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Pigmentation in the sensory organs of the ascidian larva is essential for normal behavior

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Summary

Free-living animals and their larvae utilize light and gravity as cues to navigate in open space. Detection and response to these environmental stimuli are important for the dispersal and settlement of ascidian larvae. Two pigmented structures in the brain of the ascidian larva, the ocellus and the otolith, have been shown to function as the photoreceptive and gravity sensitive organs, respectively. Here, we show that pigmentation is essential for proper phototactic and geotactic behavior in larvae of the ascidian species *Ciona savignyi*. Two recessive and complementing mutant lines of *C. savignyi*, *immaculate* and *spotless*, that specifically disrupt the pigmentation of the sensory organs during larval development are described. Homozygous mutant larvae are unable to respond properly to gravity and illumination cues while settling. Genetic analysis shows that *spotless* is caused by a point mutation within the tyrosinase gene that creates a premature stop codon, while the molecular nature of *immaculate* is unknown. Although the role of pigmentation in the ocellus of *C. savignyi* is similar to that in vertebrate visual systems, our results demonstrate a novel use of melanin in geotactic behavior.

Key words: ascidian, pigmentation, melanin, settlement, behavior, ocellus, otolith.

Introduction

Ascidians are a diverse class of the urochordates that are found in a wide range of marine environments. The ascidian *Ciona savignyi* is a sessile filter-feeder found in shallow, shaded environments (Satoh, 1994). Although by many measures, such as genome size, ascidians are less complex than their vertebrate relatives, they share many aspects of physiology and embryology. The study of ascidian development and physiology has provided valuable insight into the evolution of the chordates and the diversification of the three chordate subphyla (vertebrates, urochordates and cephalochordates) following their divergence from a common chordate ancestor.

The behavior of the ascidian larva has long been of interest to ecologists and developmental biologists (Svane and Young, 1989). Early work on ascidian larvae described conserved phototactic and geotactic behaviors across the majority of ascidian species. Free-swimming larvae are geonegative for the majority of the larval dispersal period until shortly before settlement, when they begin to swim towards gravity. The larvae become photosensitive ≈4 h after hatching, responding to decreased light intensity by swimming more actively and seeking out shaded locations (Svane and Young, 1989). Recent work has suggested that the pigmented cells of the ocellus and otolith are necessary for sensing light and gravity, respectively, although the role of the pigment itself, particularly in gravity sensation is unresolved (Sakurai et al., 2004; Tsuda et al., 2003c). The development of these cells has been studied extensively, and the lineage, structure and melanogenesis are well understood (Dilly, 1962; Dilly, 1964; Nishida and Satoh, 1989; Sato and Yamamoto, 2001; Whittaker, 1966). However, a direct proof for the essential role of pigmentation in ascidian larval physiology is still lacking. We describe here two mutant lines of *C. savignyi* that are unable to make melanin, and thus lack pigment in the larval sensory structures. The absence of melanin results in profound behavioral abnormalities in mutant larvae.

Materials and methods

immaculate

Adult *Ciona savignyi* (Herdman 1882) were collected from the Santa Barbara yacht harbor and tested for pre-existing recessive mutations by self-fertilization (*C. savignyi* are hermaphrodites). The adults were first kept in circulating filtered seawater for several days under constant light to allow for the accumulation of gametes. They were then placed

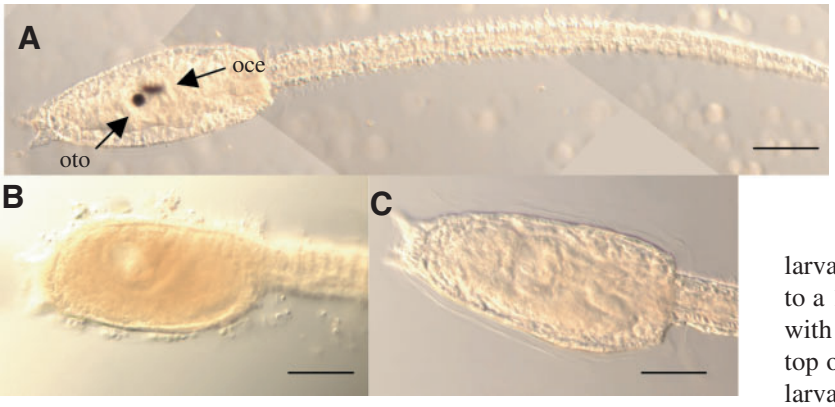

Fig. 1. Larvae of the ascidian *Ciona savignyi*. (A) Wild type, (B) homozygous *immaculate* (C) homozygous *spotless.* oto, otolith; oce, ocellus. Scale bars, 50 μm.

individually in cups of filtered seawater, kept in the dark for 8 h, and induced to spawn eggs and sperm by exposure to light. The majority (≈60%) of the wild-collected *C. savignyi* produce self-fertilized broods when spawned in this way. Self-fertilized eggs were collected from the cups and cultured at 18°C in 15 mm × 60 mm dishes containing 20 ml seawater with 50 mg ml^{-1} of kanamycin and streptomycin. Hatched larvae, ≈24 h post-fertilization, were screened for developmental defects. Those adults producing broods with developmental defects (including the unpigmented lines described here) were crossed to wild-type adults to produce a second heterozygous generation that was again screened for the phenotype.

spotless

The mutant line *spotless* was identified in a screen for ethylnitroso urea (ENU)-induced mutations (Sigma, St Louis, MO, USA). The ENU treatment was essentially as described before (Moody et al., 1999), with minor modifications. Briefly, animals to be mutagenized were first screened for pre-existing recessive mutations by self-fertilization (see above). Animals that did not carry obvious recessive mutations were injected with 50 μl of 100 mmol l^{-1} ENU into the gonads and then incubated in seawater containing 3 mmol l^{-1} ENU for 1 h. This treatment was performed once a week for 3 weeks. Animals that survived treatment were allowed to recover for 3 weeks. Sperm from ENU-treated animals was collected by dissection and used to cross to wild-type eggs to generate an F_1 generation. F_2 broods produced by self-fertilization from each F_1 individual were screened for developmental phenotypes. F_1 adults potentially carrying recessive mutations of interest were crossed to wild-type animals and the progeny re-screened.

Imaging and immunohistochemical staining

Larvae were mounted on glass slides either alive or fixed in 10% formamide in seawater. Images of the ocellus and otolith were taken at 630× or 1000× magnification on an Isoskope 2 binocular microscope (Carl Zeiss, Jena, Germany). Wild-type and mutant larvae were fixed in 10% formaldehyde in sea water at 4°C for 3 h, washed in phosphate-buffered saline (PBS) + 0.1% Triton X-100 and dehydrated to 80% ethanol. Larvae were then stained using antibodies against *Ci*-opsin1 and *Ci*-arrestin as described previously (Tsuda et al., 2003b).

Behavioral assays

Geotaxis

Wild-type and pigmentation mutant embryos were raised at 18°C in the dark. Newly hatched larvae, or embryos just before hatching, were transferred to a 15 cm Petri dish containing 60 ml filtered seawater with antibiotics. A second Petri dish was suspended on top of the first dish at a height of 1 cm to sandwich the larvae between two potential settling surfaces. Larvae were incubated for 24 h at 18°C in the dark. At the end of the incubation, larvae attached to the top or bottom of the dish were counted.

Phototaxis

Wild-type and mutant embryos were raised at 18°C in the dark until hatching. The newly hatched larvae were transferred to one half of a 15 cm Petri dish that was shaded with black tape and aluminum foil, while the other half was left transparent. The apparatus was maintained at 18°C and continuously illuminated from above. After 24 h, larvae attached to the light or shaded areas were counted.

Tyrosinase assay

Wild-type and mutant larvae were fixed in cold 70% ethanol at 4°C for 1 h. Larvae were incubated in 4 mmol l^{-1} KH_2PO_4, 11 mmol l^{-1} Na_2HPO_4 with or without 3.8 mmol l^{-1} L-dopa (Sigma) for 4 h. Unreacted L-dopa was removed by soaking in 70% ethanol for 6 h at room temperature before imaging.

Genetics

The sequence of *C. savignyi* tyrosinase was identified by a translated BLAST search of the *C. savignyi* genome (http://www.broad.mit.edu/annotation/ciona/index.html) using the *Halocynthia roretzi* tyrosinase protein sequence. Specific primers, 5′ TCAGCCCAGTTTCCAAGGAGG and 3′ AGAGCAGCAGCTCTGTTTTCT, were used to isolate *Cs*-tyrosinase cDNA from both wild-type and *spotless* embryo total RNA and cloned into pCR II (Invitrogen, Carlsbad, CA, USA). DNA sequencing was performed using the ABI Big Dye Terminator Sequencing Kit at the Iowa State University sequencing facility. The identified mutation was then confirmed by sequencing the genomic region isolated from *spt* by PCR and cloned into pCR II (PCR primers: 5′ CAACTTCACCATGTTCAGGACAGTGTTACCAG and 3′ CTGTGCAGGCTGATACAATGTCCTGTCGCCCC; sequencing primer: GTCCTATCTGCCGTTGCG).

Rescue

A 10.5 kb genomic fragment containing the *C. savignyi* tyrosinase gene and 5′ flanking DNA extending to the start of the immediate upstream putative gene was amplified by PCR (primers: 5′ GGCATTTCATGTGAAGTGATTGATATGGT-

GACC and 3′ GGGGCGACAGGACATTGTATCAGCCTG-CACAG) using LA Taq (Takara, Shiga, Japan) from wild-type genomic DNA. The PCR product was cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA, USA). This plasmid was electroporated (100 μg/electroporation) into fertilized eggs from crossed s*pt* homozygous adults as previously described (Corbo et al., 1997). The electroporated embryos were allowed to develop to the swimming tadpole stage according to standard protocols.

Results

immaculate (*imc*) is a spontaneous mutant isolated from the wild population in the Santa Barbara yacht harbor, while the mutant *spotless* (*spt*) was identified in an ENU mutagenesis screen. Larvae homozygous for either mutation appear morphologically normal, with the exception of the absence of pigmentation in the central nervous system (Fig. 1B,C). Homozygous mutant larvae swam normally and initiated metamorphosis to develop into fertile adults with no obvious abnormalities (data not shown). Homozygous mutant adults produce 100% non-pigmented offspring with no apparent additional defects, indicating neither gene has an essential maternal role. Crosses between homozygous *imc* and *spt* adults produced larvae with normal pigmentation, indicating that the two mutations disrupt different genes.

The pigmented structures seen in normal larvae (Fig. 2A) are components of two sensory organs, the otolith and the ocellus, found within the sensory vesicle. The otolith is composed of a large spherical cell attached to the ventral wall of the sensory vesicle by a narrow stalk. It contains a single pigment granule that occupies most of the cell body. The ocellus lies posterior to the otolith along the dorsal wall of the sensory vesicle. It is composed of three lens cells, a single cup-shaped cell containing numerous small pigment granules and 15-20 photoreceptor cells (Dilly, 1962; Dilly, 1964; Eakin and Kuda, 1971; Sakurai et al., 2004). In both mutants, Nomarski imaging indicated these structures are properly formed despite the absence of pigment (Fig. 2B–G). The structure of the ocellus in both mutants was also assessed by immunostaining with antibodies to opsin1 and arrestin, which stain the outer segments of the photoreceptors and the entire photoreceptor cell body, respectively. The staining patterns with these antibodies were indistinguishable between the mutants and the wild type (Fig. 3).

Complementation assays indicated that *imc* and *spt* disrupted different genes. Furthermore, while we were unable to detect any pigmentation in *spt* larvae, a trace amount of melanin could be detected in the ocellus, but not the otolith, of *imc* embryos when examined at high magnification, also suggesting that the two mutations may disrupt different steps in melanin biosynthesis (Fig. 2E). Tyrosinase (dopa oxidase) has been shown to be the key enzyme necessary for melanogenesis in the ascidian pigment cells. It is known to oxidize both L-tyrosine and L-dopa to produce dopaquinone, which in turn spontaneously forms insoluble melanin (Whittaker, 1966). Homozygous mutant larvae were assayed for tyrosinase activity by soaking them in exogenous L-dopa (Whittaker, 1966). In this assay, melanin could be detected in both the ocellus and otolith of *imc* larvae, indicating the presence of tyrosinase activity (Fig. 4B). Additionally, the level of tyrosinase gene expression

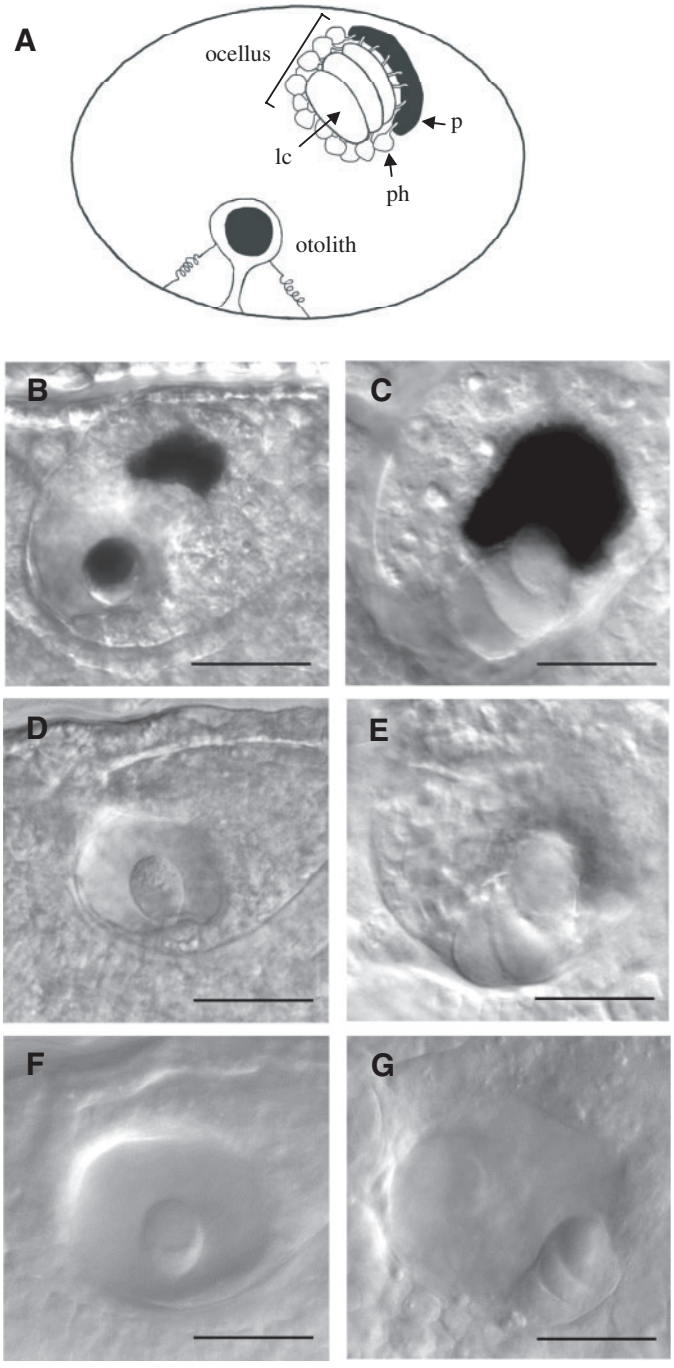

Fig. 2. Pigmentation in the sensory vesicle of *Ciona savignyi imc* and *spt* mutants is deficient. (A) Diagram of the *Ciona* sensory vesicle. (B,D,F) Nomarski images of the otolith in wild type (B), homozygous *imc* (D) and homozygous *spt* (F). (C,E,G) Nomarski images of the ocellus in wild type (C), homozygous *imc* (E) and homozygous *spt* (G). lc, lens cells; p, pigmented cell in ocellus; ph, photoreceptors; Scale bars, 20 μm.

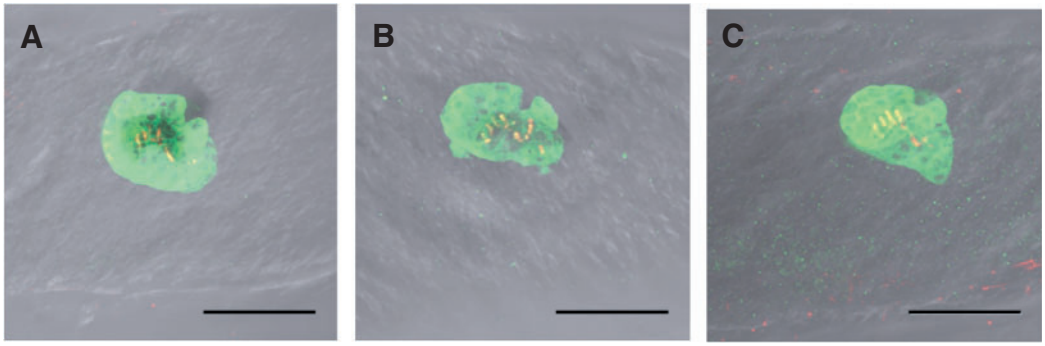

Fig. 3. Immunohistochemical staining of opsin1 (red) and arrestin (green) in wild type (A), homozygous *imc* (B) and homozygous *spt* (C) Swimming larva. Scale bars, 20 μm.

as detected by *in situ* hybridization in *imc* larvae was indistinguishable from wild type (data not shown). *spt* larvae, however, failed to synthesize melanin in the presence of L-dopa (Fig. 4C). This suggests that the pigmentation defect in *spt* is due to a deficiency in tyrosinase activity, while *imc* is a defect in some unknown aspect of the pigmentation process, most probably upstream of tyrosinase.

Three tyrosinase-like genes, tyrosinase and tyrosinase-related proteins 1 and 2, are found in the *C. savignyi* genome. cDNAs for all three genes were sequenced from homozygous *imc* embryos, and no significant differences from the nucleotide sequence provided by the *C. savingyi* genome project were found (data not shown). However, sequencing of a tryrosinase cDNA from homozygous *spt* larvae uncovered a single nucleotide substitution that results in a premature stop codon before two copper binding sites known to be essential for tyrosinase function (Oetting and King, 1999) (Fig. 5A). The presence of the nucleotide substitution was confirmed by sequencing a genomic fragment PCR-amplified from homozygous *spt* DNA. To confirm that the mutant tyrosinase gene was responsible for the *spt* phenotype, a 10.5 kb genomic DNA fragment containing the wild-type tyrosinase gene and its upstream region was electroporated into *spt* homozygous embryos. Electroporation into *Ciona* typically results in variable uptake of exogenous DNA. In the brood of electroporated *spt* homozygous embryos, we observed a range of phenotypes from no pigmentation, to pigmentation of only one of the sensory organs, to pigmentation of both sensory organs (Fig. 5B,C). The ability of the electroporated genomic fragment to rescue pigmentation led us to conclude that the *spt* phenotype was due to the mutation of the tyrosinase gene.

The major purposes of the shorted-lived larval stage of ascidians is first dispersal, then selecting an appropriate settling site, and finally attachment. To test the role of pigmentation in the behavior of the larvae, we assayed for final settling position on substrates in different gravitational orientations, and in light and dark environments. *Ciona* larvae become photosensitive ≈4 h after hatching, as indicated by their vigorous swimming response to shade (Mast, 1921; Nakagawa et al., 1999). We subjected *imc* and *spt* larvae to repeated exposures of bright and dim light and found that, like wild type, their swimming frequency increased in dim light (data not shown). To assay for phototaxis, larvae from crossed heterozygous *imc* or crossed heterozygous *spt* were placed in the shaded half of a half-shaded, half-illuminated 15 cm Petri dish (Fig. 6A,B). The larval broods, which consisted of both pigmented and unpigmented offspring of the heterozygous parents, were allowed to settle for 24 h. The number of non-pigmented offspring (homozygous mutant) and their pigmented siblings (heterozygous and homozygous wild type) that had settled on the dark and illuminated sides of the dish were counted. Pigmented larvae preferentially settled on the shaded side (81.3% and 81.9% for the *spt* and *imc* experiments, respectively), while neither homozygous *imc* nor *spt* larvae showed preference for shaded over illuminated areas, settling in the shaded area at the frequencies of 49.4% and 54.4%, respectively (Fig. 6D,E). Unpigmented larvae showed no deficiency in their ability to swim, and the assay used required larvae to swim from the shaded areas before settling in the illuminated half. This result suggests that non-pigmented larvae are unable to detect the source of light, and consequently are unable to seek out the shaded location preferred by their wild-type siblings.

In a second experiment, the geotactic behavior of the non-

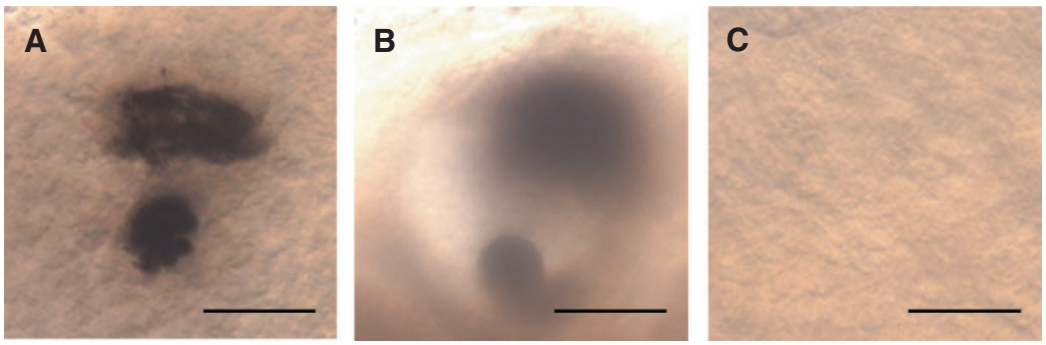

Fig. 4. L-dopa staining of the sensory vesicle in wild type (A), homozygous *imc* (B), and homozygous *spt* (C) Swimming larva. Scale bars, 20 μm.

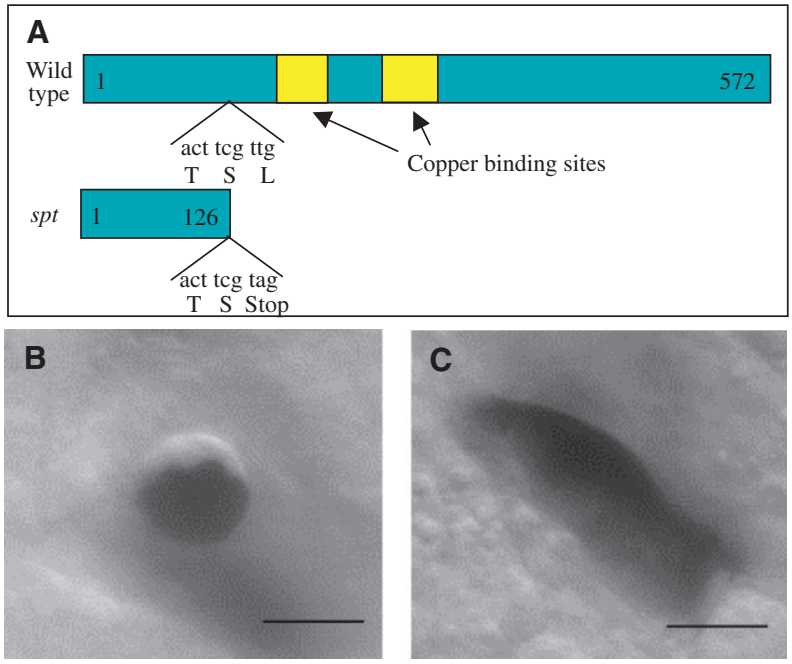

Fig. 5. *spt* mutation and rescue. (A) Schematic diagram of *Ciona savignyi* tyrosinase protein structure and the site of mutation in *spt*. (B,C) Pigmentation in otolith (B) and ocellus (C) in a homozygous *spt* tadpole rescued by electroporation of wild-type *C. savignyi* tyrosinase genomic DNA. Scale bars, 10 μm.

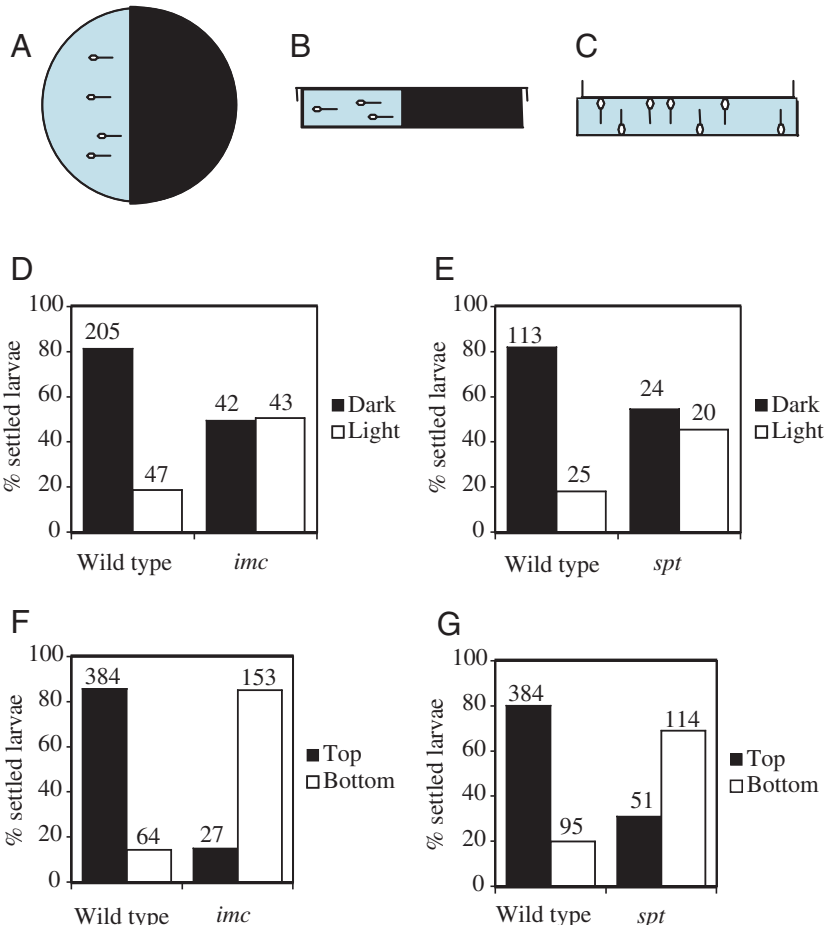

Fig. 6. Homozygous *imc* and *spt* tadpoles failed to swim towards shaded areas and against gravity. (A–C) Diagrams of the apparatus; (A) top view; (B,C) side views. (A,B) For phototaxis assays Petri dishes were half shaded (black) and larvae were placed in the shaded area and their response monitored. (C) For geotaxis assays tadpoles were placed in Petri dish containing 60 ml filtered seawater with antibiotics. A second Petri dish was suspended on top of the first dish at a height of 1 cm to sandwich the larvae between two potential settling surfaces. (D,E) Percentage of tadpoles settled on either dark (black) or illuminated (white) area. (F,G) Percentage of tadpoles settled on either top (black) or bottom (white) of the apparatus.

pigmented mutants was assayed. Newly hatched larvae from crossed heterozygous *imc* or crossed heterozygous *spt* adults were placed into an apparatus that would allow attachment either to the bottom of the lower dish or to the underside of a floating dish (Fig. 6C). The apparatus was kept in the dark for 24 h to allow larvae to settle. Pigmented larvae from the crossed heterozygous *imc* and *spt* adults attached to the upper dish at a frequency of 85.7% and 80.2%, respectively. In contrast, homozygous *imc* and *spt* larvae settled at the top of the apparatus at frequencies of only 13.5% and 30.9%, respectively (Fig. 6F,G). One would expect the majority of larvae that were indifferent to gravity to settle to the bottom, as we observed. Our data demonstrate that the pigmentation is essential for the negative geotactic behavior of *Ciona* larvae.

Discussion

Our results show that pigmentation defects alone are sufficient to cause profound behavioral defects in ascidian larvae. The *spt* phenotype is caused by a premature stop codon in the tyrosinase gene. The molecular nature of the *imc* mutation is not known. Our results show that tyrosinase transcript in homozygous *imc* embryos is present at levels comparable to that in wild-type embryos, and that if *imc* embryos are given exogenous L-dopa they can synthesize melanin. We hypothesize that the *imc* gene product may play a role in transporting substrate, or making them available in the pigment cells. Our results represent the first evidence that pigmentation is essential for ascidian larvae to respond to both light and gravitational stimuli in the dispersal and settling phase of their life cycle. Most ascidian species

are found in shallow, shaded environments, and larval behavior is highly conserved across these species.

Although the ascidian visual system is remarkably simple, it nevertheless bears many similarities to vertebrate eyes. As in the vertebrate eye, light traveling through the ascidian lens cells is focused onto the outer segments of the photoreceptor cells. The eye socket in the vertebrate skull acts to restrict the angle of light able to pass through the lens and onto the retina. In addition, the retinal pigmented epithelia in vertebrate eyes absorb any stray light that has passed through the photoreceptors, thereby preventing visual over-stimulation. In contrast to vertebrates, the ascidian larva is completely transparent. In order to detect the direction of light, the ascidian larvae must restrict the angle of light that gains access to the photoreceptor cells. The cup-shaped pigment cell of the ascidian ocellus surrounds the outer segments of the photoreceptor cells and restricts light intake to a single direction. This restriction of light, along with the spiraling motion while swimming, allows the larvae to determine the direction of light input (Mast, 1921). Our pigmentation mutants demonstrate the essential role of pigmented cells in detecting the direction of light. Our assay is different from the 'shadow response' assays employed in previous behavioral studies of ascidian larvae (Mast, 1921; Nakagawa et al., 1999; Tsuda et al., 2003a). Some investigators placed emphasis on this response in the context of the search for a dark or shaded location to settle (Svane and Young, 1989). However, other studies have challenged the importance of the shadow response by showing that fluctuating light was insufficient to induce tadpoles to locate shaded habitats in most of the solitary ascidian species tested (Young and Chia, 1985). Therefore, how ascidians seek out dark places to settle remains unknown.

The second behavioral characteristic of free-swimming ascidian larvae is their sensitivity to gravity. Strong negative geotaxis exhibited during the greater part of the swimming period is thought to contribute to the dispersal of progeny (Millar, 1971). It has been suggested that the pigmented vesicle within the otolith moves in response to gravitational forces, thereby deforming the cell body and stimulating spring-like structures that extend from the sensory vesicle wall and attach to the otolith near the stalk (Dilly, 1962; Eakin and Kuda, 1971; Sakurai et al., 2004). Here we show *C. savignyi* larvae lacking pigmentation do not behave properly in response to gravity. Our results show unequivocally that the melanization of tyrosine within the granule of the otolith is essential in this process. While melanin itself is insoluble and heavier than water, the otolith has also been shown to concentrate metal ions, which may contribute to its density (Sakurai et al., 2004). Previous studies have demonstrated that the content of the otolith is denser than the rest of the body (Dilly, 1962). We propose that the greater density achieved by melanization of tyrosine provides the crucial mass needed for the otolith vesicle to be efficiently influenced by gravitational forces.

Since gravity is a universal cue on earth, many organisms, from single cells to metazoans, utilize different georeceptors to orient themselves and to navigate. The calcareous statolith in ctenophores, calcium carbonate inclusions in snails, air bubbles trapped in certain passageways in a number of aquatic insects, and fluid flow in the mammal inner ear represent a few strategies that animals use in receiving the gravitational pull (Anken and Rahmann, 2002; Brusca and Brusca, 2003). The use of melanin in geotaxis has not been found in other metazoans, and thus appears to be unique to ascidians. How the melanization biochemical pathway was co-opted into the otolith is unknown.

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