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Transcriptional regulation of *Rab32/38*, a specific marker of pigment cell formation in *Ciona robusta*

Claudia Racioppi[a,b], Ugo Coppola[a], Lionel Christiaen[b], Filomena Ristoratore[a,*]

[a] Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn Napoli, Villa Comunale, 80121 Napoli, Italy
[b] Center for Developmental Genetics, Department of Biology New York University, New York, USA

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ABSTRACT

Through a myriad of pigments stored in different cells, animal pigmentation represents a crucial process to face disparate environmental and ecological challenges. In vertebrates, the small GTPase Rab32 and Rab38 have a conserved role in the transport of key melanogenic enzymes, as tyrosinase (tyr) and tyrosinase-related protein (tyrp), to the melanosomes in formation. We provide a survey on *Rab32/38* evolution and its regulatory logics during pigment cell formation in *Ciona robusta*. Our phylogeny supports the existence of a single *Rab32/38* gene in tunicates, which is probably the unique transporter for tyrosinase family members in this clade. Different deletions allow us to identify the minimal *cis*-regulatory element able to recapitulate the endogenous gene expression during pigment cell development in *C. robusta*. In this conserved region, we identified two putative binding sites for the transcription factor Mitf, which is known for its role as regulator of pigmentation in vertebrates. Mutational analysis revealed that both Mitf binding sites are essential for the activity of this regulatory region and we demonstrated that Mitf misexpression is able to induce ectopic activation of the *Rab32/38* regulatory region *in vivo*.

Our results strongly indicate that Mitf is involved in the regulation of *Rab32/38* activity during *Ciona* pigment cell development.

1. Introduction

Pigmentation represents one of the most fascinating phenomena inside metazoans. Albeit the animal phyla are extremely diversified, in all of them, biological pigments are stored in specialized cells with different developmental origins and functions that have been employed for their adaptation in a multitude of ecological niches.

Amongst biological pigments, melanin is, by far, the most successful during evolution, with distinct roles in prokaryotes and eukaryotes. Animals possess cells containing melanin granules involved in physiological processes that are important to face disparate ecological challenges, such as inter and intra specific behaviors as sexual display and mating (Wittkopp et al., 2002). In vertebrates, melanocytes and melanophores are melanin-containing cells, which derive from the closing neuroepithelium (Quevedo and Fleischmann, 1980). Melanocytes localized in retinal pigmented epithelium (RPE) of the eye and in pineal gland arise from the neural tube, whereas melanin-containing cells of inner ear and skin are neural crest derivatives (Quevedo and Fleischmann, 1980). Melanocyte cell specification and development is governed by diverse pathways like Wnt, BMP and FGF signaling (Fuhrmann, 2010; Yaar and Park, 2012). Similarly to vertebrates, a multitude of pigment cells with several functions is known in invertebrates, as melanin granules in the haemolymph of insects (Nappi and Christensen, 2005) and in the ink gland of cephalopods (Fiore et al., 2004).

Present in early-branching chordates as cephalochordates and urochordates, pigmented cells are mostly associated to larval stage sensory organs. In the tunicate *Ciona robusta*, two pigmented cells are localized in the anterior sensory vesicle (Tsuda et al., 2003; Horie et al., 2005): the otolith, a single melanized cell involved in gravity perception, and the photosensitive ocellus, formed by 30 photoreceptor cells, three lens cells and one cup-shaped pigment cell. Pigmented cells associated to these structures develop following a stereotypical pattern during embryogenesis showing a differential transcriptional code respect to other cells of the lineage (Racioppi et al., 2014). Moreover, *Ciona* pigment cell lineage exhibits a genetic toolkit comparable to vertebrate neural crest cells, such as the centrality of Wnt signaling (Squarzoni et al., 2011; Abitua et al., 2012).

Although melanized cells are diverse in terms of embryonic origins and functions, all of them produce melanin through the Raper-Mason pathway, a complex series of reactions starting from the phenolic amino acid

* Corresponding author.
E-mail address: filomena.ristoratore@szn.it (F. Ristoratore).

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precursor L-tyrosine (Borovansky and Riley, 2011). The metalloproteases Tyrosinase (Tyr) along with Tyrosinase-related protein 1 and 2 (Tyrp1, Tyrp2) play a crucial role in this process (del Marmol and Beermann, 1996; Slominski et al., 2004a, 2004b) and form a family conserved during metazoan evolution, with *tyrp* genes emerged at the stem of chordates (Esposito et al., 2012). For their role in melanogenesis, tyrosinase family members are considered specific markers for pigmented cells: they are expressed in melanocytes of vertebrates (Shibahara et al., 1986; Camp and Lardelli, 2001; Camp et al., 2003) and in pigment cell lineage of amphioxus and ascidians (Camp et al., 2003; Yu et al., 2008; Esposito et al., 2012; Racioppi et al., 2017). Melanin production and storage represent key features of melanocytes, deputed to membrane-enclosed organelles called melanosomes which are lysosome-related organelles (LROs) characterized by an intense vesicular trafficking (Raposo and Marks, 2007). These ultra-specialized organelles develop through a multi-step process, orchestrated by trafficking factors including some Rab small GTPases (Sitaram and Marks, 2012). Among these, Rab32 and Rab38 are localized on trans-Golgi network (TGN) vesicles inside melanocytes and their function is redundant in the transport of tyrosinase- and Tyrp1-containing vesicles to the melanosomes in formation (Slominski et al., 2004a, 2004b; Wasmeier et al., 2006).

In vertebrates, the *Rab32* of frog *Xenopus tropicalis* is localized in skin melanophores (Park et al., 2007), while zebrafish *Danio rerio Rab32a* and *Rab38a* are expressed in RPE and migrating neural crest cells that will give rise to skin pigment cells (Coppola et al., 2016). Mutations in *Rab38* gene are responsible for *Chocolate (cht)* mice mutant strain showing a diluted coat colour and oculocutaneous albinism phenotype (Loftus et al., 2002), while *Rab32* depletion has a similar effect characterized by severe hypopigmentation (Loftus et al., 2002; Wasmeier et al., 2006). Moreover, *Rab38* mutation affects *ruby* rats (Oiso et al., 2004) that is a strain presenting deficiencies similar to the ones present in Hermansky-Pudlak syndrome (HPS), a pathology with dramatic consequences on the pigmentary system (Oiso et al., 2004; Wei, 2006). Coherently, it has been demonstrated that mutations in *Rab-RP1* of *Drosophila melanogaster* (ortholog to *Rab32*) that is expressed in eye lysosomes, cause *lightoid* phenotype, showing hypopigmentation of the eye (Ma et al., 2004). Cumulatively, these findings indicate an involvement in pigmentation during evolution, as also suggested by common evolutionary history for the whole Rab32/38 subfamily in deuterostomes (Wasmeier et al., 2006; Coppola et al., 2016).

In the closest living relative of vertebrates (Delsuc et al., 2006), the tunicate *Ciona robusta*, *Rab32/38* is specifically expressed from neurula stage in four cells of pigment cell lineage (Racioppi et al., 2014) comprising the two cells that will give rise to pigmented cells of the anterior sensory vesicle (Tsuda et al., 2003; Horie et al., 2005). It has been demonstrated that *Rab32/38* is an 'hub' gene in the formation of *Ciona* pigment cells, with a crucial role for their proper development (Racioppi et al., 2014). However, no information are available on how *Rab32/38* gene is specifically regulated in the pigmented cell lineage and which could be the transcription factors that promote its transcription.

Here, we shed light on the transcriptional regulation of the single *Rab32/38* gene present in *Ciona robusta*, to garner understanding in *Ciona* pigment cell specification. We characterized the *cis*-regulatory element controlling *Cr-Rab32/38* expression identifying a small region able to recapitulate the endogenous gene expression in *Ciona* pigment cells. We analyzed two putative Mitf binding sites in this fragment concluding that the bHLH transcription factor Mitf is a strong regulator of *Ra32/38* in pigment cell precursors during *Ciona* development.

2. Materials and methods

2.1. Evolutionary analyses and Transcription Factor Binding Sites survey

The sequences employed for the evolutionary survey were retrieved from NCBI (www.ncbi.nlm.nih.gov), Ensembl (www.ensembl.org) and Aniseed (www.aniseed.cnrs.fr/aniseed) databases, using Rab32/38 of *Ciona robusta* as query sequence (Table S1). The Rab32/38 protein sequences were aligned using Clustal Omega (Sievers et al., 2011) and utilized for a phylogenetic reconstruction with the Maximum Likelihood estimation (MLE) employing MEGA6 with 1000 replicates and the WAG + γ matrix (Tamura et al., 2013). The graphical representation was performed using Dendroscope (Huson and Scornavacca, 2012).

The analysis of conservation among ascidian putative *Rab32/38* regulatory regions was performed on genomic regions from *C. robusta*, *Phallusia mammillata* and *Halocynthia roretzi* (Table S2) using the mVISTA tool (Ratnere and Dubchak, 2009), the sequence of *C. robusta* was employed as reference. We used LAGAN (global pairwise and multiple alignment of finished sequences) and the following parameters: minimum Conservation Width for CNS (40 bp), Minimum Y value (20%), Minimum Length for CNS (100 bp) for the plot. To predict putative transcription factor binding sites (TFBs) in the putative cis-regulatory region of surveyed ascidians, we employed CIS-BP (http://cisbp.ccbr.utoronto.ca) using *Ciona intestinalis* DNA-binding-domain classes database (Weirauch et al., 2014). To study the Mitf binding sites we consulted also the JASPAR (http://jaspar.genereg.net/) database (Khan et al., 2018)

2.2. Animals and embryo electroporation

Adults of *Ciona robusta* were collected from the Gulf of Naples. Gametes from several animals were collected separately for *in vitro* cross-fertilization followed by dechorionation and electroporation as previously described (Christiaen et al., 2009a, 2009b; Racioppi et al., 2014). Embryos were staged according to the developmental timeline established in Hotta et al. (2007). To visualize GFP, embryos were fixed in MEM-FA (3.7% methanol-free formaldehyde, 0.1 M MOPS pH 7.4, 0.5 M NaCl, 2 mM MgSO4, 1 mM EGTA) for 30 min and washed several times in PBS with 0.15% Triton X-100, 0.05% Tween 20. Each electroporation was performed three times and more than 150 embryos were counted for each condition.

2.3. Double in situ hybridization

Double *in situ* hybridizations were carried out essentially as described previously (Christiaen et al., 2009a; Russo et al., 2014; Racioppi et al., 2014), using DIG- and FLUO-labeled riboprobes, anti-DIG-POD andanti-FLUO-POD Fab fragments (Roche, Indianapolis, IN), and Tyramide Amplification Signal coupled to Fluorescein (Perkin Elmer, MA). The antisense riboprobes were obtained from plasmids contained in the *C. intestinalis* gene collection release I: Rab32/38 (GC40p15) and Mitf (GC28k08).

2.4. Observation and imaging

Samples were scored under a Zeiss Axio Imager M1. Imaging was performed using a Zeiss Axio Imager M1 and a Zeiss LSM 510 META confocal microscope.

2.5. Molecular biology techniques

The *cis*-regulatory elements upstream *C. robusta Rab32/38* were identified using Ensembl database (Zerbino et al., 2018) and PCR-amplified. Insertion of the products into expressing vectors was performed using TOPO-TA Cloning kit (Invitrogen). The QuickChange Site-Directed Mutagenesis Kit from Agilent was used to generate the mutations in two putative Mitf binding sites from sequence of the *pRab32/38–3* element. The *pBS/pBra700/GFP/SV40* construct (gift of Dr. Anna Di Gregorio (New York University, New York, USA and Dr. A. Spagnuolo, Stazione Zoologica Anton Dohrn, Napoli, Italy) (Corbo et al., 1997) was used to prepare the construct utilized for the Mitf overexpression in the notochord cells.

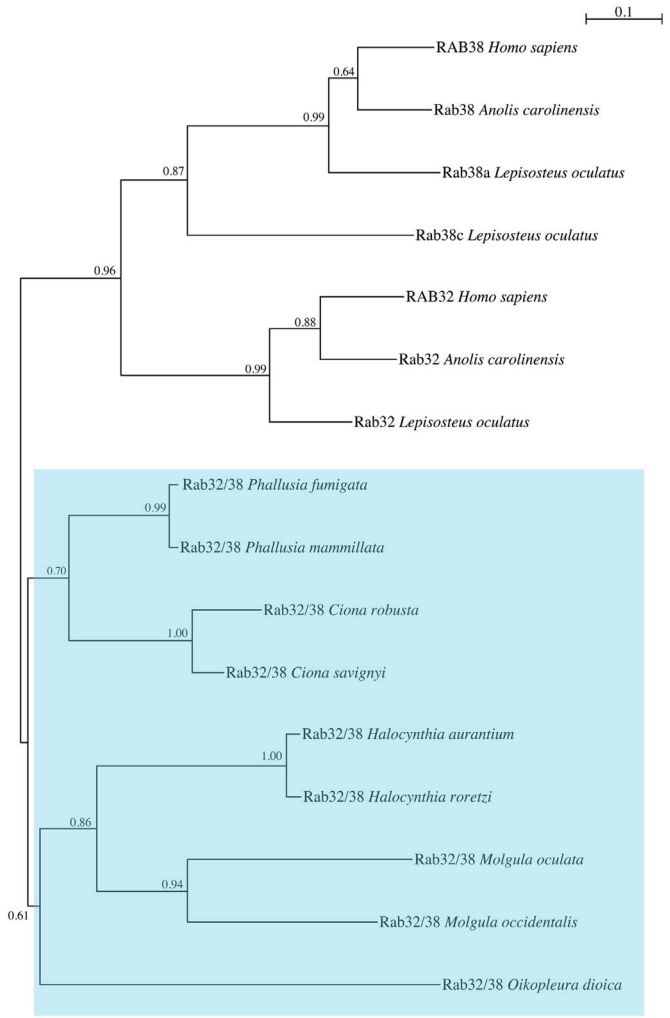

Fig. 1. Evolution of *Rab32/38* in tunicates. The phylogenetic reconstruction showed the orthology among vertebrate *Rab32* and *Rab38* and the unique *Rab32/38* gene of tunicates (light blue box). Values at the branches represent the replicates obtained using the Maximum Likelihood estimation method. The complete protein sequences were employed for tree inference. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article).

The *GFP* reporter gene was replaced with the coding sequence of *Mitf* (KH.C10.106) amplified by Phusion High-Fidelity PCR (Thermo Fisher Scientific) using *"Mitf OVER* forward and reverse" primers, and inserted in *BamHI* and *EcoRI* sites (Table S3). The sequences of the primers mentioned in the manuscript are listed in Table S4.

3. Results

3.1. Rab32/38 evolutionary history in tunicates

To shed light on the evolution of *Rab32/38* gene in tunicate subphylum, a Maximum-Likelihood phylogenetic reconstruction has been performed (Fig. 1) including protein sequences from several tunicates (*Ciona robusta*, *C. savignyi*, *Phallusia mammillata*, *P. fumigata*, *Halocynthia roretzi*, *H. aurantium*, *Molgula occidentalis*, *M. oculata*, *Oikopleura dioica*), vertebrates *Lepisosteus oculatus* (non-teleost fish), *Anolis carolinensis* (reptile) and *Homo sapiens* (mammalian). Our genome search and phylogeny strongly supported the presence of a unique *Rab32/38* gene in all the surveyed tunicates (light blue box, Fig. 1), except in the colonial species *Botryllus schlosseri* and *Botrylloides leachii*.

As we previously showed (Coppola et al., 2016), the topology of the tree evidenced the orthology among *Rab32/38* genes present in non-vertebrate deuterostomes and vertebrate *Rab32* and *Rab38* counterparts. Moreover, to support orthology of Rab32/38 members inside tunicate subphylum, we compared the available protein sequences of tunicates with human RAB32 and RAB38 (Fig. S1). The alignment evidenced high degree of conservation within peculiar Rab domains in all analyzed tunicates (P-Loop, Switch I, Switch II), with few modifications in the protein sequence.

Overall, our evolutionary survey reveals a strong phylogenetic signal for the conservation of a unique *Rab32/38* gene in tunicates.

3.2. The Rab32/38 cis-regulatory region analysis

In light of *Rab32/38* centrality to pigment cell development in *C. robusta* (Racioppi et al., 2014) and its conserved role in pigmentation dynamics during evolution (Coppola et al., 2016), we used deletion analysis to investigate the *Rab32/38* regulation in *Ciona* (Fig. 2). To gain insights in *Rab32/38* regulatory scenario in ascidians, we first surveyed the putative regulatory sequences of *C. robusta*, *H. roretzi* and *P. mammillata* (Fig. 2A) and we found conservation (using mVISTA browser) among a region of 344 bp of *C. robusta* (−398 to −54) and those of *H. roretzi* (520 bp, −600 to − 92) and of *P. mammillata* (375 bp, −73 to 448). In particular, we uncovered higher degree of conservation between *C. robusta* and *H. roretzi* (Fig. 2A). In contrast, the comparison with upstream genomic region of vertebrate *Rab32* and *Rab38* genes, did not reveal any relevant homology.

To isolate the *cis*-regulatory element controlling *Rab32/38* expression in *Ciona robusta*, we focused our attention on an intergenic non-coding region of 660 bp (−714 to −54 from the ATG) localized between *Rab32/38* and the contiguous gene (Fig. 2A), as annotated on Aniseed genome browser (Brozovic et al., 2016, 2018). To evaluate whether this element was sufficient to activate transcription of a reporter gene, we isolated this region by PCR from *C. robusta* genomic DNA and we cloned this DNA fragment (called *pRab32/38-1*) in a vector containing GFP reporter gene with *β-globin* minimal promoter (Zeller et al., 2006). By reporter gene, we detected GFP expressing embryos starting from late tailbud stage (stage 22, according to Hotta et al., 2007). In 65% of embryos, GFP expression was observed in two of the four cells expressing endogenous *Rab32/38*, corresponding to pigment cell precursors (Fig. 2B). At larval stage (stage 26), GFP expression was strongly detected around the two sensory organs pigmented cells, the otolith and ocellus (Fig. 2D).

To characterize the minimal *Rab32/38 cis*-regulatory element, we used highly conserved non-coding sequences between *C. robusta* and *Ciona savignyi* as a guide for molecular dissection (Fig. 2A). We identified two main conserved regions inside the *pRab32/38-1* element and we analyzed whether these two conserved regions contained functional elements by introducing deletions. We isolated a region of 340 bp (−714 to −374) encompassing the first conserved region, that we called *pRab32/38-2*, and another element of 344 bp (−398 to −54 bp) that includes the second conserved region, named *pRab32/38-3* (Fig. 2C). We tested both elements activity by reporter gene assay following GFP expression during *Ciona* development. Only *pRab32/38-3* was sufficient to active the transcription of GFP in around 45% of tailbud and larvae embryos, whereas no GFP expression was detected when using the *pRab32/38-2* element (Fig. 2C).

This result suggested that *pRab32/38-3* contains transcription factor binding sites necessary to activate *Rab32/38* expression in the pigment cell lineage, according to high sequence conservation of this region with distantly-related ascidians (Fig. 2A). To further analyze the minimal functional element driving reporter expression, we introduced 5' deletions of the *pRab32/38-3* element. Thus, we divided *pRab32/38-3* element in two fragments of 172 bp, *pRab32/38-3A* (−398 to −226) and *pRab32/38-3B* (−226 to −54), and tested them by reporter gene assay (Fig. 2C). Only *pRab32/38-3B*, was able to drive GFP expression in the pigment cell lineage (~20%) suggesting this region contains all the functional *cis*-regulatory elements to activate transcription on its own in a reporter assay (Fig. 2C).

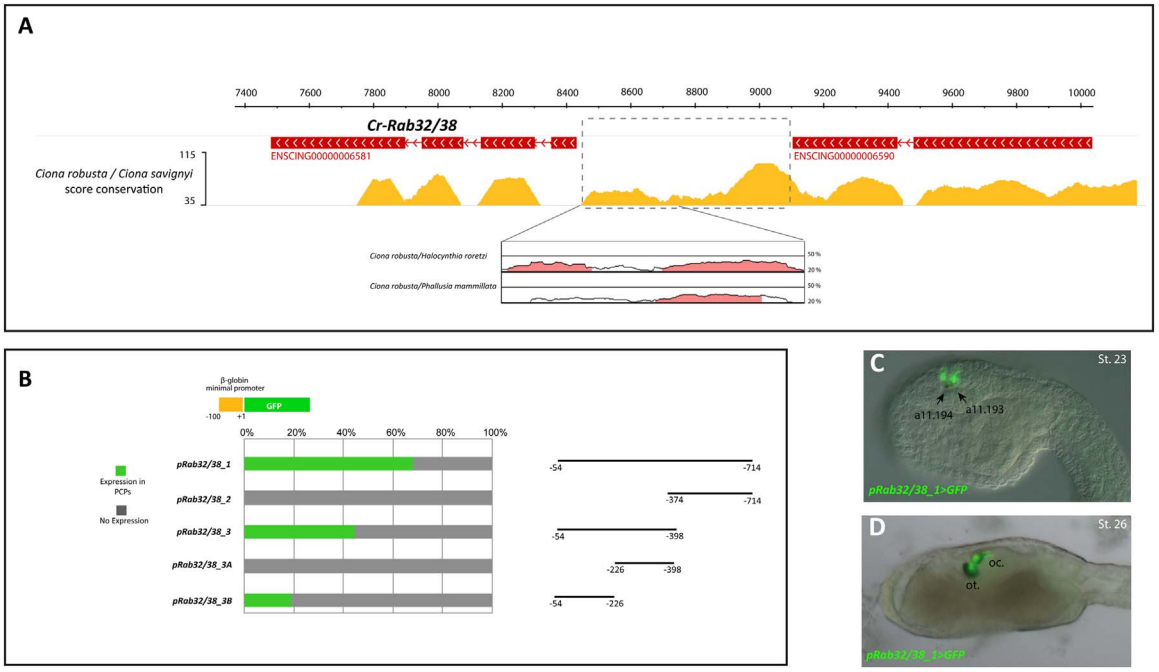

Fig. 2. Analysis of *Rab32/38 cis*-regulatory region in *Ciona robusta*. (A) Conservation score of *Rab32/38* locus between *Ciona robusta* and *Ciona savignyi* using WASHU browser (Brozovic et al., 2018); the dashed rectangle evidences the analyzed intergenic region; in the linked box VISTA plot among *C.robusta*, *Phallusia mammillata* and *Halocynthia roretzi* regions upstream *Rab32/38* orthologs (B) Percentage of embryos expressing GFP reporter in pigment cell precursors (a11.194 and a11.193 cells) along with schematic representations of the fragments dissected and analyzed by reporter gene assay. Within each bar is the combined number of larvae counted during at least three trials; n > 150 embryos scored for transgene expression. (C-D) Side view of tailbud and larva embryos (stage 23 and 26 according to (Hotta et al., 2007), respectively) electroporated with *pRab32/28_1 > GFP* plasmid. GFP expression is detected in a11.194 and a11.193 cells at stage 23 (C) and in pigmented cells of otolith and ocellus at stage 26 (D).

3.3. Mitf binding is necessary for Rab32/38 cis-regulatory activity

To further analyze the regulatory logics underlying *Rab32/38* expression in *Ciona* pigment cells, we therefore sought to identify putative transcription factor binding sites (TFBSs) in *pRab32/38-3* fragment using CIS-BP database for *C. intestinalis* motifs (Weirauch et al., 2014) and the JASPAR database for vertebrates (Khan et al., 2018).

Among the several predicted binding sites present in the *Rab32/38* non-coding regulatory sequence, we found several bHLH motifs, including *Mitf*. Given that *Mitf* (Microphthalmia-associated transcription factor) (Goding, 2000) is known to activate *Tyr* and *Tyrp* genes during the melanogenesis of neural crest-derived melanocytes in vertebrates (Curran et al., 2010) we focused our attention on this gene as potential regulator of *Rab32/38* transcription.

In *C. robusta* genome, only one gene encoding for *Mitf* has been annotated and it is expressed in the a9.49 lineage (Abitua et al., 2012) and *Mitf* ortholog is expressed in the pigment cell lineage of the tunicate *H. roretzi* as well (Yajima et al., 2003).

By double fluorescent *in situ* hybridization (FISH), we confirmed that *Mitf* and *Rab32/38* are co-expressed in the pigment cell precursors at tailbud stages (from stage 20–22), where both transcripts are detected in posterior a11.193 and a11.194 cells, which give rise to ocellus and otolith pigment cells, respectively (Fig. 3A, A"). Notably, these are the same cells where we detected *Rab32/38 cis*-regulatory activity.

To assess whether Mitf is controlling *Rab32/38* activation in *Ciona* pigment cells, we selected bHLH binding sites, with high predicted scores for Mitf, using also JASPAR database (Khan et al., 2018), and we individually mutated the core motif for each of these sites that we called Mitf1 (CACGCG) and Mitf2 (CACGTG) (yellow in Fig. 3B).

We then tested the activity of the *pRab32/38-3* fragment leading point mutations in Mitf1 and Mitf2 binding sites by reporter-gene assay (red in Fig. 3D). Only 5% embryos electroporated with *Mitf1_Mut* were expressing GFP in the pigmented sensory organs, whereas 11% of embryos were expressing the reporter in pigment cells when electroporated with *Mitf2_Mut* plasmid. These results are consistent with the presence of putative bHLH binding sites in the *Rab32/38* upstream sequences of *C. savignyi*, *H. roretzi* and *P. mammillata* analyzed by employing CIS-BP database for *C. intestinalis* (Fig. S2).

Altogether, we showed that *Mitf* and *Rab32/38* transcripts are colocalized in the two posterior pigmented cell precursors and that *Rab32/38 cis*-regulatory region activity was strongly affected when at least one copy of each putative TFBS recognized by Mitf was mutated (Fig. 3C).

3.4. Overexpression of Mitf in the notochord induced ectopic activity of Rab32/38 cis-regulatory region in vivo

To investigate the role of Mitf in regulating *Rab32/38* enhancer *in vivo*, we induced ectopic expression of Mitf in the notochord, a territory where it is normally not expressed. To this aim, we cloned the coding sequence of *Mitf* downstream of the promoter sequence of the notochord-specific gene *Brachyury* (*pBra > Mitf*) (Corbo et al., 1997; Yamada et al., 2003). Then, we electroporated *pBra > Mitf* along with the minimal *cis*-regulatory region of *Rab32/38*, comprising the two putative Mitf binding sites, driving GFP *(pRab32/38-3 > GFP)* and fixed embryos at late tailbud (st. 16) and late hatching larva stage (st. 26) for reporter gene visualization (Fig. 4).

In 23% of larvae co-electroporated with *pRab32/38–3 > GFP* and *pBra > Mitf* (26 and 30 stages), we detected GFP in some notochord cells compared to the control embryos electroporated with *pRab32/38-3 > GFP* only (Fig. 4 A-C). The reporter gene was not found in all the notochord cells due to the mosaic incorporation of the transgene (Fig. 4 B-D). To further validate the ability of Mitf to activate *Rab32/38* in the notochord cells, we co-electroporated *pBra > Mitf* along with the *wt pRab32/38-3* fragment or Mitf1 and Mitf2 mutated site constructs. The Mitf-sites-mutated constructs strongly reduced GFP expression in the notochord: we detected GFP in the notochord cells in 4% of

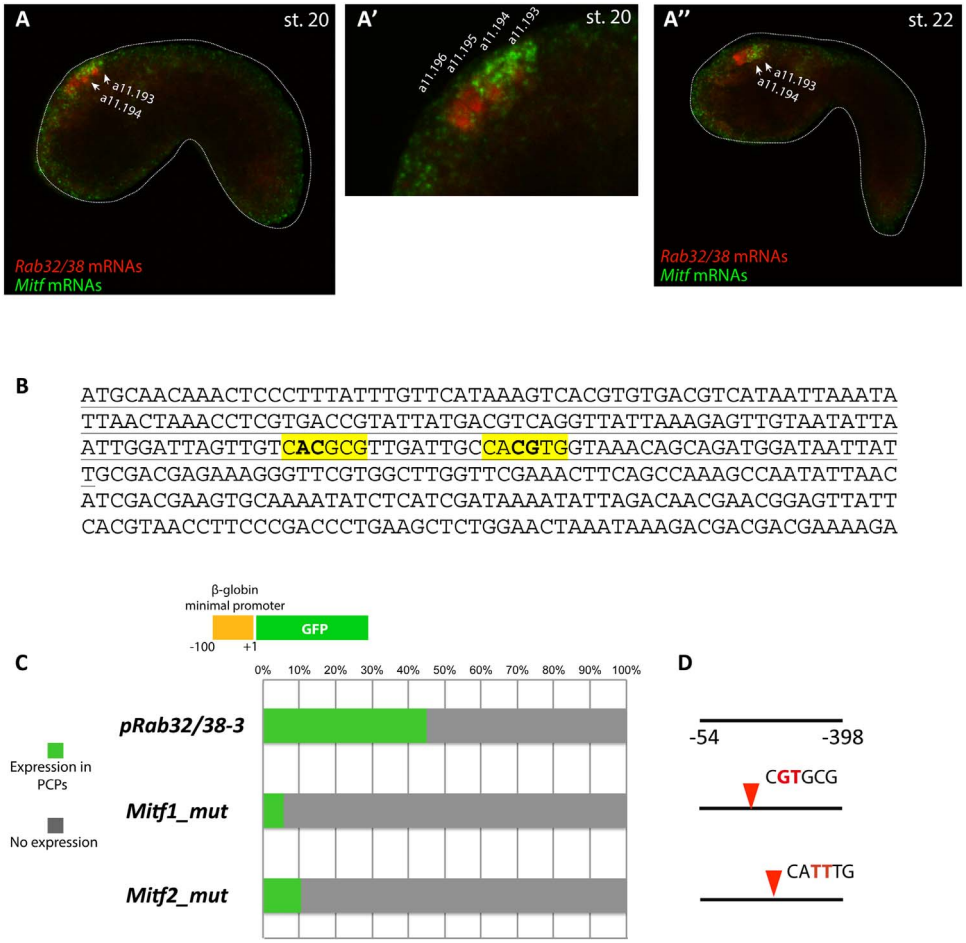

Fig. 3. Two putative Mitf binding sites control *Rab32/38 cis*-regulatory element activity. (A-A") Double whole-mount *in situ* hybridization experiments of *Rab32/38* (red) and *Mitf* (green) at tailbud stages (stage 20, A and stage 22, A"). *Rab32/38* and *Mitf* transcripts co-localize in the two posterior pigment cell precursors (a11.193 s and a11.194 s) which give rise to ocellus and otolith pigment cells, respectively (A', zoom view of co-expression domain; *Rab32/38* is expressed in all four pigment cell precursors, labeled as a11.193, a11.194, a11.195 and a11.196). (B) Sequence of *Ciona robusta pRab32/38_3* with putative Mitf binding sites highlighted in yellow; the fragment named *pRab32/38_3B* is underlined. Nucleotides selected for mutational analysis in Mitf_1 and Mitf_2 sites are reported in bold. (C-D) Percentage of embryos expressing GFP reporter in pigment cell precursors (PCPs) in the reporter gene assay. Within each bar is the combined number of larvae counted during at least three trials; n > 150 embryos scored for transgene expression. (D) Schematic representations of *pRab32/38-3* fragments, wt and with point mutation targeted in Mitf_1 and Mitf_2 putative binding sites, represented as red triangles. The mutated nucleotides are reported in red and in bold. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article).

embryos co-electroporated with *Mitf2_mut* and *pBra > Mitf* whereas only 1% when using *Mitf1_mut* and *pBra > Mitf* at both 26 and 30 stages (Fig. 4E).

Taken together, these results indicate that Mitf is able to activate the *cis*-regulatory region of *Cr-Rab32/38* through the binding of Mitf sites *in vivo*.

4. Discussion

Similar to findings in vertebrates (Loftus et al., 2002; Park et al., 2007; Coppola et al., 2016), *Rab32/38* has a key role in ascidian pigment cell development. Our phylogenetic survey (Fig. 1) confirms the scenario based on the orthology between invertebrate deuterostome *Rab32/38* and vertebrate *Rab32* and *Rab38* (Coppola et al., 2016) suggesting a role in pigmentation for this gene in tunicates, considering that vertebrate Rab32 and Rab38 are implicated in transporting tyrosinase family member,s as Tyr and Tyrp proteins, to the melanosomes in formation (Wasmeier et al., 2006). The alignment of available tunicate Rab32/38 proteins demonstrated high degree of conservation of P-Loop, Switch I and Switch II functional domains with some exceptions, i.e. the Switch I in *O. dioica* and P-Loop in *Molgula spp.* and *Halocynthia* spp. (Fig. S1). Importantly, each tunicate protein has retained an amino acid quartet downstream to Switch I (FALK) that is shared exclusively by the members of Rab32/38 subfamily (Coppola et al., 2016). The presence in tunicates of a unique *Rab32/38* gene (Fig. 1, Fig. S1) and our previous data demonstrating the crucial role for *Rab32/38* in *Ciona* pigment cell formation (Racioppi et al., 2014), suggest its centrality in pigment cell specification in the whole clade leading to surmise that Rab32/38 protein is able to transport all the melanogenic enzymes present in ascidians (Racioppi et al., 2017). Expression of *Rab32/38* in pigment cell lineage also in *P. mammillata* (Aniseed Expression Database, Dumollard Team), could support this scenario.

The implication of Rab32/38 members in pigmentation dynamics during metazoan evolution, together with its sustained expression in *Ciona* pigment cell lineage (Racioppi et al., 2014), prompted us to gain insights in *Rab32/38* regulatory scenario. The comparison among different ascidian genomes sheds light on a conserved non-coding intergenic region upstream *Rab32/38* (Fig. 2A), possibly related to the existence of shared regulatory mechanisms for this gene. Albeit tunicates are characterized by highly divergent genomes (Berná and Alvarez-Valin, 2014), these data and the evolutionarily conserved role for *Rab32/38* genes in pigmentation could subtend the presence of conserved non-coding elements also in other non-vertebrate deuterostomes, which have a single *Rab32/38* (Coppola et al., 2016). Here, we studied a non-coding element upstream the transcription starting

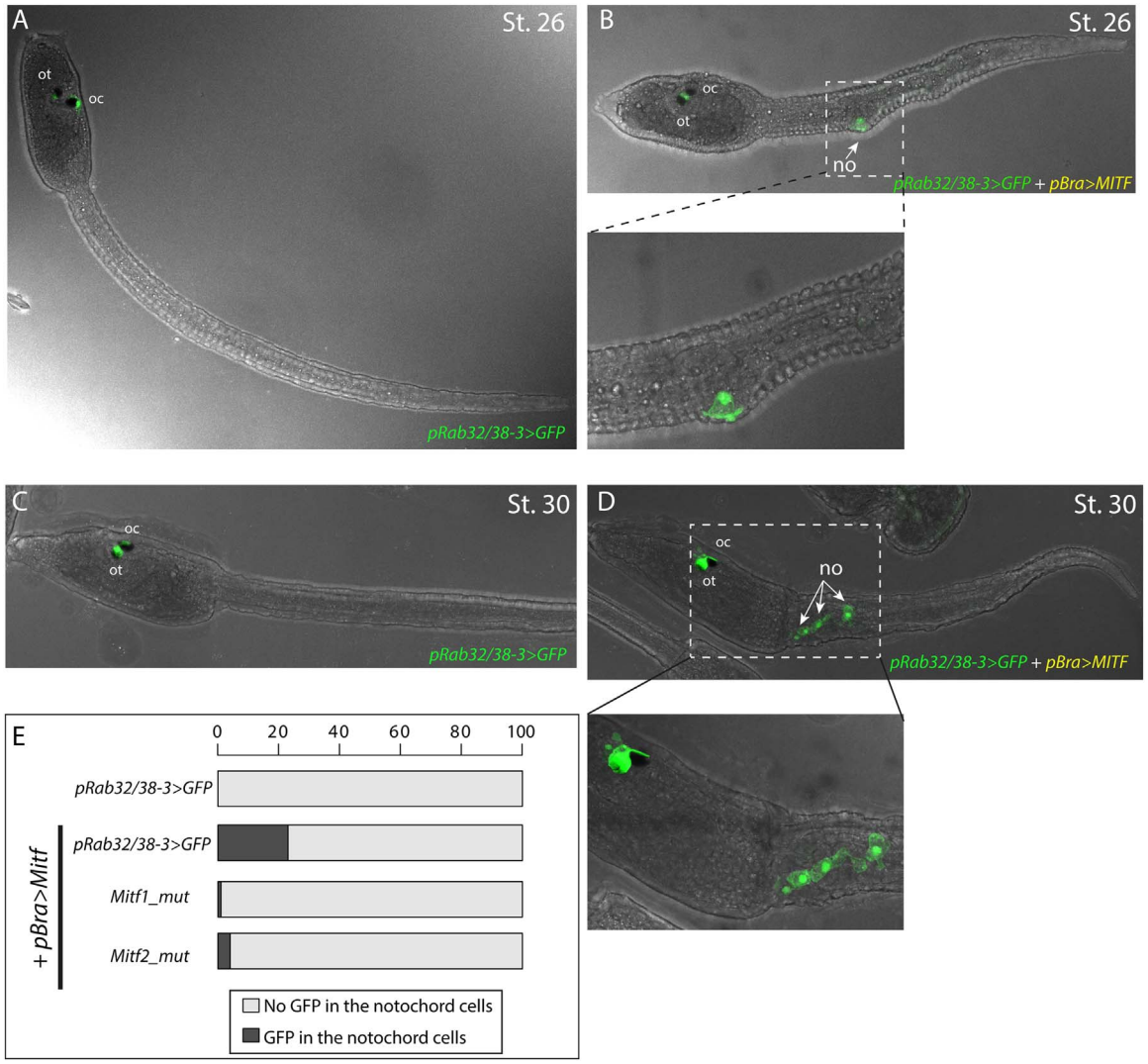

Fig. 4. Cr-Rab32/38 ectopic activity *in vivo* driven by overexpression of Cr-Mitf in the notochord. (A,D) Side view of larva embryos (stage 26 and 30 according to (Hotta et al., 2007), respectively) electroporated with *pRab32/28_3 > GFP* plasmid (A,C) and *pRab32/28_3 > GFP* along with *pBra > Mitf* (B,D) with zoom view on the notochord in the tail. GFP expression is detected in pigmented cells of otolith (ot) and ocellus (oc) and in the notochord cells (no). (E) Percentage of larvae expressing GFP reporter in the notochord cells. Within each bar is the combined number of larvae counted during at least three trials; n > 120 embryos scored for transgene expression.

site of *Rab32/38* sufficient to activate a reporter gene and recapitulate *Rab32/38* endogenous expression in pigment cells. Indeed, this region is able to activate *GFP* transcription in pigment cell precursors at tailbud stages and in the pigmented cells associated to sensory organs, both otolith and ocellus, at hatching larva (Fig. 2).

When searching for TF motifs in the *Rab32/38* non-coding regulatory sequence, we identified putative binding sites for the transcription factor Mitf. Known for its conserved role as regulator of pigmentation in vertebrates (Levy et al., 2006) and co-expressed with *Rab32/38* in the two posterior PCPs which give rise to the otolith and ocellus pigment cells in the larva sensory organs, Mitf represented a good candidate involved in regulating *Rab32/38* expression. Additionally, *Mitf* is the only bHLH transcription factor expressed in *Ciona* pigmented cells. Indeed, in our previous microarray analysis, we identified *Mitf* downregulated when FGF signaling is blocked in PCPs by using a dominant-negative form of the unique FGF receptor in *Ciona* (Racioppi et al., 2014).

Here, we sought to characterize the putative role of Mitf as a transcription regulator of *Rab32/38*. Mutational analyses of two Mitf putative binding sites reduced significantly *pRab32/38-3* activity in pigment cell precursors, pointing out an implication of Mitf in the activation of *Rab32/38* transcription during *Ciona* pigment cell development (Fig. 3). We then demonstrated that Mitf is not only necessary but also sufficient to activate *Rab32/38* enhancer *in vivo* by overexpressing Mitf in the notochord cells, a territory where this gene is normally not expressed (Fig. 4). When *Mitf* is expressed in an ectopic tissue as the notochord, it is able to recognize the binding sites present in *pRab32/38-3* region and induce GFP expression in the notochord cells. Conversely, when Mitf1 and Mitf2 binding sites were mutated in the *Rab32/38* enhancer element, the ectopic expression in the notochord cells was strongly reduced. Notably, the regions conserved amongst distantly-related ascidians contain putative binding sites for Mitf (Fig. S2), suggesting a possible conservation of its role in the regulation of *Rab32/38* in this clade that needs to be investigated. Although one of the mutated Mitf sites is not “canonical” as in vertebrates (Goding, 2000; Pogenberg et al., 2012), our searches in TFBs databases lead to suppose a certain degree of variability in E-box motif for Mitf in ascidians, as suggested for other bHLH factors like Hey in *Drosophila melanogaster* (Heisig et al., 2012).

In summary, our findings shed light on the regulation of *Rab32/38*, a gene conserved in tunicates with a key role in pigment cell development. The bHLH transcription factor *Mitf* is involved in *Rab32/38* regulation in *Ciona* and probably in ascidian pigment cell formation.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.11.013.

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