Fibroblast growth factor signalling controls nervous system patterning and pigment cell formation in *Ciona intestinalis*

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During the development of the central nervous system (CNS), combinations of transcription factors and signalling molecules orchestrate patterning, specification and differentiation of neural cell types. In vertebrates, three types of melanin-containing pigment cells, exert a variety of functional roles including visual perception. Here we analysed the mechanisms underlying pigment cell specification within the CNS of a simple chordate, the ascidian *Ciona intestinalis*. *Ciona* tadpole larvae exhibit a basic chordate body plan characterized by a small number of neural cells. We employed lineage-specific transcription profiling to characterize the expression of genes downstream of fibroblast growth factor signalling, which govern pigment cell formation. We demonstrate that FGF signalling sequentially imposes a pigment cell identity at the expense of anterior neural fates. We identify FGF-dependent and pigment cell-specific factors, including the small GTPase, Rab32/38 and demonstrated its requirement for the pigmentation of larval sensory organs.
Distinct combinations of transcription factors (TFs) and signalling molecules define the complex gene regulatory networks that control patterning and commitment of different cell types. In vertebrate embryos, pigment-producing cells originate from the closing neuroepithelium: Retinal pigment epithelium (RPE) of the eye and pigment cells of the pinnae organ arising from the neural tube; melanocytes of the inner ear, skin, derive from a population of cells migrating from the neural plate border (neural crest). Despite their different embryological origins, all vertebrate pigment cells share some basic characteristics: melanin pigments are synthesized and stored in melanosomes involving tyrosinase (tyr) and tyr-related enzymes. Melanosomes are tissue-specific lysosome-related organelles distributed in the cell by a regulated vesicular trafficking system, which requires several Rab-type small GTPases and their regulators.

The development of vertebrate RPE and neural retina involves complex interplays among cell-signalling and cell-intrinsic factors: Wnt, bone morphogenetic proteins and fibroblast growth factors (FGFs). Among the documented roles for signalling pathways in the formation of the vertebrate visual system, FGF is required to induce neural retina progenitors at the expense of RPE formation. Nevertheless, understanding the molecular impacts of cell-extrinsic components on cell-specific determinants in the developing of the retina remains a challenging task.

Ascidians belonging to the chordate group represent an attractive model system to investigate the generation of cell diversity in the central nervous system (CNS). Despite their highly divergent adult body plan and genomic architecture, phylogenomic analyses indicated that tunicates are the closest living relatives of the vertebrates. The reduced genetic redundancy found in the compact tunicate genome facilitates functional analyses of homologous TFs and signalling molecules in the context of the typical chordate body plan of embryos and larvae.

The ascidian *Ciona intestinalis* emerged as an experimental system of choice due to their simple embryos, rapid development and straightforward transgenesis methods. Because the ascidian cell lineages and fate maps are well characterized, the identification of neural precursors starting from early fate restriction facilitates the study of progressive cell fate specification in individual precursors through each division. Thus, it is possible to investigate the cellular and molecular mechanisms underlying fate specification with single-cell resolution during the development of chordate CNS.

The *Ciona* tadpole larva offers the advantage of a typical chordate body plan with a simple brain. The anterior sensory vesicle contains two distinct pigment cell containing sensory organs: the geotactic otothil, a single cell containing a melanin granule and the photosensitive ocellus, composed of 30 photoreceptor cells, three lens cells and one cup-shaped pigment cell. Ascidian pigment cells express the melanogenic enzyme-coding genes tyrosinase (tyr) and tyr-related protein, in a fashion similar to the vertebrate pigment cells. Moreover, *Ciona* cephalic melanocyte lineage expresses neural plate border and neural crest specification genes, like *Id*, *Snail*, *Ets* and *FoxD* and the signalling events and regulatory inputs leading to ascidian ocellus formation are similar to those involved in vertebrate neural crest lineage specification, including canonical Wnt and FoxD-mediated repression of *Mif*.

We recently showed that the FGF/MAPK/Ets pathway is responsible for pigmented cell precursor specification, starting from the late gastrula stage, by rendering their progenies competent to respond to a Wnt signal. However, the FGF signalling downstream targets involved in the regulatory architecture of pigment cell lineage formation remain uncharacterized. Here, we employed a transcriptomic approach by exploiting targeted interference of the FGF signalling coupled with the enrichment of cells of interest by fluorescence-activated cell sorting (FACS) to obtain pigment cell lineage-specific transcription profiles. By using ‘reverse engineering’ methods, we created a pigment lineage-specific co-regulated network to identify key effectors for pigmentation. By characterizing the transcriptional response to FGF signalling in the pigment cell lineage, we identified molecular mechanisms underlying anterior–posterior patterning of *Ciona* CNS and identified novel regulators of pigment cell specification and differentiation.

**Results**

**FGF dependent Pigment cell precursors transcription profiles.** In ascidians, the pigment cells of the otolith and ocellus arise from the paired a8.25 blastomeres. During gastrulation, these blastomeres divide and form a7.49 and a7.50 cell pairs: the a7.50s (located in the row IV of the neural plate) are progenitor cells of the anterior brain, while the a7.49s (located in the row III) become fate restricted as pigment cell precursors (PCPs) (Fig. 1a). At the mid-neurula stage (stage 13; Four-dimensional Ascidian Body Atlas), bilateral PCPs divide, forming two cell pairs (a10.98s and a10.97s, Fig. 1b,c). Taking advantage of the stability and brightness of 2xGFP reporter driven by tyrp1/2a enhancer, we observed that a10.97/a10.98s, previously described as a postmitotic pigment cell precursor, undergo an additional division forming eight cells (four on each side) (Fig. 1d). An extra a10.97 cell division was also suggested in a note added in proof of Abita et al. and recently confirmed in Haupaux et al. (see note added in proof).

Following cell nomenclature previously described in Cole and Meinertzhagen, we referred to these cells as a11.196 and a11.195 for the two anterior cells as a11.194 and a11.193 for the two posterior ones (Fig. 1d,e). Among the tyrp1/2a+ cells, only the two most posterior become otolith and ocellus pigment cells.

We recently demonstrated that FGF signalling induces PCP’s formation. We perturbed the endogenous FGF signalling by using a dominant-negative form of the unique Ci-FGF receptor and a constitutively active form of Ci-Ets1/2 (Ets:VP16), a transcriptional effector of FGF/MAP kinase cascade that is predominantly expressed in all the row III cells, including PCPs, at late gastrula stage. Targeted expression of FGFFRDN or Ets:VP16 in PCPs caused larvae to either lack pigment cells or form extra pigment cells in the sensory vesicle, respectively (Fig. 2a–c).

Here, we employed FACS and microarray analyses to obtain PCP lineage-specific transcription profiles and identify FGF downstream genes responsible for *Ciona* PCPs formation. We used the same tyrp1/2a enhancer to express FGFFRDN, Ets:VP16 and green fluorescent protein (GFP) and FAC-sort a7.49 cells and their progeny as GFP+ cells (Fig. 2d,e). To avoid a contamination by mesenchymal cells where *Ciona* enhancers are often ectopically activated, we co-electroporated the *MyoD905* > YFP (yellow fluorescent protein) construct and counter-selected YFP+ cells as previously described (Fig. 2e). To gain insight into the temporal deployment of the FGF signalling regulatory network, we examined the transcription profiles of PCPs isolated at two developmental stages: 8 hpf at ~16°C (neurala stage) corresponding to early FGF-mediated induction, and 12 hpf at ~18°C tailbud(TB) stage, where PCPs are already fate restricted as pigment cells (Fig. 2d).

The entire data set of 24 microarrays was analysed to identify new candidates involved in the PCP-specific FGF/MAPK/Ets-dependent regulatory network (Supplementary Fig. 1).
Correlation analyses of normalized expression values among replicates indicated that microarray experiments were highly reproducible (Supplementary Fig. 1).

To explore the expression data set, we reasoned that pigment cell-specific genes should be downregulated upon misexpression of FGFRDN and/or upregulated by Ets:Vp16 compared with controls (Fig. 2h), given that FGFRDN blocks pigmentation while Ets:Vp16 causes the formation of supernumerary pigment cells in the PCP lineage17 (Fig. 2a–c). The quadruplicate experiments were used to compute adjusted P values, corrected for multiple testing errors.

We first examined the expression values for a training data set consisting of known PCP markers, including Ci-tyr, Ci-tyrpl/2a14, Ci-Tcf/Lef17, Ci-Mitf, Ci-Bmp5/7 and Ci-msxb25 (Fig. 2f). When comparing their expression levels in FGFRDN versus Ets:Vp16 12hpf samples, the melanogenic genes, Ci-tyr, Ci-tyrpl/2a, the direct FGF downstream gene and transcriptional effector of canonical Wnt signalling Ci-Tcf/Lef as well as known factors involved in pigment cell formation, Ci-Mitf and Ci-Bmp5/7, were strongly downregulated (Fig. 2f). Ci-Mitf and Ci-Bmp5/7 expressions decrease between 8 and 12 hpf. This is in agreement with their early expression in the pigment cell lineage (Supplementary Fig. 2a,b; ref. 16) and is probably related to their early regulatory function.

PCP expression of the transcription regulator Ci-msxb decreased over time, while FGFRDN increased its expression at 12 hpf. These observations are consistent with in situ hybridization (ISH) assays revealing that Ci-msxb is expressed in the 8 hpf neural plate, including the PCPs, while it is excluded from most of the a9.49 derivatives in 12 hpf tailbud embryos, except for the most anterior a11.195s and a11.196s, as demonstrated by the co-localization with Ci-tyrpl/2a (Supplementary Fig. 2c,d). According to the extra cell division observed for pigment cell lineage, tyrpl/2a transcripts accumulated preferentially in a10.97s rather than a10.98s retaining a stronger expression in a10.97 progeny (a11.193s/a11.94s) than a10.98s derivatives (a11.195s/a11.96s) that maintains only a residual amount of tyrpl/2a RNAs. Thus, our microarray data recapitulated distinct tissue-specific expression dynamics: Ci-msxb is downregulated within the PCP lineage between 8 and 12 hpf, but it is maintained in surrounding non-pigmented CNS cells and therefore ectopically expressed in 12 hpf PCPs on inhibition of FGF signalling.

FGF signalling opposes the anterior neural plate fate in PCPs.

To consider all the experimental conditions used for microarray analysis, we focused on 912 probe sets that were differentially expressed when comparing the two perturbed conditions to each other and with control samples (FGFRDN versus Control, Ets:Vp16 versus Control and FGFRDN versus Ets:Vp16) at 8 and 12 hpf (Fig. 2g).

Since FGF inhibition reprogrammes PCPs into non-pigmented cells, we first sought to characterize the transcription profiles underlying this fate change. We reasoned that genes upregulated upon FGF inhibition reflect the CNS fate alternative to PCP. We found 461 probe sets corresponding to 264 unique ‘FGF-inhibited’ genes (Fig. 2h). Most of these genes were also more highly expressed at 8 than 12 hpf (Fig. 2h). Considering that CNS regionalization and cell fate specification take place in early development, we expected that genes involved in these processes would be more highly expressed at early stages. Thus, we reasoned that early ‘FGF-inhibited’ genes would reflect the respecification of a9.49 lineage and illuminate the role of FGF signalling in CNS patterning and early PCP specification.

Among the upregulated genes, that is, more highly expressed in FGFRDN samples and/or decreased expression in Ets:Vp16 in comparison with control samples, we first focused on Ci-Stx3/6,
a known transcription factor-coding gene expressed in anterior neural domains throughout the animal kingdom\textsuperscript{26,27}. In Ciona, it is expressed at late gastrula stage in the neural plate IV row of cells, including in daughters of the a9.50 cells (Fig. 3a), the sisters of a9.49 PCP precursors located on neural plate III row (Fig. 1a,b and in Moret et al.\textsuperscript{26}). Double whole-mount ISH (WMISH)
demonstrated that Ci-Six3/6 is expressed in a territory that is adjacent but not overlapping with the domain of Ci-tyrpl/2a expression at the neurula to tailbud stages (Fig. 3a,b).

To mirror microarray experiments, we performed double WMISH using Ci-Six3/6 and Ci-tyrpl/2a probes following FGF perturbations. In Ets:Vp16 electroporated embryos and control samples, we did not detect any change in Ci-Six3/6 expression (Fig. 3d), while FGF inhibition caused ectopic Ci-Six3/6 expression in a posterior domain that includes Ci-tyrpl/2a-positive a9.49 derivatives (Fig. 3c). These findings recapitulate the microarray data and indicate that FGF signalling normally inhibits Ci-Six3/6 expression in Ci-tyrpl/2a cells. To characterize the FGF regulatory network that generate cell identity in the anterior CNS of the tailbud embryos, we analysed the expression pattern of genes encoding Tfs that were significantly upregulated when comparing FGFRDN with Ets:Vp16 in 12 hpf PCPs. Among 21 genes, we selected Ci-Pax3/7, Ci-Macho-1, Ci-Foxha, Ci-mxs6 and Ci-Pax6. We included additional genes with published expression profiles in the ANISEED database28, Ci-RGR/Nut, Ci-Neurogenin (Ngn) and Ci-CO25 that are widely expressed in the CNS. We then asked whether these genes co-localize with Ci-Six3/6+ anterior neural cells in tailbud embryos. All these genes were expressed in anterior neural domains, overlapping with Ci-Six3/6 (Supplementary Fig. 3a–i).

According to published neural lineages20, this domain located anteriorly to the prospective PCP includes a10.100s, a11.198s and a11.197s, all derived from the a9.50 blas-tomeres, which already express Ci-Six3/6 in early neurula embryos and are a9.49 sister cells (Supplementary Fig. 3a). Our observations that Ci-Six3/6 and other markers of a9.50 derivatives were upregulated by FGFRDN misexpression in the a9.49 lineage indicate that FGF signalling promotes PCP specification in a9.49 derivatives by inhibiting a9.50 misexpression including Ci-Six3/6 expression, thus specifying different anterior-to-posterior fates within the neural plate.

These results indicate that the most posterior Ci-Six3/6+ cells express a unique combination of genes, which are inhibited by FGF signalling in the adjacent PCPs. As further support, we found that anterior CNS markers, as Ci-Pax3/7, Ci-Pax6, Ci-COE, are ectopically expressed posteriorly in PCPs domain when FGF signalling is specifically inhibited in these cells (Supplementary Fig. 4).

Progressive PCP induction by sequential FGF/MAPK signalling. Starting from early gastrulation, the FGF signal is released from the A-nerve cord precursors and induces the a8.25 blas-tomeres, subsequently their progenies (a9.49s), as future pigment cells17,18,29. To determine the timing of MAPK activation during PCP divisions in a10.97/a10.98s and in their progeny, we performed immunostaining assays of the dual-phosphorylated (dp) form of ERK1/2 (ref. 29). At late gastrula stage, dpERK appears in the a-line neural plate in the row III cells, including a9.49s, but not on the row IV that includes Ci-Six3/6-expressing cells, as shown in Hudson et al.29 Later in development, we detected dpERK staining in the a10.97s but not in a10.98s (Fig. 4a–c). After the second round of PCP division, dpERK staining remains in a10.97 progenies, appearing stronger in the precursors of otolith and ocellus pigmented cells (a11.193s) than in a11.194s (Fig. 4d). These data suggest that the MAPK activation is progressively restricted to the posterior PCPs, the sole to become pigmented at later stage. We next used timed inhibition of MAPK signalling to further test whether sequential induction of FGF signalling corresponds to successive steps in PCP specification. To this aim, we followed PCP division by assaying GFP reporter expression and placed embryos in the pharmacological agent U0126 to block the MEK kinase for 30 min at gastrula (a9.49s), neurula (a10.97s/a10.98s) and early tailbud (a11.193s/a11.194s and a11.195s/a11.196s) stages. We then observed the presence of pigmented cells in the larval sensory vesicle. As already demonstrated, the resulting larvae completely lacked pigment cells when embryos were treated at gastrula stage17. We found that when embryos were treated at neurula and early tailbud stages, larvae deprived of pigment cells decreased (Fig. 5a) while a little effect was observed when embryos were placed in U0126 after the final cell division (a11.193s) of pigment cell lineage. These data are in according to the differential activation of ERK1/2 in PCPs that appeared stronger in the a10.97s and their progenies than the other PCP cells.

We then examined Ci-Six3/6+ and Ci-tyrpl/2a+ cells at tailbud stage following Ci-Six3/6 and Ci-tyrpl/2a transcripts, together with GFP expression driven by tyrpl/2a enhancer (ptyrp1/2a > 2xGFP) as pigment cell lineage marker (Fig. 5b). Upon treatment at 5 h30 (a9.49s), Ci-tyrpl/2a expression is lost in PCPs (Fig. 5c) whereas it is strongly reduced when embryos were treated at the neurula stage (10.97s/a10.98s) (Fig. 5d) and appeared almost normal when the drug was applied at early tailbud stage (a11.193s/a11.194s) (Fig. 5e); by contrast, Ci-Six3/6 detection is always expanded posteriorly in the CNS, although to a lesser extent upon later treatments. The results obtained with successive U0126 treatments show that MAPK activity is necessary at each sequential PCP division (a9.49 and a10.97/a10.98 division) to direct PCPs through their final fate from gastrula to tailbud stage and became less effective when PCP became post mitotic (a11.193s/a11.194s).

Expression of new marker genes for pigment cell lineage. We next sought to identify novel genes expressed in the PCP

Figure 2 | Pigment cell lineage-specific transcription profile. Phenotype of larvae electroporated with ptyrp1/2a > LacZ (a), ptyrp1/2a > FGFRDN (b), ptyrp1/2a > Ets:Vp16 (c) observed with transmitted light. View, anterior is on the left. (d) Schematic representation illustrating the microarray experimental design in two developmental stages, neurula (8 hpf) and tailbud stage (12 hpf); PCPs-lineage cells for microarray analysis were sorted based on their GFP fluorescence. tyrpl/2a enhancer drives target expression of FGFRDN or Ets:Vp16 in 12 hpf PCPs; FGFRDN and Ets:Vp16 conditions mimic regression (light pink) or expansion (dark pink) of the pigmented cells, respectively; (e) FACs plots: ptyrp1/2a > 2xGFP (8 hpf, right), ptyrp1/2a > GFP (12 hpf, left) and pMyoD > GFP-expressing cells are distinguished by their green or yellow fluorescence, respectively (hpf, hours post fertilization). The GFP-only quadrant (GFP2) was used to sort PCPs. (f) Hierarchical clustering and heat map: training data set expression values (log2 FC) consisting of known PCP markers (Ci-tyr, Ci-tyrpl/2a, Ci-Tcf/Lef, Ci-Mitf, Ci-bmp5/7, Ci-mxs6 and Ci-FoxD). Clustering was performed taking in account all three conditions (control, FGFRDN, Ets:Vp16) at the two developmental stages (8 and 12 hpf). Expression levels are colour coded as represented by the colour range below. (g) Identification of 912 probe sets differentially expressed when comparing the two perturbated conditions with each other and with control samples (FGFRDN versus Control, Ets:Vp16 versus Control and FGFRDN versus Ets:Vp16) at 8 and 12 hpf (for more details on transcript selection, see the text); (h) Log2 FC of 912 probe sets showing an increased (upregulated) or decreased (downregulated) expressions when FGFRDN is compared with control or Ets:Vp16 conditions. Colours indicate fold change in pairwise comparisons, for example, dNGFR-Ets:Vp12, means differential expression in ptyrp1/2a > FGFRDN compared with ptyrp1/2a > Ets:Vp16 samples sorted at 12 hpf; red indicate upregulation, green downregulation, respectively. See also Supplementary Fig. 1.
downstream of FGF signalling. Since FGF signalling is implicated in Ciona pigment cell formation, we reasoned that candidate PCP-specific FGF targets should be either downregulated by FGFRDN and/or upregulated by Ets:Vp16 at 8 or 12 hpf and identified 451 probe sets matched to 207 unique annotated genes (Fig. 2h). Gene ontology (GO) terms analyses indicated that the pool of candidate PCP-specific FGF targets are enriched for genes involved in melanization with overrepresented processes such as ‘pigmentation’, ‘pigment binding’, ‘melanocyte differentiation’ and ‘eye pigment biosynthetic process’ thus confirming the validity of our approach and its potential for the discovery of novel PCP-specific candidates involved in melanization that are under the control of FGF signalling (Supplementary Table 1).

To gain insights into the FGF-dependent regulatory network controlling pigment cell formation, we analysed the expression values of candidate PCP genes encoding TFs and signalling molecules. We identified 20 regulatory genes that might contribute to PCP fate specification (Supplementary Fig. 5). We identified the conserved FGF/MAPK pathway components MAPKK7, Dusp1.2.4.5, Dusp6.9 and ELK1/3 (ref. 31), the BMP-family ligands Bmp5/7, Bmp2/4 (ref. 31) and Wnt signalling molecules such as the downstream effector Tcf/Lef, Wnt14/15, Frizzled receptor (Orphan Fz-a), the Wnt-co-receptors Lrp4 and Lrp1b and the secreted Wnt antagonist Dickkopf1 (Dkk1) (ref. 32). Interestingly, genes encoding the secreted BMP antagonists, Ci-noggin as well as Ci-BMPER, a BMP-binding antagonist, were upregulated in FGFRDN compared with Ets:Vp16 samples. Taken together, these data suggest that the FGF/MAPK pathway potentiates BMP signalling during PCP fate specification and provides further insight into the interaction between FGF and Wnt that we previously uncovered.

To find new PCP-specific markers, we selected 30 genes among the downregulated genes identified from the pairwise comparisons (FGFRDN versus Ets:Vp16 and FGFRDN versus control) for subsequent ISH analyses. We obtained a specific CNS staining for 14 of the PCP candidates. Among these, six genes (Ci-Lrp4, Ci-Bzrap1, Ci-Casz1, Ci-Mad/mnt, Ci-Slc45a2, Ci-Piwi) showing...
Figure 4 | Sequential induction mediated gradually by FGF/MAPK/ERK on PCPs. Activation of ERK1/2 visualized by dpERK1/2 antibody staining. Ciona embryos were electroporated with ptyrp1/2a > 2xGFP and stained using anti-GFP antibody (green) to label the PCPs from early neurula (a) (a10.97s and a10.98s), late neurula (b,c) (a10.97s and a10.98s in division) to early tailbud stage (d) (a11.194s, a11.195s and a11.196s). Note that at early and late neurula stages, nuclear dpERK is detected in the a10.97s and their progenies but not in a10.98s. (a,b,d) Scale bar, 50 μm; (c) scale bar, 20 μm; nuclear staining is visualized by DAPI (blue). White asterisks and arrowhead indicate a10.98s and a10.97s and their cell progenies, respectively. Number of embryos showing dpERK1/2 staining out of the total embryos scored. Each experiment was repeated at least three times.
defined PCP expression were selected for further analysis (Fig. 6a–g). Double WMISH using the pigment cell marker, Ciptrp1/2a, was performed to ascertain PCP-specific expression (Fig. 6). Detailed analysis of the expression domain showed that some genes are expressed in all the eight a9.49-derived cells (Ci-Mad/Mnt, Ci-Piwi), while Ci-Casz1 is localized in the posterior six cells; others are exclusively expressed in the posterior two presumptive pigment cells (Ci-Bzrap1, Ci-Slc45a2, Ci-Lrp4) (Fig. 6). Furthermore, in situ assays on embryos where FGF signalling was perturbed confirmed that the expression of some candidates (Ci-Casz1, Ci-Slc45a2 and Ci-Bzrap1) depends upon proper FGF signalling in PCPs (Fig. 7). Thus, our results reveal that each PCP lineage cell at the dorsal midline of the tailbud stage exhibits a unique combination of transcripts. These combinations constitute specific molecular signatures that define individual cell identities in the dorsal CNS.

**Figure 5** | Sequential FGF-MAPK signalling is required to inhibit expression of anterior CNS markers in PCP territories. (a) Summary model of the timed inhibition of MAPK signalling using UO126 treatment. Wild-type in vitro fertilized embryos were treated with UO126 at the gastrula, neurula and early tailbud stages for 30 min at room temperature. Ciona embryos were electroporated with ptyrp1/2a > 2xGFP to label the PCPs and stained with anti-GFP (green) antibody. (b) Control embryos incubated with dimethylsulphoxide showing Ci-Six3/6 (yellow) and Ci-tyrp1/2a (red) expression at tailbud stage. Dashed line indicates the border among Ci-Six3/6 and Ci-tyrp1/2a endogenous expression pattern. (c) Sequential treatment with UO126 at 5h 30 (a9.49 PCPs), at 6h 20 (d) (a10.97s and a10.98s) and 6h 50 (e) (a11.193s, a11.194s, a11.195s and a11.196s) showing alteration in Ci-tyrp1/2a (red) and Ci-Six3/6 (yellow) expression in PCPs. Lateral view, anterior towards the left. Scale bar, 50 μm; scale bar, 20 μm, for zoomed embryos. Number (n) of embryos showing the observed phenotype out of the total counted embryos, referred to one experiment that was repeated at least three times. Nuclear staining is visualized by DAPI (blue).
Figure 6 | Expression of Ciona candidate PCP genes. Expression pattern at tailbud stage of PCP candidate genes (red): Ci-bzrap (a), Ci-Slc45a2 (b), Ci-Lrp4 (c), Ci-Mnt (d), Ci-Casz1 (e), Ci-Piwi (f) and Ci-doc2a (g); co-localization (merge) of these PCP markers with Ci-tyrp1/2a (green), higher magnification on the right. Schematic representation of a9.49-derived cells in the neural tube of Ciona tailbud stage showing Ci-tyrp1/2a^+ cells (green) and PCP candidates expressing cells (red), adapted from Cole and Meinertzhagen, 2004. Tyrp1/2a transcripts are accumulated mostly in a10.97 progeny (a11.193s/a11.94s, green) than a10.98s derivatives (a11.195s/a11.96s, light green) that maintains only a residual amount of tyrp1/2a RNAs. Lateral view, anterior is on the left. Scale bar, 50 µm; scale bar, 20 µm for zoomed embryo; nuclear staining by DAPI (blue). Number of embryos scored was higher than 30; each experiment was repeated at least two times.
**Ci-Rab32/38 as ‘hub’ gene in Ciona pigment cell formation.** To identify key effector genes necessary for Ciona PCPs formation and controlled by FGF signalling, we used a ‘reverse engineering’ approach. This method identifies co-regulated genes, that is, genes that are co-expressed across a set of experimental conditions. Co-regulated genes may be part of the same pathway, regulated by the same transcription factor, and/or part of the same protein complex. The reverse engineering approach we used is based on computing correlations across pairs of genes from gene expression profiles. Specifically, we computed the Spearman correlation coefficient (SCC) values for each pair of probe sets across the 24 microarrays to build a network of co-regulated genes for Ciona PCP (Methods). The resulting gene network consists of 4,755,086 significant (P value < 0.01) predicted connections among 30,969 probe sets. To identify groups of candidate FGF target genes in early PCPs, we built a smaller subnetwork by selecting only the top 50 differentially expressed genes (when comparing FGFRDN to control samples at 8 hpf) together with their co-regulated genes from the main network (gene neighbours). In a co-regulatory network, most of the genes are connected to few other genes. Nevertheless, it is possible to identify a relatively small number of genes with a large number of neighbours, called hubs. In the subnetwork comprising early FGF targets, we identified a set of ‘hub genes’ and ranked them according to the number of neighbours (Supplementary Table 2). The top two hub genes were annotated as unknown protein in the Ciona genome, even though for the second one we found a blast homology with mouse C3a anaphylatoxin chemotactic receptor, part of the rhodopsin family of G protein-coupled receptor. The remaining hub genes were the transcription factor Ci-NF001, the Ras-related protein Ci-Rab32/38, the Notch co-activator mastermind, Ci-mam and the Melanophilin, Ci-Mlph, a Rab effector involved in melanosome transport along microtubules (Supplementary Table 2).

Among the selected hub genes, we focused on the small GTPase Ci-Rab32/38. In vertebrate melanocytes, the two homologous proteins, Rab32 and Rab38, localize to secretory vesicles of the trans-Golgi network where they function redundantly to transport Tyrosinase- and Tyrp1-containing vesicles to the forming melanosomes. The C. intestinalis genome encodes a unique ortholog, Ci-Rab32/38/rab-rp1/ltd (Ci-Rab32/38), showing a significant differential expression when comparing FGFRDN and control at 8 hpf, as confirmed by *in situ* assays on embryos electroporated with FGFRDN and Ets:Vp16 (Supplementary Fig. 6a–c).

**Figure 7 | FGF signalling controls the expression of new PCP marker genes.** Double fluorescent WMISH of Ci-Casz1, Ci-Slc45a2 and Ci-Bzrap1 (red) with Ci-tyrp1/2a (green) coupled with immunostaining of GFP (light blue, in zoomed embryos). Embryos are electroporated both with ptyrp1/2a>LacZ (a–c) and ptyrp1/2a>FGFRDN (d–f) and ptyrp1/2a>GFP. Lateral view, anterior is on the left. Scale bar, 50 µm; scale bar, 20 µm, for zoomed embryos; nuclear staining by DAPI (blue); number (n) of embryos showing the observed phenotype out of the total counted embryos, referred to one experiment, that was repeated at least three times.
Figure 8 | Rab-type small GTPases as ‘hub’ gene in Ciona pigment cell formation. (a) Early PCP-specific subnetwork inferred from the analysis of microarrays. ‘Hub’ genes are represented as grey spots, numbered from 1 to 5, by ranking them according to the number of neighbours. Ci-Rab32/38 is evidenced in red. (b) Ci-Rab32/38 expression (green) in PCPs during Ciona neurulation: neurula, initial TB, early TB, middle and late TB stages. (c) Double WMISH for Ci-Rab32/38 (green) and Ci-tyrp1/2a (red) from neurula to middle TB stage; nuclear staining by DAPI (blue); n > 50 embryos scored for gene expressions.
Starting at the early neurula stage, Ci-Rab32/38 is specifically expressed in two cells that correspond to a10.97s and subsequently in a11.193s and a11.194s at tailbud stage as confirmed by the co-localization with Ci-tyrp1/2a (Fig. 8b,c).

The specific and early expression of Ci-Rab32/38 in the pigment cell lineage suggested a role in Ciona melanization by regulating vesicle dynamics during pigment cell formation. In keeping with this hypothesis, we observed that a group of genes also implicated in vesicular trafficking and melanosome biogenesis were differentially expressed when FGF signalling is blocked in PCPs between 8 and 12 hpf. This group includes the Rab GAP Wdr67, the Rab effector MyRip/Exophilin and other genes involved in exocytosis and vesicle docking (RABEPK, Rab6– into Ci-Rab32/38 targeted expression of Rab32/38 G19V in PCPs using the tyr enhancer14 altered both otolith and ocellus pigmentation. In more than 70% of larvae, pigmented sensory organs appeared either not or partially melanized (Fig. 9a). Considering that mouse Rab38G19V is unstable because it lacks posttranslational lipid modification that results in premature degradation14, we introduced another point mutation in Ci-Rab32/38 altering a second residue in the highly conserved GTPase fold domain (Rab32/38G19V + T23N) creating a dominant-negative mutated form (T23N)45. Targeted expression of Ci-Rab32/38G19V + T23N in pigment cell lineage caused a drastic reduction of the proportion of larvae (5%) showing two pigmented cells within the sensory vesicle, with more than 90% of perturbed larvae had alteration in at least one of the two pigmented cells (Fig. 9a). To further confirm the role of Ci-Rab32/38, we used short hairpin RNA (shRNA)-mediated RNA interference approach46 to knock down Ci-Rab32/38 activity and analyze the effect on pigment cell formation. We used combinations of shRNA constructs targeting two sites in the Ci-Rab32/38 coding region. Double WMISH assays on embryos electroporated with either the U6>shRab32/38-C and -D or U6>shRab32/38-E and -F combination, showed that Ci-Rab32/38-targeting shRNAs markedly reduced its endogenous expression in PCPs (Fig. 9c,d).

Expression of two pairs of constructs

**Role of Rab32/38 in Ciona pigment cell formation.** In vertebrates, mutations in several Rab GTPases and their regulators result in pigmentation defects due to altered platelet and melanosome formation42. The chocolate (cht) mouse, characterized by a recessive Gly19 to Val mutation, displayed dramatically reduced numbers of melanosomes in adult RPE, in addition to a mild phenotype in skin melanocytes43,44. Depletion of Rab32 in mildly hypopigmented cht melanocytes severely impairs pigmentation and melanosome morphology indicating that the hypopigmentation defects in cht melanocytes was due to a functional compensation of Rab32 (ref. 40). To investigate a possible function of the single Ci-Rab32/38 ortholog gene during Ciona pigmentation process, we first studied the effects of a dominant-negative Ci-Rab32/38 mutant (Rab32/38G19V). PCP-targeted expression of Rab32/38G19V in PCPs using the tyr enhancer14 altered both otolith and ocellus pigmentation. In more than 70% of larvae, pigmented sensory organs appeared either not or partially melanized (Fig. 9a). Considering that mouse Rab38G19V is unstable because it lacks posttranslational lipid modification that results in premature degradation14, we introduced another point mutation in Ci-Rab32/38 altering a second residue in the highly conserved GTPase fold domain (Rab32/38G19V + T23N) creating a dominant-negative mutated form (T23N)45. Targeted expression of Ci-Rab32/38G19V + T23N in pigment cell lineage caused a drastic reduction of the proportion of larvae (5%) showing two pigmented cells within the sensory vesicle, with more than 90% of perturbed larvae had alteration in at least one of the two pigmented cells (Fig. 9a). To further confirm the role of Ci-Rab32/38, we used short hairpin RNA (shRNA)-mediated RNA interference approach46 to knock down Ci-Rab32/38 activity and analyze the effect on pigment cell formation. We used combinations of shRNA constructs targeting two sites in the Ci-Rab32/38 coding region. Double WMISH assays on embryos electroporated with either the U6>shRab32/38-C and -D or U6>shRab32/38-E and -F combination, showed that Ci-Rab32/38-targeting shRNAs markedly reduced its endogenous expression in PCPs (Fig. 9c,d). Expression of two pairs of constructs

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**Figure 9 | Role of Rab32/38 in Ciona pigment cell biogenesis.** Histograms showing the percentage of larvae with two pigment cells (red), one pigment cell (violet) and no pigment cells (orange) after electroporation of dominant-negative forms of Ci-Rab32/38 under control of PCP enhancer (ptyr > Rab32/38G19V or ptyr > Rab32/38G19V + T23N) (a) or short hairpin inhibiting construct against Ci-Rab32/38 mRNA (U6 > shRab32/38-C-D and U6 > shRab32/38-E-F) (b) compared with control larvae electroporated with ptyr > LacZ. Merged bright field/fluorescent images of transgenic larvae. (c,d) Double fluorescent WMISH was performed to test the ability of U6 > shRab32/38 used to knock down the endogenous Ci-Rab32/38. The expression of Ci-Rab32/38 (red) is compared with Ci-tyrp1/2a (green) and with anti-GFP antibody to follow expression of the electroporated constructs. Embryos were electroporated with ptyr1/2a > GFP plus ptyr1/2a > LacZ (c) and ptyr1/2a > GFP plus U6 > shRab32/38-C-D (d). U6 > shRab32/38 is able to knock down the endogenous Ci-Rab32/38 transcripts (n = 33/59, d) and to induce defects in pigmented sensory organ melanization (a,b), while Ci-tyrp1/2a expression resulted not affected (c) as compared with the control embryos (n = 73/80, c). Anterior to the left; nuclear staining by DAPI (blue). Scale bar, 50 µm; scale bar, 20 µm for zoomed embryos. Within each histogram is the combined number of larvae counted during at least three trials; n ≥ 150 embryos scored for transgene expression; error bar, s.e.m.; oc, ocellus, ot, otolith.
(U6 > shRab32/38-C, -D and U6 > shRab32/38-E, -F) resulted in up to 90% larvae showing defective pigment cell melanization (Fig. 9b). Taken together, these data indicate that the complete melanization of the pigment cell is thus significantly affected by Ci-Rab32/38G19V + T23N and Ci-Rab32/38 knockdown experiments, suggesting that proper Ci-Rab32/38 activity has an essential role in pigment cell terminal differentiation.

Discussion

The combination of cell sorting and transcriptome analyses identified pigment cell lineage candidate genes and provided new insights into the mechanisms of CNS patterning in Ciona. We have shown that FGF signalling functions as successive inputs rather than continuously during induction of PCPs. Sequential FGF inputs influence defined regulatory networks successively controlling early fate specification, the selection of definitive pigment cells within the PCP lineage and the processes that underlie pigment cell differentiation at later stages (summarized in Fig. 10).

Our data elucidate the roles of FGF signalling pathway during differential fate specification and anteroposterior patterning of the Ciona CNS. We show that, in the absence of FGF signalling, PCPs change their fate into anterior CNS cells, as judged by ectopic expression in the a9.49-derived PCPs of genes, which are normally expressed in 9.50s derivatives. According to these observations, we propose that FGF signalling imposes a pigment cell identity at the expense of the anterior CNS cell fate, distinguishing between anterior and posterior identities during the formation of Ciona nervous system. This is reminiscent of the situation in vertebrates, where FGF, together with Wnt signalling act to induce a posterior identity on neural tissue during neurulation.

In chick, FGF derived from the lens ectoderm is necessary to maintain the retina domain in the distal optic vesicle. Moreover in FGFR9 mouse mutants, the RPE expands into the presumptive retina suggesting that FGF9 is required to define the boundary between retina and RPE. Our analyses suggest that in Ciona FGF signalling plays an initial role during the induction of

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**Figure 10** | **FGF/MAPK inputs sequentially govern PCP versus anterior CNS formation.** Summary of pigmented cell development within CNS; embryonic stage drawings are indicated below the time line (hpf, hours post fertilization). Black bars connect sister cells. At 6 hpf embryo, the bilateral a9.50s are marked in blue; a9.49 cell pair (in pink) are in close proximity to two sources of FGF signalling molecules, Ci-FGF8/17/18 (light green) and Ci-FGF9/16/20 (dark green) in the underneath row, respectively. Cell lineage scheme indicate one side of the embryo and the colour code of cells remains the same used in the upper drawings. Red arrows indicate FGF/MAPK signalling mediated inductions. a8.25-derived cells with specific gene expression patterns: boxes represent a9.50- and a9.49-derived cells with the same colour code used in the upper drawings. FGFRDN: dominant-negative form of FGF receptor; Ets:Vp16: constitutive active form of Ets1/2.
pigmented versus CNS cells. This could be related to the position of photoreceptor and pigmented cell precursors on the III row cells of the neural plate, both in close proximity to FGF signalling molecules, Ci-FGF8/17/18 and Ci-FGF9/16/20 (refs 11, 18) (Fig. 10). Although all PCPs have the potential to complete pigment cell differentiation, only the two posterior a11.133s will form the otolith and melanocyte of the ocellus. It is likely that specific transcription profiles impose their cell identity to uniquely defined PCP derivatives. Microarray data identified new candidates involved in Ciona pigment cell formation and showing a distinct gene expression profiles among PCPs (summarized in Fig. 10). Although some factors, such as Rab32/38 and Ci-Slc45a2, are associated with melanogenesis in vertebrates3,5, to the best of our knowledge, the others (Ci-Mad/Mnt, Ci-Piwi, Ci-Lrp4, Ci-Brazap1, Ci- Doc2a) have not been implicated in pigmentation before. Interestingly, Ci-tyr, Ci-tyr1p1/2a and Ci-tyr1p1/2b are expressed in PCPs long before melanization begins4 (Fig. 10). This precocious expression of tyr in the pigment cell lineage is a feature shared with the vertebrates13. However, it is not clear why only the two posterior PCPs, a11.193 cell pairs, are finally melanized although all the cells express melanogenic genes. It has been suggested that this could be related to the differential tyr activity in the presumptive pigment cells, where it is finally lost in the most anterior PCP cells that do not become pigmented52. Additional melanocyte-specific constituents influence melanosome biogenesis, such as the availability of the tyr substrate, D-tyrosine, as well as the accumulation of the DOPA cofactor that governs its enzymatic activity. Perhaps the permeability of PCP cell membrane controls the time and rate of access of the tyr substrate and cofactor to the enzyme. Here, we showed that the expression of a subset of transcripts encoding membrane transporters (that is, Ci-Slc2a6, Ci-Slc12a9, Ci-Slc7a6, Ci-Slc25a23) was decreased when FGF signalling was blocked in PCPs. In particular, Ci-Slc45a2, the homologue of SLC45A2, which is responsible for ocucutaneous albinism type 4 in humans53, is selectively expressed in the posterior a11.193 cell pairs. These data suggest that FGF signalling could regulate melanization in the two posterior PCP cells by increasing the permeability and regulating the ionic composition of these cells. Here, we modelled a pigment cell lineage-specific transcriptional network to identify key effector genes involved in the early PCP induction and identified PCP-specific Ci-Rab32/38 as a hub gene. In mice, Rab32 and Rab38 control the trafficking of melanogenic enzymes (Tyr and Tryp1) and are critical for melanosome maturation40. Moreover, it has been demonstrated that loss of Rab38 function alone causes a dramatic reduction in melanosome number within adult mouse RPE cells43. Also in Drosophila, mutation of Rab32/38 ortholog, Rab-RPI, causes eye pigmentation defects55. These evidences parallel our finding in C. intestinalis where targeted perturbations of Ci-Rab32/38 activity interfere with a terminal melanization of the sensory organs. Considering the evolutionary relationship between the vertebrate small Rab GTPase members, Rab32 and Rab38, and the unique Ciona homologue, Ci-Rab32/38, ascidian pigment cells provide a simple and suitable system to identify the interacting partners for Rab32/38 and to study the functions of their regulators as well as the functional relationships with other factors during melanosome maturation.

We showed that FGF signalling is involved in the early pigment cell type-specific regulation of several components of melanosome vesicular trafficking such as Ci-Rab32/38 and the melanophin, Ci-Mlp, which are differential expressed in all pairwise comparisons at 8 hpf. Further studies are required to investigate the role of FGF signalling in the regulation of Ci-Rab32/38 and other melanosome-specific factors, thus offering insights on the molecular events underlying pigment cell biology. Furthermore, our microarray data revealed that FGF regulates the expression of known target-associated albinism or reduced pigmentation, such as HPS1 and LYST (Hermansky–Pudlak and Chédiak–Higashi syndromes34,35). Therefore, it appears that Ciona pigment cells are endowed with the basic molecular machinery that underlies vertebrate melanosome biogenesis and that Ciona melanosomes may thus follow a biogenesis process similar to that observed in more complex organisms. In summary, the importance of Ci-Rab32/38 function during pigment cell formation together with the identification of Ci-Rab32/38 and Ci-Mlp as central hub genes in the inferred 8 hpf network, strongly suggest that FGF signalling regulates the expression of key effectors required to prime the molecular machinery for melanization since the early FGF-mediated induction on PCPs.

Although detailed dissection of the different steps of Ciona melanosome maturation will require more in-depth analyses, Ciona pigment cells provide an opportunity to analyse the conserved molecular basis of melanosome organization in vivo with high spatiotemporal resolution avoiding redundancy and complications of vesicular trafficking components as well as the endocytic network that make difficult to dissect these cellular processes in a more complex system like vertebrates.


Methods

Animals and embryo electroperoration. Adult C. intestinalis were collected from the Gulf of Naples and, for microarray experiments, obtained from M-REP (San Diego, CA, USA). Ripe oocytes and sperm were collected surgically and kept separately until in vivo fertilization. We used a chemical dechorination to eliminate chorion and follicular cells surrounding and protecting the eggs. Dechorionated eggs were then in vitro fertilized from different individuals. Fertilized eggs were washed in fully-filtered sea water (MFSW) transferred in the alternative setting (77°C) overnight, and with the amount of DNA used for electroporation that was typically 50-80 pg of each plasmid. The electroporation was carried out in Bio-Rad Gene Pulser 0.4 cm cuvettes, using Gene Pulser II (Bio-Rad)56,57. Each experiment was performed at least three times. Embryos were staged according to the developmental times established in Hotta et al.58. Embryo imaging was performed with a Zeiss Axio Imager M1 and a Zeiss LSM 510 META confocal microscope.

Cell dissociation and FACS. Embryonic cell suspensions for FACS were obtained as follows: stage-selected embryos were rinsed three times in artificial calcium and magnesium-free sea water (CFM-ASW; 449 mM NaCl, 33 mM Na2SO4, 9 mM KCl, 2.15 mM NaHCO3, 10 mM Tris-Cl pH 8.2, 2.5 mM EGTA) at room temperature. Embryos were dissociated by thorough pipetting for 2–3 min in 1 ml CFM-ASW supplemented with 0.1% bovine serum albumin (BSA, Sigma, A-3311). Cell suspensions were then rinsed three times with ice-cold CFM-ASW + BSA (cell were pelleted at 4°C, by centrifugation at 3,000 r.p.m. (~500–700 g), for 2 min). Cell suspensions were maintained on ice and filtered into 5 ml polystyrene round bottom tubes equipped with a 40 μm cell strainer cap (Falcon) before cell sorting59. Fluorescent-FACS of ptyrp1/2a & ptyrp1 was performed using a Zeiss Axio Imager M1 and a Zeiss LSM 510 META confocal microscope.

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We also built a smaller early responsive to FGF signalling pathway subnetwork by starting from the top 50 differentially expressed transcripts (when comparing FGFRD® versus control at 8 hpf) and the transcripts connected to in the main network (gene neighbours). To identify ‘hub genes’ in this subnetwork, we ranked all the probe sets in the subnetwork according to their degree (that is, the number of connections a probe set has in the network).

In situ hybridization. The following ISH probes were obtained from plasmids contained in the \textit{C. intestinalis} gene collection \textit{I}: trypt1/2a (GC31h05), Bmp5/7 (GC46m03), Bzrap1(GC05h08), Doc2a (GC21m06), Lrp4 (GC42f11), Mad/Mnt (GC02d23), Rab32/38 (GC40p15), S6X3 (GC11m13), FoxH (GC32f03), Neurogenin (GC07n07), Pax3/7 (GC42e02), COE (GC02z14), Macho-1 (GC18g07), Mxsx (GC42h24), Pwi (GC20f14) and 1 ORF Gateway-compatible clone: Caslt (VSE66_L10), RGR/Nat2 (VSE94_E20), Pax6 (VSE87_B20).

PCR amplification of transcription templates was then performed with the following oligos: Slc45a2 5′ (5′-CTCATGGATGATGTTGAC-3′) and Slc45a2 rev (5′-TTATTGGTGTGCAAGACAGGA-3′). DIG- and FLUO-labelled probes were transcribed at 16°C overnight. Embryos were washed in PBT and then incubated with donkey anti-mouse secondary antibody (1:1,500) and Alexa Fluor (Life Technologies) and anti-rabbit secondary antibody (1:1,500) for 1 h at 4°C.

Embryo manipulation. For antibody staining, embryos were fixed in 4% MEM-PFA for 30 min, rinsed in several times in PBT (PBS with 0.1% Tween 20) and incubated with anti-dpERK antibody (Rabbit mAb, Cell Signalling technology, 1:100) and anti-GFP (Mouse mAb, Roche, 1:100) with 2% normal goat serum in PBT at 4°C overnight. Embryos were washed in PBT and then incubated with donkey anti-mouse secondary antibody (1:1,500) and Alexa Fluor 488 (Life Technologies) and anti-rabbit secondary antibody (1:1,500) in PBT at 2% normal goat serum for 1 h at room temperature, then washed in PBT.

Drug treatments. Embryos were obtained by in vitro fertilization, then chemically dechorionated and grown in MFSW at room temperature, until the desired stage. Experimental embryos were treated with the 4 μM MEK inhibitor compound U0126 (Sigma) in MFSW (U0126 stock solution: 2 mM in dimethylsulphoxide) (Sigma) for 30 min 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 ISH or immunohistochemistry.

Construct preparation. For cell sorting, PCPs were labelled using ptyr1/2 > 2Xgfp (for 8 hpf) and ptyr1/2 > GFP (for 12 hpf), the ptyr1/2a minimal enhancer region is the same described in Esposito and coauthors. The ptyr1/2 > GFP, the GFP coding sequence was digested with Xhol and Smal restriction enzymes and fused downstream from ptyr1/2a promoter, replacing mCherry in the ptyr1/2a > mCherry vector, previously digested with Xhol-EcoRV to eliminate mCherry.

For ptyr1/2a > 2Xgfp and ptyr1/2a > LacZ, the ptyr1/2a enhancer was cloned upstream of the 2Xgfp coding sequence and in the in the pBlueScript 1230 vector using NotI and EcoRI restriction sites. For ptyr > Rab32/38, the CDNA of Rab32/38 was amplified by PCR using as template the cDNA clone (N. Satoh Gene Collection 1 ID: CCG40p15), with the oligonucleotides Rab32/38_F (5′-AAGCCGAAAACAGCCTGCTAACGTATT GACGCGAAAGAAGTAGA-3′) and Rab32/38_EcoRI REV (5′-CCCGGATCTTCC GGTATCTGCAAACACCGCTTTTCCT-3′). The Ct-Rab32/38 CDNA, digested with NotI/EcoRI was inserted in place of mCherry in the construct ptyr1/2a > mCherry, previously digested with NotI/EcoRI to eliminate mCherry.

For each different site-directed mutagenesis of ptyr > Rab32/38 construct were induced to test Ct-Rab32/38 function. The first (ptyr > Rab32/38 (G19V)) was prepared by a Gly to Val substitution in position 19 in the highly
conserved phosphate/Mg$^{2+}$ domain, where GTP binding pocket is located. The second point mutation was a threonine to asparagine substitution in position 23 (ptyr > Rab32/38[199 + 22N]), that disrupts the Mg$^{2+}$ binding site, resulting in greatly reduced affinity for guanine nucleotides. The point mutations were performed using (Quick Change Site-Directed Mutagenesis Kit from Stratagene/Agilent) using the following oligonucleotides: 

Rab32/38[199F] (5'-ggctgagattgcagtgattgggagctggacct-3'),
Rab32/38[22N] (5'-aatgacgtcctcctacaactatccgatcagaccag-3'),
Rab32/38[22T] (5'-cgagagctttgagggagtctgctaaattagat-3'),
Rab32/38[22R] (5'-atcattttgacgctccctacaactacgct-3').

shRNA-mediated RNA interference knockdown of Rab32/38 function. The coding sequence of Ci- Rab32/38 was used to identify shRNA target sites with the DSIR and Public TRC Portal (http://www.broadinstitute.org/rnai/public/seq/search) algorithms. Candidate 21-mer target sites were trimmed to 20 mer, and sequences were reverse complemented, and sense and antisense sequences were generated (that is, 16 nucleotide between positions 2 and 17 on the antisense). Target sense sequences were combined and were used to select and commercialize probes.

References


47. Rollins, C. R., L.A.C. contributed reagents/materials/analysis tools. C.R. L.A.C. F.R. wrote the analysis of microarray data. G.G., D.d.B. did transcriptional networking analysis. F.R.-K., E.D.L., R.S., L.A.C., F.R. analysed the data. R.S. did bioinformatical experiments. C.R., A.K.K., R.S., L.A.C., F.R. conceived and designed the experiments. C.R. and A.K.K. performed the preparing constructs. C.R. and A.K.K. have been supported by SZN PhD fellowship. C.R. has been supported by a short-term fellowship from EMBO. This work is supported by the NIGMS/NIH (grant R01GM096032).


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Author contributions

C.R., L.A.C., F.R. conceived and designed the experiments. C.R. and A.K.K. performed the preparing constructs. C.R. and A.K.K. have been supported by SZN PhD fellowship. C.R. has been supported by a short-term fellowship from EMBO. This work is supported by the NIGMS/NIH (grant R01GM096032).