CHAPTER

13

The planarian *Schmidtea mediterranea* as a model for studying motile cilia and multiciliated cells

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CHAPTER OUTLINE

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Abstract

In the past few years, the freshwater planarian *Schmidtea mediterranea* has emerged as a powerful model system to study the assembly and function of cilia. *S. mediterranea* is a free-living flatworm that uses the beating of cilia on its ventral epidermis for locomotion. The ventral epidermis is composed of a single layer of multiciliated cells highly similar to the multiciliated cells that line the airway, the brain ventricles, and the oviducts in humans. The genome of *S. mediterranea* has been sequenced and efficient methods for targeting gene expression by RNA interference (RNAi) are available. Locomotion defects induced by perturbing the expression of ciliary genes can be often detected by simple visual screening, and more subtle defects can be detected by measuring locomotion speed. Cilia are present in large numbers and are directly accessible, which facilitates analyses by immunofluorescence and electron microscopy. Here we describe a set of methods for maintaining planarians in the lab. These include gene knockout by RNAi, cilia visualization by immunofluorescence, transmission electron microscopy, and live imaging.

INTRODUCTION

Planarians are free-living flatworms best known for their amazing ability to regenerate whole animals from minuscule body fragments. The regeneration abilities of planarians were first reported 250 years ago but only in the last 15 years these animals were reestablished as a model for studying regeneration and stem cells thanks to the development of molecular tools (Newmark & Sánchez Alvarado, 2002). Another very interesting characteristic of planarian flatworms is that their locomotion depends on a ventral multiciliated epidermis similar to tissues lining the airway, the brain ventricles, and the oviducts in humans (Figure 1(A)−(C),(G)) (Azimzadeh, Wong, Downhour, Sánchez Alvarado, & Marshall, 2012; Rink, Gurley, Elliott, & Sánchez Alvarado, 2009; Rompolas, Patel-King, & King, 2010; Sánchez Alvarado & Newmark, 1999). The planarian ventral epidermis is monostratified with multiciliated, columnar cells directly apposed to a basement lamina. Each cell assembles approximately 80 motile cilia, which beat within a plane parallel to the long axis of the animal with an effective stroke directed toward the posterior end (Azimzadeh et al., 2012). The cilia beat sequentially along this axis, forming wave-like patterns called metachronal waves as seen in vertebrate multiciliated epithelia (Krugelis McRae, 1967; Rompolas et al., 2010). In addition to the ventral surface, multiciliated cells are found at the surface of the pharynx, the feeding organ of planarians (Figures 1(D)−(F),(J) and 2(A,B)), and within protonephridia, where they are called flame cells (Azimzadeh et al., 2012; Rink, Vu, & Sánchez Alvarado, 2011). Planarians also form ciliated sensory neurons involved in chemotaxis and likely also mechanosensation (Krugelis McRae, 1967). In contrast to sensory neurons in *Drosophila* and *Caenorhabditis elegans*, which are immotile and lack a central pair of microtubules (9 + 0 axoneme), planarian sensory cilia display a central pair of microtubules (9 + 2 axoneme) (Krugelis McRae, 1967). Whether planarian sensory cilia are indeed

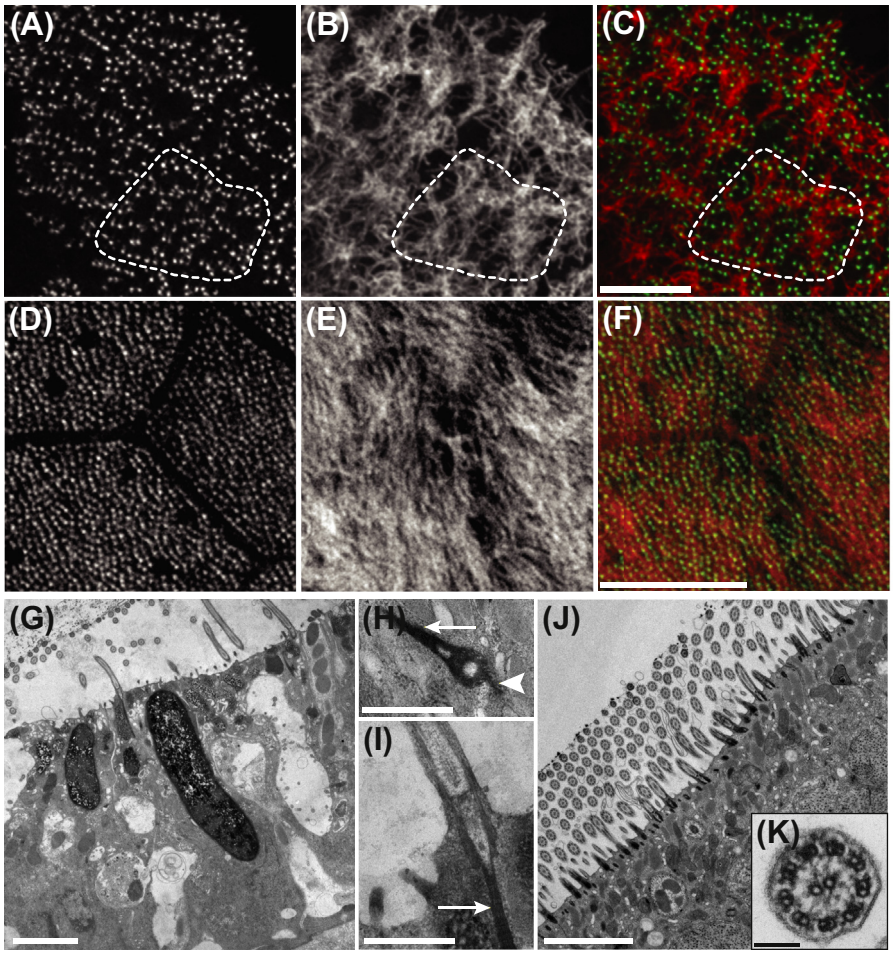

FIGURE 1 Motile cilia are present at the surface of multiciliated cells in the planarian epidermis.

(A–F) Immunofluorescence staining of ventral (A–C) and pharyngeal epidermises (D–F) after methanol fixation. The tissues were labeled with antibodies that revealed centrioles anti-SMED-CEP135 (left panel, in green in the merged panel) (A, D) and cilia antiacetylated tubulin (middle panel in red in the merged panel) (B, E) and merge images (C, F). The dashed line in (A–C) delineates the boundary of one cell. (G–K) Transmission electron microscopy micrographs showing the ventral (G–I) or pharyngeal (J, K) epidermis. (H, I) High magnification view of centrioles seen in cross-section (H) or longitudinal section (I). Arrows points to striated ciliary rootlets and arrowhead to the basal foot. (K) Axoneme of a motile cilium seen in cross-section. Bar is 10 μm in (C) and (F), 2 μm in (G) and (J), 0.5 μm in (H) and (I), 0.1 μm in (K). (See color plate)

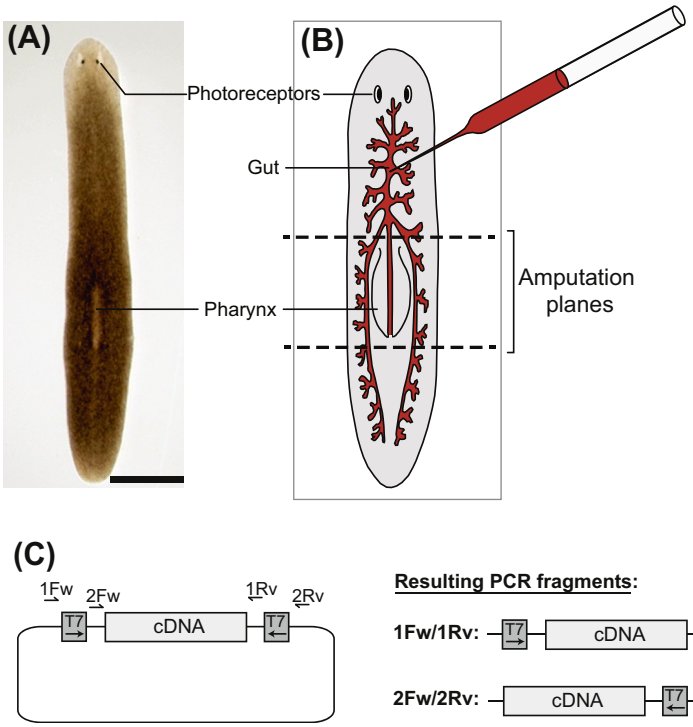

FIGURE 2 RNA interference (RNAi) in planarians.

(A) Top view of a *S. mediterranea* individual of the asexual strain. The photoreceptors and the region of the pharynx are indicated. Bar is 2 mm. (B) Schematic representation showing the approximate position of the gut and where to place the injection tip for microinjection of double-strand RNAs (dsRNA) solution (in red (gray in print versions)) (Section 2.2.2, Step 9). Dashed lines represent the planes where amputations are performed following RNAi (Section 2.2.1, Step 19, Section 2.2.2, Step 12) or for colony expansion (Section 1.4, Step 5). (C) Schematic representation of dsRNA expression vector used for RNAi by bacterial feeding (Section 2.1.1, item 1) or microinjection (Section 2.2.1, item 1). Two T7 promoters in opposite orientation are found at each side of the cloning site containing the target gene cDNA. The position of the two pairs of primers (1Fw/1Rv and 2Fw/2Rv) used for generating the DNA templates required for in vitro synthesis of dsRNA (Section 2.2.1, item 2), and the resulting PCR fragments are shown.

motile and the biological significance of this structural characteristic remains unknown, however. At the ultrastructural level, planarian multiciliated cells appear similar to their vertebrate counterparts. Centrioles (or basal bodies) at the base of each cilium possess a basal foot pointing in the direction of the effective stroke and a striated ciliary rootlet pointing in the opposite direction (Figure 1(H)) (Park, Mitchell, Abitua, Kintner, & Wallingford, 2008). A second ciliary rootlet oriented perpendicular to the apical surface is also present (Figure 1(I)).

The model planarian flatworm *Schmidtea mediterranea* (Figure 2(A)) is a freshwater, nonparasitic species originating from southern Europe and Northern Africa. *Schmidtea mediterranea* has two distinct strains, a sexual and an asexual strain. Sexual *S. mediterranea* are cross-fertilizing hermaphrodites that can reach up to 2 cm in length. They lay large egg capsules containing several embryos that produce hatchlings resembling adult animals (Newmark & Sánchez Alvarado, 2002). The genome of the sexual strain, which is diploid (2N = 8), has been entirely sequenced and Blast searches of target genes are available through the *S. mediterranea* Genome Database Web site (Robb, Ross, & Sánchez Alvarado, 2008) (Smed DB: http://smedgd.neuro.utah.edu/). The asexual strain is a naturally occurring strain that results from a chromosome translocation between chromosomes 1 and 3. Individuals of the asexual strain are smaller, reaching only up to 1.3 cm in length. Asexuals never form gonads and reproduce by transverse fission in posterior two-third of the animal. Following fission, each fragment regenerates the missing tissues, producing two well-proportioned animals of different sizes. The whole regeneration process takes 10−15 days. The genome sequence of the asexual strain is not yet available but comparison between sexual and asexual transcriptomes reveals that the two strains are mostly identical, despite the presence of polymorphism and differences in the level of expression of some genes (Lázaro et al., 2011; Resch, Palakodeti, Lu, Horowitz, & Graveley, 2012).

Gene inactivation by RNA interference (RNAi) works very efficiently in *S. mediterranea* and has been used to screen up to 1065 genes at a time (Reddien, Bermange, Murfitt, Jennings, & Sánchez Alvarado, 2005). RNAi can be performed either by feeding or microinjection of long double-strand RNAs (dsRNA) (Newmark, Reddien, Cebria, & Sánchez Alvarado, 2003; Sánchez Alvarado & Newmark, 1999). In addition, both ventral and pharyngeal epidermis are easily accessible and form cilia at high density and in known orientation, which greatly facilitates analyses by immunofluorescence, electron microscopy as well as live imaging (Azimzadeh et al., 2012; Rompolas, Azimzadeh, Marshall, & King, 2012; Rompolas et al., 2010).

In the present chapter, we describe protocols for establishing and maintaining a planarian colony in the lab, as well as for gene inactivation by RNAi, preparation of samples for whole-mount immunolocalization, and transmission electron microscopy (TEM) and live imaging.

1. MAINTAINING PLANARIANS IN THE LAB

Planarians are usually found in ponds and rivers. They are carnivores and exhibit a negative phototaxis behavior, i.e., they tend to move away from light sources. In the lab, planarians are fed on calf liver and are kept in the dark. Here, we describe how to establish and maintain a colony of the asexual strain of *S. mediterranea*. The reason we favor the asexual strain for studying cilia is that individuals are smaller, which is an advantage for most of the protocols detailed below. These animals are available as

clonal populations derived from single individuals and can be obtained from most current planarian laboratories.

1.1 MATERIALS

1. **Planarian medium:** (1.6 mM NaCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.1 mM $MgCl_2$, 0.1 mM KCl, 1.2 mM $NaHCO_3$). This medium corresponds to a 1X solution of Montjuïc salts prepared in Milli-Q water as described earlier (Cebrià & Newmark, 2005). We prepare a large batch of 4X stock of planarian medium using Milli-Q water. The pH of the 4X solution must be adjusted to 7.2 using 2 N HCl. Note that the planarian medium described by Cebria and Newmark is adjusted to pH 7.0 but we consistently found that the flatworms seem to prefer pH 7.2. When accidentally exposed to pH < 6.5, the animals will crawl off the water, which can lead to rapid dehydration and death. When this behavior is observed, check the pH of the 4X stock of planarian medium and adjust to 7.2 if needed. Prepare a new 1X dilution and immediately use to replace the medium in the flatworm containers. The 4X stock solution can be autoclaved and kept for several months at 4 °C. Slight precipitation of the stock solution is normal.
2. **Plastic containers:** Use plastic, food grade containers such as Tupperware boxes to house planarians. Wide containers with a flat bottom and removable lid are ideal. Do not close the lids to allow for oxygenation of the medium. Adapt container size to the number of individuals. For instance, we use $12 \times 12 \times 5$ cm (L, W, H) containers with 300 mL planarian medium to host 50−100 individuals and $25 \times 15 \times 8$ cm (L, W, H) containers with 1.5 L planarian medium to host up to 500 individuals. Prior to use, the containers must be thoroughly rinsed with deionized water. We do not use detergent, as detergent residues can be toxic to planarians.
3. **Calf liver homogenate:** Prepare from fresh, good quality organic calf liver. One ∼200 g slice of liver is usually sufficient to feed four $25 \times 15 \times 8$ cm (L, W, H) containers (∼2000 individuals total), during several months. The slice of liver is trimmed of all major vessels, fat, and connective tissue and homogenizing in a blender at 4 °C. Blend until an homogeneous paste is obtained but avoid introducing air bubbles, otherwise the liver homogenate will float and feeding (see below) will be uneasy. The homogenate can be aliquoted into 35 mm petri dishes and stored at −80 °C for up to 6 months.
4. Transfer pipettes (e.g., Fisher Scientific Cat. No. 13439108).
5. Autoclavable 10 L carboy with spigot (e.g., Thermo Scientific Nalgene Cat. No. 2319-0020).
6. Disposable scalpels (e.g., Swann-Morton No. 22, product code 6608).

1.2 CULTURE CONDITIONS

1. Fill approximately half of the volume of a plastic container with planarian medium.

2. Transfer planarians from the shipping tube or flask to the container using a plastic transfer pipette. Planarians are usually either attaching solid substrates or gliding along them. To detach the flatworms, use the transfer pipette to squirt some medium on them and then aspirate within the pipette. When exposed to flow or movement, planarians tend to round up their bodies and individuals of up to 1.5 mm can then go through an opening of approximately 2 mm diameter without injury
3. Put the lid of the container back **without closing it completely** to allow for gas exchange.
4. Keep the planarians in the dark at 18−20 °C, ideally in a temperature-controlled incubator with sufficient airflow. A cabinet in a temperature-controlled room is a possible alternative.

1.3 FEEDING AND CLEANING

Once a colony of a sufficient number of individuals is established (we maintain a colony of ~1500−2000 individuals in four 25 × 15 × 8 cm containers), planarians are fed once a week. However, planarians can support starvation during several weeks through their ability to "degrow," i.e., to decrease their overall size (Newmark & Sánchez Alvarado, 2002). Conversely, feeding more frequently allows increasing the size of planarians, which combined with amputation can be used to expand the colony (Section 1.4). Cleaning following feeding is imperative to prevent contamination of the planarians' environment.

1. Thaw an aliquot of calf liver homogenate until it reaches room temperature.
2. Using a clean spatula, drop a few chunks of liver homogenate into the container. Do not allow the spatula to contact the medium to avoid spreading possible contaminants. Make sure the liver chunks sink to the bottom where flatworms can easily access it. If the liver chunks float due to trapped air bubbles, use a clean transfer pipette to bring it to the bottom.
3. Planarians normally react very quickly to the presence of food. It usually takes less than 15 min to have most of the individuals gathering around the liver chunks. Failure to observe this behavior is a sign of stress and should be treated appropriately (Step 10).
4. Allow planarians to feed for at least 1H and up to 2H. Individuals that are done eating swim away from the food and usually appear lighter in color because of the pale pinkish color of the ingested food.
5. Remove uneaten liver homogenate with a transfer pipette and then carefully pour out the medium. Planarians usually stay attached to the container and will not come off but in case they do, gently squirt some medium to bring them down to the bottom of the container.
6. Tilt the container and flush the planarians in one corner with fresh medium. We use planarian medium straight out of the polypropylene carboy by placing the container with the planarians directly under the spigot. Alternatively, use a

squeeze bottle filled with planarian medium or a transfer pipette. Leave enough medium to submerge the planarians. Clean the mucus and food leftovers from the bottom and sides of the container using paper wipes.

7. Flush the planarians to the opposite corner using fresh medium to finish wiping the container.
8. Pour out the medium and refill the container with fresh planarian medium.
9. Clean the containers after every feeding or at least once a week.
10. Watch for changes in behavior and culture conditions that can be due to contamination or toxicity of the medium. If planarians present signs of stress such as firmer attachment to the substrate, increased mucus secretion, loss of appetite, body lesions, lysing animals, or unpleasant smell, quarantine the corresponding container. Split into more containers if the population density is high and change the medium frequently. Add 50 μg/mL gentamicin in the medium to prevent bacterial infection. Avoid feeding as food by-products contribute to spoiling the medium and stressed planarians usually lack appetite. Resume feeding after approximately a week or when the population starts recovering, as this will accelerate recovery. Toxicity of the planarian medium (due to abnormal pH in particular) will often result in planarians crawling out of the water. In this case, quickly replace using a new batch of medium.

1.4 COLONY EXPANSION

The asexual strain of *S. mediterranea* naturally propagates by fission. Fission occurs posteriorly to the pharynx, the feeding organ of planarians, to generate a larger anterior fragment and a smaller posterior fragment. Both fragments will eventually regenerate the missing tissues and organs to produce two whole animals. Spontaneous fission frequency depends on a range of factors, in particular food availability (larger worms are more likely to undergo fission) and planarian density (crowding negatively regulates fission). Increasing feeding frequency and splitting the planarians into additional containers can thus allow increasing the number of individuals. A faster way to expand the colony is to perform manual amputation, as each fragment generated by amputation will eventually regenerate a whole animal.

1. Feed planarians every other day during a week.
2. Stop the feeding 2 days before amputation to allow time for full digestion.
3. Soak a piece of Whatman paper with planarian medium and place to the bottom of a petri dish.
4. Using a plastic transfer pipette, pick about 10 large individuals (0.8 to 1.3-cm long for the asexual strain) and place on the piece of Whatman paper. Decreasing the temperature helps immobilizing planarians in a stretched-out posture, which greatly facilitates amputation. To do so, place the petri dish on ice during the time required for amputation. Make sure to minimize exposure to cold temperature as this can be detrimental to *S. mediterranea.*

5. Using a disposable scalpel, crosscut the planarians anteriorly and posteriorly to the pharynx to generate three fragments (Figure 2(B)). Perform clean cuts as this greatly facilitates wound healing and viability of the amputated fragments.
6. Transfer the fragments into a separate container by squirting fresh medium from a transfer pipette or a squeeze bottle.
7. Repeat Steps 4–6 as required.
8. Feed every other day during 1 week starting 1 week after amputation and perform additional rounds of amputation when the flatworms are ~1-cm long.

2. RNA INTERFERENCE

Two alternative protocols, involving either bacterial feeding (Newmark et al., 2003) or injection for the delivery of dsRNAs (Sánchez Alvarado & Newmark, 1999) are available. Bacterial feeding is easier and thus best adapted when targeting large number of genes. Injection of dsRNA requires additional equipment and is more time-consuming. However, dsRNA injection often leads to better RNAi efficiency so it should be considered for target genes that are not well inactivated by bacterial feeding.

2.1 RNAi VIA BACTERIAL FEEDING

2.1.1 Materials

1. **Cloning vector for dsRNA expression:** Full-length cDNAs or cDNA fragments corresponding to target genes are cloned between two opposing T7 promoters in a vector designed for bidirectional transcription by bacteriophage T7 RNA polymerase (Figure 2(C)). We use pPR-T4P (Liu et al., 2013), which possesses a site for ligation-independent cloning (Aslanidis & de Jong, 1990) in between the T7 promoters. Other options include pDONR-dT7 (from which pPR-T4P is derived), which carries gateway cloning sites (Reddien et al., 2005), and pL4440 (Timmons & Fire, 1998) for cloning using restriction enzymes.
2. **HT115 (DE3) *Escherichia coli* competent cells:** This strain of *E. coli* expresses bacteriophage T7 RNA polymerase in an IPTG-inducible fashion and is deficient for RNAseIII activity, which is required to prevent the degradation of the dsRNA (Timmons, Court, & Fire, 2001). The HT115 strain is tetracycline resistant.
3. **Calf liver homogenate:** Prepare as in Section 1.1, item 3.
4. **LB medium:** (10 g/L Bacto Tryptone, 5 g/L Bacto Yeast Extract, 10 g/L NaCl): Prepare with ultrapure water, adjust pH to 7.0 with 5 N NaOH and sterilize by autoclaving.
5. **Antibiotics:** Kanamycin (50 mg/mL in water), tetracycline (5 mg/mL in 70% ethanol).
6. **Planarian medium:** Prepare as in Section 1.1, item 1.
7. **Red food dye:** 0.5% Allura red AC (Sigma, cat. number: 458848) in water.

2.1.2 Feeding protocol

1. Clone a cDNA fragment corresponding to your gene of interest between the T7 promoters of a dsRNA expression vector such as pPR-T4P. The cDNA fragment should be at least 200 bp in length and up to full-length. Using longer cDNA fragments tends to increase RNAi efficiency for some target genes.
2. Transform HT115 (DE3) *E. coli* competent cells with the resulting plasmid.
3. Select positive clones on LB plates supplemented with 50 μg/mL kanamycin (if using pL4440 vector, use 100 μg/mL ampicillin instead of kanamycin).
4. Inoculate a 5 mL starting culture of LB Medium supplemented with 50 μg/mL kanamycin and 20 μg/mL tetracycline and grow O/N in a shaker at 37 °C.
5. Use starter culture to inoculate 0.5 L LB medium supplemented with antibiotics and let the cells grow to $OD_{595} = 0.6$.
6. Induce expression of dsRNA by adding 1 mM IPTG for 2H.
7. Centrifuge at 3000 g during 15 min at 4 °C to pellet bacteria.
8. Discard supernatant and resuspend the bacterial pellet in 10 mL ice-cold planarian medium.
9. Transfer to a 15 mL conical tube and centrifuge at 3000 g during 10 min at 4 °C to pellet bacteria.
10. Dilute approximately 1 mL of thawed calf liver homogenate by adding 0.5 mL planarian medium.
11. Estimate the volume of the bacterial pellet and mix it with two volumes of diluted calf liver homogenate.
12. Add 10 μL of red food dye to monitor dsRNA-liver ingestion.
13. Prepare 100 μL aliquots into microfuge tubes to be used immediately or stored at −80 °C.
14. Transfer 10−20 medium-sized (∼1 cm) planarians into a plastic petri dish with planarian medium. We have observed that planarians sometimes have difficulties moving and feeding when placed in a new petri dish. In this case, transfer the flatworms into a new petri dish 1 day before the first feeding is planned.
15. Completely thaw an aliquot of bacteria/liver mix. Cut the tip of a 200 μL pipette tip to widen it and use it to place about 50 μL bacteria/liver mix at the bottom of the petri dish. The pipet tip should touch the bottom of the dish so that the food mix adheres to the substrate and remains well accessible to the flatworms.
16. Cover the petri dish to keep it dark and allow planarians to feed for 1−2 h. The flatworms should get a red shade due to the presence of dye in the ingested food.
17. Remove uneaten food mix. Carefully pour out most of the medium and gather the flatworms on one side of the dish by squirting medium with a transfer pipette. Keep just enough medium to cover the flatworms and clean the bottom of the dish using Kimwipes. Fill halfway with fresh planarian medium and transfer back in the incubator.
18. Repeat Steps 15−17 every other day for a total of three feedings.

19. We usually amputate planarians at this stage because it triggers formation of new tissues depleted from the target gene and allows faster observation of phenotypes. Two days after the last feeding (this delay ensures that the food mix has been totally digested), crosscut the flatworms pre- and post-pharyngeally (Figure 2(B)) as described in Section 1.4, Steps 3–6.
20. Clean daily and monitor the behavior of planarians. Locomotion phenotypes are usually visible 3–7 days following amputation. Strong locomotion defects induce a so-called inchworming phenotype, whereby planarians move slowly by waves of whole-body contraction and extension (Azimzadeh et al., 2012; Rink et al., 2009; Rompolas et al., 2010). Milder defects can result in a gliding motion slower than in control animals. When ciliary function is strongly impaired, planarians also exhibit bloating due to impaired function of the flame cells necessary to excretion (Reddien et al., 2005; Rink et al., 2011). This is usually lethal ~3 weeks after amputation.

2.2 RNAi VIA INJECTION OF dsRNA

2.2.1 Materials

1. **Cloning vector:** We recommend using a dsRNA expression vector such as pPR-T4P, pDONR-dT7, or pL4440 (Section 2.1.1, item 1). This way, the resulting construct can be used for both microinjection and bacterial feeding. If you are not planning to use bacterial feeding then any vector can be used to clone target gene cDNAs.
2. **PCR Primers:** Design two sets of primers that will allow amplifying the cloned cDNA fragment with a T7 promoter at either end (Figure 1(C)). When using pPR-T4P or pDONR-dT7, the following primers can be used: **Forward primer 1** (AAC CCC TCA AGA CCC GTT TAG A) and **Reverse primer 1** (GAA TTG GGT ACC GGG CCC), **Forward primer 2** (CCA CCG GTT CCA TGG CTA GC) and **Reverse primer 2** (GAG GCC CCA AGG GGT TAT GTG). When using other cloning vectors, a T7 promoter can be added to the primer sequences: **Forward primer 1** (TAA TAC GAC TCA CTA TAG G—16 to 20 gene specific nucleotides), **Reverse primer 1** (20–25 cDNA specific nucleotides), **Forward primer 2** (20–25 cDNA specific nucleotides) and **Reverse primer 2** (TAA TAC GAC TCA CTA TAG G—16 to 20 cDNA specific nucleotides).
3. Proteinase K.
4. Sodium dodecyl sulfate (20% solution).
5. Phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA.
6. Absolute ethanol, RNAse-free.
7. 70% ethanol, RNAse-free.
8. RNAse-free water.
9. **dsRNA synthesis kit:** T7 RiboMAX Express RNAi System (Promega Cat. No. P1700) or equivalent.

10. **Nanoject II Auto-Nanoliter Injector** (Drummond Scientific Cat. No. 3-000-204) equipped with Micromanipulator (Drummond Scientific Cat. No. 3-0000-024-L/R) and support base (Drummond Scientific Cat. No. 3-0000-025-SB).
11. **Injection tips:** Prepared by pulling borosilicate capillaries (Drummond Scientific Cat. No. 3-000-203-G/X) using a micropipette puller (e.g., P-97 Flaming/Brown micropipette puller, Sutter instrument) and breaking the micropipette tip with fine tweezers to increase the tip diameter.
12. Light mineral oil (Sigma Cat. No. 5904).
13. **Red food dye:** 0.5% Allura red AC (Sigma Cat. No. 458848) in water.
14. Stereoscope

2.2.2 Microinjection protocol

1. Clone a cDNA fragment (minimum size ~200 bp) corresponding to your gene of interest into a dsRNA expression vector.
2. Set the two PCR reactions to amplify the cloned cDNA with a T7 promoter at one of the two ends.
3. Remove contaminant RNAse by adding Proteinase K and SDS to the PCR reactions to a final concentration of 0.5 mg/mL and 0.5% respectively. Incubate 30 min at 37 °C.
4. Extract with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitate by adding 1/10th volume of 3 M ammonium acetate, pH 5.2 (RNAse-free, provided with T7 RiboMAX Express RNAi System) and 2.5 volumes absolute ethanol. Perform two washes with 70% ethanol, centrifuging 15 min at Vmax in a tabletop centrifuge and carefully discarding the supernatant between each step.
5. Dissolve the DNA pellet in RNAse-free water. You will need approximately 1 μg of template DNA per reaction for RNA synthesis.
6. Synthesize sense and antisense RNA strands separately using the two different PCR reactions as template using an in vitro dsRNA synthesis kit. Anneal the two strands and treat with RNAse A (provided with T7 RiboMAX Express RNAi System) to remove nonannealed RNA as recommended by the manufacturer. Dissolve the RNA pellet in 50−100 μL RNAse-free water.
7. Backfill an injection tip with mineral oil and position it on the Nanoject injector. Pipet 3 μL of dsRNA solution on a clean piece of parafilm and add 0.5 μL of 0.5% Allura red. Position the piece of parafilm under the stereoscope and slowly aspirate the dsRNA solution within the injection tip. It is important to avoid air bubbles, as this can interfere with injection.
8. Set up to five ~0.5−1 cm planarians as described in Section 1.4, Steps 3−4 and place under the stereoscope.
9. Inject at slow speed 3 × 32 nL of dsRNA solution (~100 nL total) into the gut of 0.5−1 cm planarians (Figure 2(B)). For larger individuals, it is possible to inject up to 5 × 32 nL (~150 nL total). To inject into the gut, the micropipette tip is placed in the area between the photoreceptors and the pharynx along the

midline. Successful injection results in a red shade within the branched gut of the planarians (Figure 2(B)).

10. Transfer the injected planarians into a clean petri dish filled with planarian medium.
11. Repeat Steps 6–9 twice, for a total of three injections over three consecutive days to obtain maximal levels of inactivation.
12. One day after the last injection, amputate planarians pre- and postpharyngeally as described in Section 1.4, Steps 3–6 (Figure 2(B)) to accelerate the appearance of the phenotype (see Section 2.1.2, Steps 19–20).
13. For certain target genes, it is possible to obtain higher levels of inactivation by performing a second round of injection/amputation. This is done 1 week after amputation by injecting the trunk fragments obtained following the first round of injection/amputation. Inject at slow speed 3 × 13.6 nL dsRNA solution (~40 nL total) within the gut and amputate pre- and postpharyngeally the following day.

3. WHOLE-MOUNT IMMUNOFLUORESCENCE STAINING

We routinely perform whole-mount immunofluorescence staining using either methanol or formaldehyde for fixation. Both fixation methods work well for visualizing the cilia by staining acetylated tubulin (Figure 1(A)–(F)). The main peculiarity of planarians compared to other model systems is the presence of a thick layer of mucus at the surface of the epidermis that must be removed to allow antibody penetration. This is achieved by treating the animals with the mucolytic agent *N*-acetyl cysteine prior to fixation (Pearson et al., 2009).

3.1 MATERIALS

1. **5% NAC solution:** Prepared freshly by dissolving 5% *N*-acetyl cysteine (NAC, Sigma Cat. No. A7250) in 1X PBS. NAC is used both to kill the planarians and remove the mucus. Alternatively, a 1 N HCl solution prepared in PBS can be used instead of the NAC solution.
2. **PBST:** 1X PBS containing 0.5% Tween-20.
3. **PBSTB:** 1X PBS containing 0.5% Tween-20 and 3% bovine serum albumin.
4. **Methanol (*for methanol fixation*)**, precooled at −20 °C.
5. **Formaldehyde fixative (*for formaldehyde fixation or postfixation*):** prepared fresh for each experiment by dilution of a 16% formaldehyde stock solution (Electron Microscopy Sciences Cat. No. 15710) into PBST.
6. **Bleaching solution:** 6% hydrogen peroxide (30% stock; Sigma Cat. No. H1009) in methanol. This solution is used to bleach the planarians, which have a brown pigmentation (Pearson et al., 2009), but we also found it to improve staining efficiency, possibly because it helps removing the mucus.

7. **Reduction solution:** Freshly prepared by diluting 50 mM DTT, 1% NP-40, 0.5% SDS, in 1X PBS. This solution helps permeabilizing the specimens to facilitate antibody penetration (Pearson et al., 2009).
8. **Rehydration methanol series:** 75%, 50%, and 25% methanol diluted in PBST (vol:vol).
9. **Antiacetylated tubulin antibody:** We use mouse monoclonal antibody, clone 6-11B-1 (Sigma Cat. No. T7451) for staining of cilia.
10. **Fluorescent-labeled anti-mouse secondary antibody:** Alexa Fluor 488 Goat Anti-Mouse IgGs (Life Technologies, Cat. No. A11008) or Alexa Fluor 555 Goat Anti-Mouse IgGs (Life Technologies, Cat. No. A21422).
11. **Immunofluorescence mounting medium:** We use Vectashield mounting medium containing DAPI (Vector Laboratories, Cat. No. H-1000) to stain the nuclei.
12. Nail polish.
13. **Labtop cooler:** (Nalgene, Sigma Cat. No. C2437), precooled at −20 °C.

3.2 IMMUNOFLUORESCENCE STAINING PROTOCOL USING METHANOL FIXATION

1. Transfer up to 20 small planarians (2−4 mm) into a 1.5 mL microfuge tube containing planarian medium. Use planarians that have been starved for a week to avoid possible cross-reactions with the ingested food.
2. Aspirate the planarian medium and replace by 1 mL NAC solution to kill the flatworms and remove the mucus. Incubate at room temperature during 1−2 min with gentle agitation. Optimal incubation time depends on specimen size and experimental conditions such as agitation speed. Prolonged incubation in NAC (or any other low pH solution) affects the integrity of the ciliated epidermal layer. We recommend that you optimize incubation time to determine the shortest amount of time required for bright antibody staining in your experimental conditions.
3. Carefully aspirate all the NAC solution and fix the specimens by addition of 1.3 mL of prechilled, −20 °C methanol. To prevent samples from warming-up, place in the labtop cooler immediately after addition of the cold methanol. Incubate 2−4 h at −20 °C.
4. Aspirate the methanol and replace 1 mL bleach solution. Incubate O/N under direct light, for instance, by placing the specimens under a desk lamp.
5. Rehydrate specimens by incubating successively in 75%, 50%, and 25% methanol dilutions, then in PBST, 5 min each time, with gentle rocking.
6. Proceed to reduction step by adding ∼1 mL reduction solution preheated at 37 °C. Incubate 10 min at 37 °C. Homogenize by gently flipping the tubes (specimens are very fragile at this step) every 2−3 min.
7. Aspirate the reduction solution and wash twice with ∼1 mL PBSTB, 5 min each time, with gentle rocking. Replace by ∼1 mL fresh PBSTB and block

1H at room temperature, with gentle rocking, to prevent nonspecific binding of antibodies.

8. Dilute primary antibody in PBSTB. To stain cilia, we dilute antiacetylated tubulin antibody (clone 6-11B-1) to a final concentration of 2 μg/mL. Incubate 2−4 h at room temperature, with gentle rocking. Alternatively, incubate O/N at 4 °C.
9. Wash a minimum of four times with 1.3 mL PBSTB, during 30 min each time, with gentle rocking.
10. Replace last wash by secondary antibody diluted in PBSTB (1:500 vol/vol) and incubate 2−4 h at room temperature, with gentle rocking. Alternatively, incubate O/N at 4 °C.
11. Wash a minimum of four times with 1.3 mL PBST, during 30 min each time, with gentle rocking.
12. For some antibodies, a brighter staining can be obtained by performing post-fixation. For this, we incubate the specimens in formaldehyde fixative during 30 min, at room temperature, with gentle rocking. The specimens are then washed two to three times in PBST, during 30 min each time.
13. Carefully aspirate the PBST and add ~50 μL (one drop) Vectashield. Incubate a few hours to O/N at 4 °C.
14. Using a polypropylene transfer pipet with a ~2 mm wide opening, transfer the specimens to a microscope slide.
15. Place the slide under a stereoscope and use fine tweezers or a fine needle to position the planarians with the ventral epidermis facing up. Avoid damaging the ventral surface. To identify the ventral surface, observe the photoreceptors. They can be seen through the ventral epidermis on bleached specimens, but they appear lighter in color. Place the specimens so that the side where the photoreceptors appear less dark is facing up.
16. Aspirate the excess mounting medium and replace with fresh Vectashield.
17. Under the stereoscope, place a coverslip on top of the specimens using tweezers. To do so, position the coverslip at an angle and bring it down slowly to avoid moving the specimens and trapping air bubbles.
18. Let rest for a few hours at room temperature or O/N at 4 °C so the specimens slowly flatten under the coverslip. Aspirate excess mounting medium and seal using nail polish.

3.3 IMMUNOFLUORESCENCE PROTOCOL USING FORMALDEHYDE FIXATION

1. Pick up 2 to 4-mm long planarians and treat with NAC as described in Section 3.2, Steps 1−2.
2. Carefully aspirate all the NAC solution and fix the specimens by addition of 1.3 mL formaldehyde fixative. Incubate 2−4 h at room temperature, or O/N at 4 °C.
3. Rinse three times with 1 mL PBST, during 10 min each time.

4. Aspirate the PBST and replace with 1 mL bleach solution. Incubate O/N under direct light by placing the specimens under a desk lamp.
5. Process the specimens as described in Section 3.2, Steps 5–18.

4. ELECTRON MICROSCOPY

Here we describe a method for sample preparation that requires a microwave tissue processor. This protocol allows rapid fixation and is well adapted for observing not only cilia and centrioles (Figure 1(G)–(K)), but also more dynamic cytoskeletal structures such as cytoplasmic microtubules (Azimzadeh et al., 2012). Protocols for high-pressure freezing and a standard protocol for chemical fixation have also been described (Brubacher, Vieira, & Newmark, 2014; Salvenmoser, Egger, Achatz, Ladurner, & Hess, 2010).

4.1 MATERIALS

1. **Cacodylate buffer** (100 mM sodium cacodylate, pH 7.4): Prepare from premade 200 mM cacodylate (Polysciences Cat. No. 18661) using ultrapure water.
2. **Primary fixative A** (4% paraformaldehyde, 2% glutaraldehyde, ~100 mM sodium cacodylate, pH 7.4): Prepared freshly by diluting 200 mM sodium cacodylate, pH 7.4, 16% (wt/vol) paraformaldehyde (Electron Microscopy Sciences Cat. No. 15710) and 8% (vol/vol) glutaraldehyde (Electron Microscopy Sciences Cat. No. 16020) using ultrapure water.
3. **Primary fixative B** (3% glutaraldehyde, ~100 mM sodium cacodylate, pH 7.4): Prepared freshly by diluting 200 mM sodium cacodylate, pH 7.4 and 8% (vol/vol) glutaraldehyde using ultrapure water.
4. **Osmium tetroxide (secondary fixative)** (1% wt/vol OsO_4, 100 mM sodium cacodylate, pH 7.4): Prepared freshly by diluting 4% OsO_4 stock solution and 200 mM sodium cacodylate, pH 7.4 using ultrapure water. Unused 4% stock can be stored at −20 °C in an airtight container and protected from light. ***Osmium tetroxide is highly volatile and toxic. Wear personal protective equipment and handle solutions only under a fume hood.***
5. **Absolute ethanol:** Use a freshly opened container for each experiment to ensure that ethanol remains anhydrous. The remaining ethanol can be used to prepare graded ethanol series (see below).
6. **Acetone:** Use a freshly opened container for each experiment.
7. **Epon/Araldite resin mixture without accelerant:** Using Epon/Araldite embedding kit with benzyldimethylamine (BDMA) (Ted Pella Cat. No. 18028), prepare resin mixture without accelerant by measuring by mass into a disposable beaker: 31 g Eponate 12, 22.2 g Araldite 502, and 61 g DDSA (included in the Epon/Araldite kit). Stir thoroughly until homogeneously mixed. Prepare sufficient resin for the whole procedure (about 12 mL per

specimen tube) to ensure that all solutions used for infiltration and embedding draw from a consistent batch.

8. **Epon/Araldite resin mixture with accelerant:** Add 350 μL BDMA (included in the Epon/Araldite kit) per 10 g resin mixture prior to the final step of infiltration and stir thoroughly.
9. Microwave tissue processor (PELCO Biowave Pro, Ted Pella Inc., Cat. No. 36500).
10. Flat embedding mold, 24 cavities (e.g., Ted Pella, Cat. No. 105).
11. Oven set at 60 °C.

4.2 MICROWAVE-BASED TEM SAMPLE PREPARATION

1. Transfer one to five planarians into a 35 mm petri dish containing ~5 mL of planarian medium using a transfer pipette. Place the dish on ice until the worms stop moving and stretch.
2. Slowly aspirate the medium to avoid perturbing the animals and flood the animals with ~5 mL primary fixative A. Gently swirl the dish to prevent the worms from sticking to the bottom. Incubate for 5 min then cut the specimens into 3 to 4-mm long fragments.
3. Transfer the fragments to a microfuge tube containing 1 mL fresh primary fixative A.
4. Leaving the lid of the microfuge tube open, fix in the microwave tissue processor at 150 W during 1 min with vacuum at 20 inHg, then 1 min with vacuum only. Close the tubes, and flip them upside down gently several times. Repeat Step 4 two times.
5. Aspirate the primary fixative A and replace with 1 mL primary fixative B, incubate 4H to O/N at 4 °C.
6. Wash three times with 1 mL 100 mM cacodylate buffer. Incubate 10 min at room temperature on an orbital shaker set to ~100 rpm.
7. Replace the cacodylate buffer with 1 mL secondary fixative (OsO_4 solution). Open tube lids and fix in the microwave tissue processor at 150 W during 1 min with vacuum at 20 inHg, then 1 min with vacuum only. Repeat once as before then twice at 350 W during 1 min with vacuum at 20 inHg followed by 1 min with vacuum only. Close tube lids and flip the tubes upside down several times between each run in the microwave processor. Incubate the specimens 1H at room temperature, protected from light.
8. Wash the fragments 10 min at room temperature two times with 100 mM cacodylate buffer then three times with ultrapure water.
9. Leaving the fragments in the same microfuge tube, perform low-temperature dehydration by successively incubating during 10 min each time in the following prechilled ethanol solutions: 35% ethanol at 4 °C, 50% ethanol on ice, 75% ethanol at −20 °C, 80% ethanol at −20 °C, 95% ethanol on dry ice, twice in absolute, anhydrous ethanol on dry ice.

10. Replace ethanol by anhydrous acetone and incubate 10 min at room temperature.
11. Prepare the following graded series of resin mixture without accelerant diluted in acetone (vol/vol): 10%, 25%, 30%, 40%, 60%, 80%, and 100%. Place the planarian fragments successively in the resin mixture dilutions and process in the microwave tissue processor at 250 W during 3 min with vacuum at 20 inHg, with tube lids open. Repeat one time for each of the last three dilutions using fresh resin mixture.
12. Change to fresh 1 mL 100% resin mixture with accelerant (BDMA) and allow the specimens to infiltrate for 6H at room temperature, with gentle rocking. Alternatively, specimens can be stored O/N in 100% resin mixture without accelerant prior to incubation with resin mixture containing accelerant.
13. Fill embedding molds (silicone flat molds) with resin mixture with accelerant.
14. Transfer one specimen into each well and orient as desired.
15. Place the molds in an oven set at 60 °C and allow the resin to cure for 48–72 h.

5. LIVE IMAGING

The body of *S. mediterranea* is completely opaque and it is thus not possible to directly visualize the cilia on the ventral surface. However, motile cilia can be imaged very easily from the lateral part of the epidermis using differential interference contrast (DIC) microscopy (Rompolas et al., 2010, 2012). Importantly, the protocol developed by Rompolas et al. (2010) allows confining planarians to restrain their movement, and thus keep them under the same plan of view, while keeping them under physiological conditions.

5.1 MATERIALS

1. Upright microscope (Olympus BX51 or equivalent) equipped with DIC optics and a 60× objective, and coupled with a high frame-rate CMOS camera (for instance, S-PRI F1 high speed camera from AOS Technologies).
2. Microscope slides.
3. Square microscope coverslips, e.g., 22 × 22 mm.
4. Parafilm.
5. One-hole punch (available in office supply stores).

5.2 SAMPLE PREPARATION AND IMAGING

1. Prepare a parafilm spacer by punching a hole in a 1 × 1 cm square of parafilm using the one-hole puncher. Place the parafilm spacer on a clean microscope slide and carefully flatten the spacer to form a watertight imaging chamber.
2. Select a small individual (1–3 mm in length) and place it at the center of the parafilm hole in a drop of planarian medium.

3. Carefully place a clean coverslip on top of the parafilm spacer and gradually press to remove the extra medium and flatten the body of the planarian to be observed. The width of the parafilm spacer must allow immobilizing small individuals without causing injury.
4. Place on the microscope stage and focus on the lateral sides of the planarian body with the 60× objective. For maximum contrast, orient the flatworm by rotating the microscope stage so that cilia are parallel to the shear axis of the DIC microscope.
5. Rotate the camera so that the cilia are vertical to the field of view in the preview monitor and select a frame rate of 250 frames/s.
6. Start acquisition. Check on the acquired video that the cilia are intact and form metachronal waves. If the coverslip is pressed too hard on the parafilm spacer, the epidermis can be damaged and the cilia then look disorganized. On the contrary, if the coverslip is not close enough, the animal will still move and the frame will shift. In either of these situations, start again with a different animal.

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REFERENCES

Aslanidis, C., & de Jong, P. J. (1990). Ligation-independent cloning of PCR products (LIC-PCR). *Nucleic Acids Research, 18*(20), 6069−6074.

Azimzadeh, J., Wong, M. L., Downhour, D. M., Sánchez Alvarado, A., & Marshall, W. F. (2012). Centrosome loss in the evolution of planarians. *Science, 335*(6067), 461−463.

Brubacher, J. L., Vieira, A. P., & Newmark, P. A. (2014). Preparation of the planarian *Schmidtea mediterranea* for high-resolution histology and transmission electron microscopy. *Nature Protocols, 9*(3), 661−673.

Cebrià, F., & Newmark, P. A. (2005). Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development, 132*(16), 3691−3703.

Krugelis McRae, E. (1967). The fine structure of sensory receptor processes in the auricular epithelium of the planarian, *Dugesia tigrina*. *Zeitschrift fur Zellforschung und Mikroskopische Anatomie, 82*, 479−494.

Lázaro, E. M., Harrath, A. H., Stocchino, G. A., Pala, M., Baguñà, J., & Riutort, M. (2011). *Schmidtea mediterranea* phylogeography: an old species surviving on a few Mediterranean islands? *BMC Evolutionary Biology, 11*, 274.

Liu, S. Y., Selck, C., Friedrich, B., Lutz, R., Vila-Farré, M., Dahl, A., et al. (2013). Reactivating head regrowth in a regeneration-deficient planarian species. *Nature, 500*(7460), 81−84.

Newmark, P. A., Reddien, P. W., Cebria, F., & Sánchez Alvarado, A. (2003). Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians. *Proceedings of the National Academy of Sciences USA, 100*, 11861−11865.

Newmark, P. A., & Sánchez Alvarado, A. (2002). Not your father's planarian: a classic model enters the era of functional genomics. *Nature Reviews Genetics, 3*(3), 210−219.

Park, T. J., Mitchell, B. J., Abitua, P. B., Kintner, C., & Wallingford, J. B. (2008). Dishevelled controls apical docking and planar polarization of basal bodies in ciliated epithelial cells. *Nature Genetics, 40*(7), 871−879.

Pearson, B. J., Eisenhoffer, G. T., Gurley, K. A., Rink, J. C., Miller, D. E., & Sánchez Alvarado, A. (2009). Formaldehyde-based whole-mount in situ hybridization method for planarians. *Developmental Dynamics, 238*(2), 443−450.

Reddien, P. W., Bermange, A. L., Murfitt, K. J., Jennings, J. R., & Sánchez Alvarado, A. (2005). Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. *Developmental Cell, 8*(5), 635−649.

Resch, A. M., Palakodeti, D., Lu, Y. C., Horowitz, M., & Graveley, B. R. (2012). Transcriptome analysis reveals strain-specific and conserved stemness genes in *Schmidtea mediterranea*. *PLoS One, 7*(4), e34447.

Rink, J. C., Gurley, K. A., Elliott, S. A., & Sánchez Alvarado, A. (2009). Planarian Hh signaling regulates regeneration polarity and links Hh pathway evolution to cilia. *Science, 326*(5958), 1406−1410.

Rink, J. C., Vu, H. T., & Sánchez Alvarado, A. (2011). The maintenance and regeneration of the planarian excretory system are regulated by EGFR signaling. *Development, 138*(17), 3769−3780.

Robb, S. M., Ross, E., & Sánchez Alvarado, A. (2008). SmedGD: the *Schmidtea mediterranea* genome database. *Nucleic Acids Research, 36*(Database issue), D599−D606.

Rompolas, P., Azimzadeh, J., Marshall, W. F., & King, S. M. (2012). Analysis of ciliary assembly and function in planaria. *Methods in Enzymology, 2013*(525), 245−264.

Rompolas, P., Patel-King, R. S., & King, S. M. (2010). An outer arm Dynein conformational switch is required for metachronal synchrony of motile cilia in planaria. *Molecular Biology of the Cell, 21*(21), 3669−3679.

Salvenmoser, W., Egger, B., Achatz, J. G., Ladurner, P., & Hess, M. W. (2010). Electron microscopy of flatworms: standard and cryo-preparation methods. *Methods in Cell Biology, 96*, 307−330.

Sánchez Alvarado, A., & Newmark, P. A. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proceedings of the National Academy of Sciences USA, 96*, 5049−5054.

Timmons, L., Court, D. L., & Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene, 263*(1−2), 103−112.

Timmons, L., & Fire, A. (1998). Specific interference by ingested dsRNA. *Nature, 395*(6705), 854.