The Cis-Regulatory Code for Kelch-like 21/30 Specific Expression in Ciona robusta Sensory Organs

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The tunicate Ciona robusta is an emerging model system to study the evolution of the nervous system. Due to their small embryos and compact genomes, tunicates, like Ciona robusta, have great potential to comprehend genetic circuitry underlying cell specific gene repertoire, among different neuronal cells. Their simple larvae possess a sensory vesicle comprising two pigmented sensory organs, the ocellus and the otolith. We focused here on Klhl21/30, a gene belonging to Kelch family, that, in Ciona robusta, starts to be expressed in pigmented cell precursors, becoming specifically maintained in the otolith precursor during embryogenesis. Evolutionary analyses demonstrated the conservation of Klhl21/30 in all the chordates. Cis-regulatory analyses and CRISPR/Cas9 mutagenesis of potential upstream factors, revealed that Klhl21/30 expression is controlled by the combined action of three transcription factors, Mitf, Dmrt, and Msx, which are downstream of FGF signaling. The central role of Mitf is consistent with its function as a fundamental regulator of vertebrate pigment cell development. Moreover, our results unraveled a new function for Dmrt and Msx as transcriptional co-activators in the context of the Ciona otolith.

Keywords: tunicates, otolith, Mitf, Klhl family evolution, pigment cells, CRISPR/Cas9, cis-regulatory regions

INTRODUCTION

Understanding the developmental logics that orchestrate specific gene expression inside the nervous system represents a fascinating challenge in cell and developmental biology. However, the identification of the molecular processes underlying cell specific expression among different neuronal cells is very difficult using vertebrate models, due to the relative complexity of vertebrate embryos and genomes, as well as the numbers of genes involved.

Within the chordates, the tunicate subphylum is the sister group of vertebrates, forming with them the clade Olfactores (Delsuc et al., 2006). Due to their small, invariant embryos and compact genomes (Berná and Alvarez-Valin, 2014), tunicates have great potential to help to uncover the genetic circuitry regulating chordate-specific mechanisms of neural development. The larva of the tunicate Ciona robusta possesses two distinct pigmented sensory organs, the otolith and the ocellus, contained in the anterior sensory vesicle, a structure evolutionarily related to the forebrain of vertebrates (Moret et al., 2005; Dufour et al., 2006). The ocellus is formed by 30 photoreceptors, three lens cells and one melanized cup-shaped cell (Horie et al., 2005). Due to its association with photoreceptors, the ocellus has been compared to the vertebrate eye (Kusakabe et al., 2001; Nakagawa et al., 2002; Nakashima et al., 2003; D’Aniello et al., 2006). In contrast, the otolith is a
single spherical cell containing melanin granules and attached to the sensory vesicle floor through a tight stalk (Dilly, 1962, 1969). It has been proposed that the displacement of the otolith within the fluid-filled sensory vesicle lumen stimulates a putative mechanosensory antenna cell in the adjacent brain, thus playing a key role in negative geotropism and gravitational orientation (Tsuda et al., 2003; Sakurai et al., 2004). Supporting this model, an ammonium channel regulates the sensory vesicle fluid composition and otolith functioning (Marino et al., 2007). In addition, the pigmentation of these sensory organs is fundamental for normal photo- and geotactic behaviors in the close sibling species C. savignyi (Jiang, 2005).

During embryonic development, ocellus and otolith pigment cells derive from a pair of left/right pigment cell precursors (PCP) in the neural plate that intercalate at the dorsal midline of the neural tube during neurulation (Cole and Meinertzhagen, 2004). Later, during neural tube closure, PCPs divide twice and give rise to a total of 8 cells at early tailbud stage, which express melanogenic genes belonging to the Tyrosinase (Tyr) family (Tief et al., 1996; Caracciolo et al., 1997; Esposito et al., 2012; Haupaix et al., 2014; Raciotto et al., 2014, 2017). The loss of Tyr function in Ciona results in pigment-free larvae (Sordini et al., 2008; Crocetta et al., 2015), whereas some tunicate species (e.g., Molgula occulta) have lost Tyr family genes and lack melanin pigmentation altogether (Racioppi et al., 2017).

The majority of the transcription factors and cell signaling molecules implicated in otolot and ocellus differentiation are expressed in the whole PCP lineage, even though only two cells will become pigmented. A FGF-dependent transcriptional code for the formation of Ciona PCPs was recently partially elucidated (Racioppi et al., 2014). Among the two pigmented cells the anterior-posterior order of intercalation orchestrates ocellus versus otolith pigment cell determination: the anterior cell always becomes the otolith while the posterior cell always gives rise to the ocellus pigment cell. These mechanisms, which are still not well studied, involve a Wnt and FoxD-mediated suppression of Pax3/7-dependent activation of the Microphthalmia-associated transcription factor gene (Mitf) (Abitua et al., 2012).

Here, we focused on the transcriptional control of Klhl21/30, a gene that we found to be specifically expressed in the ocellus and otolith precursors, during embryogenesis. Klhl21/30 belongs to the Kelch-like (Klhl) family of genes, encoding for proteins characterized by the presence of multiple Kelch motifs, which are evolutionarily conserved, but poorly characterized, short domains implicated in protein-protein interactions (Adams et al., 2000). In mammalian cells, Klhl21 is thought to be involved in E3-ubiquitination during cytokinesis and regulation of cortical dynamics (Maerki et al., 2009; Courthieux et al., 2016) and is implicated in diverse types of carcinoma (Shi et al., 2016; Chen et al., 2018). We provide the most updated evolutionary reconstruction of Klhl family, demonstrating that Klhl21/30 is ultra-conserved in chordates. This gene has dynamic expression pattern in Ciona PCPs, becoming restricted to the otolith during embryogenesis. Klhl21/30 transcription is governed by a complex regulatory code, in fact, we have identified the minimal key cis-regulatory element able to drive Klhl21/30 expression in the C. robusta otolith, containing functional binding sites for the transcription factors Mitf, Msx, and Dmrt. Using tissue-specific CRISPR/Cas9-mediated gene knockouts, we found that Mitf is central to Klhl21/30 expression in the otolith, as it is for many pigmentation markers in the pigment cells of vertebrates (Levy et al., 2006), with Msx and Dmrt acting as co-activators.

RESULTS
Kelch-like Gene Family Evolution in Chordates
A lineage-specific transcription profiling of genes downstream of fibroblast growth factor signaling (FGF), was used to find novel players involved in pigment cell formation (Racioppi et al., 2014). Among these, we found a Kelch-like (Klhl) gene family member (Kyoto Hoya gene model KLHL84.23), which exhibited similar expression values of known PCP markers, including Tyr, Tyrp.a and Rab32/38 and that, by reciprocal BLASTs, is similar to vertebrate Kelch-like 21 (Klhl21) and Kelch-like 30 (Klhl30).

To shed light on the evolutionary origins of the Klhl21/30 gene and to gain insights into the poorly studied Klhl family in metazoans, we performed an evolutionary analysis of these proteins in chordates. First, we analyzed their domain organization in PROSITE (de Castro et al., 2006) and Ensembl databases. This revealed the presence of one BTB/POZ domain, one BACK domain and five Kelch repeats (Supplementary Figure S1) in both C. robusta Klhl21/30 and Homo sapiens KLHL21 (Dhanoa et al., 2013). To confirm the orthology of C. robusta Klhl21/30 (KH.L84.23) and study the evolution of Kelch-like genes in chordates, we performed a phylogenetic survey employing a manually curated database (Supplementary Table S1). Our phylogenetic reconstruction included 118 Kelch-like protein sequences from the cephalochordate amphioxus Branchiostoma belcheri, tunicates Oikopleura dioica and Ciona robusta, and human Homo sapiens as representative of vertebrates (Figure 1). Sequences with high degree of molecular divergence have been excluded from phylogeny and listed in the Supplementary Table S2. In total, we identified 25 Klhl subfamilies, conserved throughout chordate evolution from cephalochordates to vertebrates, with a unique exception (KLHL5L) that is tunicate-specific (Figure 1). Genome search and phylogenetic tree defined the complete Kelch-like toolkit of B. belcheri (37), C. robusta (32), O. dioica (14), H. sapiens (42): this confirms the previous survey performed in human (Dhanoa et al., 2013) and represents the first step into understanding Kelch-like evolution in all three chordate subphyla. Though the number of Kelch-like genes in the amphioxus genome has been shaped by various amphioxus-specific duplications (as in the case of Klhl42 duplications), our survey is coherent with relative genomic stasis of this slow-evolving branch (Putnam et al., 2008). In contrast, C. robusta retained 32 Klhl genes and 16 subfamilies, whilst O. dioica maintained 14 genes and 10 subfamilies. This is coherent to the tendency of the fast-evolving Oikopleura dioica to lose large portions of gene families (Berná and Alvarez-Valin, 2014; Albala and Cañestro, 2016; Martí-Solans et al., 2016; Coppola et al., 2019). Otherwise, we also detected different...
FIGURE 1 | Phylogenetic tree of Kelch-like proteins in chordates. Maximum likelihood (ML) reconstruction of 118 Kelch-like members from four key chordate species: amphioxus Branchiostoma belcheri, larvacean Oikopleura dioica, ascidian Ciona robusta, human Homo sapiens. The phylogeny demonstrates the existence of a robust Klhl21/30 subfamily (orange box). Numbers at the branches indicate replicates obtained using the ML estimation method.
tunicate-specific duplications of certain Kelch-like genes in either species (Figure 1).

In particular, our phylogenetic tree clustered Ciona KH.L84.23 with one O. dioica protein, two B. belcheri proteins (deriving from a local duplication) and H. sapiens KLHL21 and KLHL30, forming a protein class with robust support that we named Klhl21/30 (orange box; Figure 1). The presence of two related genes in human speaks in favor of a common origin for vertebrate Klhl21 and Klhl30, which could have derived from a local duplication or a whole-genome duplication event in the vertebrate ancestor (Abi-Rached et al., 2002; Dehal and Boore, 2005). In contrast, invertebrate chordates possess a single Klhl21/30 gene, similar to other invertebrates such as the nematode Caenorhabditis elegans (data not shown). Remarkably, Klhl21/30 is one of the few Kelch-like genes not lost by O. dioica and therefore could be functionally relevant for conserved developmental processes. Moreover, we analyzed the Klhl21/30 locus in several available tunicate genomes, finding high degree of local synteny surrounding Klhl21/30 in C. robusta, C. savignyi, Phallusia mammillata, and Halocynthia roretzi (Supplementary Figure S2). Our survey prompted us to describe an ancestral cluster of 11 genes in tunicates, nearly all conserved between C. robusta and P. mammillata. Interestingly, all surveyed species exhibited linkage between Klhl21/30 and Nph4 (nephrocytin-4) genes, which is also observed in human between KLHL21 and NPH4. Conversely, this linkage has been lost for KLHL30 (Supplementary Figure S3). The comparison of Klhl21 and Klhl30 genome environment of species as coelacanth Latimeria chalumnae, spotted gar Lepisosteus oculatus, frog Xenopus tropicalis and human, demonstrated the conservation of flanking genes during gnathostome evolution (Supplementary Figure S3). Moreover, the presence of orthologous genes on both the surveyed chromosomal regions, strongly indicated Klhl21 and Klhl30 as paralogs deriving from one of the events of whole-genome duplication (WGDs) occurred at the root of vertebrate radiation (Dehal and Boore, 2005).

In sum, we have reported here the first evolutionary study dedicated to Kelch-like family in chordates, focused on the seemingly indispensable Klhl21/30 subfamily, which was present in all chordate species examined.

**Klhl21/30 Dynamic Expression in Otolith**

The analysis of the spatio-temporal expression pattern of Klhl21/30, by whole mount *in situ* hybridization (WISH) in embryos at different developmental stages (Figures 2A–D), showed that during C. robusta embryogenesis, Klhl21/30 is expressed from initial tailbud stage onward in the pigment cell lineage (Figures 2A–D). In particular, at initial tailbud stage it is expressed in two cells (Figures 2A,B), possibly the ones originating the otolith and ocellus pigmented cells of the larva. Later in development, from the middle tailbud stage onward, Klhl21/30 transcript became restricted specifically to one cell (Figures 2C,D). Double WISH using the pigment cell marker, Tyrp1/2a, was performed to better define PCP-specific expression. Detailed and thorough analysis of the expression domain proved that Klhl21/30 is expressed specifically in the a11.193 pair, i.e. the most posterior cells marked by Tyrp1/2a (Haupaix et al., 2014; Racioppi et al., 2014) (white arrow in Figures 2E–F”), later, starting from middle tailbud stage, the Klhl21/30 expression became restricted to the otolith precursor (white arrow in Figures 2G–G”), while it disappears from the ocellus pigment cell precursor. Thus, Klhl21/30 represents the earliest-expressed otolith specific gene described so far. Its expression is maintained in the otolith pigment cell precursor while it is downregulated in the ocellus pigment cell precursor, even before expression of the βγ-crystallin, which is expressed specifically in the otolith starting from the larval stage (Shimeld et al., 2005). Klhl21/30 expression in PCPs is consistent with microarray data (Racioppi et al., 2014), in which Klhl21/30 expression was observed to decrease between 8 and 12 hpf. Furthermore, its expression was shown to be dependent on the FGF signaling, which is required for PCPs specification. Coherently, *in situ* hybridizations performed using Klhl21/30 probe on tailbud embryos electroporated with a construct, Tyrp > dnFGFγδ, able to drive a dominant-negative form of the FGF receptor in the PCPs (Squarzoni et al., 2011), showed a sharp decrease in the number of tailbud embryos expressing Klhl21/30 in PCPs compared to control (Supplementary Figure S4). Altogether, these data identified Klhl21/30 as the earliest marker of the Ciona otolith and the first Klhl member described in tunicates.

**Cis-Regulatory Analysis of Klhl21/30 Expression**

Since Klhl21/30 was identified as a specific marker for PCPs as well as the earliest otolith marker in Ciona robusta, we sought to study its transcriptional regulation to understand the regulatory logics underlying differentiation of these structures (Figure 3). In order to discover the cis-regulatory element involved in the control of Klhl21/30 expression, we took advantage of the genome conservation tracks in the ANISEED genome browser (Brozovic et al., 2018) to select a 949-bp non-coding region upstream Klhl21/30 (“KIA”, −1044 to −95 from the start codon), a region presenting conserved peaks with the sibling species C. savignyi (Figure 3A). This KIA fragment was cloned in a vector containing a GFP reporter gene downstream of a human β-globin minimal promoter (Zeller et al., 2006). Once electroporated the KIA > GFP reporter plasmid into Ciona embryos (Corbo et al., 1997), we detected otolith-specific GFP fluorescence in 70% of larvae at stage 26 (Hotta et al., 2007; Figures 3B,C). By WISH using GFP probe, we registered transcription of KIA > GFP at the middle tailbud stage in one cell of the sensory vesicle, possibly corresponding otolith precursor (Supplementary Figure S5). This indicates that the absence of GFP fluorescence until the larval stage is due to a delay in GFP maturation and accumulation. Our reporter plasmid thus recapitulates the endogenous expression of Klhl21/30 at least in the otolith, confirming the restriction of its expression to the otolith precursor in the late embryogenesis. To dissect the regulatory logics underlying the expression of Klhl21/30, we focused on two smaller, highly conserved regions within the KIA sequence: KIB (385 bp long, −918 to −553) and KIC (441 bp
Figure 2 | Expression of Klhl21/30 during Ciona robusta embryogenesis. Klhl21/30 expression was detected by in situ hybridization in the C. robusta pigment cell lineage at initial tailbud (A), early tailbud (B), middle tailbud (C), late tailbud (D). Double WISH with Tyrp1/2a that colocalizes precisely Klhl21/30 among PCPs. (E–F") Early tailbud I (E–E") and early tailbud II (F–F") stages, white arrow indicates the two cells expressing Klhl21/30 that colocalizes with the posterior two Tyrp1/2a positive cells. (G–G") Middle tailbud stage. One white arrow indicates the sole cell expressing Klhl21/30 at this stage that colocalizes with otolith precursor as shown in (G–G").

long, −536 to −95). While KIC did not drive GFP expression, 43% of larvae electroporated with KIB > GFP recapitulated the strong GFP signal in otolith, suggesting that KIB fragment retains the minimal regulatory information necessary to drive Klhl21/30 expression (Figure 3C). When we divided KIB roughly into two halves, KID (199 bp long, −918 to −719) and KIE (166 bp long, −719 to −553), neither was sufficient to drive GFP expression (Figure 3C). However, we found that two smaller fragments centered around KIB, which are KIF (257 bp long, −810 to −553) and KIG (321 bp long, −874 to −553), drove GFP expression in 10 and 22% of larvae, respectively (Figure 3C). These fragments therefore represent the minimal cis-regulatory elements sufficient to recapitulate Klhl21/30 expression. We hypothesized that the KIB region contains transcription factors binding sites (TFBS) crucial for the sustained activation of Klhl21/30 in pigment cell precursors. Therefore, we searched
in this fragment putative TFBS using Genomatix software and CIS-BP database with *C. intestinalis* motifs (Weirauch et al., 2014) and these results have been compared to JASPAR database (Khan et al., 2018; Figure 4). Among several predicted binding sites, we focused our attention on two bHLH-binding motifs. In *Ciona* 44 bHLH genes have been found (Satou et al., 2003) and, for most of them, expression pattern during development has been described (ANISEED database). We selected *Mitf* as possible factor binding the bHLH sites in the 

region (yellow in Figure 4A) because of involvement of *Mitf* in eye development and in the activation of melanogenic markers *Tyr* and *Tyrp/DCT* in vertebrates (Goding, 2000; Curran et al., 2010) and its specific expression in the pigment cell lineage of *Ciona* (Curran et al., 2010; Abitua et al., 2012) and *Halocynthia roretzi* (Yajima et al., 2003). We demonstrated that *Mitf* is co-expressed with *Klhl21/30* in both otolith and ocellus pigmented cells precursors at early tailbud stage (Figures 4B–B’’) and that both became restricted to otolith precursor starting from

![Figure 3](image-url)
A

Coppola et al. (2020) examined the regulatory logic underlying Klhl21/30 expression in Ciona Otolith. They analyzed the Klhl21/30 region with transcription factor binding sites (TFBS) as follows:

(A) Transcription factor binding sites (TFBS) analyzed: Mitf (yellow-orange), Dmrt (violet), Msx (blue).

(B) Co-expression in pigment cell precursors of Klhl21/30 with Mitf at early tailbud (B–B''') and middle tailbud (C–C'''), and Msx (D–D''''), respectively.

(E) Mutational analysis with percentages of larvae expressing GFP, employing KIB > GFP as control; the TFBS are shown using rectangles with the same color code reported for the sequence, while the mutated sites are represented with white rectangles. Experiments were repeated at least three times and 200 embryos were counted each time; all the mutated constructs were significant versus the control KIB (Fisher exact test, p < 0.00001).

FIGURE 4 | Regulatory logic underlying Klhl21/30 expression. (A) KIB region with the transcription factor binding sites (TFBS) analyzed: Mitf (yellow-orange), Dmrt (violet), Max (blue), (B) Co-expression in pigment cell precursors of Klhl21/30 with Mitf at early tailbud (B–B''') and middle tailbud (C–C'''), and Msx (D–D''''), respectively. (E) Mutational analysis with percentages of larvae expressing GFP, employing KIB > GFP as control; the TFBS are shown using rectangles with the same color code reported for the sequence, while the mutated sites are represented with white rectangles. Experiments were repeated at least three times and 200 embryos were counted each time; all the mutated constructs were significant versus the control KIB (Fisher exact test, p < 0.00001).

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middle tailbud stage (Figures 4C–C”). Previous experiments showed also that Mitf is downregulated upon perturbation of FGF signaling (Racioppi et al., 2014). To test the relevance of these two putative sites for Mitf (CACGTG), we mutated them individually in the KlB reporter, naming these mutant constructs K_Mitf_mut1 and K_Mitf_mut2 (Figure 4E). When each of these constructs was electroporated, the percentage of larvae showing GFP signal in the otolith was close to zero (2.5 and 3%, respectively, Figure 4E). These data strongly support these binding sites for Mitf as involved in KlB activity, leading to the hypothesis that Mitf activates Klhl21/30 in Ciona robusta.

We also identified a well-supported binding site in KlB for Dmrt (TTACAT, violet in Figure 4A), a transcription factor expressed in the early a-line neural plate (Wagner and Levine, 2012). C. savignyi Dmrt mutants present abnormalities in the development of the larval sensory vesicle (Tresser et al., 2010) and the expression of the C. robusta ortholog is under early FGF control (Imai et al., 2006). The KlB fragment harboring a mutated Dmrt site (K_Dmrt_mut) drives GFP expression only in 20% of electroporated larvae, roughly half of the activity of wild-type KlB (Figure 4E). Additionally, two binding sites attributed to the homeodomain transcription factors caught our attention (ATTA, blue in Figure 4A). Among TFs able to bind these sites we focused on Mxs because of its early expression in C. robusta pigment cell precursors (Aniello et al., 1999; Russo et al., 2004), and given that it is sharply down-regulated when FGF is blocked (Racioppi et al., 2014). We detected co-expression of Mxs and Klhl21/30 in the PCPs at early tailbud stage (Figures 4D–D”). As development proceeds, the expression of Mxs in PCP decrease becoming excluded from most of the a9.49 derivatives in 12 hpf tailbud embryos as already described (Racioppi et al., 2014). This early expression suggests that Mxs might be activating early Klhl21/30 transcription, even though Mxs factors normally have been described to act as repressors in Ciona (Roure and Darras, 2016). We individually mutated each putative Mxs binding site in KlB, resulting in two mutant constructs, which we termed K_Mxs_mut1 and K_Mxs_mut2. The mutated reporters caused a decrease in the percentage of GFP-expressing larvae of 24 and 19%, respectively, with respect to the wild-type (Figure 4E). Mutating both putative Mxs sites simultaneously caused a strong reduction in GFP expression, observed in only 4% of larvae (Figure 4E, K_2xMxs_mut). Taken together, our data suggest that also Dmrt and Mxs could be involved in the transcriptional activation of Klhl21/30 with a novel role in Ciona.

Functional Analysis of Trans-Acting Factors by CRISPR/Cas9

To test the potential role of Mitf, Dmrt and Mxs as transcriptional activators of Klhl21/30, we used CRISPR/Cas9-mediated mutagenesis to knock out these factors in C. robusta embryos (Stolfi et al., 2014; Gandhi et al., 2017, 2018). To design primers to generate sgRNAs for Mitf, Dmrt, and Mxs, we used CRISPOR v4.0 portal (Haeussler et al., 2016), which takes in account the potential single-nucleotide polymorphisms (SNPs) and the specificity of each sgRNA and provides their predicted “efficacy” (predicted efficiency of their ability to induce specific DSBs) by the Fusi/Doench algorithm (Doench et al., 2016). Using a series of candidate sgRNAs, we targeted different parts of the coding regions of the selected genes (Supplementary Table S3), synthesized U6 > sgRNA cassettes through One-Step Overlap PCR (OSO-PCR) and validated them by peakshift analysis (Gandhi et al., 2017; see section “Materials and Methods” for details; Supplementary Table S3).

We co-electroporated each gene-specific U6 > sgRNA cassettes, (targeting either Mitf, Dmrt, or Mxs) together with Fos > Cas9, which drives Cas9 expression in the a-line blastomeres through the Fos promoter (Rothbacher et al., 2007), and the KIB > GFP reporter plasmid to verify the effect of the experiments on Klhl21/30 isolated regulatory region. As a control, we co-electroporated Fos > Cas9 and KIB > GFP with an sgRNA cassette targeting the mesoderm-specific transcription factor Mesp.5 (Davidson et al., 2005), which is not expressed in the pigment cell lineage. While the control sample had a proportion of GFP-expressing larvae comparable to electroporation with KIB > GFP alone (43% Figure 5A, compared with Figure 2C), we detected a loss of GFP fluorescence in larvae electroporated with sgRNA cassettes targeting the three putative regulators of Klhl21/30 (Figure 5A). When Mitf was knocked out, KIB > GFP was expressed in only 3% of larvae, a sharp decrease compared to the control condition. Moreover, the knockout of Dmrt and Mxs also reduced GFP expression, with only 10 and 4% of larvae showing the KIB > GFP expression, respectively (Figure 5A). Because of the strong effect of Mitf loss-of-function on KIB > GFP activity in C. robusta, and the evolutionary conservation of Mitf function in pigment cell development (Goding, 2000; Levy et al., 2006), we performed a complementary Mitf gain-of-function experiment. We co-electroporated KIB > GFP along with Ebf −2.6 kb > H2B:mCherry and Ebf −2.6 kb + 15 STOP > Mitf to overexpress Mitf in Ebf + neural progenitors during development. These resulted in ectopic KIB > GFP expression in Ebf + cells in the central nervous system (Figures 5B–B”), suggesting that Mitf drive the transcription of Klhl21/30 in these cells.

Thus, these data strongly suggest that, downstream the FGF signaling, Klhl21/30 is under the control of Mitf, with Dmrt and Mxs acting as important co-activators.

DISCUSSION

Our work sheds light on a novel Ciona robusta marker of pigment cell development, the gene Klhl21/30, which represents the earliest gene specifically restricted to the otolith pigmented cell during development. We show that Klhl21/30 is a highly conserved member of the poorly studied Klhl family, whose components have been implicated in various protein-protein interaction and ubiquitination functions. Although human genome encompasses 42 Kelch-like genes (Dhana et al., 2013; Figure 1), our phylogenetic analysis revealed the basic chordate toolkit of Klhl genes (Figure 1), whose roles, during development, have yet to be fully explored. We described the Klhl toolkit of C. robusta (32), the larvacean tunicate O. dioica (14), and...
FIGURE 5 | Functional approach on Klhl21/30 regulators. (A) Histogram with phenotypic assays for otolith-specific loss of KlB > GFP in F0 embryos. Larvae were electroporated with 35 µg Fog > Cas9, 60 µg KlB > GFP plus 50 µl of OSO-PCR-based sgRNA cassettes U6 > Mesp.5 (control), U6 > Mitf-ex3 106, U6 > Dmrt-ex2 76, and U6 > Msx-ex3 209, respectively. (B,C) Mitf overexpression driving ectopic KlB > GFP expression: control (60 µg KlB > GFP, 10 µg Ebf –2.6 kb > H2B:mCherry), overexpression experiment (60 µg KlB > GFP, 10 µg Ebf –2.6 kb > H2B:mCherry, 50 µg Ebf –2.6 kb/ +15 STOP > Mitf). (B–B”) Ectopic expression of KlB > GFP in EBF + cells expressing Mitf ectopically. (C–C”) Control larva showing KlB > GFP expression in the otolith (C,C”) but no ectopic expression in EBF + cells (C”). Experiments were performed at least three times and 100 embryos were scored each time; each site-directed mutagenesis was significant versus the control Mesp.5 (Fisher exact test, p < 0.00001).
the cephalochordate (amphioxus) B. belcheri (37). Thus, we
deduced that 25 ancestral subfamilies were present at the
stem of chordates and are conserved in human, plus one (Klhl5L)
tunicate-specific. Despite the tendency of tunicates to lose genes
and the relative genomic stasis of cephalochordates (Putnam
et al., 2008), we detected several local duplications in these
groups. For instance, in tunicates the subfamily Klhl9/13 has
undergone tremendous increase, while in amphioxus we found
Klhl21/30 duplication and a dramatic expansion of Klhl42 genes.
With respect to the ancestral chordate Klhl toolkit, larvaeceans lost
more than 50% of subfamilies, while we registered an expansion
of the family in amphioxus, Ciona and human. Interestingly,
the Klhl increase in invertebrate chordates has been driven
by different events of gene duplication, whilst WGDs (Abi-
Rached et al., 2002; Dehal and Boore, 2005) could have been a
major role in modeling human (and vertebrate) Klhl repertoire.
Further investigations in other species might be important to
understand the exact role exerted by gene duplications, WGDs
and losses in shaping the Klhl toolkit, especially in vertebrates.
Taken together, our data represent the most detailed analysis of
the evolutionary landscape of Klhl family in Chordata so far.
Strikingly, Klhl21/30 is one of the most conserved subfamilies
and our syntenic survey demonstrates its conservation in all the
tunicates (Supplementary Figure S2). Furthermore, Klhl21/30
represents one of few subfamilies retained by the gene-loser
O. dioica, hinting at its relative importance. Moreover, we
detected a WGD-origin of human (and vertebrates) KLHL21 and
KLHL30 genes (Supplementary Figure S3).

There is scarcity of information about the function and
expression of genes of the Klhl21/30 subfamily. Klhl21 is known
to bind E3 ubiquitin ligase through Cul3 (Maerki et al., 2009;
Courteoux et al., 2016) and Klhl30 has been identified as a
circadian pathway gene involved in the onset of glioma
(Madden et al., 2014). Like many other Klhl members, Klhl21
is implicated in diverse types of carcinoma, probably due to
its role in cell division (Shi et al., 2016; Huang et al., 2017;
Chen et al., 2018). Our findings represent the first expression
data for Klhl21/30 subfamily in the chordate nervous system.
In C. robusta, Klhl21/30 is initially transcribed in two cells
of the pigment cell lineage alongside well-known markers of
pigmented cells such as Tyr, Typr, and Rab32/38 (Coppola
et al., 2016; Racioppi et al., 2019), which are conserved in
chordate pigment cell evolution (Esposito et al., 2012; Coppola
et al., 2016). Interestingly, Klhl21/30 became restricted to
the otolith precursor from middle taillbud stage onward. This renders
Klhl21/30 the earliest marker of Ciona otolith so far identified,
well before the activation of β-crystallin in the otolith at
the larval stage (Shimeld et al., 2005). Expression data from
invertebrates and vertebrates could be crucial to understand
the potential functional conservation during evolution of poorly
studied Klhl21/30 genes.

The sustained expression of Klhl21/30 in the otolith prompted
us to investigate the transcriptional regulation of this gene.
Taking advantage of genome conservation with the sibling species
C. savignyi, we identified the smallest region containing the
TFBS crucial for Klhl21/30 expression in the otolith. Our cis-
regulatory mutational analyses suggested the involvement of
three transcription factors (Mitf, Dmrt, Msx) in the activation
of Klhl21/30 (Figure 3). The tissue-specific, CRISPR/Cas9-
mediated loss-of-function of Mitf, Dmrt and Msx resulted
in highly penetrant downregulation of the Klhl21/30 reporter
in electroporated larvae, suggesting that Klhl21/30 expression
depends on these trans-acting factors (Figure 5A). Moreover,
a gain-of-function experiment confirmed Mitf as the main
regulator for Klhl21/30 reporter expression in other cells of
the nervous system. Due to the fact that some Ebf + cells also
express Msx, it may be that also Msx collaborates in the ectopic
activation of Klhl21/30 reporter. On the other hand, the low
efficiency of ectopic expression of Klhl21/30 in Ebf > Mitf ectopic
expression could reflect lack of the Msx or Dmrt in these cells
or low penetrance of CRISPR/Cas9 for these genes. We therefore
conclude that Mitf, a key regulator of melanocyte development
and melanoma in vertebrates (Levy et al., 2006), seems to be
an indispensable transcription factor for Klhl21/30 expression
in Ciona, with Dmrt and Msx acting as potential co-factors.
However, these transcription factors could also work without
a direct interaction with the regulatory region responsible for
Klhl21/30 expression in pigment cell lineage.

Interestingly, Mitf shows sustained expression in the otolith
at later developmental stages (Figure 4; Abitua et al., 2012)
confirming that this factor could be the final node of the
regulatory network underlying the fate determination between
otolith and ocellus pigment cells in Ciona as already suggested
by Abitua et al. (2012). Besides, Mitf controls also the expression
of Rab32/38, a conserved melanogenic marker (Racioppi et al.,
2019). The role of Mitf in pigment cell development has been
observed also in Drosophila melanogaster (Hallsson et al., 2004),
and the function of Mitf as master regulator of genes related
to pigment cells and pigmentation is conserved in vertebrates
(Levy et al., 2006; Vachet and Borovansky, 2010). However,
in this latter Mitf plays a crucial role also in other processes
as mast cell development (Mori, 2004; Putnam et al., 2008)
and osteoclast biogenesis (Hershey and Fisher, 2004), while
in tunicates this bHLH factor seems to be involved only in pigment
cell development, and otolith specification, as suggested also by
its specific expression during embryogenesis (Figures 4B–C”;
Yajima et al., 2003). If we consider also the specific expression
of Mitf in the first pigmented spot of the early-branching
amphioxus (Yu et al., 2008), we can speculate that the ancestral
Mitf function in early development of chordates was related
only to pigment cells and, in vertebrates, it acquired new
specializations. However, it is not known if tunicates possess
similar cell types homologous to mast cells or osteoclasts, especially
in the poorly studied adult phase. Therefore, a more thorough
understanding of Mitf functions (and the existence of other
PCPs-specific genes regulated by Mitf) throughout the entire life
cycle of invertebrate chordates will be necessary to corroborate
this hypothesis.

Our data hinted at Dmrt and Msx as positive regulators
of Klhl21/30 (Figures 4, 5). Activated very early by FGF
signaling (Imai et al., 2006), the Dmrt gene is crucial for otolith
development in the sister species C. savignyi, in which Dmrt
mutants typically possess a single ocellus pigment cell but lack
an otolith (Tresser et al., 2010). Our results further suggest that
Dmrt is a co-activator of an otolith-specific gene, Klhl21/30. With respect to Msx, this transcription factor is traditionally considered a repressor in Ciona (Roure and Darras, 2016) and in different vertebrates (Takeda et al., 2000; Takahashi et al., 2001; Xie et al., 2013). However, in mouse, Msx1 and Msx2 are the transcriptional activators of the proneural gene Atoh1 during spinal cord patterning (Duval et al., 2014). Our findings reveal that the function as activator for Msx might be present also in tunicates.

Importantly, while Mitf has clearly a role in Klhl21/30 activation in Ciona, the fragment containing solely Mitf binding sites (KIE) is not sufficient to drive expression of Klhl21/30 in otolith. In contrast, fragments that include Dmrt and Msx binding sites in addition to Mitf binding sites are sufficient for reporter gene activity and one of them (KIB > GFP) has been utilized for Mitf gain-of-function experiment (Figures 5B–C”). Together with our cis-regulatory mutational and CRISPR/Cas9-mediated knockout data, this suggests that all three factors could be key activators. Alternatively, the much earlier expression of Dmrt and Msx in PCPs evokes that their early binding to the Klhl21/30 cis-regulatory region might be required for later transcriptional activation by otolith specifier Mitf, acting as a so-called “pioneer factors” (Zaret and Carroll, 2011), involved in keeping the chromatin accessible to recruit other late transcriptional activators. Novel functional data regarding Dmrt and Msx in Ciona could unveil their function as activators and/or pioneer factors, and if this role is restricted to pigment cells or if it extends to more broadly anterior neural plate derivatives.

In sum, we provided the first description of a Klhl member in Ciona robusta, identifying it as the earliest otolith pigment cell marker and we elucidated the gene regulatory network (GRN) controlling its expression.

**MATERIALS AND METHODS**

**Evolutionary Analyses and Transcription Factor Binding Site Analysis**

Kelch-like protein sequences from vertebrate Homo sapiens were used as queries in BLASTp and tBLASTn searches in ANISEED (Brozovic et al., 2018), NCBI or Ensembl genome databases of selected species. Orthology was initially assessed by reciprocal best blast hit (RBBH) approach and supported by phylogenetic analyses. Evolutionary reconstructions were performed using ML inferences calculated with PhyML v3.0 and automatic modality of selection of substitution model (Guindon et al., 2010) using protein alignments generated with MUSCLE (Edgar, 2004) and ClustalX (Larkin et al., 2007) programs. Alignment of Supplementary Figure S1 has been carried out employing ClustalX. Full protein sequences were used in our analysis. Accession numbers and names for phylogenetic tree of Figure 1 are listed in Supplementary Table S1, while those excluded are encompassed in Supplementary Table S2. The analysis of synteny conservation (shown in Supplementary Figures S2, S3) was performed by employing ANISEED, Ensembl and Genomicus databases (Louis et al., 2015). To predict putative transcription factor binding sites (TFBS) in the surveyed cis-regulatory regions, we utilized the MatInspector module of the Genomatix Software Suite and CIS-BP employing a Ciona intestinalis (former name for Ciona robusta) DNA-binding-domain classes database (Weirauch et al., 2014). We also used the JASPAR database (Khan et al., 2018) to recognize the potential binding sites.

**Animals and Embryo Electroporation**

Adults of Ciona robusta were collected from the Gulf of Naples, or from San Diego, CA, United States, by M-REP. Gametes from many animals were gathered separately for in vitro cross-fertilization followed by dechorionation and electroporation as previously illustrated (Christiaen et al., 2009a; Racioppi et al., 2014). Electroporated plasmid amounts (e.g., 10 µg) were per 700 µl of total volume. Embryos were staged according to the developmental timeline shown 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-NH4Cl and in PBS containing 0.05% Triton X-100. Each electroporation of Figures 3, 4 was carried out using 60 µg of plasmid. The statistical significance of electroporations associated to Figure 4 was validated using Fisher exact test. The statistical significance of electroporations of Supplementary Figure S4 was evaluated employing Chi-square test for trend.

**In situ Hybridization**

Single and double in situ hybridization experiments were performed out essentially as described previously (Christiaen et al., 2009b; Racioppi et al., 2014), using DIG- and FLUO-labeled riboprobes, anti-DIG-POD and anti-FLUO-POD Fab fragments (Roche, Indianapolis, IN), and Tyramide Amplification Signal with Fluorescein (Perkin Elmer, MA). The antisense riboprobes were obtained from plasmids contained in the C. intestinalis gene collection release I: Klhl21/30 (KH2012:KH.L84.23, GC17e22), Tyrp1/2a (KH2012:KH.C8.537, GC31h05), Mitf (KH2012:KH.C10.106, GC28k08), Dmrt (KH2012:KH.S543.3, GC02f18), and Msx (KH2012:KH.C2.957, GC42h24) (Satou et al., 2002).

**Molecular Cloning**

The cis-regulatory elements upstream Klhl21/30 were PCR-amplified from genomic DNA and their localization on upstream sequence is shown in Supplementary Figure S6. Insertion of the products into pSP72 vector containing GFP (Zeller et al., 2006) was carried out using TOPO-TA Cloning kit (Invitrogen). The QuickChange Site-Directed Mutagenesis Kit (Agilent) was employed to generate the mutations inside the putative binding sites (Mitf, Dmrt, Msx) identified in the sequence of the KIB element (Figure 4). All the oligos used for cloning the putative regulatory regions and for mutational experiments are listed in Supplementary Table S4.
**Functional analysis by CRISPR/Cas9 and Gain-of-Function**

*Cas9* and *sgRNA* expression vectors were constructed or used as previously described (Stolfi et al., 2014; Gandhi et al., 2017). The predictive algorithm used for designing *in vivo*-transcribed *sgRNAs* has been the Fusi/Doench (Doench et al., 2016), available on CRISPOR portal (Haeussler et al., 2016). The target sequences have been selected not too close to the translational start, to have the higher impact on the function of protein of interest (Gandhi et al., 2017). DNA oligos used to generate *sgRNAs* are listed in **Supplementary Table S3**. One-step overlap PCR (OSO-PCR) was employed for the fast synthesis of a U6 > *sgRNA* cassettes through a single PCR reaction, performed using Platinum Pfx Polymerase (Invitrogen). The products were cloned into pCESA plasmid using Gibson Assembly Cloning Kit (NEB). Before the electroporation, the products were purified utilizing AMPure XP (Agencourt).

*Mitf*, *Msx*, and *Dmrt* *sgRNAs* were validated by PCR amplification and Sanger sequencing of the targeted region as previously described (Gandhi et al., 2018). Pooled larvae were lysed for 30 min in 180 µl buffer + 5 µl of Proteinase K from QIAamp DNA Micro Kit (Qiagen) and eluted in 20 µl of water. Approximately 200 ng/µl of genomic DNA extracted from hatched larvae was used for “touchdown” PCRs with Platinum Pfx Polymerase (Invitrogen), as described in Gandhi et al. (2017). The genomic oligos (“peakshift oligos”) employed for “touchdown” PCRs were selected at 150–500 bp away from the primer (**Supplementary Table S3**), to ensure a proper peakshift. Electroporations were performed as single biological replicates. Electroporation mix recipes can be found in **Supplementary Table S3**. One-step overlap PCR (OSO-PCR) was employed for the fast synthesis of a U6 > *sgRNA* cassettes through a single PCR reaction, performed using Platinum Pfx Polymerase (Invitrogen). The products were cloned into pCESA plasmid using Gibson Assembly Cloning Kit (NEB). Before the electroporation, the products were purified utilizing AMPure XP (Agencourt). *Mitf*, *Msx*, and *Dmrt* *sgRNAs* were validated by PCR amplification and Sanger sequencing of the targeted region as previously described (Gandhi et al., 2018). Pooled larvae were lysed for 30 min in 180 µl buffer + 5 µl of Proteinase K from QIAamp DNA Micro Kit (Qiagen) and eluted in 20 µl of water. Approximately 200 ng/µl of genomic DNA extracted from hatched larvae was used for “touchdown” PCRs with Platinum Pfx Polymerase (Invitrogen), as described in Gandhi et al. (2017). The genomic oligos (“peakshift oligos”) employed for “touchdown” PCRs were selected at 150–500 bp away from the primer (**Supplementary Table S3**), to ensure a proper peakshift. Electroporations were performed as single biological replicates. Electroporation mix recipes can be found in **Supplementary Table S3**. One-step overlap PCR (OSO-PCR) was employed for the fast synthesis of a U6 > *sgRNA* cassettes through a single PCR reaction, performed using Platinum Pfx Polymerase (Invitrogen). The products were cloned into pCESA plasmid using Gibson Assembly Cloning Kit (NEB). Before the electroporation, the products were purified utilizing AMPure XP (Agencourt). *Mitf*, *Msx*, and *Dmrt* *sgRNAs* were validated by PCR amplification and Sanger sequencing of the targeted region as previously described (Gandhi et al., 2018). Pooled larvae were lysed for 30 min in 180 µl buffer + 5 µl of Proteinase K from QIAamp DNA Micro Kit (Qiagen) and eluted in 20 µl of water. Approximately 200 ng/µl of genomic DNA extracted from hatched larvae was used for “touchdown” PCRs with Platinum Pfx Polymerase (Invitrogen), as described in Gandhi et al. (2017). The genomic oligos (“peakshift oligos”) employed for “touchdown” PCRs were selected at 150–500 bp away from the primer (**Supplementary Table S3**), to ensure a proper peakshift. Electroporations were performed as single biological replicates. Electroporation mix recipes can be found in **Supplementary Table S3**. One-step overlap PCR (OSO-PCR) was employed for the fast synthesis of a U6 > *sgRNA* cassettes through a single PCR reaction, performed using Platinum Pfx Polymerase (Invitrogen). The products were cloned into pCESA plasmid using Gibson Assembly Cloning Kit (NEB). Before the electroporation, the products were purified utilizing AMPure XP (Agencourt).

**DATA AVAILABILITY STATEMENT**

All datasets presented in this study are included in the article/Supplementary Material.

**AUTHOR CONTRIBUTIONS**

UC and FR: conceptualization. UC and AK: investigation. UC, AS, and FR: data curation. AS and FR: supervision. UC: writing. AS and FR: review and editing. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.569601/full#supplementary-material

**FIGURE S1** | Domain conservation among *Klhl21/30* proteins of *Ciona robusta* and Homo sapiens. BTB/POZ (blue), BACK (underlined), Kelch-repeat 1 (green), Kelch-repeat 2 (yellow), Kelch-repeat 3 (turquoise), Kelch-repeat 4 (magenta), Kelch-repeat 5 (gray). Domains mapped using Smart classification available on Ensembl database (H. sapiens KHLH30: ENSP00000386389.1; H. sapiens KHLH21: ENSP00000366886.4; C. robusta Klhl21/30: ENSCINP0000014893.3). Ciona *Klhl21/30* shares the same domain architecture of human KHLH (according to Dhana et al., 2013).

**FIGURE S2** | *Klhl21/30* synteny analysis in Olfactores. Identification of an ancestral cluster in ascidians, comprising *Klhl21/30* gene (orange boxes). Ascidians and human share the gene duplet formed by *Klhl21/30* and Nphp4 (red rectangle). Each orthologous gene has been represented using the same color code and black arrows for transcription direction.

**FIGURE S3** | *Klhl21/30* synteny analysis in Gnathostomes. Conservation of genes flanking gnathostome *Klhl21* and *Klhl30* (orange boxes) comparing *Latimeria chalumnae*, *Lepisosteus oculatus*, *Xenopus tropicalis* and *Homo sapiens*. Each orthologous gene has been represented using the same color code and black arrows for transcription direction.

**FIGURE S4** | Expression of *Klhl21/30* in embryos electroporated with *dnFgfr* probe on tailbud embryos electroporation with *KIA > GFP*. Evaluation of percentage of embryos showing expression in the otolith respect to wild-type (wt). The experiment was repeated three times, showing a significant trend respect to control (Chi-square test for trend, p < 0.0001; n = 200).

**FIGURE S5** | GFP transcription in tailbud embryos electroporated with *KIA > GFP*. In situ hybridization experiment using GFP probe on tailbud embryos electroporation with *KIA > GFP construct (A,B)*.

**FIGURE S6** | Localization of regions used for cis-regulatory analysis (**Figure 3**) inside upstream region of *Ciona robusta* *Klhl21/30* ([KHLH4.23]). Different colors indicate the selected regions and the oligos used for cloning, with respective combinaciones.

**FIGURE S7** | Electroporation mix recipes and constructs used for functional experiments.

**TABLE S1** | List of *KH* protein sequences employed for phylogeny of **Figure 1**.

**TABLE S2** | *KH* protein sequences excluded from phylogeny for their high degree of molecular divergence. Their names were assessed by genome search and reciprocal BLASTs.

**TABLE S3** | List of oligonucleotides used for functional assays. Names contain indication on the position of the target sequence inside the selected exon (e.g., *Mitf-ex3 106* indicates that the target sequence is within exon3 starting from its nucleotide 106). In bold and capital letters the *sgRNA* N (19) target sequences. In
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Table 54 List of oligos used for cloning and mutational experiments (in red are shown the mutated nucleotides, with respect to wt sequence).
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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