fig. 6D). Thus, ERK1/2 is continuously differentially activated in the pigment cell lineage following each successive A–P oriented cell division, consistent with an ongoing and direct requirement for MEK/ERK dependent signals for pigment cell specification (Figs. 5 and 6).

Eph/ephrin signals restrict the spatial extent of ERK1/2 activation during the penultimate division of the pigment cell precursors

Differential ERK1/2 activity is involved in differential fate specification at both the 3-row and the 6-row neural plate stages (Fig. 2) (Wagner and Levine, 2012). We showed that Eph/ephrin signals are not involved in the spatial control of ERK1/2 or neural plate patterning at these stages (Fig. 2; Supplementary Fig. 2). However, we found that Eph/ephrin signals are required to restrict the number of pigment cells (Fig. 3). We also found that ERK1/2 is continuously activated in the pigment cell lineage during neurulation, until the early tailbud stage (Fig. 5). Focusing on the 12-row neural plate stage, we found that Eph/ephrin signals are required to restrict ERK1/2 activation to the posterior daughter cells of row III and IV (row IIIp and row IVp) (Fig. 6B). In *Dmrt::Eph3ΔC*-embryos, ERK1/2 activation was observed in both the anterior and posterior daughter cells of rows III and IV. This effect was particularly prominent in the pigment cell lineage with strong ectopic ERK1/2 activation in a10.98, the anterior sister cell of a10.97. Consistent with a critical role for Eph/ephrin signals in restricting pigment cell fate to the a10.97 lineage, we observed ectopic expression of *Trp* in a10.98 in *Dmrt::Eph3ΔC*-embryos (Fig. 7). In these experiments, we also occasionally (3% of embryo halves) observed persistence of *Trp* expression in row IV cells. It is not clear to us why this happens, since we do not see ectopic expression of row III genes in row IV at the 6-row stage in *Dmrt::Eph3ΔC* embryos. Similar results were obtained using *Fog::Eph3ΔC* (Supplementary Fig. 2). Conversely, treatment of embryos with U0126 from the 6-row neural plate stage resulted in a strong reduction of *Trp* expression (Fig. 7). Thus, Eph/ephrin signals are required to restrict ERK1/2 activity at the 12-row neurula stage, but not at earlier stages (3-row and 6-row).

The spatial control of ERK1/2 activation by Eph/ephrin signals is mediated by p120RasGAP in *Ciona* (Haupaix et al., 2013). This is also likely to be the case during pigment cell specification since electroporation of a dominant negative version of RasGAP, *RG (R818E)*, resulted in a similar phenotype of supernumerary pigment

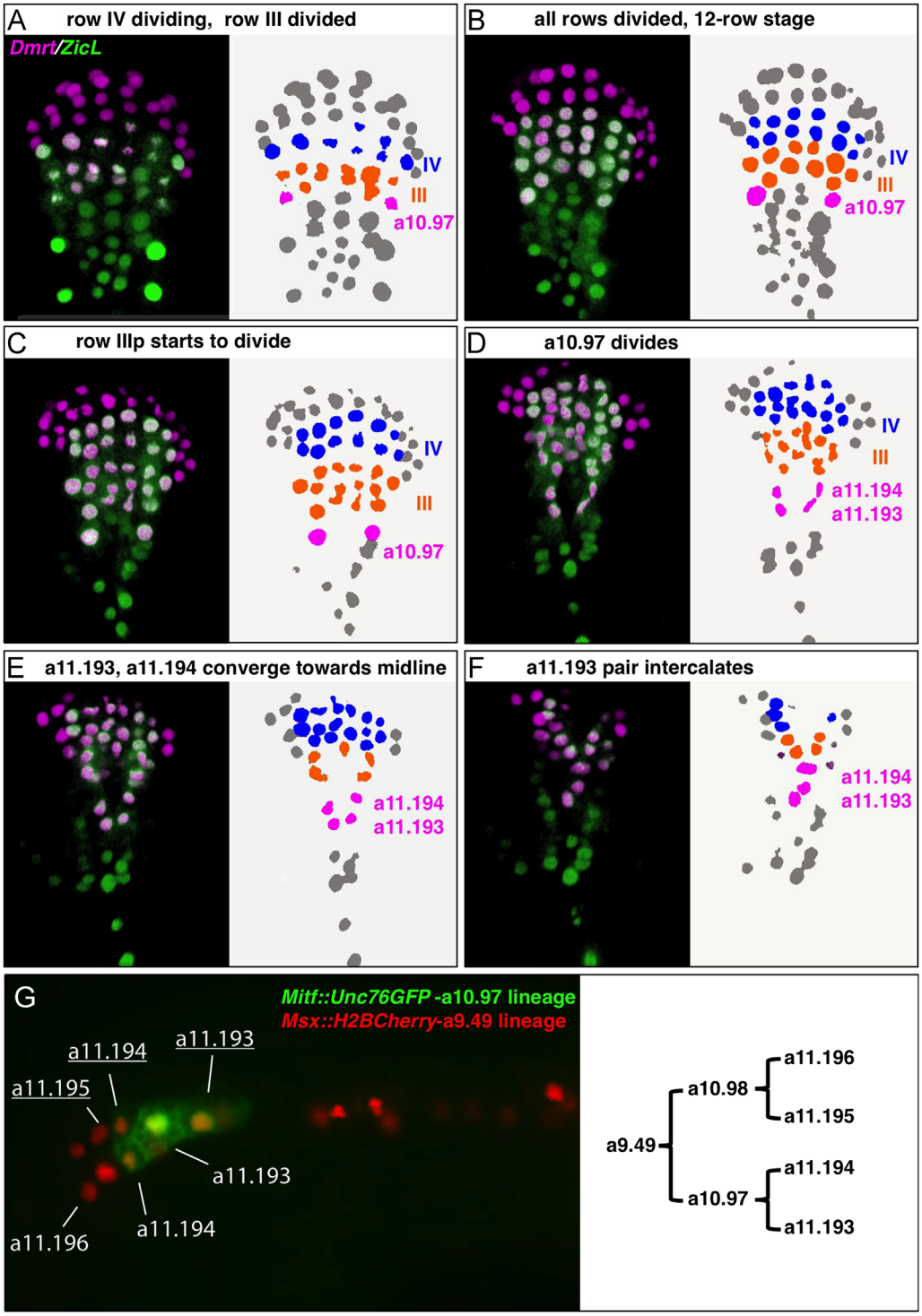

Fig. 4. A revised pigment cell lineage. This figure accompanies Supplementary movie 1. (A–F) Embryos were electroporated with *ZicL::H2BCFP* (green) to label rows I–IV and *Dmrt::H2BYFP* (magenta) to label rows III–VI. Still images from the movie are shown on the left. On the right are the same still images with false-labeled nuclei to highlight the positions of row III and IV derivatives in orange and blue respectively. The a10.97 lineage is marked in pink, highlighting the extra division (D). (G) An embryo that has been electroporated with *Mitf::Unc76GFP* (green), *Msx::H2BmCherry* (red) to show the lineage derivatives of a10.97 and a9.49 respectively. The position of each cell is labeled on the tailbud stage embryo.

cells in larvae and ectopic activation of ERK1/2 in 12-row neurula, but not 6-row neural plate stage embryos (Figs. 2A, 3 and 6C).

A role for Eph/ephrin at the 12-row stage is consistent with the formation of more than five pigmented cells that we frequently observed in *Dmrt::Eph3ΔC*-larvae, since transformation of a10.98 into a a10.97-like fate should result in a pool of a maximum of eight *Trp*-positive cells instead of the usual four. The presence of more than four pigmented cells also suggests that the subsequent fate choice between a11.194 and a11.193 is also perturbed in many *Dmrt::Eph3ΔC*-larvae.

Finally, we did not find any evidence that the choice between ocellus and otolith fates requires Eph/ephrin signals. In a pool of embryos electroporated with *Dmrt::Eph3ΔC*, we counted an average of 1.5 otolith and 1.4 ocellus-type pigmented cells ($n=299$)

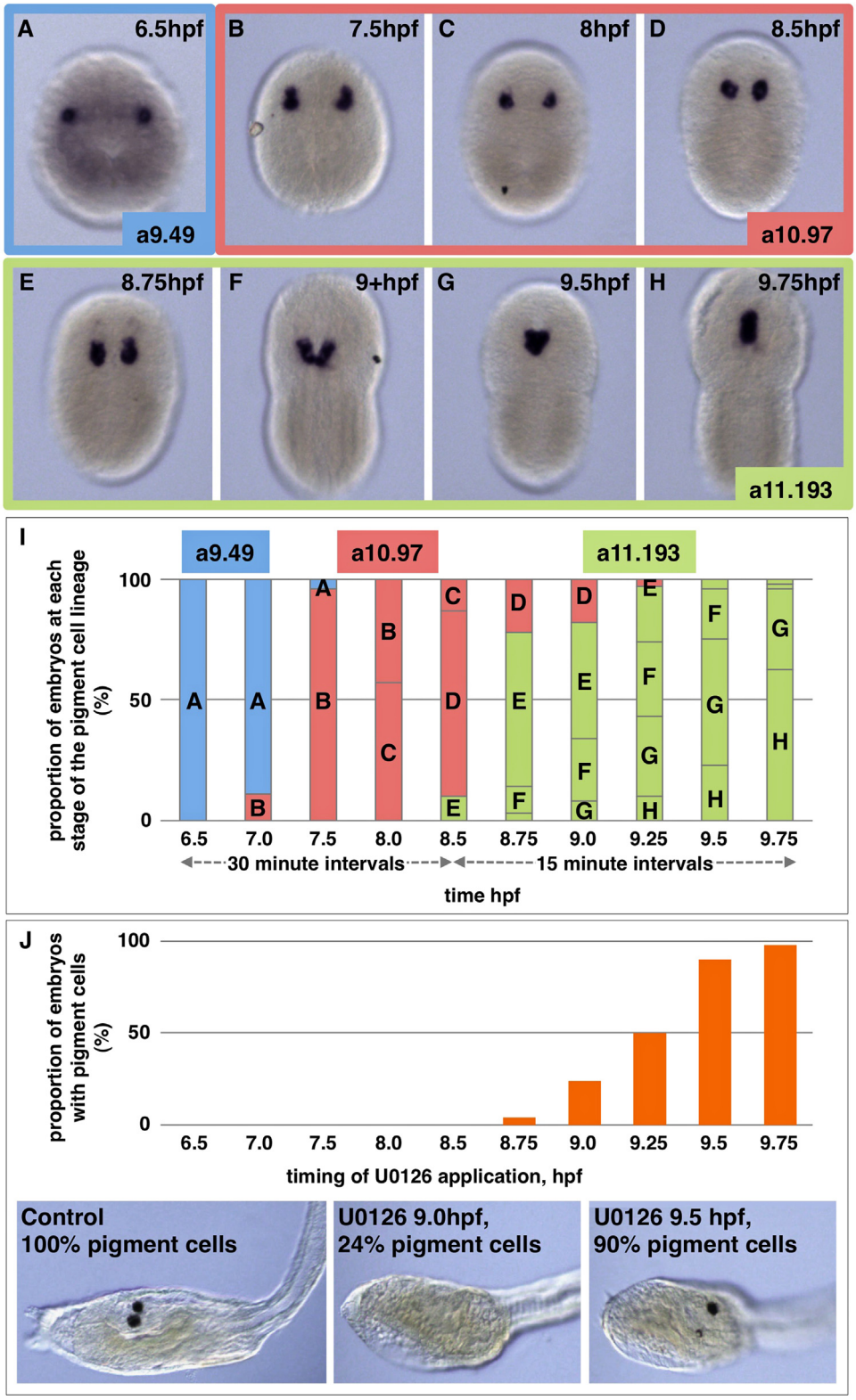

Fig. 5. Pigment cell formation depends on MEK1/2 signals until early tailbud stage. (A–H) Expression of *Trp* at different stages of the pigment cell lineage as indicated by the colored boxes. See text for details. Time points are hours post fertilization (hpf) at 18 °C; the time indicated reflects the embryological stage seen for the majority of embryos at each time point (as shown in I). (I–J) In these experiments, one half of the embryos were fixed for *Trp* in situ hybridization (I) to determine the precise stage of U0126 treatment (n=minimum 36), the other half were treated with U0126 until larval stages in order to monitor pigment cell formation (J) (n=minimum 34). (I) The color code of the graph represents the stage of the pigment cell lineage, defined as a9.49 (blue), a10.97 (red) and a11.193 (green). Bars are further broken down into the proportion of embryos representing the precise embryological stage, as represented in A–H. (J) Bars represent the proportion of embryos with any pigmented cells at each time point. Representative resultant larvae after treatment with U0126 from the time points indicated are shown.

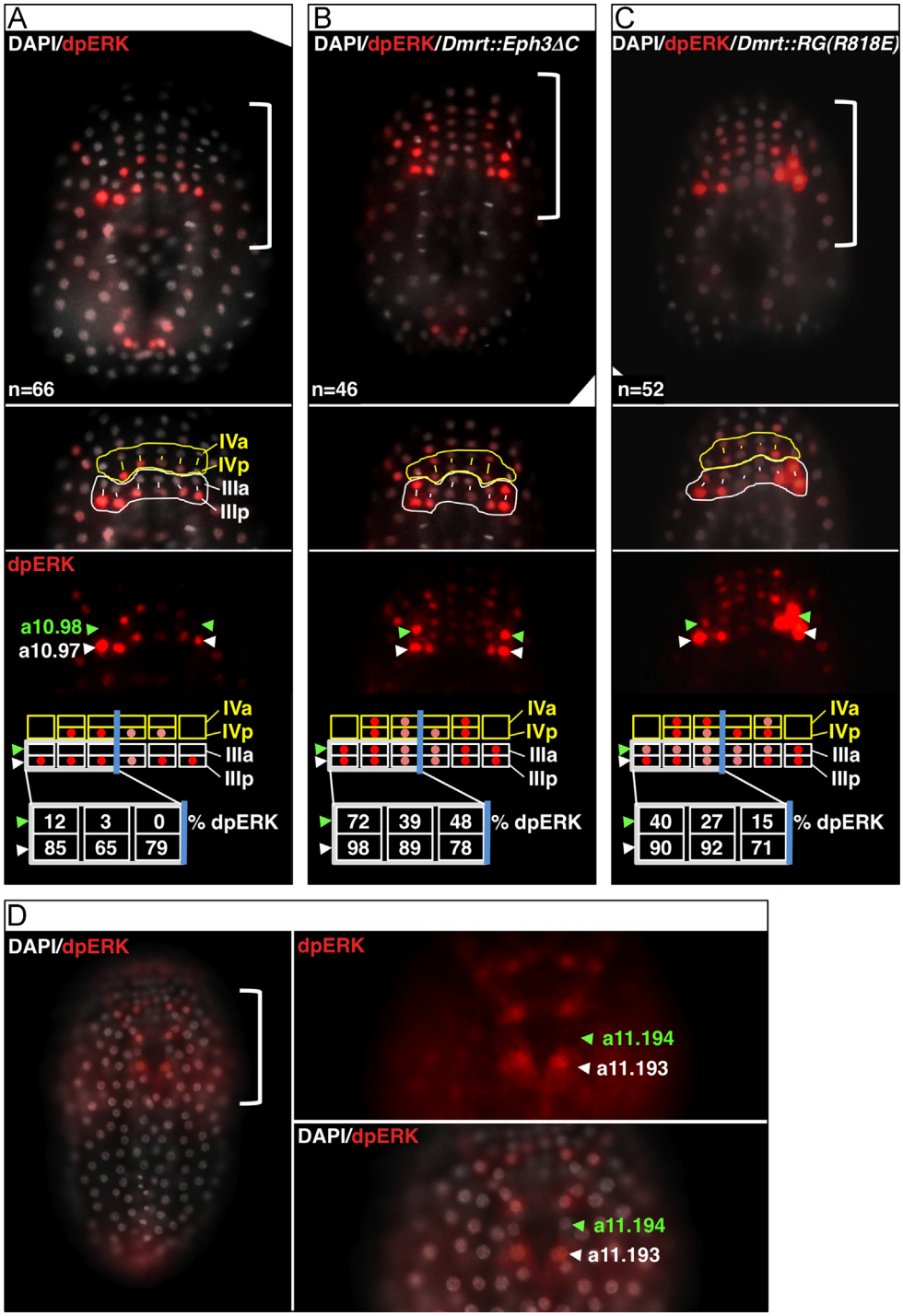

Fig. 6. Eph/ephrin signals control the spatial extent of ERK1/2 activation at the 12-row neurula stage. (A–C) Experimental conditions are indicated on the top panels. Embryos were stained for activated ERK1/2 (dpERK1/2) in red, with nuclei (DAPI) in white. For this analysis embryos were also co-electroporated with *Msx::LacZ* or *Dmrt::LacZ* followed by LacZ immunofluorescence to ensure our cell identification was correct (not shown). The top panel shows the entire embryo and the bracket indicates the annotated region in the lower panels. The divided rows III and IV are outlined in white and yellow respectively in the middle panel. At the stage of analysis, the lateral precursors of row IV have not yet divided. In the lower panel, only the dpERK immunofluorescence is shown for clarity. The interpretation of the dpERK immunofluorescence intensity is depicted by red and pink dots on the schematic of divided rows III and IV. The percentage of dpERK1/2 detection in each cell of row IIIa and IIIp is indicated on the lower schematic, representing independent scoring for each embryo half. n=number of half embryos scored. (D) Early tailbud stage embryo stained for dpERK1/2 (red) and DAPI (white). Enlarged region is indicated by bracket on whole embryo panel.

compared to control embryos, which exhibited an average of 0.8 otolith and 1.2 ocellus-type pigment cells (n=324). The preferential increase in otolith-type pigment cells is consistent with a previous study where the ectopic activation of Ets results in extra pigments cells of the otolith-type. The choice between ocellus and otolith fate is governed by Wnt7, which serves as a positional cue for specifying the posterior ocellus pigment cell (Abitua et al., 2012).

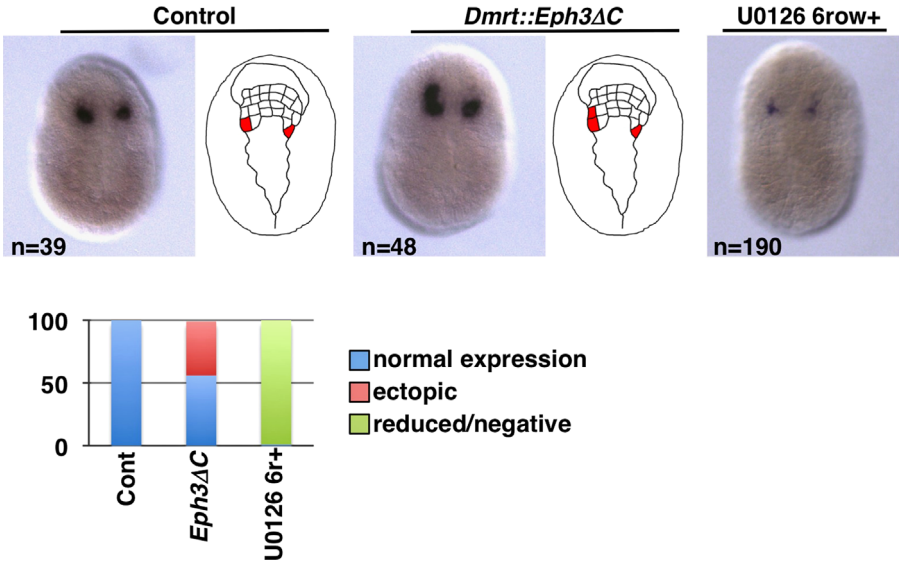

Fig. 7. Ectopic *Trp* expression in a10.98 following inhibition of Eph/ephrin signals. Expression of *Trp* at 12-row neurula stage in control embryos, embryos electroporated with *Dmrt::Eph3ΔC* or embryos treated with U0126 just after the 6-row stage (U0126 6row+). In these experiments, U0126-treatment began once embryos started to leave the 6-row neural plate stage and the cells in row II begin to divide. Each embryo half was scored independently and the quantification is shown in the graph.

Discussion

We document an additional division of the pigment cell lineage, which was previously described to become post-mitotic following nine cell divisions after fertilization in *Ciona* (Whittaker, 1979). We found that disruption of Eph/ephrin signals results in supernumerary pigment cells in larvae. Our results show that ephrin/Eph/RasGAP signaling is required during the 12-row neural plate stage, but not at earlier neural plate stages, to restrict ERK1/2 activation to the posterior daughters of row III (IIIp, as well as row IVp). Removal of this control mechanism results in ectopic activation of ERK1/2 in row IIIa and ectopic *Trp* expression in a10.98.

Reiterative differential ERK1/2 associated with A-P oriented cell division in the developing nervous system

FGF/MEK/ERK-driven cell fate choices generate cell diversity at multiple steps during ascidian embryogenesis (Bertrand et al., 2003; Davidson et al., 2006; Hudson et al., 2003, 2007; Imai et al., 2002a; Kim and Nishida, 2001; Picco et al., 2007; Shi and Levine, 2008; Shimauchi et al., 2001; Squarzoni et al., 2011; Stolfi et al., 2011; Yasuo and Hudson, 2007). Even within the a-line neural lineage, there are many examples. At the 32-cell stage, the a5.3 cell divides to produce the a6.6 cell and the a6.5 cell. The a6.5 cell exhibits ERK1/2 activation in response to the neural inducing FGF signal and adopts a neural fate while the a6.6 cell, with no ERK1/2 activation, adopts an epidermal fate (Bertrand et al., 2003; Hudson et al., 2003). Restriction of ERK1/2 activation to the neural precursor depends upon ephrin signals (Ohta and Satou, 2013). At this stage, a-line neural cells contain both CNS and palp lineages. These cells are prevented from precociously activating the CNS-specific program by a time delay mechanism involving a pair of Blimp1-like zinc finger repressors (Ikeda et al., 2013). From the 3-row neural plate stage, differential ERK1/2 activation is used repeatedly to specify the posterior-most cell fates during at least four successive rounds of A–P oriented cell divisions in the a-line neural lineages. During each division, the pigment cell lineage segregates into the posterior cells. At the 3-row neural plate stage, CNS lineages (row III/IV) segregate from palp lineages (row V/VI) with FGF/MEK/ERK promoting CNS over palp fate in row III/IV (Wagner and Levine, 2012). Differential activation of ERK1/2 between rows III/IV and V/VI may be achieved simply by the restricted expression of the inducing ligand, since *Fgf9/16/20* is expressed in row I/II cells, adjacent to the row III/IV cells (Supplementary Fig. 1) (Imai et al., 2006). At the 6-row neural plate stage, ERK1/2 activation is restricted to row III of the a-line neural plate. Localized expression of FGF ligands (*Fgf9/16/20* and *Fgf8/17/18*) in row II might also account for the differential activation of ERK1/2 between rows III and IV (Supplementary Fig. 1) (Hudson et al., 2007; Squarzoni et al., 2011). At this stage, differential ERK1/2 activation also drives the cell fate choices between row I and row II sister cells of the A-line neural plate with ERK1/2 activated in row I cells, though it is not clear how this differential activation pattern is established (Hudson et al., 2007).

Here we described an additional differential activation of ERK1/2 in row III and row IV derivatives following their division into rows IIIa, IIIp, IVa and IVp, whereby ERK1/2 activation is again associated with the posterior daughter cells in row IIIp and row IVp (Fig. 6). We show that ERK1/2 activation is spatially restricted to the posterior rows by Eph/ephrin signals. This mechanism is unlikely to be confined to the pigment cell lineage choice between a10.97 and a10.98 fates, since ectopic ERK1/2 activation is seen broadly in rows IIIa and IVa following inhibition of Eph/ephrin signals. It is not yet clear which ephrin ligands are involved in this process. *ephrin-Ad* is expressed in rows V–VI from the 6-row neural plate stage and may therefore be involved in the restriction of ERK1/2 activity between rows IVa and IVp (Wagner and Levine, 2012) (Supplementary Fig. 1). *ephrin-Ac* and *ephrin-Ab* are expressed in discrete domains of the neural plate in close proximity to row III and thus might be involved in the spatial control of ERK1/2 activation between row IIIa and IIIp cells (Supplementary Fig. 1). Early in development the *Eph3* receptor is expressed ubiquitously, then shortly after the 6-row stage it is upregulated in the pigment cell lineage. Interestingly, this upregulation depends on MEK/ERK signaling, suggesting that MEK/ERK signals render this cell lineage particularly sensitive to Eph/ephrin signals during subsequent stages (Supplementary Fig. 1). Based on *ephrin* and *Fgf* ligand and receptor expression profiles, it is unclear how such a precise spatial pattern of ERK1/2 activation is achieved at the 12-row neural plate stage. It is possible that other mechanisms are also involved, however our data show that Eph/ephrin plays an essential role in establishing this pattern (Fig. 6).

Following the MEK/ERK-mediated specification of a10.97 as the pigment cell lineage, these cells divide into a11.194 and a11.193 and the posteriorly positioned a11.193 cells become pigmented.

It is not yet clear how this fate choice between a non-pigmented and pigmented cell fate is achieved, although differential ERK1/2 may be involved since ERK1/2 is preferentially activated in a11.193. Differentially-expressed gene markers of a11.193 and a11.194 would be required to address this issue. To determine whether Eph/ephrin signals are directly involved will necessitate a means to block signaling specifically after the 12-row neural plate stage. The fact that five or more pigment cells are frequently observed in *Dmrt::Eph3ΔC* larvae, suggests that this fate choice is also perturbed in this experimental context.

Finally, the two a11.193 cells from both sides of the neural plate intercalate at the midline and adopt either an ocellus or otolith fate. The post-mitotic pigment cell precursors have been shown to form an equivalence group in another ascidian species, *Halocynthia roretzi*. These cells intercalate at random and the posteriorly positioned cell adopts the ocellus fate (Nishida, 1987; Nishida and Satoh, 1989). We did not find any evidence that Eph/ephrin signals are involved in this final fate choice. In *Ciona,* this fate choice is controlled by Wnt7 signals, whereas BMP-Chordin antagonism has been implicated in *Halocynthia* (Abitua et al., 2012; Darras and Nishida, 2001).

If the pigment cell lineage resembles the evolutionary precursors of the vertebrate neural crest, it is relatively easy to envisage how these cell types could both increase in number and adopt a migratory capacity. Simple deregulation of signaling pathways leads to quite a dramatic increase in pigment cell number (Fig. 3) (Abitua et al., 2012; Squarzoni et al., 2011). A subset of these cells would then need to acquire the expression of a mesenchyme-promoting factor such as *Twist* in order for a primordial neural crest to develop (Abitua et al., 2012).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.07.010.

Note:

Some of the findings reported in this article have also been reported elsewhere. Claudia Racioppi, Ashwani K. Kamal, Florian-Razy-Krajka, Gennaro Gambardella, Laura Zanetti, Diego di Bernardo, RemoSanges, Lionel A. Christiaen and FilomenaRistoratore. Fibroblast Growth Factor signals sequentially govern nervous system patterning and pigment cell formation in Ciona intestinalis. Nature Communications. In Press.

References

Abitua, P.B., Wagner, E., Navarrete, I.A., Levine, M., 2012. Identification of a rudimentary neural crest in a non-vertebrate chordate. Nature 492, 104–107.
Bertrand, V., Hudson, C., Caillol, D., Popovici, C., Lemaire, P., 2003. Neural tissue in ascidian embryos is induced by FGF9/16/20, acting via a combination of maternal GATA and Ets transcription factors. Cell 115, 615–627.
Christiaen, L., Jaszczyszyn, Y., Kerfant, M., Kano, S., Thermes, V., Joly, J.-S., 2007. Evolutionary modification of mouth position in deuterostomes. Semin. Cell Dev. Biol. 18, 502–511.
Christiaen, L., Wagner, E., Shi, W., Levine, M., 2009. Electroporation of transgenic DNAs in the sea squirt Ciona. Cold Spring Harb. Protoc. 2009 (pdb.prot5345).
Cole, A.G., Meinertzhagen, I.A., 2004. The central nervous system of the ascidian larva: mitotic history of cells forming the neural tube in late embryonic Ciona intestinalis. Dev. Biol. 271, 239–262.
Conklin, E.G., 1905. The organisation and cell lineage of the ascidian egg. J. Acad. Natl. Sci. Phila., 1–119.
Darras, S., Nishida, H., 2001. The BMP/CHORDIN antagonism controls sensory pigment cell specification and differentiation in the ascidian embryo. Dev. Biol. 236, 271–288.
Davidson, B., Shi, W., Beh, J., Christiaen, L., Levine, M., 2006. FGF signaling delineates the cardiac progenitor field in the simple chordate, Ciona intestinalis. Genes Dev. 20, 2728–2738.
Guillemot, F., Zimmer, C., 2011. From cradle to grave: the multiple roles of fibroblast growth factors in neural development. Neuron 71, 574–588.
Haupaix, N., Stolfi, A., Sirour, C., Picco, V., Levine, M., Christiaen, L., Yasuo, H., 2013. p120RasGAP mediates ephrin/Eph-dependent attenuation of FGF/ERK signals during cell fate specification in ascidian embryos. Dev. Camb. Engl. 140, 4347–4352.
Hébert, J.M., 2011. FGFs: neurodevelopment's jack-of-all-trades – how do they do it? Front. Neurosci. 5, 133.
Hotta, K., Mitsuhara, K., Takahashi, H., Inaba, K., Oka, K., Gojobori, T., Ikeo, K., 2007. A web-based interactive developmental table for the ascidian *Ciona intestinalis*, including 3D real-image embryo reconstructions: I. From fertilized egg to hatching larva. Dev. Dyn. Off. Publ. Am. Assoc. Anat. 236, 1790–1805.
Hudson, C., Darras, S., Caillol, D., Yasuo, H., Lemaire, P., 2003. A conserved role for the MEK signalling pathway in neural tissue specification and posteriorisation in the invertebrate chordate, the ascidian *Ciona intestinalis*. Dev. Camb. Engl. 130, 147–159.
Hudson, C., Lotito, S., Yasuo, H., 2007. Sequential and combinatorial inputs from Nodal, Delta2/Notch and FGF/MEK/ERK signalling pathways establish a grid-like organisation of distinct cell identities in the ascidian neural plate. Dev. Camb. Engl. 134, 3527–3537.
Hudson, C., Kawai, N., Negishi, T., Yasuo, H., 2013. β-Catenin-driven binary fate specification segregates germ layers in ascidian embryos. Curr. Biol. CB 23, 491–495.
Ikeda, T., Matsuoka, T., Satou, Y., 2013. A time delay gene circuit is required for palp formation in the ascidian embryo. Dev. Camb. Engl. 140, 4703–4708.
Imai, K.S., Satoh, N., Satou, Y., 2002a. Early embryonic expression of FGF4/6/9 gene and its role in the induction of mesenchyme and notochord in *Ciona savignyi* embryos. Dev. Camb. Engl. 129, 1729–1738.
Imai, K.S., Satou, Y., Satoh, N., 2002b. Multiple functions of a Zic-like gene in the differentiation of notochord, central nervous system and muscle in *Ciona savignyi* embryos. Dev. Camb. Engl. 129, 2723–2732.
Imai, K.S., Hino, K., Yagi, K., Satoh, N., Satou, Y., 2004. Gene expression profiles of transcription factors and signaling molecules in the ascidian embryo: towards a comprehensive understanding of gene networks. Dev. Camb. Engl. 131, 4047–4058.
Imai, K.S., Levine, M., Satoh, N., Satou, Y., 2006. Regulatory blueprint for a chordate embryo. Science 312, 1183–1187.
Imai, K.S., Stolfi, A., Levine, M., Satou, Y., 2009. Gene regulatory networks underlying the compartmentalization of the Ciona central nervous system. Dev. Camb. Engl. 136, 285–293.
Irvine, S.Q., Cangiano, M.C., Millette, B.J., Gutter, E.S., 2007. Non-overlapping expression patterns of the clustered Dll-A/B genes in the ascidian *Ciona intestinalis*. J. Exp. Zool. B: Mol. Dev. Evol. 308, 428–441.
Ivashkin, E., Adameyko, I., 2013. Progenitors of the protochordate ocellus as an evolutionary origin of the neural crest. EvoDevo 4, 12.
Jiang, D., Tresser, J.W., Horie, T., Tsuda, M., Smith, W.C., 2005. Pigmentation in the sensory organs of the ascidian larva is essential for normal behavior. J. Exp. Biol. 208, 433–438.
Kim, G.J., Nishida, H., 2001. Role of the FGF and MEK signaling pathway in the ascidian embryo. Dev. Growth Differ. 43, 521–533.
Lamb, T.D., Collin, S.P., Pugh Jr, E.N., 2007. Evolution of the vertebrate eye: opsins, photoreceptors, retina and eye cup. Nat. Rev. Neurosci. 8, 960–976.
Lemaire, P., Bertrand, V., Hudson, C., 2002. Early steps in the formation of neural tissue in ascidian embryos. Dev. Biol. 252, 151–169.
Nicol, D., Meinertzhagen, I.A., 1988. Development of the central nervous system of the larva of the ascidian, Ciona intestinalis L. I. The early lineages of the neural plate. Dev. Biol. 130, 721–736.
Nishida, H., 1987. Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. Dev. Biol. 121, 526–541.
Nishida, H., Satoh, N., 1989. Determination and regulation in the pigment cell lineage of the ascidian embryo. Dev. Biol. 132, 355–367.

Ohta, N., Satou, Y., 2013. Multiple signaling pathways coordinate to induce a threshold response in a chordate embryo. PLoS Genet. 9, e1003818.
Picco, V., Hudson, C., Yasuo, H., 2007. Ephrin-Eph signalling drives the asymmetric division of notochord/neural precursors in Ciona embryos. Dev. Camb. Engl. 134, 1491–1497.
Rothbächer, U., Bertrand, V., Lamy, C., Lemaire, P., 2007. A combinatorial code of maternal GATA, Ets and beta-catenin-TCF transcription factors specifies and patterns the early ascidian ectoderm. Dev. Camb. Engl. 134, 4023–4032.
Sardet, C., McDougall, A., Yasuo, H., Chenevert, J., Pruliere, G., Dumollard, R., Hudson, C., Hebras, C., Le Nguyen, N., Paix, A., 2011. Embryological methods in ascidians: the Villefranche-sur-Mer protocols. Methods Mol. Biol. Clifton NJ 770, 365–400.
Sato, S., Yamamoto, H., 2001. Development of pigment cells in the brain of ascidian tadpole larvae: insights into the origins of vertebrate pigment cells. Pigment Cell Res. Spons. Eur. Soc. Pigment Cell Res. Int. Pigment Cell Soc. 14, 428–436.
Satou, Y., Yamada, L., Mochizuki, Y., Takatori, N., Kawashima, T., Sasaki, A., Hamaguchi, M., Awazu, S., Yagi, K., Sasakura, Y., et al., 2002. A cDNA resource from the basal chordate *Ciona intestinalis*. Genesis 33, 153–154.
Shi, W., Levine, M., 2008. Ephrin signaling establishes asymmetric cell fates in an endomesoderm lineage of the Ciona embryo. Dev. Camb. Engl. 135, 931–940.
Shi, W., Peyrot, S.M., Munro, E., Levine, M., 2009. FGF3 in the floor plate directs notochord convergent extension in the Ciona tadpole. Dev. Camb. Engl. 136, 23–28.
Shimauchi, Y., Murakami, S.D., Satoh, N., 2001. FGF signals are involved in the differentiation of notochord cells and mesenchyme cells of the ascidian *Halocynthia roretzi*. Dev. Camb. Engl. 128, 2711–2721.
Squarzoni, P., Parveen, F., Zanetti, L., Ristoratore, F., Spagnuolo, A., 2011. FGF/MAPK/Ets signaling renders pigment cell precursors competent to respond to Wnt signal by directly controlling Ci-Tcf transcription. Dev. Camb. Engl. 138, 1421–1432.
Stolfi, A., Wagner, E., Taliaferro, J.M., Chou, S., Levine, M., 2011. Neural tube patterning by Ephrin, FGF and Notch signaling relays. Dev. Camb. Engl. 138, 5429–5439.
Stuhlmiller, T.J., Garcia-Castro, M.I., 2012. FGF/MAPK signaling is required in the gastrula epiblast for avian neural crest induction. Dev. Camb. Engl. 139, 289–300.
Taniguchi, K., Nishida, H., 2004. Tracing cell fate in brain formation during embryogenesis of the ascidian *Halocynthia roretzi*. Dev. Growth Differ. 46, 163–180.
Tassy, O., Dauga, D., Daian, F., Sobral, D., Robin, F., Khoueiry, P., Salgado, D., Fox, V., Caillol, D., Schiappa, R., et al., 2010. The ANISEED database: digital representation, formalization, and elucidation of a chordate developmental program. Genome Res. 20, 1459–1468.
Tsuda, M., Sakurai, D., Goda, M., 2003. Direct evidence for the role of pigment cells in the brain of ascidian larvae by laser ablation. J. Exp. Biol. 206, 1409–1417.
Veeman, M.T., Newman-Smith, E., El-Nachef, D., Smith, W.C., 2010. The ascidian mouth opening is derived from the anterior neuropore: reassessing the mouth/neural tube relationship in chordate evolution. Dev. Biol. 344, 138–149.
Wagner, E., Levine, M., 2012. FGF signaling establishes the anterior border of the Ciona neural tube. Dev. Camb. Engl. 139, 2351–2359.
Whittaker, J.R., 1979. Quantitative control of end products in the melanocyte lineage of the ascidian embryo. Dev. Biol. 73, 76–83.
Yasuo, H., Hudson, C., 2007. FGF8/17/18 functions together with FGF9/16/20 during formation of the notochord in Ciona embryos. Dev. Biol. 302, 92–103.