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SYMPOSIUM

Loss of collagen gene expression in the notochord of the tailless tunicate *Molgula occulta*

Sydney Popsuj[*], Anna Di Gregorio[†,1], Billie J. Swalla[‡,2] and Alberto Stolfi[*,3]

[*]School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA 30332, USA; [†]Department of Molecular Pathobiology, New York University College of Dentistry, New York, NY 10010, USA; [‡]Department of Biology, University of Washington, Seattle, WA 98195, USA

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[1]E-mail: adg13@nyu.edu
[2]E-mail: bjswalla@uw.edu
[3]E-mail: alberto.stolfi@biosci.gatech.edu

Synopsis In tunicates, several species in the Molgulidae family have convergently lost the tailed, swimming larval body plan, including the morphogenesis of the notochord, a major chordate-defining trait. Through the comparison of tailless *M. occulta* and a close relative, the tailed species *M. oculata,* we show that notochord-specific expression of the *Collagen Type I/II Alpha (Col1/2a)* gene appears to have been lost specifically in the tailless species. Using CRISPR/Cas9-mediated mutagenesis in the tailed laboratory model tunicate *Ciona robusta,* we demonstrate that *Col1/2a* plays a crucial role in the convergent extension of notochord cells during tail elongation. Our results suggest that the expression of *Col1/2a* in the notochord, although necessary for its morphogenesis in tailed species, is dispensable for tailless species. This loss is likely a result of the accumulation of *cis*-regulatory mutations in the absence of purifying selective pressure. More importantly, the gene itself is not lost, likely due to its roles in other developmental processes, including during the adult stage. Our study further confirms the Molgulidae as an interesting family in which to study the evolutionary loss of tissue-specific expression of indispensable genes.

Introduction

Tunicates are non-vertebrate chordates and comprise the sister group to vertebrates (Delsuc et al. 2006). Most species have a distinct biphasic life cycle divided between a tailed, swimming larva and a sessile, filter-feeding adult (Fodor et al. 2021a). They are key organisms amenable to studies of the evolution and the development of chordate traits (Lemaire 2009, 2011). Their tailed, tadpole-like larvae swim in search of a place to settle and undergo metamorphosis thanks to key traits shared with vertebrates, such as a dorsal central nervous system and a notochord flanked by paraxial muscles (Nishino 2018). However, they develop distinct body plans for the swimming larval phase and the sessile adult phase. The larval body plan is eventually degraded when larvae settle and undergo metamorphosis to give rise to a new adult body plan (Karaiskou et al. 2015).

In some tunicate species, mostly ascidians of the family Molgulidae, the swimming larval phase has been lost, and instead, development proceeds through tailless, non-swimming larvae before metamorphosis begins (Lacaze-Duthiers 1874; Maliska et al. 2013; Fodor et al. 2021b). The evolutionary loss of the swimming larval body plan posits an interesting case for the study of developmental trait loss. In some cases, a gene exclusively required for the larval stage can become pseudogenized in non-swimming species, as it is the case with *Tyrosinase,* which is required for the melanization of the sensory organ pigment cells of swimming larvae (Racioppi et al. 2017). However, many other genes cannot be entirely lost, due to their roles in forming the adult body plan, yet appear downregulated during the development of tailless larvae relative to their tailed counterparts (Fodor et al. 2021b).

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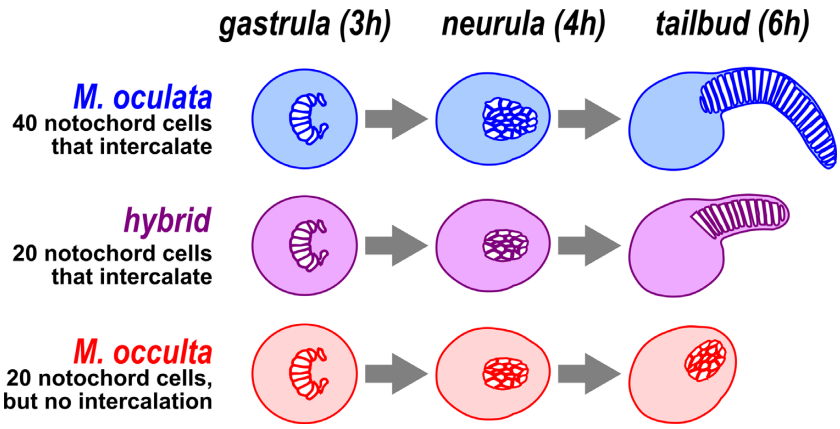

Fig. 1. Notochord development in *Molgula*. Diagram comparing notochord morphogenesis in tailed swimming (*M. oculata*) and tailless non-swimming (*M. occulta*) species and their interspecific hybrids (*M. occulta* eggs fertilized with *M. oculata* sperm). "*h*" = hours post-fertilization.

When gene expression is evolutionarily lost in a specific tissue or cell type in one species, but the gene is not lost from its genome, an interesting set of questions emerge regarding the molecular mechanisms underlying this change. Evolutionary loss of tissue-specific gene expression might also indicate that the gene plays an important role in a developmental process that is no longer needed. One of the key chordate traits is the presence of a notochord, a group of mesodermal cells that intercalate to form a rod-like single-file row of cells during embryogenesis. In the larvae of most tailed ascidian species, the notochord is formed by exactly 40 cells (Fig. 1) (Satoh 2013). These cells, much like in vertebrates, undergo convergent extension to form a rod-shaped notochord that will later become a hollow tube through extensive vacuolization, serving ultimately as a hydrostatic skeleton for larval swimming (Miyamoto 1985; Jiang and Smith 2007; Denker and Jiang 2012). In the tailless species *Molgula occulta*, only 20 notochord cells are specified and they fail to undergo convergent extension (Fig. 1) (Berrill 1931; Swalla and Jeffery 1990). Notochord development is partially rescued in interspecific hybrids formed by the fertilization of *M. occulta* eggs with sperm from the closely related, tailed species *M. oculata* (Swalla and Jeffery 1990). While there are still only 20 notochord cells in the hybrid, these cells undergo convergent extension, suggesting that zygotic gene expression is sufficient to drive this process in development (Swalla and Jeffery 1990). Ultimately, *M. occulta* provides a unique opportunity for studying notochord development and evolutionary loss of gene expression. The purpose of the present study is two-fold (1) To identify the molecular mechanisms underlying evolutionary loss of tissue-specific gene expression in *M. occulta*, and (2) to use *M. occulta* as a "natural" genetic screen for genes that may be essential for notochord morphogenesis in tailed species.

Previously analyzed transcriptomic data from *M. occulta*, *M. oculata,* and their hybrids, identified a list of notochord-expressed genes whose expression is lost or reduced in the tailless embryos (Lowe et al. 2014; Lowe et al. 2021; Fodor et al. 2021b). Many appeared to be involved in the production or processing of collagen, a major component of the notochord extracellular matrix in both tunicates and vertebrates (Miller and Mathews 1974; Zhang and Cohn 2006; Kugler et al. 2011; Yasuoka 2020). In this study, we set out to investigate the connection between loss of expression of the *Collagen Type I/II Alpha (Col1/2a)* gene, and the lack of notochord convergent extension in *M. occulta*. We show that *M. occulta* embryos lack distinct expression of *Col1/2a* in the notochord, but not in other tissues. This was consistent with transcriptomic data from hybrid embryos, which showed enrichment of transcripts from the *M. oculata* (tailed) parental *Col1/2a* allele relative to the *M. occulta* (tailless) allele. Due to the difficulty of DNA transfection and gene knockout approaches in *Molgula,* we carried out functional tests in the laboratory model tunicate *Ciona robusta* (formerly *intestinalis* Type A) instead. CRISPR/Cas9-mediated-disruption of *Col1/2a* in the *Ciona* notochord revealed a key role for this gene in notochord intercalation in tailed larvae. However, *cis*-regulatory analyses of *Molgula Col1/2a* genes were inconclusive, likely due to the well-documented phenomenon of developmental system drift between *Molgula* and *Ciona* (Stolfi et al. 2014b; Coulcher et al. 2020).

Methods

Molgula embryo collection

Molgula occulta and *M. oculata* can be collected simultaneously in the waters surrounding the Station Biologique de Roscoff, France. Gravid during July and August, these animals were collected and dissected to obtain the eggs and sperm necessary for their fertilization, and for the generation of interspecific (*M. oculata* sperm × *M. occulta* eggs) hybrids. Embryos were raised, dechorionated, and fixed as previously described (Swalla and Jeffery 1990). Images were then acquired on an inverted fluorescence DM IL LED or DMI8 microscopes (Leica). RNAseq data were generated and previously published (Lowe et al. 2014; Fodor et al. 2021b). *In situ* hybridizations were performed as previously described (Lowe et al. 2021).

Electroporations for *Ciona*

Ciona robusta (intestinalis Type A) adults were collected in San Diego, CA, USA, by M-REP. Animals were then dissected to isolate gametes for *in vitro* fertilization as previously described (Christiaen et al. 2009b). Dechorionated zygotes were then transfected with plasmids *via* electroporation as described (Christiaen et al. 2009a). Embryos were raised at 20°C and fixed for 15 min in MEM-FA fixative (3.7% formaldehyde, 0.1 M MOPS pH 7.4, 0.5 M NaCl, 1 mM EGTA, 2 mM MgSO4, and 0.1% Triton-X100), rinsed first in 1X PBS, 0.4% Triton-X100, 50 mM NH4Cl, then in 1X PBS, 0.1% Triton-X100, before being mounted in 2% DABCO/1X PBS/50% glycerol mounting solution. Images were then acquired as described above for *Molgula.*

CRISPR/Cas9-mediated genome editing

Single-chain guide RNA (sgRNA) plasmids were designed with the assistance of the algorithm CRISPOR (Haeussler et al. 2016). Cas9 and sgRNA ("F + E") scaffold sequences used and cloning procedures were previously published (Stolfi et al. 2014a; Gandhi et al. 2018). *Col1/2a* sgRNAs were tested by co-electroporating 50 μg of each individual sgRNA expression plasmid in combination with 30 μg *Foxa.a > Cas9* (Di Gregorio et al. 2001; Gibboney et al. 2020), 45 μg *Ebf > Unc-76:: mCherry* (Stolfi and Levine 2011), and 45 μg *Ebf > TESTR1:: GFP* per 700 μl of total electroporation volume. The "TESTR1" sequence in this case contained target sites for all *Col1/2a* sgRNAs tested. Loss of GFP signal in *Ebf* + cells (labeled by Unc-76:: mCherry) was interpreted as efficient mutagenesis by the particular sgRNA tested. Using this method, we settled on *U6 > Col1/2a.658* and *U6 > Col1/2a.694* sgRNAs (Supplementary Fig. 1). All sequences, published and unpublished, and electroporation protocols can be found in the Supplementary Sequences File.

Results

Loss of *Col1/2a* expression in the notochord of tailless *M. occulta*

Previous analysis of RNA sequencing (RNAseq) data from *M. occulta, M. oculata,* and their interspecific hybrids revealed the reduced expression of several putative notochord-expressed genes (Lowe et al. 2014; Lowe et al. 2021; Fodor et al. 2021b). The orthologs of these genes are known to be expressed in the notochord of *Ciona,* thanks to the extensive characterization of the notochord gene regulatory network in this species (Kugler et al. 2008; Kobayashi et al. 2013; Di Gregorio 2020). Many of these genes with reduced expression in *M. occulta* appeared to be involved in collagen production or processing, including *Col1/2a,* previously called *Fibrillar collagen 1* (*Ci-FCol1)* in *Ciona* (Katikala et al. 2013). According to the tunicate sequence database ANISEED (Dardaillon et al. 2020), tunicate *Col1/2a* is equally similar to human *COL1A1, COL1A2,* and *COL2A1*, all of which, especially *COL2A1*, have been reported as markers of the notochord or notochord-derived *nucleus pulposus* in humans and other vertebrates (Su et al. 1991; Yan et al. 1995; Peck et al. 2017). Because of the enrichment for collagen pathway genes in our RNAseq and the homology to vertebrate notochord-expressed *collagen* genes, we reasoned that *Col1/2a* would be a good candidate gene to study further.

Our RNAseq analysis indicated that *Col1/2a* expression is reduced in *M. occulta* during tail elongation, in comparison to *M. oculata* (Fig. 2A). While *Col1/2a* expression is significantly upregulated in the tailed *M. oculata* between neurula and tailbud stage, when tail extension is happening, the expression in *M. occulta* did not significantly change. Similarly, interspecific hybrids between *M. occulta* and *M. oculata* showed enriched expression of their tailed *M. oculata* parental allele relative to the *M. occulta* parental allele at 6 hpf, indicating that changes in *cis* could be contributing to the divergence in the level of *Col1/2a* gene expression between the two species.

Because it is known that *Col1/2a* is also expressed in tail muscles in *Ciona* (Kugler et al. 2010), we next performed whole-mount *in situ* hybridizations to determine whether Col1/2a expression was reduced in these tissues. As expected, tailed *M. oculata* embryos showed expression of *Col1/2a* in both tail muscles and notochord, the latter of which also expressed the notochord marker *Islet* (Fig. 2B). However, tailless *M. occulta*

A Differential *Col1/2a* expression analysis from RNAseq

	oculata		occulta		Hybrid		oculata vs. occulta allele	
Staging	**LogFC**	**p**	**LogFC**	**p**	**LogFC**	**p**	**LogFC**	**p**
Neur vs. Gast	1.8	0.0274	0.1	0.88	2.1	0.01	3.3 (neur)	0.0001
Tb vs. Neur	4.7	0.0000003	-0.7	0.4	0.5	0.5	3.1 (tb)	0.0003

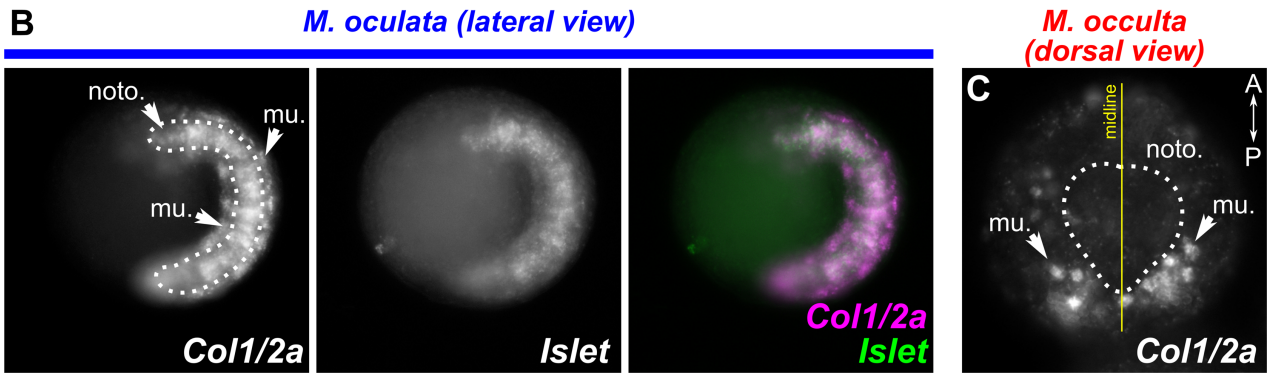

Fig. 2. Loss of *Col1/2a* expression in the notochord of *M. occulta*. (A) RNAseq data from Fodor et al. 2021b, focusing on the *Col1/2a* gene in *M. oculata*, *M. occulta*, and *M. oculata* × *M. occulta* hybrids. LogFC = Log2 fold-change of reads measured between different stages of development: Gastrula ("Gast," 3 hours post-fertilization), Neurula ("Neur," 4 hpf), and Tailbud ("Tb," 6 hpf). Positive values indicate upregulation at the later stage indicated (neurula relative to gastrula, or tailbud relative to neurula). Negative values indicate downregulation. *P* = *p*-value. For allele-specific data, LogFC = Log2 fold-change of reads mapped specifically to one species' genome or the other in hybrid embyos at the indicated stage (neurula or tailbud). Positive values = more *M. oculata* reads than *M. occulta* reads. (B) Two-color, whole-mount fluorescent mRNA *in situ* hybridization of *Col1/2* and *Islet* in *M. oculata* tailbud embryo showing expression of *Col1/2a* in notochord ("noto.," dashed outline) and muscles ("mu."). Embryo positioned laterally, showing tail bud extending posteriorly and ventrally. (C) *In situ* hybridization of *Col1/2a* in *M. occulta*, showing expression in muscles but not notochord (dashed outline). Nearly spherical *M. occulta* embryo positioned dorsally, with anterior (A) to the top and posterior (P) to the bottom. Embryonic midline shown as yellow line. Compare to *Islet* expression in Supplementary Fig. 2.

clearly showed expression only in muscle cells, while notochord cells in the center of the embryo were negative for *Col1/2a* expression (Fig. 2C), though positive for *Islet* (Supplementary Fig. 2). Taken together, these results confirmed that *Col1/2a* expression has been specifically lost in the notochord of tailless *M. occulta* embryos.

Divergence between putative *M. occulta* and *M. oculata Col1/2a cis*-regulatory sequences

The evolutionary loss of expression of a given gene in a specific tissue or cell type can be due to the loss of regulatory sequences in *cis* (e.g., mutations in *cis*-regulatory sequences), or to the loss of expression or function of regulatory proteins in *trans* (e.g., loss of an upstream transcription factor). To further decipher the molecular basis of reduced *Col1/2a* expression in the notochord, we used published genome assemblies (Stolfi et al. 2014b) to align *M. occulta* and *M. oculata* sequences upstream of *Col1/2a* (Fig. 3A). We focused on a ~630 bp fragment upstream of the *Col1/2a* gene, which in *C. robusta* contained the *cis*-regulatory modules (CRMs, or enhancers) that are sufficient to drive expression separately in notochord and muscle (Kugler et al. 2010; Katikala et al. 2013). By aligning *Col1/2a* upstream sequences from *M. occulta/oculata* using the online service MAFFT (Katoh et al. 2019), we found a high sequence identity through much of the sequence, except for a gap of high divergence from roughly −475 bp to −298 bp upstream of the start of exon 1 (Fig. 3A and B), which prompted us to further analyze this region both manually and using the transcription factor binding site search algorithm JASPAR (Castro-Mondragon et al. 2022) (see Supplementary Sequence File). We found two predicted Brachyury binding sites arranged in tandem in tailed *M. oculata* but absent in tailless *M. occulta,* which were identified using the mouse Tbxt (Bra) position weight matrix (Fig. 3C). This was of potential interest because Brachyury is the key transcription factor regulating notochord specification in *Ciona* (Di Gregorio 2020). These predicted binding sites were rearranged and divergent in *M. occulta* to the point where JASPAR predicted for them a much lower affinity score (see Supplementary Sequence File). Interestingly, a roughly equivalent region in the *Col1/2a* upstream sequence of *C. robusta* comprises the notochord-specific CRM and its required Brachyury binding sites (Fig. 3D) (Katikala et al. 2013). There were additional predicted Brachyury binding sites in conserved regions both proximal to and distal from the divergent region. However, some of these are also potential binding sites

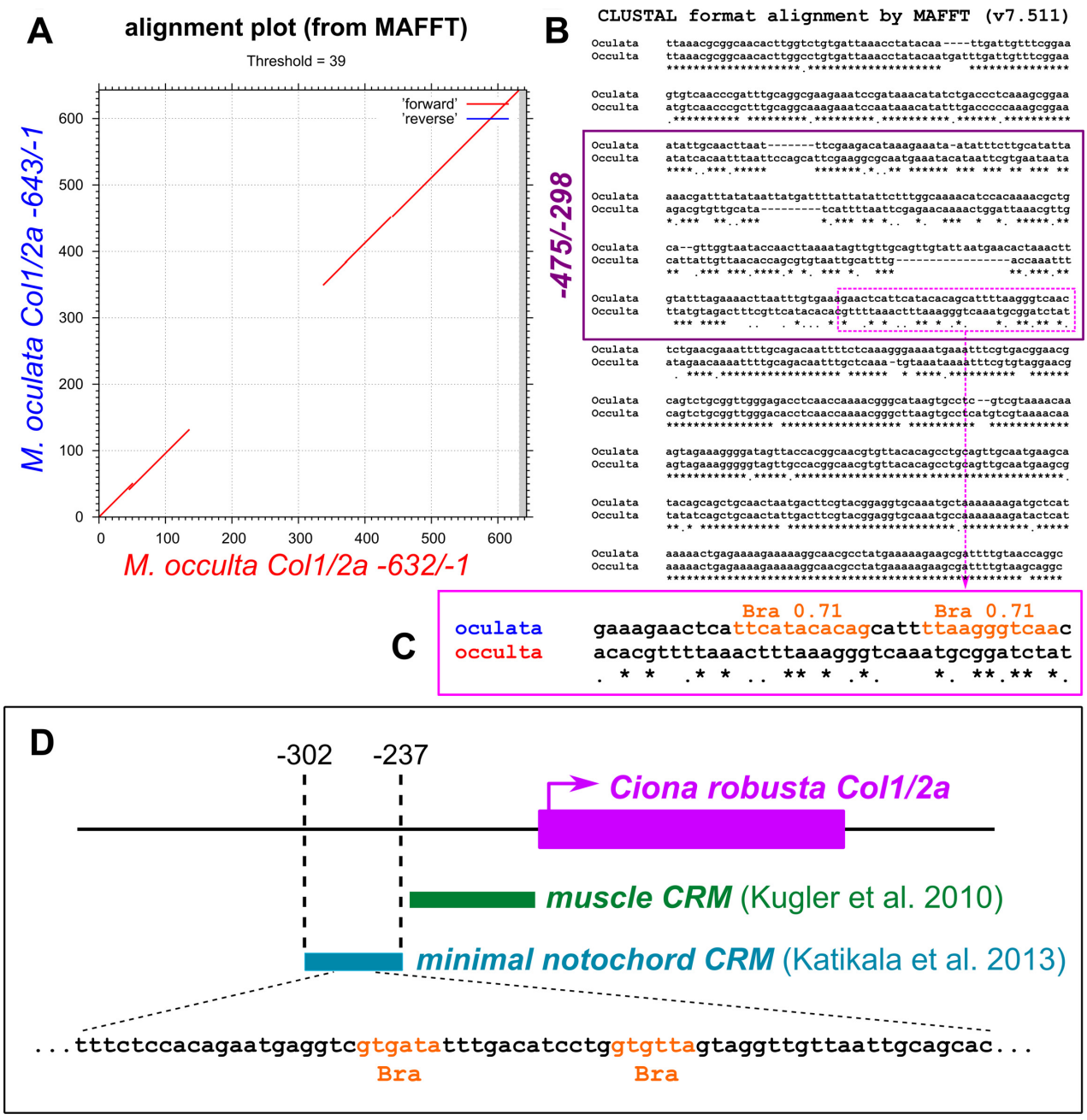

Fig. 3. *Col1/2a cis*-regulatory sequence analysis. (A) Alignment plot from MAFFT, indicating loss of alignment (red line) between *M. oculata* and *M. occulta Col1/2a* upstream sequences roughly between −475 and −298 upstream of the start of exon 1. (B) MAFFT alignment of the *Col1/2* upstream sequences, with the −475/−298 region of low identity indicated by the purple outline box. (C) Magnified view of smaller region in pink dotted outline showing low sequence identity between *M. oculata* and *M. occulta,* with predicted mouse Brachyury (Bra) sites and their affinity scores in the *M. oculata* sequences highlighted. (D) Diagram of *Col1/2a* upstream region in *C. robusta,* showing known CRM previously characterized, including a minimal notochord CRM with functionally validated *Ciona* Brachyury binding sites highlighted.

for Tbx6, which has been shown to regulate *Col1/2a* in the tail muscles of *Ciona* (Kugler et al. 2010).

Unfortunately, we were not able to test these sequences in *Molgula,* due to technical limitations (see in section "Discussion"). Thus, we attempted to test these sequences in much more tractable *C. robusta* embryos. Electroporation in *Ciona* embryos revealed *Molgula Col1/2a* reporter expression only in tail muscles, regardless of whether the sequence was from *M. oculata* (*−643/−1* upstream of the start codon) or *M. occulta* (*−632/−1* upstream) (Fig. 4). This suggests that the *cis*-regulatory logic of *Col1/2a* expression in the notochord is highly divergent between these distantly related genera due to developmental system drift, as we have

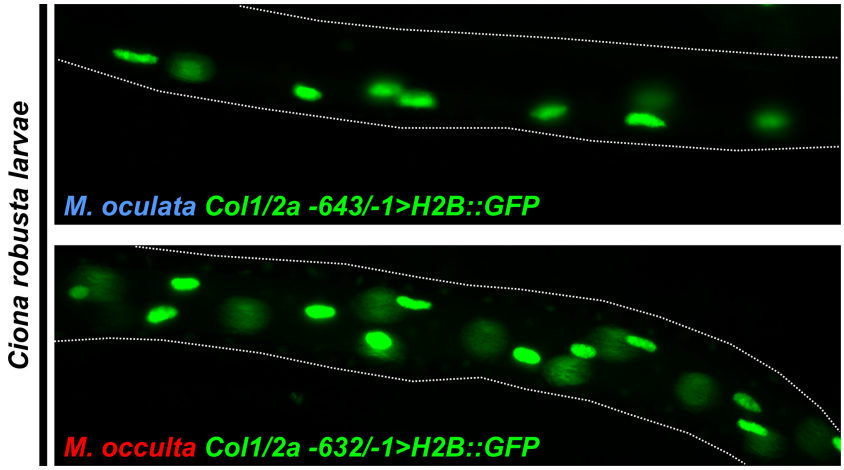

Fig. 4. *Molgula Col1/2a* reporter activity in *Ciona* larvae. Images of the tails of *C. robusta* larvae electroporated with *M. oculata* (top) and *M. occulta* (bottom) *Col1/2a* H2B:: GFP reporter plasmids. H2B:: GFP expression (green nuclei) was observed in tail muscles with both *M. oculata* or *M. occulta* reporters, but no notochord labeling was seen with either construct. This suggests that the notochord CRM might be "unintelligible" between these distantly related tunicate genera (i.e., *Molgula* vs. *Ciona*). Tail outlines are indicated by dashed lines.

previously shown for other CRMs (Stolfi et al. 2014b; Lowe and Stolfi 2018). Alternatively, the full *cis*-regulatory sequences required for notochord expression may not be contained in our reporters. However, we find this explanation less likely, as the *C. robusta* notochord CRM is located between 302 and 237 bp upstream of *Col1/2a* (Fig. 3D), and *Molgula* genomes are even more compact than that of *Ciona* (Stolfi et al. 2014b).

A role for *Col1/2a* in notochord morphogenesis revealed by CRISPR in *Ciona*

Evolutionary loss of the larval tail in several *Molgula* species is thought to be due to a lack of any selective advantage for swimming larvae in certain habitats, especially for those species inhabiting mud or sand flats at northern latitudes (Huber et al. 2000). While species with swimming larvae such as *Ciona* swim upward to attach to the undersides of outcroppings or other suspended substrates, *M. occulta* larvae fall to the ocean floor and live buried in the sand as adults (Berrill 1931). As the hollow, rod-like notochord likely functions as a hydrostatic skeleton for the tail muscles during swimming (Stemple 2005), a convergent extension of the notochord appears to be dispensable for tunicate species whose larvae do not need to swim in order to disperse and settle. Therefore, *M. occulta* may have lost the expression of genes important for notochord morphogenesis simply due to a lack of purifying selection. To show that *Col1/2a* expression in the notochord is important for its morphogenesis in tailed species, we turned to tissue-specific CRISPR/Cas9-mediated mutagenesis in *C. robusta.*

Notochord-specific disruption of *Col1/2a* was performed by co-electroporating a *Brachyury > Cas9::Geminin-N* plasmid (Corbo et al. 1997; Song et al. 2022) together with two *Col1/2a*-targeting guide RNA plasmids (Fig. 5A) and *Brachyury > Venus(YFP)* to assay notochord morphogenesis. *Col1/2a* disruption resulted in notochord intercalation defects compared to a negative control condition omitting the *Col1/2a*-specific guide RNAs (Fig. 5B and C). The severity of the notochord defects observed correlated with mosaic incorporation of plasmid DNA, as assayed by YFP fluorescence, consistent with the fact that Col1/2a protein secreted by unaffected notochord cells might be able to partially rescue *Col1/2a* mutations in neighboring cells (Fig. 5C). Taken together, these data suggest that *Col1/2a* is essential for proper notochord morphogenesis in tunicate species with tailed, swimming larvae.

Discussion

We have presented evidence supporting the hypothesis that the expression of genes important for notochord morphogenesis has been lost over the course of the evolution of tunicates with non-swimming larvae such as *M. occulta.* RNAseq and *in situ* hybridization data show that expression of the extracellular matrix component Col1/2a is reduced in the notochord of *M. occulta* when compared to that in its close tailed relative *M. oculata.* Differential parental allele-specific expression of *Col1/2a* in *M. occulta* X *M. oculata* hybrids supports the hypothesis that this reduction is due to changes in *cis*-regulatory sequences, which is further supported by bioinformatic alignment and transcription factor binding sequence analysis. Using CRISPR/Cas9 in the tailed laboratory model tunicate *Ciona,* we show that *Col1/2a* is likely involved in the morphogenesis of the notochord.

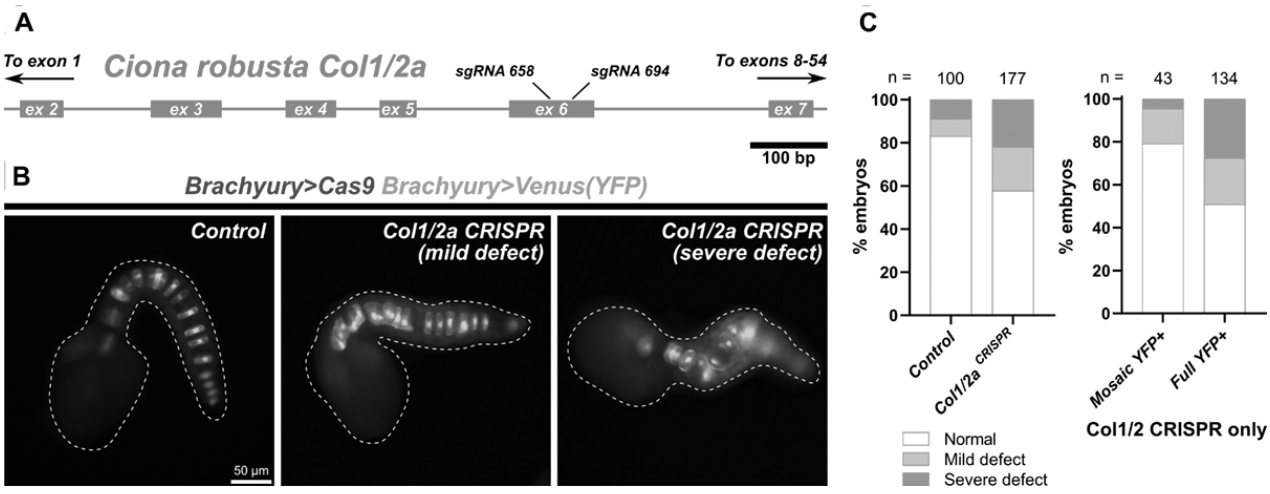

Fig. 5. CRISPR/Cas9-mediated disruption of *Col1/2a* in *Ciona* impairs notochord morphogenesis. (A) Diagram of part of the *Col1/2a* gene in *C. robusta*, indicating the target sites of sgRNAs used for CRISPR/Cas9-mediated mutagenesis in this study. (B) Embryos subjected to notochord-specific CRISPR/Cas9-mediated mutagenesis of *Col1/2a*, compared to a control embryo. Examples of mild and severe notochord morphogenesis defects are shown. Notochord cells labeled by *Brachyury* > *Venus* (green). (C) Scoring data of notochord defects observed in CRISPR and negative control embryos, one trial each. Left: all YFP + embryos in both conditions. Right: *Col1/2a* CRISPR scoring data broken down by left/right mosaic expression of YFP (due to mosaic incorporation of electroporated plasmids) and "full" YFP expression.

However, we did not test the requirement of *Col1/2a* in tailed *Molgula* species, as CRISPR/Cas9 has not been adapted to these species yet. Furthermore, we were unable to test differential *cis*-regulatory sequence activity, due to our inability to access *Molgula* embryos for electroporation, and due to *cis*-regulatory incompatibility between *Molgula* and *Ciona* (Lowe and Stolfi 2018). We were not able to routinely obtain *M. oculata/occulta* specimens, as these species overlap in an extremely limited geographic region and spawn only during one month of the year (Berrill 1931; Huber et al. 2000). Furthermore, 2022 was the hottest European summer in recorded history (van Daalen et al. 2022), and this has likely affected the timing and duration of gamete production (Bates 2005). In the future, it will be important to overcome these technical hurdles to rigorously investigate the *cis*-regulatory basis of tail loss in *Molgula*.

It is important to note that, unlike other genes such as *Tyrosinase* (Racioppi et al. 2017), the *Col1/2a* gene itself has not been lost from *M. occulta*. This is probably due to the important roles of collagens in the adult tissues (Rychel and Swalla 2007). One interesting observation is that *Col1/2a* expression is still observed in the otherwise non-functional larval tail muscles of *M. occulta* (Fig. 2C). This might be due to a requirement for *Col1/2a* in adult muscles. If the same *cis*-regulatory element was required to drive the expression of *Col1/2a* in both larval and adult muscles, then that would preclude the loss of expression in larval tail muscles. In contrast, the notochord is a tissue that is not present in any adult ascidian (being lost at metamorphosis), and thus notochord-specific *cis*-regulatory elements are not expected to be under the same evolutionary constraints. In sum, the tailless larvae of *M. occulta* remain an intriguing model in which to study molecular and cellular principles of evolutionary losses. Future progress will depend greatly on the ability to further adapt electroporation and CRISPR techniques to directly test *cis*-regulatory and protein-coding sequences in *Molgula*.

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Supplementary data

Supplementary data available at *ICB* online.

Conflict of interest

The authors declare that they have no competing financial interests.

Data availability

The data underlying this article will be shared upon request to the corresponding authors.

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