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Evolution of Developmental Control Mechanisms

Regulative capacity for eye formation by first quartet micromeres of the polychaete *Capitella teleta*

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ABSTRACT

The stereotypic cleavage pattern shared by spiralian embryos provides unique opportunities to compare mechanisms of cell fate specification of homologous blastomeres, and can give insights into how changes in fate may have influenced the evolution of novel structures and morphological diversity. The potential of cells to undergo regulation and the timing of cell fate specification were investigated during early development in the polychaete annelid, *Capitella teleta*. Targeted laser deletions of the first quartet micromeres were performed, with a focus on the eye-forming cells 1a and 1c. Most of the larvae resulting from deletion of the 1a or 1c micromeres lack both the pigment cell and sensory cell of the eye as predicted by the *C. teleta* fate map. In a minority of cases, however, both left and right larval eye spots develop, suggesting that other blastomeres within the embryo regulate for loss of these cells. Deletion of the 1a and 1c derivatives, $1a^1$ or $1c^1$, also largely result in larvae with one pigment spot, although there are larvae with two eye spots, suggesting that the ability to regulate for loss of an eye-generating cell persists for an additional cell cycle. Cell deletion in conjunction with intracellular labeling indicates that all four quadrants retain the ability to generate eyes, including those that normally do not. Deletion of all four first quartet micromeres provides evidence that only the first quartet micromeres have eye-forming potential. Additionally, in contrast to the right side of the head where larval and adult eye sensory cells are derived from the same cell (1c), on the left side, the larval and adult eye sensory cells are generated by different embryonic lineages. We hypothesize that cell–cell interactions and cell position are important for regulative ability in *Capitella*. To our knowledge, this is one of the first detailed deletion studies of the first quartet micromeres and the first convincing example of regulation in polychaetes, which are often thought to be non-regulative in nature.

1. Introduction

In organisms that undergo highly stereotyped patterns of early development, each blastomere is born in a predictable sequence and position, providing unique opportunities to investigate fate specification of each identified cell. When a broad range of taxa shares a stereotyped cleavage program, comparative developmental studies allow comparisons to be made of the mechanisms of specification that may contribute to our understanding of the evolution of novel structures and body forms.

Early development in spiralians—a diverse protostome clade comprising molluscs, annelids, nemerteans, flatworms, and several other animal phyla—is highly stereotyped, so that cells in the early embryo can be identified and homologized across taxa (Henry and Martindale, 1999; Lambert, 2010). After the first two meridional cell divisions give rise to the four primary quadrants of the body (A, B, C, and D), the third division occurs obliquely along the animal–vegetal axis of the embryo. This third division denotes the beginning of the "spiral" cleavage program and results in a smaller, animal-pole tier or quartet of cells, termed micromeres. When viewed from the animal pole, these micromeres are typically born in a clockwise orientation compared to the larger, vegetal-pole quartet of cells, which are termed macromeres. The birth of each quartet of micromeres then alternates between counter-clockwise and clockwise divisions of the macromeres. Each quartet is named according to its birth order (e.g. the first set of four micromeres are called the first quartet micromeres).

The regularity of the cleavage program in spiralian embryos makes them amenable to cell lineage and fate mapping studies.

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Such studies have become increasingly comprehensive with the advent of intracellular lineage tracing techniques, which have made it possible to compare the fates of homologous blastomeres across phyla. For example, the first quartet micromeres generate head ectoderm in molluscs, polychaete annelids, and a turbellarian flatworm (Ackermann et al., 2005; Boyer et al., 1998; Dictus and Damen, 1997; Hejnol et al., 2007; Henry and Martindale, 1994; Meyer et al., 2010; Render, 1991). Although numerous conserved cell fates of homologous blastomeres have been identified in spiralians, detailed investigations also reveal differences (Henry et al., 2004; Meyer et al., 2010). For example, although the eyes of most spiralians are derived from two of the first quartet micromeres, 1a and 1c, in chitons, the eyes are instead generated by two other blastomeres, 2a and 2c (Henry et al., 2004).

Experimental deletion or isolation of identified embryonic cells in spiralians has revealed surprising differences in developmental potential and mechanisms of cell fate specification. Some of the earliest studies of development in spiralians suggested that their embryos have low regulative ability, and that cell fate specification is mainly driven by segregation of cytoplasmic determinants into individual blastomeres. For example, blastomeres isolated from early stage embryos fail to regulate and instead form only the tissues they normally give rise to in the intact embryo (Costello, 1945; Novikoff, 1938; Render, 1983; Wilson, 1904). Additional studies have shown that both cell autonomous and inductive signaling play important roles in cell fate specification in multiple spiralian phyla, including turbellarian flatworms, nemerteans, and molluscs (Arnolds et al., 1983; Boyer, 1989; Martindale and Henry, 1995; Morrill et al., 1973; Sweet, 1998; van Dam and Verdonk, 1982). For example, specification of the eyes in the gastropod *Ilyanassa obsoleta* relies on inherited factors and cell position as well as inductive signaling (Sweet, 1998).

Most studies of cell fate specification in spiralians have been performed on molluscan and nemertean species. In contrast, data for polychaetes are limited to blastomere isolation, polar lobe deletion, or transplantation experiments prior to the start of spiral cleavages (Henry, 1986; Novikoff, 1938; Render, 1983; Wilson, 1904). Annelids, which include the polychaetes, constitute a diverse clade and can serve as an important point of comparison with other spiralian phyla for understanding the developmental potential and patterns of cell fate specification in the early embryo as well as the extent of regulative ability. The experiments in this study were conducted in *Capitella teleta*, a polychaete annelid model for evolutionary developmental biology. A detailed cell fate map for *C. teleta* is available and the ontogenetic origins of nearly all larval tissues are known (Meyer et al., 2010). Many cell fates in *C. teleta* are conserved in comparison with fate maps of other spiralians, e.g., the eyes are generated by the first quartet micromeres 1a and 1c, and the body ectoderm from the primary somatoblast 2d. An organizing activity from the 2d somatoblast is required to induce eye formation in the micromeres 1a and 1c (Amiel et al., 2013). In addition, morphology of the larval pigment-cup eyes of *C. teleta* has been described in detail (Rhode, 1993). The larval eyes are an easily scored morphological marker following embryonic cell deletion experiments, and are the subject of the experiments described in this manuscript. This is one of few studies to date to investigate the potential of cells to regulate during early development in a polychaete annelid. We utilized a recently developed technique for targeted single-cell laser deletion (Amiel et al., 2013; Pernet et al., 2012) to examine cell fate specification of the first quartet micromeres, using the red-pigmented eyes as a marker. We address whether the other quadrants in the early *C. teleta* embryo have the potential to generate eyes after an eye-generating blastomere (1a or 1c) is deleted. In addition, cell deletions were performed on the derivatives of 1a and 1c to examine regulative potential as the cells divide to the next cleavage stage. Our data indicate that the B and D quadrants (most likely the first quartet micromeres), not just A and C, have the ability to generate eyes.

2. Materials and methods

2.1. Animal care

Embryos were obtained either by sieving through mud for females with brood tubes, or from putting together males and females in a mating dish. Gravid females and sexually mature males were isolated from each other and kept in organically enriched mud for 3–7 days, then mixed together overnight. Embryos were dissected from female brood tubes (Seaver et al., 2005) and rinsed with 0.2 μm filtered seawater (FSW). Following experimental manipulations, embryos were raised to larval stages at 19 °C in FSW with 60 μg/mL penicillin (Sigma-Aldrich) and 50 μg/mL streptomycin (Sigma-Aldrich), exchanged 1–2 times daily. Animals were staged according to a previously published staging system for *C. teleta* (Seaver et al., 2005). Embryos were raised to stage 6 larvae for scoring. All fixations of larval stages were carried out as follows: pretreated in a 1:1 solution of FSW:0.37 M $MgCl_2$ for 10 min, fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in FSW for 1 h at room temperature, rinsed three times in phosphate buffered saline (PBS), and stored at 4 °C for up to one week before scoring.

2.2. Deletions

Deletions were performed using the XYClone system (Hamilton Thorne) with a 20X objective. Embryos were oriented on a slide in a drop of FSW with the animal pole up, and covered with a modified cover slip (Lyons et al., 2012). Two strips were cut from a cover slip, thickness no. 1½ (Electron Microscopy Sciences), and held to an intact cover slip, also thickness no. 1½, by a thin film of melted dental wax (Electron Microscopy Sciences) to create a small open chamber in which the embryos sit. For each blastomere deletion, the animal-most focal plane of the micromere was targeted, and the infrared laser pulsed 2–3 times to damage the egg envelope and cell membrane.

For all deletions, the XYClone laser was set at 100% power, with the following pulse times: −1a, −1b, −1c, −1ac, −1ab, −1bc, −1abcd, $-1a^1$, $-1c^1$ – 270 to 310 μs; −1d, $-1b^1$ – 150 or 270 μs. For the combined lineage tracing and deletion experiments, the pulse time was set to 150 μs. Differences in pulse times were due to cell size differences, time within the cell cycle (shorter pulses are sufficient shortly after cell birth), or variation in the distance of the target cell from the top cover slip (e.g., a slightly more vegetal position due to orientation of the embryo). All embryos were visually monitored to confirm that the cytoplasm had been discharged from the deleted blastomere, and there was no visible damage to the surrounding blastomeres. After first quartet micromere deletions, embryos were observed to confirm that the associated macromere (e.g. 1A for the 1a deletion) underwent normal cell division to produce the second quartet micromere daughter. For deletions of the first quartet micromere daughter cells, only embryos in which the other first quartet micromere daughter cells divided were kept. At least 30 unmanipulated sibling embryos were raised as a control for brood health for each set of deletions; experiments were scored only if greater than 90% of the unmanipulated controls were healthy and formed a distinct head, trunk, and pygidium as well as a prototroch, telotroch, neurotroch, eye spots and mouth. The overall background variability in the number of eyes in unmanipulated larvae was assessed by counting the number of eye pigment spots for 50 randomly-

chosen larvae from each of 10 independent morphologically normal broods. Of these, 495/500 (99%) had two eye pigment spots.

2.3. Lineage tracing

Prior to lineage tracing by pressure injection of dye, embryos at the 2-cell stage were pre-treated to soften the egg membrane by exposing the embryos to a freshly made, 1:1 solution of 1 M sucrose and 0.25 M sodium citrate for 30 s, following Meyer et al. (2010). This mixture was removed by several rinses in FSW. All injections were carried out at the 4-cell stage, from 10–15 min after the reabsorption of the polar lobe until prior to the division of the D blastomere. The B or D blastomere was individually pressure-injected with the lipophilic dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI). An ethanol saturated solution of DiI was mixed with soybean oil (Wesson) at a ratio of 1:19 for the working injection solution according to established methods (Meyer et al., 2010). At stage 6, unmanipulated control, injected control, and experimental larvae were fixed in 0.05 M EDTA plus 3.7% formaldehyde in FSW for 1 h, and then washed in PBS. Addition of 0.05 M EDTA preserves DiI during fixation (Meyer and Seaver, 2009). Larvae were then incubated overnight in 1 μg/μL Hoechst-33342 (Molecular Probes) and 1:200 phalloidin Alexa 488 (Molecular Probes), washed several times in PBS, and cleared in 90% glycerol in PBS.

2.4. Scoring and analysis

Larvae were scored for eye presence or absence based on visualization of the red pigment cell in live mounts or in fixed preparations mounted in 90% glycerol. The left–right position of the eye was determined based on its position with regard to the mouth and neurotroch. Medial eyes were not assigned a left–right position and are indicated as "position unclear," but are included as part of an expected phenotype for quantifying deletions of 1a and 1c. Statistical analyses were performed using a two-tailed Fisher's exact test with a 2×2 contingency table in the software package GraphPad QuickCalcs (http://www.graphpad.com/quickcalcs/).

2.5. Antibody labeling

For antibody labeling of 8-cell stage embryos, either control embryos or −1a embryos immediately following blastomere deletion were pre-treated for 3 min with a 1:1 mixture of 1.0 M sucrose and 0.25 M sodium citrate (both Sigma), and fixed for one hour in 4% paraformaldehyde (Electron Microscopy Sciences) in FSW. Fixative was removed by rinsing several times with PBS. Antibody labeling was performed according to previously established protocols (Meyer and Seaver, 2009). Briefly, animals were blocked in 10% goat serum in PBS+0.1% Triton X-100 (PBT) and incubated overnight at 4 °C in 1:250 mouse anti-histone primary antibody (Millipore), washed several times in PBT over 2 h at room temperature, incubated overnight in 1:300 donkey anti-mouse Alexa Fluor 546 secondary antibody (Molecular Probes) at 4 °C, washed in PBT, incubated in 1:200 phalloidin Alexa Fluor 488 (Molecular Probes) for 2–4 h, washed in PBS, and cleared in 90% glycerol in PBS.

To visualize the sensory cells of the eye, larvae were exposed to the 22C10 monoclonal antibody (deposited to the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA by Seymour Benzer and Nansi Colley (DSHB Hybridoma Product 22C10)). The 22C10 monoclonal antibody was originally generated by immunizing mice with homogenates of adult *Drosophila melanogaster* heads (Zipursky et al., 1984). The 22C10 antibody recognizes the *futsch* gene in *D. melanogaster*, which codes for a microtubule associated protein (Hummel et al., 2000). *C. teleta* larvae resulting from embryos in which either 1a or 1c was deleted were exposed to a 1:1 solution of FSW:0.37 M $MgCl_2$ for 10 min prior to fixation for one hour in 4% paraformaldehyde in FSW. Fixative was removed by rinsing several times with PBS. Immediately after washing out the fixative, larvae were scored for presence or absence of eye pigment. All one-eyed larvae with the expected eye missing were further processed for antibody labeling. Following several rinses into PBS+0.1% Triton X-100 (PBT), and exposure to 10% goat serum in PBS+0.1% Triton X-100 (PBT) for one hour, larvae were incubated in a 1:10 dilution of the 22C10 monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) overnight at 4 °C following Yamaguchi and Seaver (2013). The primary antibody was washed out with 4×30 min PBT washes. A 1:300 dilution of a donkey anti-mouse Alexa 488 (Life Technologies) secondary antibody was incubated with the samples overnight at 4 °C, washed out with 4×30 min washes in PBT, and then cleared in 90% glycerol in PBS for imaging.

2.6. Imaging and microscopy

Larvae were imaged by compound light microscopy using an Axioskop 2 mot *plus* (Zeiss) with a stem-mounted SPOTFlex camera and SPOT Basic Imaging Software (both Diagnostic Instruments, Inc.). Where noted, multiple focal planes were merged with Helicon Focus (Helicon Soft Ltd.). Confocal microscopy of embryos was performed with an LSM 710 (Zeiss), and Z-stack projections generated with ImageJ64 (NIH). Images for figures were prepared in Photoshop CS4 and figures were constructed in Illustrator CS4 (Adobe).

3. Results

3.1. Overview of Capitella teleta development and eye formation

A staging system for the embryonic and larval development of *C. teleta* has been previously described (Seaver et al., 2005). Following fertilization, *C. teleta* embryos undergo unequal cleavage via formation of a small polar lobe, and early cell divisions follow a conserved spiral cleavage program. A standard nomenclature is used to name individual blastomeres after Conklin (Conklin, 1897), and this system is broadly used for annelids, molluscs, nemerteans and other spiralian phyla. In the 4-cell embryo, the largest cell is the D cell, with the A, B, and C cells oriented clockwise when viewed from the animal pole. The next division produces the smaller first quartet micromeres, 1a–1d, and the larger first quartet macromeres, 1A–1D (Fig. 1A). Micromeres are named with a small letter that refers to its embryonic quadrant of origin, along with a number indicating the first through fourth quartet of micromeres. Large letters refer to macromeres, along with a number to indicate the order of quartet birth. Subsequent cleavages in the *C. teleta* embryo follow the standard spiralian pattern in which a set of second, third and fourth quartet micromeres are generated by unequal cleavages of the macromeres. Blastomere divisions, and relative size and position of blastomeres have been described in detail by Eisig for *Capitella capitata* (Eisig, 1899), and the details closely correspond to the division program in *C. teleta*. Following additional cleavages and gastrulation, larval stages are initiated at stage 4 by the appearance of two ciliary bands, the prototroch and telotroch, which demarcate the boundaries between the head, trunk, and posterior end (Fig. 1B). The two larval eyes appear at stage 5, and are positioned lateral and adjacent to the brain lobes and immediately anterior to the prototroch (Fig. 1B). The larval eyes each consist of three cells: a pigment cell, sensory cell, and

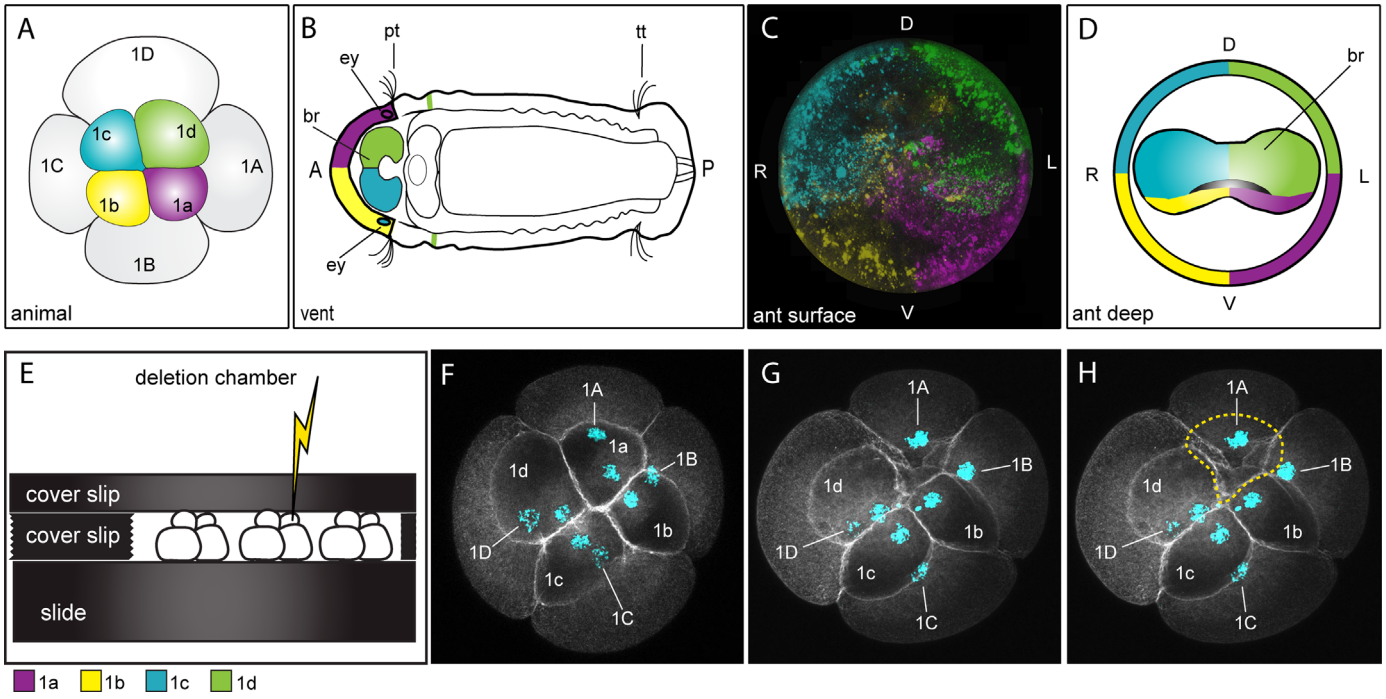

Fig. 1. *C. teleta* development and laser deletion technique. (A–D) Positions of first quartet micromeres and their contributions to the larval body as derived from the cell fate map of *C. teleta*. Each blastomere and its descendant clone have the following color code as denoted at bottom left of figure: 1a, magenta; 1b, yellow; 1c, turquoise; 1d, green. (A) Schematic of an 8-cell stage embryo, animal view. (B) Depiction of larva showing each micromere contribution to the brain, head ectoderm, and eye pigment. (C) Anterior ectodermal surface view of a stage 6 larva. Image is a composite overlay of single channel confocal image Z-stacks from four separate individuals. Each color represents a separate injection at the eight-cell stage of the following cells: 1a, 1b, 1c and 1d (adapted from Meyer et al. (2010)). (D) Graphic of first quartet micromere contributions to the brain and surrounding head epidermis. Schematic shown is a deeper set of focal planes relative to image shown in panel C. (E) Schematic showing embryo chamber for performing laser deletion of targeted blastomeres. (F, G, H) Confocal z-stack projections of 8-cell stage embryos, animal view. Cell outlines (white) and nuclei (cyan) are labeled with Alexa Fluor-488 phalloidin and anti-histone antibody, respectively. (F) Unmanipulated control 8-cell embryo. (G, H) Embryo in which blastomere 1a has been deleted. (H) Area formally occupied by 1a is outlined with a yellow dotted line. Identity of blastomeres is denoted in A, and F–H. Schematics in B and D are adapted from Meyer et al. (2010). A, ant, anterior; D, dorsal; ey, eye; L, left; P, posterior; pt, prototroch; R, right; tt, telotroch; V, vent, ventral.

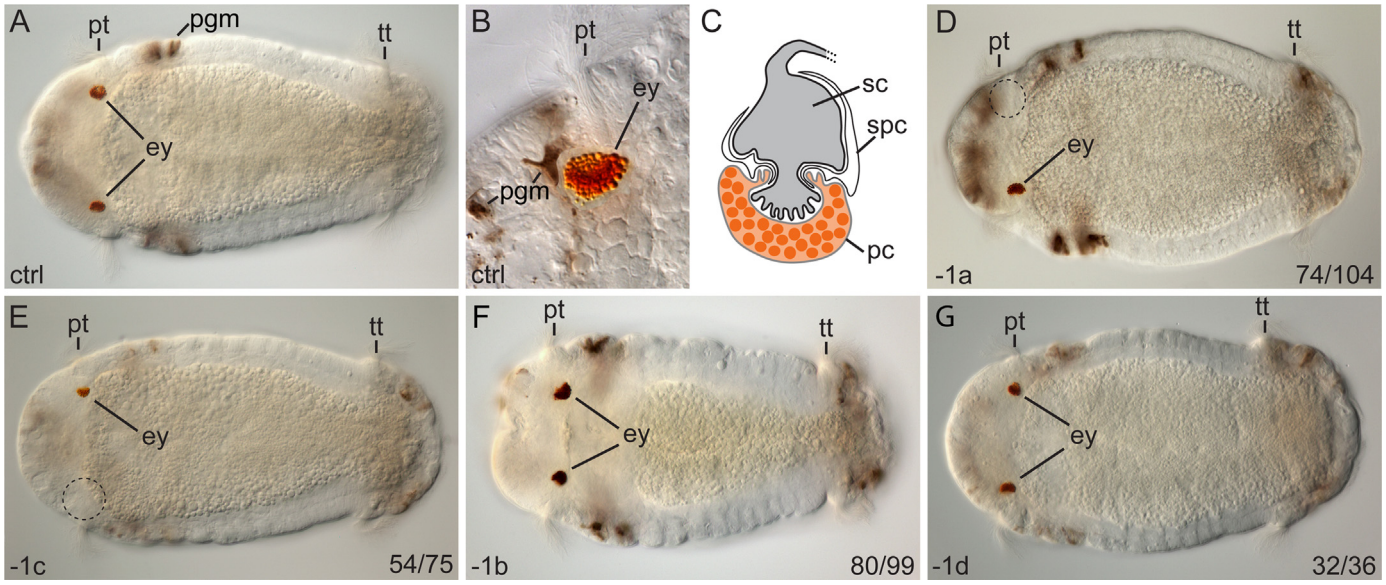

Fig. 2. Larval phenotypes following deletion of individual first quartet micromeres 1a–1d. All images are stage 6 larvae oriented in a ventral view with anterior facing left. Dotted lines mark position of missing eye. (A) Unmanipulated control larva. (B) Enlarged view of left larval eye. (C) Schematic of left larval eye showing the arrangement of the pigment cell (orange, pc), supporting cell (spc) and the sensory cell (gray, sc). The initial portion of the neuronal projection of the sensory cell is depicted towards the top of the schematic (left and posterior in larva). Adapted from Rhode (1993). (D) The left eye is missing in 71% of larvae following deletion of 1a. (E) Deletion of 1c, which generates the right eye, results in 72% of one-eyed larvae. (F) Larvae are two-eyed after 1b deletion. The panel shown exhibits synophthalmia, in which the eyes are closer together than in controls. (G) 1d deletion results in two-eyed larvae. Merged image generated from multiple focal planes. Bottom right in D - G denotes number of cases for result shown in panel. ctrl, control; ey, eye pigment cell; pc, eye pigment cell; pgm, pigment; pt, prototroch; sc, sensory cell; spc, supporting cell; tt, telotroch.

supporting cell (Fig. 2C) (Rhode, 1993). The pigment cell forms a concave cup around the distal end of the sensory cell. The pigment cell is the only visible eye cell in live or unstained specimens, and the orange color and granular texture of the eye pigment distinguish it from brown extra-ocular pigment, which is present in numerous regions of the body and appears non-granular (Fig. 2A–

B, compare 'pgm' with 'ey'). The sensory cell of the eye can be visualized using the 22C10 antibody (Yamaguchi and Seaver, 2013).

Previous intracellular fate mapping studies have shown that the first quartet micromeres generate the tissues of the larval head, including the head ectoderm, brain lobes, eyes, and prototroch in *C. teleta* (Fig. 1B–D) (Meyer and Seaver, 2010). 1a generates the sensory cell and pigment cell of the left larval eye. The sensory cell and pigment cell of the right larval eye are generated by the blastomere 1c. The origin of the support cell has not been directly determined by lineage tracing. It should be noted that 1a and 1c do not generate mirror clones as they do in other spiralians such as *C. fornicata* or *I. obsoleta* (Hejnol et al., 2007; Render, 1991). For example, 1c descendants form most of the right brain lobe in *C. teleta* whereas 1a generates only a small number of cells on the left side of the brain. The left eye is positioned adjacent to 1d descendants, and the 1c-generated right eye is positioned within 1c descendants.

3.2. Regulation of eye pigment and eye sensory cell following deletion of 1a and 1c micromeres

To investigate cell fate specification of the first quartet micromeres, we asked whether embryos of *C. teleta* could regulate for structures lost when a blastomere is deleted. The precise deletion of individual blastomeres in early-stage embryos of *C. teleta* can be performed using an infrared laser (Fig. 1E) (Amiel et al., 2013; Pernet et al., 2012). When compared with control 8-cell stage embryos (Fig. 1F), only the target cell lyses in embryos in which an individual first quartet micromere (1a–1d) is deleted, and the remaining seven cells are undamaged (Fig. 1G, H). Following the deletion of any first quartet micromere, the processes of cell division, gastrulation, and the formation of a larva generally proceed normally. Following targeted deletions, embryos were raised to stage 6, and presence of the left and right eye pigment or the eye sensory cell was scored. The eyes are representative morphological features generated by the blastomeres 1a and 1c, respectively (Meyer et al., 2010), and the appearance of the red eye pigment was scored a full day after their appearance to account for possible developmental delay of the eyes.

In general, when an eye-generating cell is deleted, the resulting larva is missing the eye pigment normally generated by that blastomere, whereas if a non-eye-generating cell is deleted, the resulting larva has both eye pigment spots (Table 1). Deletion of 1a, which generates the left larval eye, results in 71% ($n=74/104$) of larvae with the left eye pigment missing (i.e. right-eyed; Fig. 2D). In contrast, 27% ($n=28/104$) of 1a deletions result in two-eyed larvae. In the remaining two cases, one eye pigment spot is missing but the left–right position of the remaining eye pigment spot cannot be determined. Deleting 1c, which generates the right larval eye, results in 72% ($n=54/75$) of the larvae missing the right eye pigment spot (Fig. 2E). In 24% ($n=18/75$) of larvae resulting from deletion of 1c, both eyes are present. The remaining cases include one case of each of the following: left eye pigment spot is missing, both eye pigment spots are missing, or the left–right position of the eye cannot be determined. The penetrance of the one-eyed phenotype from deleting 1c (76%, $n=57/75$) is not significantly different from that of larvae with one pigment spot following 1a deletion (73%, $n=76/104$) (Fisher's exact test, $p=0.7312$).

Deletion of a non-eye-generating blastomere (1b or 1d) most often results in a larva with two eye pigment spots (Fig. 2F, G). When 1b is deleted, 81% ($n=80/99$) of the resulting larvae have two eye pigment spots and sometimes exhibit synophthalmia, in which the eyes appear closer together (Fig. 2F, Table 1). In 18% of cases ($n=18/99$) the larvae have one pigment spot; 10% ($n=10/99$) are missing the right eye spot, 5% ($n=5/99$) are missing the left eye spot, and in 3% ($n=3/99$), the position of the eye is unclear. In one other case ($n=1/99$, 1%) the larva is missing both eye pigment spots. When 1d is deleted, 89% ($n=32/36$) of resulting larvae possess both eye spots (Fig. 2G). In 3% ($n=1/36$) of the resulting larvae, the left eye pigment is missing, and in 8% ($n=3/36$) of larvae there are three eye spots, with a duplicated left eye (see Table 1).

The 1b and 1d deletions serve as controls to confirm that the phenotypes resulting from 1a or 1c deletion are the result of deleting these specific cells, rather than an artifact of the deletion itself. When the number of cases of the right-eyed phenotype after 1b deletion ($n=5/99$) is compared with the same phenotype after 1a deletion ($n=74/104$), there is a statistically significant difference between the deletion sets, such that the right-eyed phenotype is observed more frequently following 1a deletion than 1b deletion (Fisher's exact test, $p<0.0001$). Similarly, when number of cases of the left-eyed phenotype is compared between the larvae resulting from 1b deletion ($n=10/99$) versus 1c deletion ($n=54/75$), the difference between the two sets is statistically significant (Fisher's exact test, $p<0.0001$). The results are similar for 1d deletion: the right-eyed phenotype is statistically more frequent following 1a deletion (-1d vs. -1a, $p<0.0001$), and the left-eyed phenotype is statistically more frequent following -1c deletion (-1d vs. -1c, $p<0.0001$). Thus, deleting an eye-generating blastomere (1a or 1c) results in the loss of the left or right

Table 1
Summary of larval eye phenotypes resulting from micromere deletions.

Cell(s) deleted	−1a	−1b	−1c	−1d	−1a[1]	−1b[1]	−1c[1]	−1ac	−1ab	−1bc	−1abcd
Total *N*	104	99	75	36	44	39	34	20	27	21	49
No. experiments	7	7	8	5	4	3	5	4	3	3	3
Eyes present											
Two, normal	23	65	17	9	8	8	3	–	–	–	3
Two, synophthalmia	5	15	1	23	2	30	–	–	1	1[a]	–
One											
Left	–	10	54	–	–	–	30	3	–	20	–
Right	74	5	1	1	33	1	–	–	25	–	–
Position unclear	2	3	1	–	–	–	1	1	1	–	1[c]
None	–	1	1	–	1	–	–	16	–	–	45
Three	–	–	–	3[b]	–	–	–	–	–	–	–

[a] Right eye missing, second eye duplicated and adjacent to left eye.
[b] Duplicated left eye.
[c] Eye position not scored.

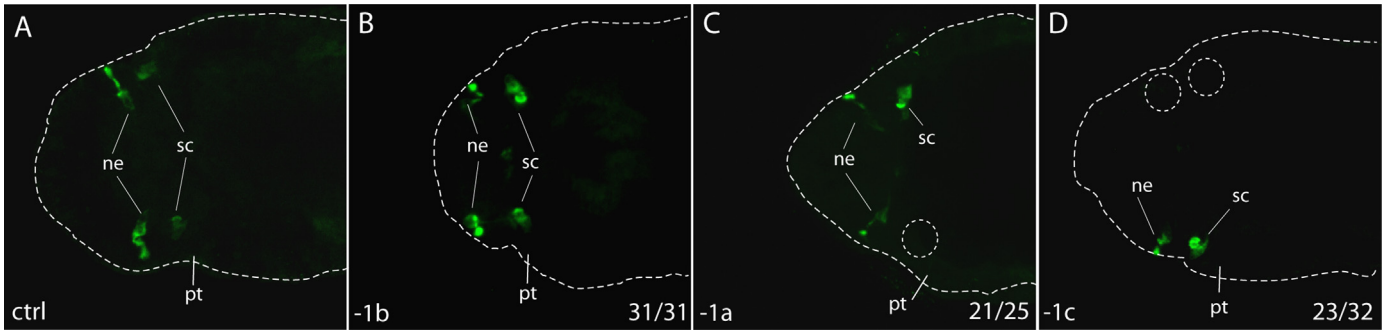

Fig. 3. Visualization of eye sensory cells in larvae by 22C10 antibody labeling. All images are confocal z-stacks oriented with anterior to the left and show a dorsal view. A dashed line indicates the boundary of the larva determined from a DIC image for each specimen, and dashed line circles mark approximate position of missing 22C10$^+$ cells. (A) Control image shows four 22C10$^+$ cells: anterior left, anterior right, posterior left, and posterior right. The posterior cells are the sensory cells of the larval eye. (B) Larva resulting from a 1b deletion showing four 22C10$^+$ cells in the head. (C) Larva resulting from an embryo in which the 1a micromere was deleted. The larva has three 22C10$^+$ cells: left anterior, right anterior, and right posterior. The left eye pigment cell is absent. (D) Larva resulting from embryos in which 1c was deleted. The larva has two 22C10$^+$ cells: left anterior and left posterior, and the right eye pigment cell is absent. Bottom right states number of cases for result shown in panel. Ctrl, control; ne, neuron; sc, sensory cell; pt, prototroch.

eye, respectively, when compared to control deletion of a non-eye-generating blastomere (1b or 1d).

To investigate whether deletion of 1a and 1c results in loss of only the eye pigment cell or of multiple cells that compose the eye, we utilized an antibody marker, monoclonal antibody (mAb) 22C10, that specifically recognizes the larval and juvenile eye sensory cells in *C. teleta* (Yamaguchi and Seaver, 2013). In stage 6 larvae, four neurons in the head show immmunoreactivity with the mAb 22C10 (22C10$^+$); the posterior pair of neurons are the sensory cells of the larval eye and the anterior pair likely give rise to the juvenile eye sensory cell (Fig. 3A) (Yamaguchi and Seaver, 2013). As expected, when the non-eye forming micromere 1b is deleted by laser ablation, all of the resulting larvae with two eye pigment spots have four 22C10$^+$ neurons in the head, including the two larval eye sensory neurons (n=31/31, 100%) (Fig. 3B). In contrast, when 1a is deleted, almost all of the resulting larvae lacking the left pigment cell also lack an eye sensory cell on the left side (n=23/25, 92%). In the remaining two cases, all four 22C10$^+$ cells are present. Similarly, when 1c is deleted, the majority of resulting larvae missing the right pigment cell also lack an eye sensory cell on the right side (n=31/32, 97%). In the remaining 1c deletion case, all four 22C10$^+$ cells are present. Thus, when the deletion of the 1a and 1c blastomeres results in loss of a pigment cell, the larval sensory cell on the same side is also missing. This demonstrates that 1a and 1c deletions generally result in loss of the eye, rather than only loss of the pigment cell.

Interestingly, we recovered two classes of 22C10$^+$ patterns in one-pigment-spot larvae following 1a or 1c deletions. There were either two or three 22C10$^+$ neurons in larvae, and the proportion of these two classes differed between the 1a and 1c deletions. Following 1a deletions, most resulting larvae lacking a left pigment spot have three 22C10$^+$ neurons, with only the left larval eye sensory cell missing (n=21/25, 84%) (Fig. 3C). In two cases, both 22C10$^+$ neurons are missing on the left side of the head (n=2/25, 8%). In contrast, following deletion of the 1c micromere, most animals have two 22C10$^+$ neurons, both located on the left side of the head (n=23/32, 72%) (Fig. 3D). Eight other cases from 1c deletions have three 22C10$^+$ neurons, with only the right eye larval sensory cell missing (8/32, 25%).

3.3. *Deletion of first quartet micromere derivatives 1a^1 and 1c^1*

During the early cleavage stages of *C. teleta*, the first quartet micromeres (1q) divide asymmetrically to form two daughter cells, one of which is nearer the animal pole (1q^1) and one is positioned more vegetally (1q^2). The animal daughter cells 1a^1 and 1c^1 generate the larval eyes (Meyer et al., 2010). Individual deletions of 1a^1 and 1c^1 were performed in 16-cell stage embryos to test whether regulation can occur at a later stage (Table 1). The control deletion of the non-eye forming blastomere, −1b^1, results in 97% (n=38/39) larvae with two pigment spots, most of which exhibit synophthalmia (n=30; Fig. 4A). In the one remaining larva (3%), the left eye spot is missing. In the majority of cases, deletion of 1a^1 results in larvae missing the left eye pigment spot (75%, n=33/44) (Fig. 4B); in one case the larva is missing both eye spots (2%), and the remaining larvae (n=10/44, 23%) possess both eyes. The proportion of one-eyed larvae following deletion of 1a^1 is not significantly different when compared with the deletion of its parent cell 1a (Fisher's exact test, p=0.6849). When 1c^1 is deleted, 88% (n=30/34) of larvae are missing the right eye pigment spot (Fig. 4C), and in one larva the position is unclear for the one eye spot present (3%); both eye spots are present in 9% of larvae (n=3/34). Deletion of 1c^1 does not give a significantly different proportion of numbers of cases of larvae with the one pigment spot phenotype when compared to deletion of 1c (Fisher's exact test, p=0.0699). Similar to the 1a or 1c deletions, the difference in the

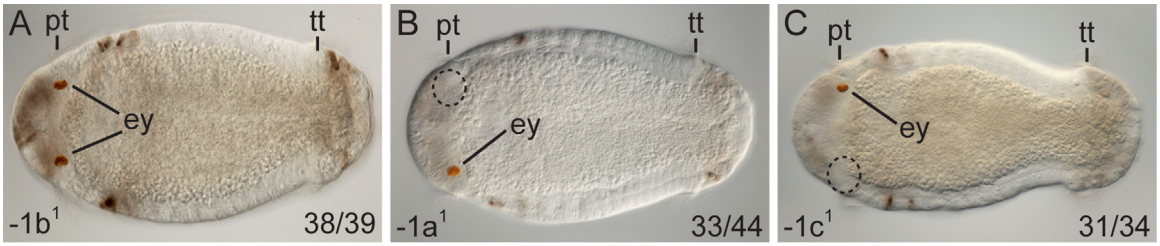

Fig. 4. Regulation following single deletion of 1a^1 and 1c^1 blastomeres. Larvae are oriented in a ventral view with anterior to the left. Dotted line circles mark position of missing eye. (A–C) Deletion of the animal daughter, 1q^1. (A) Deletion of 1b^1 results in larvae with two eye spots in 97% of cases. The example shown exhibits synophthalmia, in which the eyes are closer together than in unmanipulated controls. (B) 1a^1 deletion results in 75% of larvae with the left eye missing. (C) Larvae from 1c^1 deletion with the right eye missing in 91% of cases. Bottom right states number of cases for result shown in panel. ctrl, control; ey, eye pigment cell; pt, prototroch; tt, telotroch.

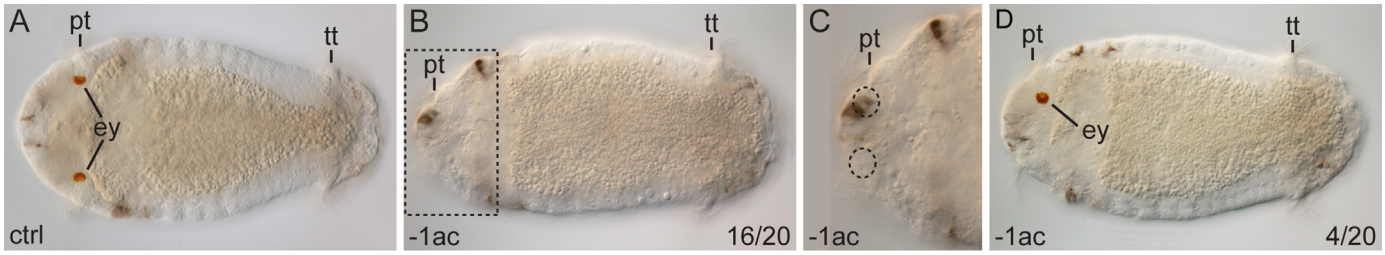

Fig. 5. Larval eye phenotypes resulting from double deletion of 1a and 1c. Stage 6 larvae shown in ventral view with anterior facing left. (A) Unmanipulated control. (B) 80% of larvae are missing both eyes. Boxed area is enlarged in C. (C) Same larva as in B showing enlarged view of head (boxed area in B) with reduced head ectoderm and no eyes. Dotted line circles indicates position of missing eyes. (D) 20% of larvae possess one eye, in this case the left eye. Bottom right in B and D indicate number of cases for result shown. ctrl, control; ey, eye pigment cell; pt, prototroch; tt, telotroch.

proportion of one-eyed phenotypes between $1a^1$ and $1c^1$ is not statistically significant (Fisher's exact test, $p=0.0799$). There is a statistically significant difference in the frequency of one versus two eye spot phenotypes observed between the numbers of cases of larvae resulting from 1b and $1b^1$ deletions (Fisher's exact test, $p=0.0138$), such that the two-eyed phenotype is observed more frequently following $1b^1$ deletion compared with 1b deletion.

3.4. Double deletion of eye-generating micromeres

To determine to what extent regulation can occur following the loss of both eye-generating cells, and whether one or more of the remaining blastomeres regulates for the deletion of these two cells, double deletion of 1a and 1c (denoted as −1ac) was performed and the resulting larvae analyzed. Unmanipulated control larvae are morphologically normal and two-eyed (Fig. 5A). After deletion of 1ac, no eyes are present in 80% ($n=16/20$) of larvae (Fig. 5B–C), and the remaining 20% of larvae possess a single eye ($n=4/20$; Fig. 5D) (Table 1). Among the one-eyed larvae, the left eye is present in 3 out of 4 cases, and in 1 out of 4 cases the position of the eye is undetermined. No larvae have both eyes present following the 1ac double deletion. Deletion of 1ac also results in larvae that are missing a larger part of the head ectoderm when compared to a single first quartet micromere deletion, based on the position of the prototroch relative to the anterior edge of the larva (compare Fig. 5A with 5B–C). These results are consistent with the fate map for *C. teleta*, since 1a and 1c both generate part of the head ectoderm (Meyer and Seaver, 2010).

3.5. B and D quadrants can regulate to form eyes

A substantial proportion of larvae have two eyes following deletion of one eye-generating cell (−1a or −1c), and larvae resulting from double deletion of both eye forming cells (−1ac) can form an eye. We therefore hypothesized that one of the remaining blastomeres can regulate to generate an eye. Transplantation experiments in the mollusc *I. obsoleta* show that 1b has a nearly equal potential to 1a or 1c to generate an eye (Sweet, 1998). To test a similar hypothesis for *C. teleta*, two additional sets of double deletions were performed, −1ab and −1bc. The 1ab deletions result in 96% of larvae ($n=26/27$) that are one-eyed, with a significant portion of the head ectoderm missing (compare Fig. 6A, B). When the left-right position of the eye can be determined, only the right eye remains (Table 1). The number of larvae with one pigment spot following 1ab deletion is significantly different from deleting 1a alone (Fisher's exact test, $p=0.0084$), indicating that regulation occurs more frequently in −1a embryos than in −1ab embryos. In the one remaining larva that possesses two eyes (4%, $n=1/27$), the eyes are adjacent to each other and in a medial position within the head, and it is unclear whether there are two

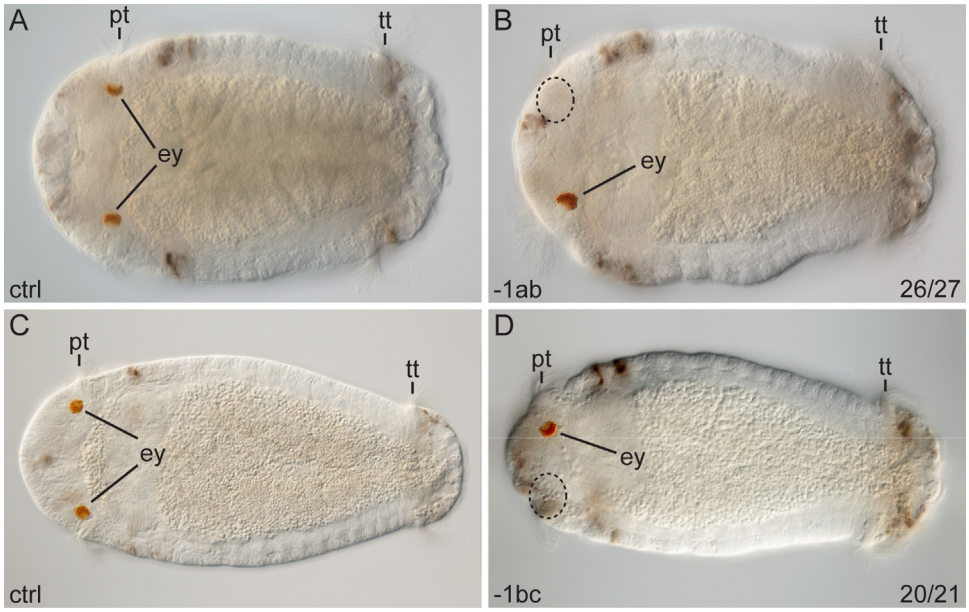

Fig. 6. Larval eye phenotypes resulting from double deletion of 1b plus 1a. Stage 6 larvae are shown in a ventral view with anterior facing left. Dotted line circles indicate position of the missing eye. (A) Unmanipulated control. (B) −1ab double deletion. 96% of resulting larvae are one-eyed. (C) Unmanipulated control. (D) −1bc double deletion. 95% of resulting larvae are missing the right eye. Bottom right in B and D indicate number of cases for result shown in panel ctrl, control; ey, eye pigment cell; pt, prototroch; tt, telotroch.

distinct eyes or one fragmented eye (not shown). Following 1bc deletion, 95% ($n=20/21$) of the resulting larvae are one-eyed and also missing much of the head ectoderm (compare Fig. 6C, D), with the left eye remaining when the eye position can be determined. One larva is missing the right eye (5%) and appears to possess a second left eye, although it is difficult to determine whether the latter is a duplicated eye or a part of one fragmented left eye (not shown). Statistical comparison of the number of cases of one-eyed phenotype resulting from −1c versus −1bc is not significantly different (Fisher's exact test, $p=0.0632$); however, it is still striking that no larvae are observed that clearly possess both the left and right eyes.

The substantial increase in the proportion of a one eyed phenotype when 1b is deleted along with an eye-forming cell relative

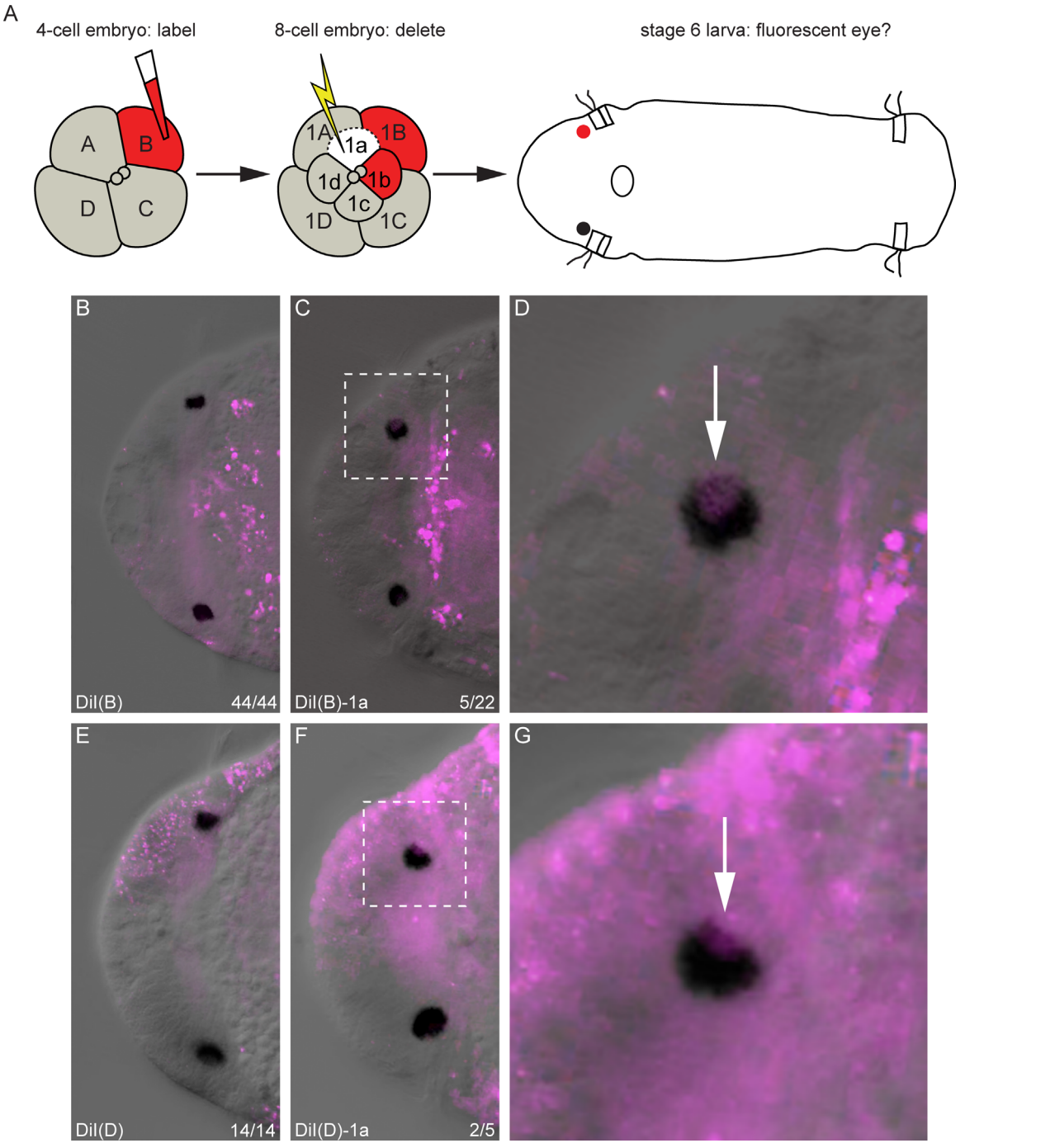

Fig. 7. Regulation by either the B or D quadrant to generate the eye sensory cell. (A) Diagram illustrating experimental design combining lineage tracing and blastomere deletions. The B quadrant was labeled at the 4-cell stage with the lineage tracer DiI. At the next cleavage cycle (8-cell embryo), the 1a blastomere was deleted, and embryos were raised to stage 6 larvae. Two-eyed larvae indicate that a cell in the embryo regulates for the loss of 1a. In B–G, differential interference contrast (DIC) images are overlaid with magenta fluorescent DiI images of larvae in ventral view. (B) Control image of a two-eyed, normal larva resulting from lineage tracing of the B quadrant, DiI(B). The fluorescent clone has a similar pattern to that previously shown by the *C. teleta* fate map for the B quadrant. (C) Of 22 two-eyed larvae, 5 have DiI labeling in the left eye (boxed area). (D) Close-up of the left eye in C. The DiI labeling is nested within the pigment cell, corresponding to the position of the sensory cell of the eye (arrow). (E) Control image of a two-eyed, normal larva resulting from labeling of the D quadrant (DiI(D)). (F) Of 5 two-eyed larvae, 2 have DiI labeling in the left eye (boxed area), indicating that the D quadrant has the potential to regulate for the deletion of 1a. (G) Enlarged view of the left eye in F. There is DiI labeling in a position corresponding with the sensory cell of the eye (arrow). Bottom right of panels B, C, E, F indicate number of cases for result shown in panel.

to deleting an eye-forming cell alone strongly suggests that 1b can regulate to form an eye. To confirm that 1b can regulate to form an eye following 1a or 1c deletion, lineage tracing and laser deletion techniques were combined. The lineage tracer DiI was injected into the parent cell, B, at the 4-cell stage (referred to as DiI(B)), and the 1a blastomere was subsequently deleted in the same embryo at the 8-cell stage (referred to as DiI(B)-1a). If 1b can regulate, it is expected that the left eye should be fluorescent in the two-eyed larvae resulting from DiI(B)-1a (Fig. 7A). In all cases ($n=44/44$), the control larvae resulting from DiI(B) injections are fluorescent in the right ventral head ectoderm and prototroch (Fig. 7B), anterior mouth, endoderm, and other structures consistent with the B quadrant clone in the *C. teleta* fate map, but are not fluorescent in the eyes (Meyer et al., 2010). Two-eyed larvae were obtained from DiI(B)-1a, of which only 23% ($n=5/22$) clearly had DiI within the sensory cell of the left eye (Fig. 7C, D). DiI labeling abuts the pigment cell along its lateral edge (Fig. 7D, arrow, see Fig. 2C for reference), in a position that directly corresponds with the position of the sensory cell of the eye (Rhode, 1993). In the remaining 17 two-eyed larvae, the left eye was not fluorescent (not shown). These data demonstrate that the B quadrant is regulating to form the eye following 1a deletion, and suggests that in some cases a cell from another quadrant regulates to form an eye. Regardless of whether the left eye is fluorescent in DiI(B)-1a larvae, some additional differences are observed in the normal fate of the B quadrant following 1a deletion. For example, the position of the labeled head ectoderm and prototroch shift to the left so that the clone is ventral and medial rather than ventral and right (compare Fig. 7B, C), possibly due to spreading of epidermal tissue into the domain of the missing tissue.

Because the B quadrant appears to generate an eye in only a fraction of cases, we also tested the eye-forming ability of the D quadrant, which normally does not give rise to an eye (Meyer et al., 2010). The D blastomere was injected with DiI at the 4-cell stage (DiI(D)), and 1a was deleted at the 8-cell stage (DiI(D)-1a) in these same embryos. Control DiI(D) larvae reflect the normal D quadrant clone, including the left dorsal head ectoderm ($n=14/14$; Fig. 7E), majority of the trunk ectoderm (not shown), but the eyes are not fluorescent in any case. Five two-eyed larvae were recovered following the DiI(D)-1a experiment. In two of the five two-eyed larvae, there is fluorescence in a position corresponding with the position of the sensory cell of the eye (Fig. 7F, G, arrow, compare with Fig. 2C), This indicates that in some instances the D quadrant regulates for the deletion of 1a. In the DiI(D)-1a larvae, the position of the trunk ectoderm, left head, and other structures generated by the D quadrant remain quite consistent with controls. From the combined lineage tracing and blastomere deletion experiments, we demonstrate the potential of both B and D quadrants—which normally do not generate eyes—to regulate and generate at least the sensory cell of the eye following deletion of the 1a micromere.

3.6. Embryos lacking the first quartet of micromeres do not form eyes

To investigate the potential of additional blastomeres to form an eye, particularly the second, third and fourth quartet micromeres, we deleted all four first quartet micromeres in 8 cell stage embryos (Fig. 8A). In larvae resulting from these deletions, 92% ($n=45/49$) have no eye pigment (Fig. 8C). These larvae are also missing most head structures, including the prototroch and brain, and the mouth exits along the anterior edge of the larva, likely due to loss of more anterior structures (compare Fig. 8D with E). These defects are expected since the four first quartet micromeres generate nearly all tissues of the head. Of the remaining cases resulting from −1abcd, three have two eye pigment spots (6%, $n=3/49$) and one has one eye spot (2%, $n=1/49$). The number of cases of no-eyed larvae following deletion of −1abcd is not significantly different from the number of cases expected if the first quartet of cells are the only cells in the embryo with the potential to generate an eye (X^2, $p > 0.05$). These results, in combination with our results demonstrating that both the D and B quadrant can generate an eye following deletion of 1a, provide evidence that all and only the first quartet of micromeres have eye-forming potential.

4. Discussion

4.1. Regulation following deletion of 1a and 1c micromeres in spiralian embryos

C. teleta is an example of an unequal-cleaving spiralian species in which early embryonic cleavages segregate the cytoplasm of the egg asymmetrically at the first two divisions through formation of a polar lobe. Most of the larvae resulting from deletion of 1a or 1c have only one eye pigment cell and one eye sensory cell, consistent with the fate map of these cells (Meyer et al., 2010). $1a^1$ or $1c^1$ deletions also show similar proportions of larvae with one eye spot, as expected from the fates of these cells (Meyer et al., 2010).

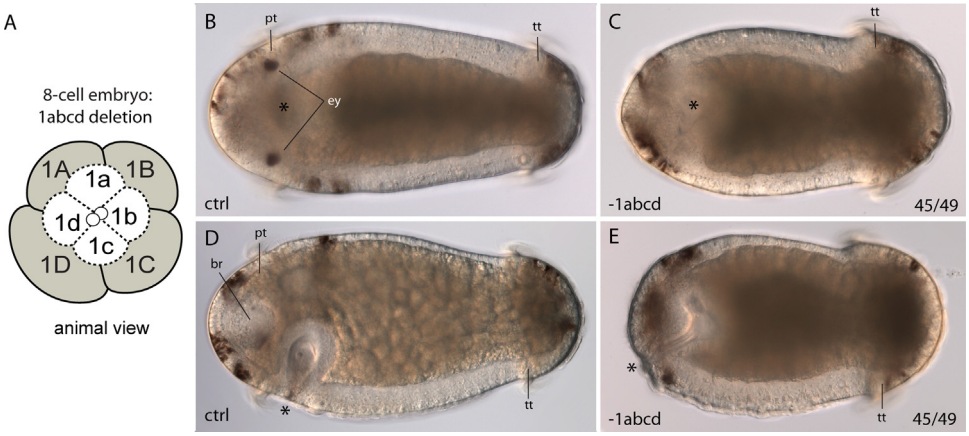

Fig. 8. −1abcd deletion results in larvae lacking eye spots. (A) Diagram illustrating experimental design in which all four first quartet micromeres are deleted in the 8 cell stage embryo (white). (B–E) Larvae are imaged live. (B) Control larva in ventral view. (C) Larva resulting from deletion of −1abcd in a ventral view. The resulting larva is missing the prototroch and both eye pigment cells ($n=45/49$). (D) Control larva imaged in lateral view. (E) Larva from −1abcd deletion shown in lateral view. The resulting larva is missing the prototroch and brain (45/49). Note the position of the mouth. ctrl, control; ey, eye pigment cell; pt, prototroch; tt, telotroch; Asterisk marks position of the mouth in D and E.

However, for all four of these deletions, both larval eye spots do develop in a significant number of cases, suggesting that another blastomere within the embryo regulates when one of these cells is missing. Following the double deletion of both eye-generating blastomeres 1a and 1c (−1ac), the only observed phenotypes are no-eyed (80%, $n=16/20$) or one-eyed (20%, $n=4/20$) larvae. In no case is a two-eyed larva observed, suggesting that only one of the remaining blastomeres has the potential to generate an eye. Three of the four one-eyed larvae observed following the double deletion −1ac generated the left eye, while in the remaining case it is unclear which eye is present. These data are consistent with the idea that the compensating cell may have greater potential to generate the left eye.

When individual first quartet micromeres are deleted in other spiralian embryos, a range of percentages of one-eyed larvae is observed. In *I. obsoleta*, which forms a large polar lobe, no regulation occurs following 1a or 1c deletion, and all veliger larvae are one-eyed (Clement, 1967). Interestingly, regulation occurs in half embryos of *I. obsoleta* generated by double deletion of the macromeres A and C at the four-cell stage, and the right eye is more commonly observed in the veliger larvae, and two eyes are observed in 10% of the total resulting larvae (McCain and Cather, 1989). In contrast, two-eyed larvae occur in up to 40% of veligers following deletion of 1a or 1c in another unequal-cleaving gastropod, *Bithynia tentaculata* (van Dam and Verdonk, 1982). In equal-cleaving spiralians, there is also evidence for regulation. Although the four quadrants are not yet distinguishable at the 8-cell stage, it is possible to determine whether a first quartet blastomere is 1a or 1c versus 1b or 1d. 1a and 1c meet along a furrow at the animal pole and together are identified as the lateral first quartet micromeres. Both eyes are often observed following deletion of a single lateral first quartet micromere (−1a/c) at the 8-cell stage in the gastropod *L. stagnalis* (Arnolds et al., 1983), and nearly 65% of larvae form both eyes in the turbellarian flatworm *Hoploplana inquilina* (Boyer, 1987). On the other hand, regulation of eye formation does not occur after single-cell deletions at the 4-cell stage of the direct-developing nemertean *Nemertopsis bivittata* (Martindale and Henry, 1995). Thus, there are a number of documented examples of regulation for loss of 1a and 1c in spiralian embryos, the degree of regulation is variable across species, and regulative ability does not appear to be correlated with the presence of asymmetric divisions at the first two cleavages.

4.2. Regulation following deletion of $1a^1$ and $1c^1$ micromeres in C. teleta

Following deletion of individual first quartet micromeres, the resulting larvae lost either the left eye (−1a) or the right eye (−1c) in the majority of cases. Deletion of $1a^1$ or $1c^1$ also results in the loss of the corresponding larval eye spot in most cases. We provide evidence that the embryo can regulate for the loss of both $1a^1$ and $1c^1$, since in 23% ($n=10/44$) and 9% ($n=3/34$) of cases, respectively, the resulting larvae have two pigment spots. The proportion of cases with two eye spots is not statistically different between deletions of $1a^1$ and the parent micromere 1a, or the deletion of $1c^1$ and its parent micromere 1c. Thus, it appears that the ability of the embryo to regulate for loss of an eye-generating cell persists for an additional cell cycle.

4.3. 1a and 1c micromeres generate different head sensory cells

Using the 22C10 mAb to visualize the sensory cell of the larval eye, we found that deletion of either the 1a or 1c micromere results in loss of both the pigment and larval sensory cell, demonstrating that both cells originate from the same blastomere in the early stage embryo. In the head of these larvae, there are either two or three remaining $22C10^+$ neurons, compared with four $22C10^+$ neurons in unmanipulated larvae. Interestingly, the proportion of larvae with either two or three $22C10^+$ neurons differs between the 1a and 1c micromere deletions, and this difference may be explained by differences in the embryonic origins of the $22C10^+$ cells. The fact that most larvae resulting from 1c micromere deletions lack the two $22C10^+$ neurons on the deleted side is consistent with the idea that 1c generates both the anterior and posterior $22C10^+$ neurons on the right side of the larval head. In contrast, most larvae resulting from 1a deletions have three $22C10^+$ neurons, suggesting that 1a generates only the larval sensory neuron, and that the anterior $22C10^+$ cell originates from a different blastomere. Given that there are four $22C10^+$ neurons in larvae resulting from 1b deletions, and all head tissues arise from the four blastomeres 1a, 1b, 1c and 1d (Meyer et al., 2010), it is most likely that the anterior $22C10^+$ neuron on the right side of the head is generated by the 1d micromere. Previous fate mapping studies using lineage tracers have demonstrated that 1a and 1c do not generate mirror descendant clones, and our findings add another difference between 1a and 1c fates. Furthermore, since 1c generates a substantial portion of the right brain lobe, the 1d micromere gives rise to the majority of the left brain lobe, and 1a makes only a small contribution to the brain, generation of the anterior $22C10^+$ neuron by the 1c micromere is consistent with it having a greater neurogenic potential. The fact that the anterior and posterior $22C10^+$ neurons on the left side of the head likely originate from different first quartet micromeres represents an example of an uncoupling of the larval versus adult developmental origins of components of the same structure, the eye, and has implications for life history evolution studies.

4.4. Multiple cells can regulate to form eyes

Double deletions in *C. teleta* revealed the importance of 1b in the ability of the embryo to regulate for loss of an eye. Following 1ab deletion, 96% ($n=26/27$) of the resulting larvae have one eye, compared with 73% ($n=76/104$) following single deletion of 1a. In the one case in which the −1ab larva has two eyes, the eyes are medial and very close together, and may be an example of what previous authors observe to be a “fragmented” eye (Martindale and Henry, 1995). The difference in the one-eyed phenotype between 1a and 1ab deletions is statistically significant, supporting the idea that the double deletion eliminates a regulating cell, 1b. Although the difference in the production of one-eyed larvae following 1bc or 1c deletion was not statistically significant (95% (n=20/21) compared with 75% ($n=56/75$), respectively), all but one of the larvae resulting from −1bc are one-eyed, providing additional evidence that 1b may be able to regulate for loss of eyes. These results observed in *C. teleta* contrast with the results obtained from equal-cleaving embryos. When two adjacent first quartet micromeres are deleted (one eye-forming plus one non-eye-forming blastomere) in the gastropod *L. palustris*, regulation occurs such that all but one of the embryos that survive past gastrulation develop into normal adult snails with two eyes (Morrill et al., 1973). In *H. inquilina*, double deletion of two adjacent first quartet micromeres results in 22% of larvae with two or more eyes (Boyer, 1989). Whereas regulation occurs following double deletion of adjacent micromeres in some other spiralian species, regulation is minimal in *C. teleta* when 1b is deleted along with an eye-forming cell, and suggests that the presence of 1b is required for regulation of eyes following 1a or 1c deletion. One caveat to this comparison is that because of the inability to uniquely identify first quartet micromeres at the 8-cell stage in equal cleaving species, one cannot confirm that adjacent micromere deletions included deletion of 1b.

To further examine the identity of the regulating cell(s), we performed additional deletion experiments, and also took

advantage of the ability to perform combined lineage tracing and laser deletion in *C. teleta*. Of the two-eyed larvae resulting from labeling the B macromere and then deleting 1a (DiI(B)-1a), 23% ($n=5/22$) of the two-eyed larvae have a fluorescent sensory cell. Taken together with our demonstration that both the pigment and sensory cell originate from the same blastomere, and only first quartet micromeres appear to have eye-forming potential, this result provides evidence that the 1b blastomere has the potential to generate an eye. We also labeled the D macromere and then deleted 1a (DiI(D)-1a) to test whether the other non-eye-generating quadrant can regulate to form an eye. These results demonstrate that the D quadrant can give rise to an eye following 1a deletion in 40% of cases (2/5). From these experiments, only 63% of two-eyed larvae have a fluorescent eye. It is possible that descendant clones from the B and D macromeres were not always bright enough to detect a fluorescent sensory cell, leading to an underestimate in the number of cases in which the sensory cell was generated by either the B or D quadrant. Alternatively, the second eye may have been generated from a different blastomere, namely 1c. Taken together, all four quadrants in *C. teleta* have the potential to generate an eye, and when 1a is deleted, either of the non-eye-forming quadrants, B and D quadrants can regulate to form an eye.

In larvae resulting from deletion of all four first quartet micromeres, 92% ($n=45/49$) have no eye pigment. These results provide evidence that only the first quartet of micromeres have eye-forming potential in *C. teleta*. Combined with our results demonstrating that both the B and D quadrant can generate an eye, we suggest that 1b and 1d regulate to form an eye following the deletion of 1a. Similar to our results in *C. teleta*, when all four micromeres are deleted at the eight cell stage in *Ilyanassa* and in *Bithynia*, eyes do not form (van Dam and Verdonk, 1982). Thus, it appears that eye forming potential is restricted to first quartet micromeres in multiple spiralians, with a notable exception in the chiton *Chaetopleura apiculata*, in which the larval eyes are generated by the second quartet micromeres 2a and 2c (Henry et al., 2004).

4.5. Fate specification and eye-forming potential

The development of eyes in *C. teleta* and other spiralians appears to depend upon a combination of segregation of determinants as well as the presence of an inductive signal. The segregation of determinants is reflected in the early restriction of cell fate potential to the first quartet micromeres in the 8-cell stage embryo. In addition, an inductive signal, which induces eye development in cells that have eye-forming potential and are competent to receive the signal, is playing a role in the irreversible determination of cell fates. In examined spiralians, an “organizer” cell, usually the 3D or 4d blastomere, is responsible for inducing the fates of the first quartet micromeres of the embryo, such as the eyes, in *I. obsoleta*, *Crepidula fornicata*, and *L. stagnalis* (Clement, 1962; Henry et al., 2006; Martindale, 1986). Single cell deletions in *C. teleta* have also demonstrated the presence of organizing activity from the D quadrant (Amiel et al., 2013). Specifically, when the somatoblast 2d is deleted, the resulting larvae lack eyes, consistent with data from molluscs demonstrating the necessity of inductive signaling for the formation of eyes.

When the 1a or 1c micromere is deleted in *C. teleta*, it is possible that the 1b or 1d micromere shifts toward the original position of the 1a or 1c micromere. When an inductive signal is sent from the D quadrant in *C. teleta*, the translocated 1b or 1d micromere may receive a signal and generate an eye. Shifts in micromere position may also explain the proportion of one-eyed larvae resulting from 1b deletions or three-eyed larvae following deletion of 1d. In the case of 1b deletions, 1a or 1c may move *away* from the D quadrant and the inductive signal, near or into the position of the original position of the 1b micromere, and thus not receive sufficient signal to induce formation of an eye. It is interesting to note that following $1b^1$ deletions, 97% ($n=38/39$) of resulting larvae have two eye spots, suggesting that one cell division later, $1a^1$ or $1c^1$ do not have the same ability to move out of the range of an inductive signal. In the case of 1d deletions, if there is a shift in position of the remaining first quartet micromeres towards an inducer derived from the D quadrant, then 1b would receive a stronger signal than normal, and produce a third eye. Shifting of micromeres following cell deletions has been observed in *L. stagnalis*, and the authors suggest that this physical movement of cells affects quadrant fate in this equal-cleaving mollusc (Arnolds et al., 1983).

Our results from the annelid *C. teleta* largely fit the model proposed by Sweet (1998) to explain first quartet micromere specification in the mollusc *I. obsoleta*. This model proposes that all of the first quartet cells have eye forming potential, although 1d has less eye forming potential compared with 1a, 1b and 1c. One important feature of the model is that cell position is critical, and that distance from the inducing D quadrant influences the ability to generate eyes. When the 1b blastomere, which is located opposite from the D quadrant and normally does not generate an eye in *I. obsoleta* (Render, 1991), is transplanted into the normal position of 1a, it generates an eye at a statistically similar rate to a homotopic control transplant (Sweet, 1998). The lack of eye development in 1d descendants in *I. obsoleta* is explained by inheritance of inhibitory factors from the polar lobe material, and therefore 1d does not respond even though it is within the range of the inductive signal. One important feature of this model that does not fit the data from *C. teleta* is the very limited eye forming potential by 1d. The frequency at which eye regulation occurs from 1d at the 8-cell stage is substantially greater in *C. teleta* compared with that in *I. obsoleta*, suggesting that in *C. teleta*, 1d may not inherit inhibitors of eye formation.

Cell deletion experiments in *I. obsoleta* show that both cell-contact dependent inhibition and inheritance of polar lobe material can prevent eye development by the 1d cell even though inductive signaling might occur later (Goulding, 2003; Sweet, 1998). In *I. obsoleta*, removal of the A and C quadrants at different time points during the 4-cell stage demonstrates that these two cells can ultimately suppress the 1d micromere from generating an eye through their contact with the D cell (Goulding, 2003). In addition, isolation of the first quartet micromeres in *H. inquilina* results in abnormal larvae of which 76% have more than two eyes, suggesting that a signal inhibiting eye formation is coming from the macromeres (Boyer, 1989). We hypothesize that inhibitory signaling through cell–cell contacts rather than inheritance of inhibitory factors may play a role in preventing 1d from normally forming an eye in *C. teleta*.

5. Conclusions

Our experiments investigating cell fate specification and regulative ability in the polychaete *C. teleta* show that there are similarities with some other spiralians in that regulation can occur following deletion of a blastomere at the 8-cell stage. We demonstrate that all four quadrants have eye-forming potential, and the first quartet micromeres appear to be the only blastomeres that have the ability to form eyes. Either the 1b or the 1d blastomere may be able to shift its position so that it occupies the position previously held by 1a, and subsequently adopts the 1a fate. Therefore, eye forming potential is initially inherited by more than two cells in the first quartet, and our results are consistent with the idea that both cell position and signaling are important

for normal development of the first quartet derivatives in embryos undergoing spiral cleavage.

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