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TECHNOLOGY REPORT

Identification of Immunological Reagents for Use in the Study of Freshwater Planarians by Means of Whole-Mount Immunofluorescence and Confocal Microscopy

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Summary: In recent years, interest in planarians as a model system for the study of metazoan regeneration, adult stem cell biology, and the evolution of metazoan body plans has been growing steadily. The availability of RNA interference (RNAi), BrdU-labeling of planarian stem cells, and thousands of planarian cDNA sequences soon to be released into public databases has opened planarians to molecular dissection. However, the successful application of large-scale RNAi-based screens, for example, will depend in part on the availability of markers to characterize the resulting phenotypes. Given the paucity of antibodies available for the study of planarian biology, we have screened various public and commercial antibody resources to identify immunoreagents capable of cross-reacting with planarian tissues. Here we report the identification and characterization of 33 such antibodies recognizing a wide variety of tissues in freshwater planarians. genesis 32:293–298, 2002.

INTRODUCTION

Planarians (class Turbellaria, phylum Platyhelminthes) are a classic model for the study of metazoan regeneration. As early as 1898, it was shown that a fragment 1/279th the size of the organism is capable of regenerating a complete animal (Morgan, 1898; Montgomery and Coward, 1974). This remarkable developmental plasticity is driven by a population of free mesenchymal stem cells known in the literature as neoblasts (Randolph, 1892). These small (5–8 μ), undifferentiated cells are found distributed throughout the organism, with the exception of the pharynx and the area in front of the photoreceptors (Brøndsted, 1969; Newmark and Sánchez Alvarado, 2000). Neoblasts are the only mitotically active cells in the flatworm and are used to replenish cells lost to normal physiological turnover and/or injury (Newmark and Sánchez Alvarado, 2000).

Exactly how these cellular activities are choreographed into a regenerative response is not completely understood. One approach being employed to further our understanding of planarian regeneration is RNA interference (RNAi) (Sánchez Alvarado and Newmark, 1999). This method specifically silences the expression of targeted genes by a yet unknown mechanism involving double-stranded RNA (dsRNA) (Sijen *et al.,* 2001). However, the characterization of loss-of-function phenotypes produced by this technology requires the use of appropriate protein markers. Unfortunately, visualization of protein patterns in planarians is limited to a handful of antibodies: Tcen (Bueno *et al.,* 1996), Tnex (Fernández-Rodríguez *et al.,* 2001), Tmus (Cebria *et al.,* 1997), DjSyt, DjPC2, and VC-1 (Agata *et al.,* 1998), which severely restricts our ability to effectively screen for and characterize RNAi-induced phenotypes.

In an attempt to overcome this obstacle, antibodies generated against proteins from other species were screened to determine if they would cross-react with planarian tissues. Antibodies screened were selected based on (1) interphyletic and/or interspecies cross-reactivity as reported in the literature and (2) the use of conserved protein motifs as antigens for antibody production. Also, pre-immune sera from chicken, rabbit, and guinea pig were tested initially, and no cross-reactivity with planarian tissues was detected (data not shown). All antibodies were tested at least three times using no less than eight animals per antibody. Of 138 antibodies examined, 33 recognized specific epitopes (≈24%). The efficiency of the screen may be a reflection of using Carnoy's as a fixative because many epitopes, especially those found in small molecules, may be lost during sample processing.

Even though more traditional fixation agents such as paraformaldehyde and/or gluteraldehyde have been used with some success in planarians (Bueno *et al.,*

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Table 1
Antibodies That Cross-React Reproducibly with Planarian Tissues

Source	Antibody ID	Antibody description	Planarian cell types/organs recognized
Developmental Studies, Hybridoma Bank	01C1D4	Germline specific P Granules	Mesenchymal cells[a]
	1E8	Schwann cell (avian) marker	Subepithelial cells[a]; GVS[a]
	22C10	Neurons	CNS[a]
	3A10	Neurofilament-assoc antigen	Mesenchymal cells[a]
	40.2D6	Islet-1 -homeobox	Free mesenchymal cells[b]
	40.3A4	Islet-1	Free mesenchymal cells[b]
	4D4	wingless	Dorsal epithelial cells[a, c]
	50.5A5	lmx1	Subepithelial cells[a]
	D-β3	Active TGFβ3	Subepithelial cells[a,c]
	E7	β-tubulin	Ciliated epithelium[a]
	F2F4	Cyclin B	Mitotic neoblasts[a,c]
	G-β3	Latent TGFβ3	Subepithelial cells[a]
	H5	Vimentin	Epithelial gland cells[a]
	JLA20	actin	Body-wall musculature[a]
	MF14	Myosin heavy chain, adult	Body-wall musculature[a]
	PAX6	Pax6	Cerebral ganglia (weak)
	SUK4	kinesin	Epithelium[a,b]
	SV2	Synaptic vesicles	CNS[a]
M. Fuller, Stanford University	3A5	α-tubulin	Submuscular nerve plexus[a]
NeoMarkers	Ab-4	α + β tubulin	Ciliated epithelium; neurons[a]
Santa Cruz	BMP2/4	Bone morphogenetic proteins	Epithelium[c]; subepithelial cells[c]
	c-kit(c 19)	Cytokine	Subepithelial cells[c]
	DHH	Desert hedgehog	Epithelium[c]; subepithelial cells[c]
	FYN	Tyrosine kinase	Subepithelial cells[c]
	gata 4	Transcription factor	GVS[a]
	mos	Serine/threonine kinase	Mesenchymal cells[c]
	patched	Patched receptor	Body-wall musculature[c]; ventral mesenchymal cells[c]
	SHH	Sonic hedgehog	Body-wall musculature[c]
	TEC	Nonreceptor tyrosine kinase	Subepithelial cells[a]
Sigma	anti-α tub	Clone B-5-1-2	Ciliated epithelium[a]
D. McClay, Duke University	β-cat	β-catenin	Central secretory cells[a]
Upstate Biotechnology	H_3P	Phosphorylated histone H3	Mitotic neoblasts[a,c]
Kiyokazu Agata, Riken Center for Developmental Biology	VC-1	Arrestin	Photoreceptor neurons[a]

Dilutions and secondary antibodies used, as well as a more detailed list of reagents, which failed to identify epitopes in planarians, can be found at http://planaria.neuro.utah.edu. Cellular localization of the antibody signal in planarians is (a) cytosolic; (b) nuclear; (c) membrane associated. GVS, gastrovascular system; CNS, central nervous system.

1997), we chose to work with Carnoy's because it is the only fixative that enables the detection of gene expression by whole-mount in situ hybridizations (Sánchez Alvarado and Newmark, 1999; Umesono *et al.,* 1997). Utilizing a single fixative for both whole-mount immunohistology and in situ hybridization allows for the simultaneous deployment of these techniques (Sánchez Alvarado, unpublished results) and simplifies high throughput analyses of gene expression and protein localization. Details of the results for the antibody screen, in particular for those antibodies that tested negative, can be found at http://planaria.neuro.utah.edu.

The antibodies scored as positives (Table 1) identified a variety of planarian cells (see Fig. 1 for examples). Cells of the central nervous system, epithelium, musculature, and secretory tissues were labeled, respectively, with the following markers: ab-4, 3A5, SHH, and H5. Putative secretory (β-cat, G-β3) and mitotically active (F2F4) cells were also identified in this screen. However, these and most other antibodies tested were produced to identify proteins from organisms that are not closely related to planarians (eg, chicks, *Xenopus*, sea urchins, *Drosophila*).

Accordingly, the proteins being recognized in flatworms may not necessarily be the orthologs to the original antigens utilized to generate the primary antibodies. Nevertheless, the 33 antibodies tallied as positives do cross-react specifically with discrete planarian cell types and in some cases are found in their expected subcellular localizations. For instance, an antibody against islet, a

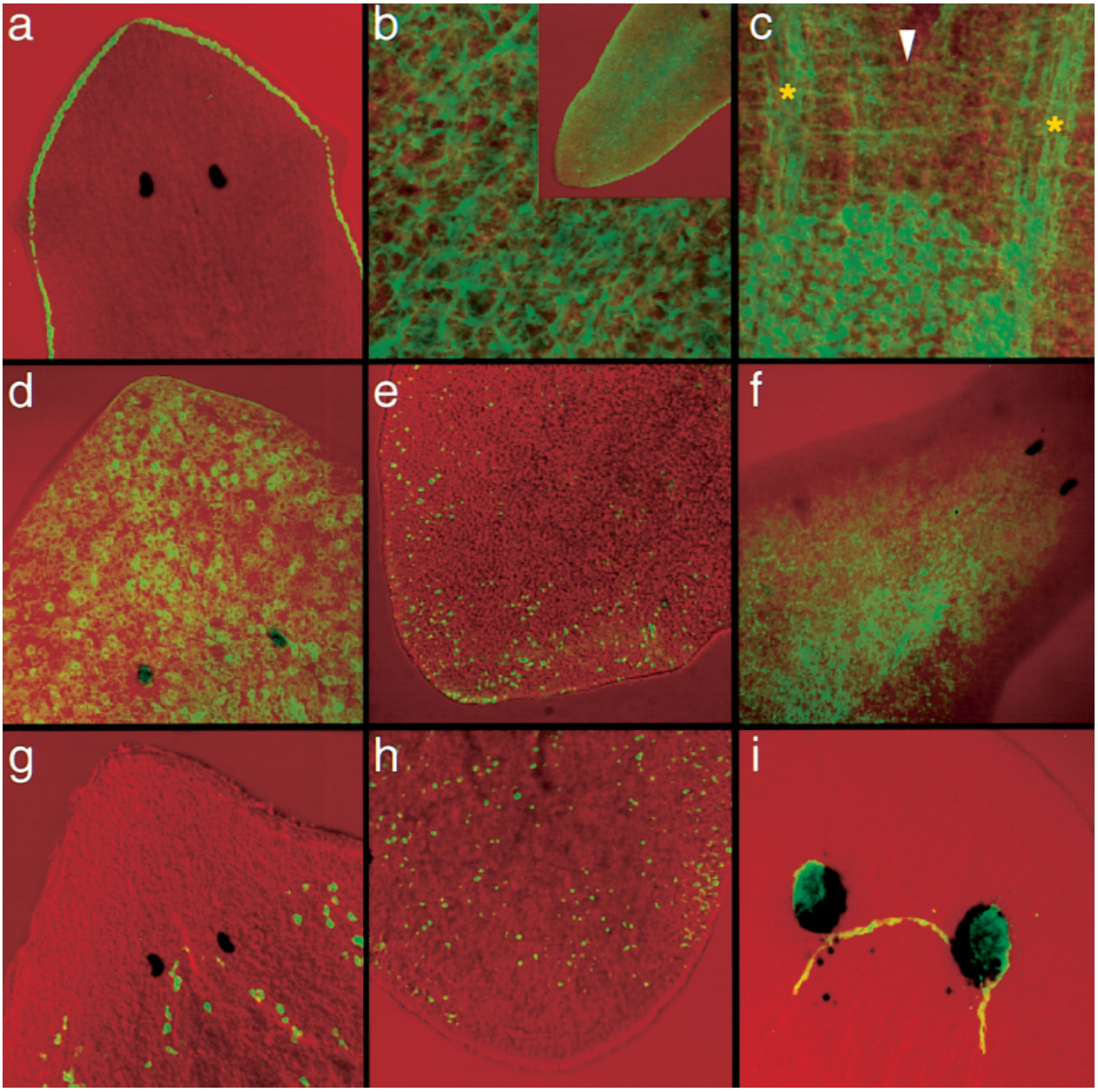

FIG. 1. Representative results of the antibody screen. All panels are confocal projections superposed on their respective phase-contrast images. Primary antibodies utilized are as follows: (**a**) Anti-vimentin (chicken; H5), 10×, 16 sections at 3.5-μ intervals; (**b**) Anti-α-tubulin (Dr. M.T. Fuller; 3A5; Piperno and Fuller, 1985), 80×, 16 sections at 8-μ intervals and inset at 10×, 16 sections at 3-μ intervals; (**c**) Anti-α-β tubulin (chicken; Ab-4), 20×, 16 sections at 0.8-μ intervals. Asterisks indicate the longitudinal ventral chords and the arrowhead, commissural neurons; (**d**) Anti-TGFβ active (chicken; D-β3), 20×, 16 sections at 2-μ intervals; (**e**) Anti-TGFβ latent (chicken; G-β3), 20×, 18 sections at 6-μ intervals; (**f**) Anti-wingless (*Drosophila*; 4D4), 10×; (**g**) Anti-cyclin b (*Drosophila*; F2F4), 20×, 16 sections at 1.3-μ intervals; (**h**) Anti-islet (chicken; 40.3A4), 20×, 16 sections at 1-μ intervals; (**i**) Anti-arrestin (Dr. Kiyokazu Agata; VC-1), 10×, 12 sections at 3.6-μ intervals.

transcription factor, identifies a nuclear localized epitope in *D. dorotocephala*, whereas an antibody against wingless is found mostly associated with the cytosol and cytosolic membranes of epithelial cells (Fig. 1f, h, and Fig. 2).

On the other hand, a number of antibodies produced specific and reproducible labeling patterns that are hard to reconcile with the known function and cellular localization of the proteins against which they were generated. Examples of such results are shown in Figure 3. An antibody produced against the active domain of the chicken TGFβ protein (D-β3; Boyer *et al.,* 1999) labels specific subcellular domains associated with the cytosolic membrane (Fig. 3a). In mice, such morphological

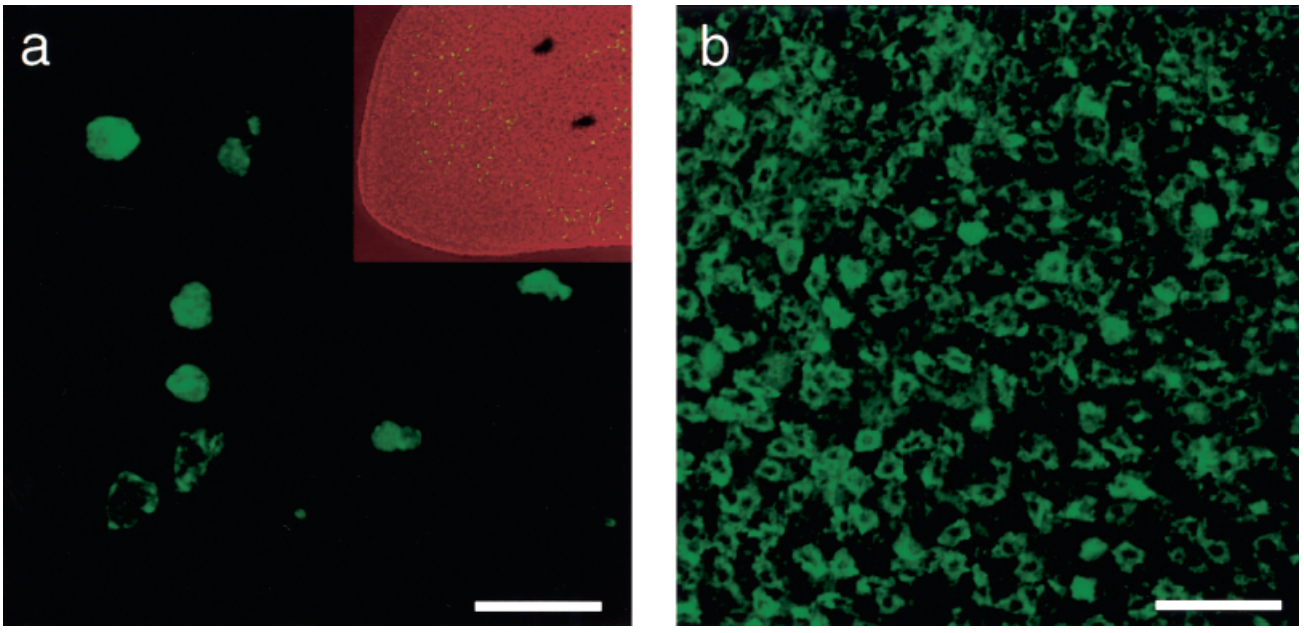

FIG. 2. Examples of antibodies likely to cross-react with the appropriate planarian ortholog antigens. Both panels are confocal projections. (**a**) Anti-islet antibody (chicken; 40.3A4) labels the nuclear compartment of primarily dorsal mesenchymal cells (200×; 10 optical sections at 0.69-μ intervals). (**b**) Anti-wingless antibody (*Drosophila*; 4D4) cross-reacts with cytosolic membrane-associated epitopes in epithelial planarian cells (40×; 16 optical sections at 0.32-μ intervals). Scale bars: 10 μ (a) and 50 μ (b).

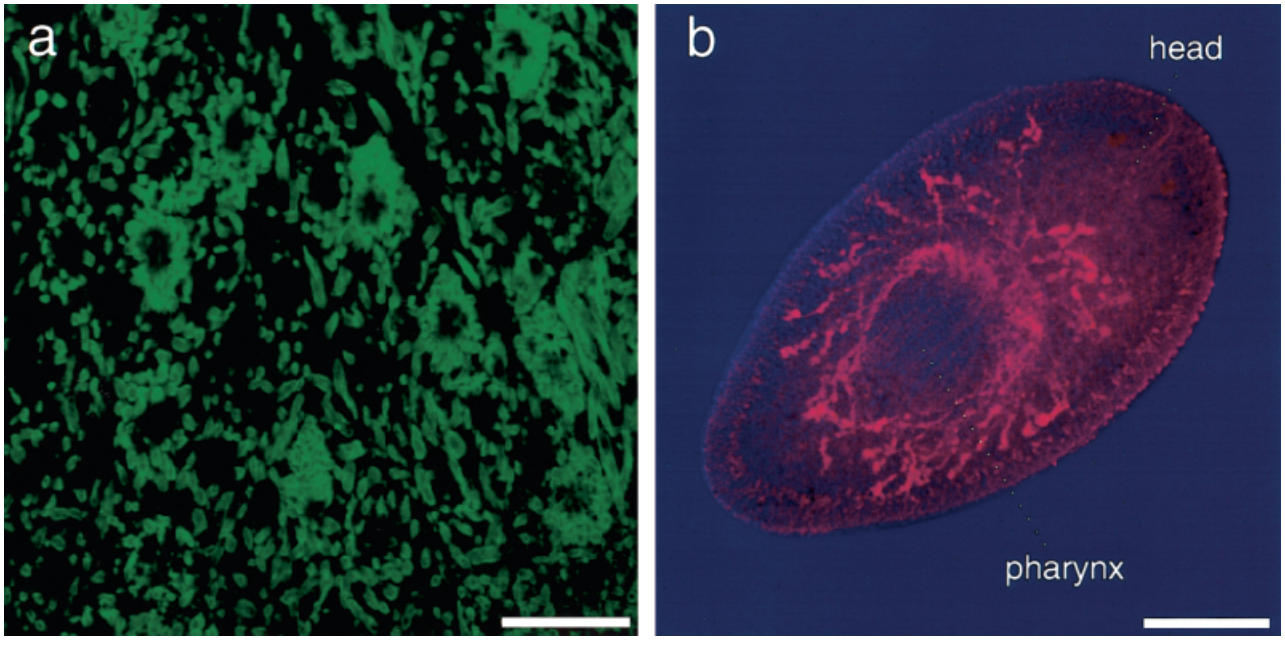

FIG. 3. Examples of antibodies not likely to cross-react with the appropriate planarian ortholog antigens. Both panels are confocal projections; b is superposed on its phase-contrast image. (**a**) Anti-TGFβ active (chicken; D-β3) labels cytosolic processes of unknown function in epithelial and subepithelial cells throughout the organism (100×; 4 optical sections at 0.3-μ intervals). (**b**) Anti-β-catenin (sea urchin; β-cat) labels a group of secretory cells surrounding the pharynx of the animal (10×; 8 optical sections at 3-μ intervals). Scale bars: 20 μ (a) and 200 μ (b).

subepithelial distribution of signal has not been reported, at least for the epithelial cells of the murine cardiac compartment (Boyer *et al.,* 1999). Likewise, the labeling obtained with a sea urchin anti-β–catenin antibody was both robust and reproducible, and defined a group of secretory cells surrounding the pharynx (Fig. 3b). The signal, however, did not appear to be nuclear localized, even though this antibody has been used ex-

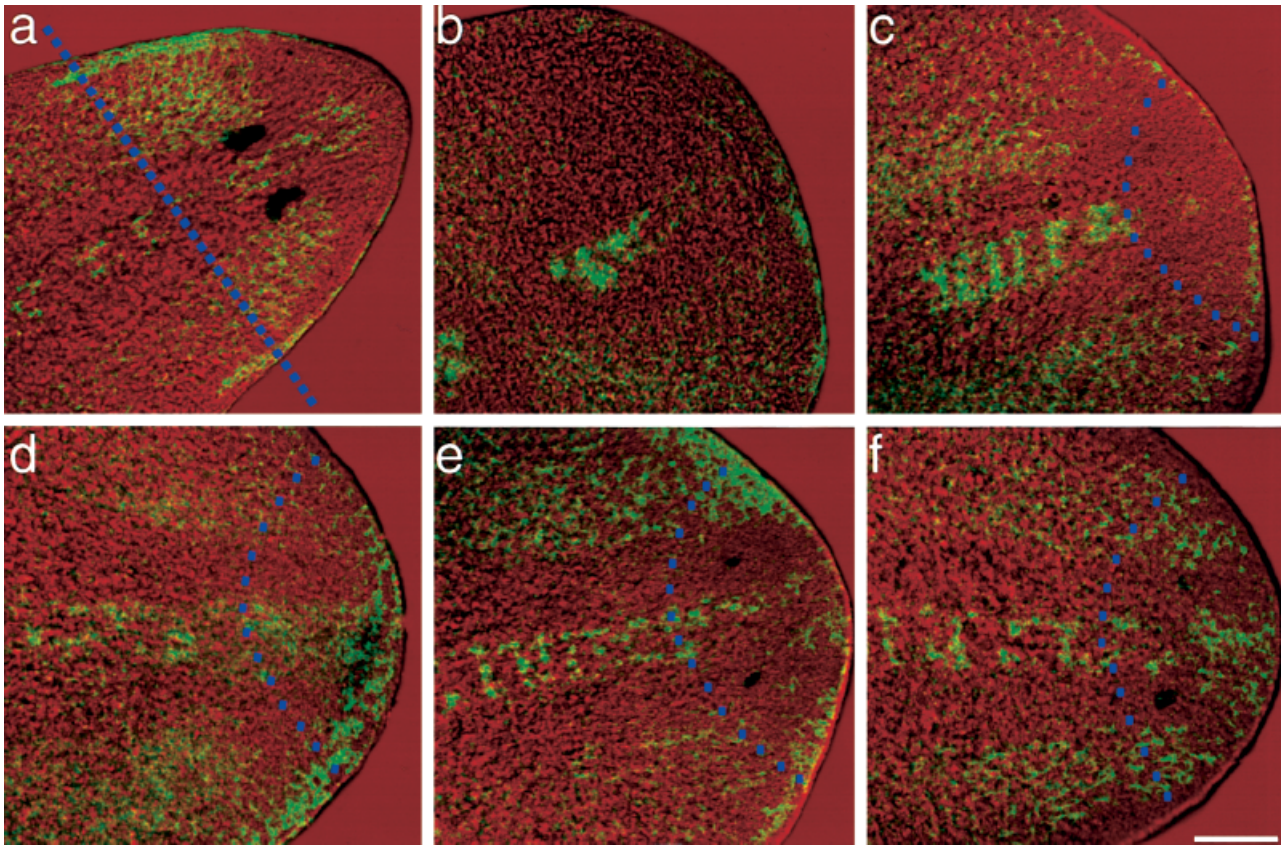

FIG. 4. Regeneration series of the dorsal epithelium in *D. dorotocephala* visualized with the anti-acetylated tubulin antibody (sea urchin; T-6793). Approximate amputation plane (a) and regeneration boundaries (c–f) are shown by dotted blue lines. (**a**) Intact organism (32 optical sections at 1-μ intervals). (**b**) Day 2 (20 optical sections at 1-μ intervals). (**c**) Day 3 (16 optical sections at 0.6-μ intervals). (**d**) Day 4 (16 optical sections at 0.6-μ intervals). (**e**) Day 5 (16 optical sections at 0.8-μ intervals). (**f**) Day 6 (16 optical sections at 0.8-μ intervals). Magnification 20×. Scale bar: 100 μ.

tensively to study the role of β-catenin nuclear localization in the establishment of all vegetal cell fates and micromere-derived signals during sea urchin embryogenesis (Logan *et al.,* 1999). Because it is not known how many members of the catenin family of proteins may exist in planarians, even the possibility that the sea urchin anti-β-catenin antibody may be cross-reacting with other catenins (such as those involved in anchoring membrane cadherins) remains speculative at best.

Although epitope characterizations will be necessary to verify the target peptide sequence of the planarian-positive antibodies reported here, the reproducibility of the patterns obtained allows us to use these reagents to follow morphological changes in these organisms. This is clearly illustrated in Figure 4 where, after decapitation, the ontogeny of the dorsal stripe of sensory cilia can be followed with the anti-acetylated tubulin antibody (T-6793) as the head regenerates. The dramatic change in tubulin distribution on the regenerating tissue as it begins to differentiate can be gauged by comparing days 3 (Fig. 4c) and 6 (Fig. 4f). Thus, it should now be possible, in combination with BrdU labeling of neoblasts (Newmark and Sánchez Alvarado, 2000), as well as gene expression detection by in situ hybridizations, to begin a systematic delineation of cell fates and dynamics of differentiation in planarians.

There is no doubt that in the end, it will be necessary to develop planarian-specific antibodies to study protein function in these organisms. However, until a critical mass of resources and laboratories working on planarians is cemented, the screening method described here remains a viable alternative for identifying useful immunological reagents for use in planarians. It is expected that the antibody characterization reported here, and the detailed screening information found at http://planaria.neuro.utah.edu will help advance the dissection of the molecular and cellular biology of these remarkable organisms.

MATERIALS AND METHODS

Two species of freshwater planarians, *Schmidtea mediterranea* and *Dugesia dorotocephala* (Carolina Biological, Burlington, NC), were used for the antibody screens. *S. mediterranea* was cultured at room temperature (RT; 21–22°C) in dechlorinated tap water, which was supple-

mented with 0.1 mM KCl, 2 mM NaCl, 0.1 mM $MgSO_4$ and 0.12 mM $NaHCO_3$. The *D. dorotocephala* were maintained at RT in dechlorinated tap water. Planarian stocks were fed beef liver purée twice a week and starved for 1 week prior to experimentation.

The planarians were killed in 2% HCl for 30 sec and then fixed in Carnoy's (six parts ethanol, three parts chloroform, one part glacial acetic acid) for 2 h at RT followed by three methanol rinses. The animals were bleached overnight in 6% hydrogen peroxide in methanol, rinsed briefly, and stored in 100% methanol at −20°C. Samples were rehydrated through a methanol series (75%; 50%; 25% methanol) in phosphate-buffered solution + 0.3% Triton X-100 (PBST; Sigma, St. Louis, MO). After rehydration, animals were rinsed in PBST, and the specimens were blocked for 2 h (*D. dorotocephala*) or 6 h (*S. mediterranea*) at RT in PBST + 0.25% bovine serum albumin (PBSTB). Samples were separated into small 8-ml scintillation glass vials (RPI, Mt. Prospect, IL) and incubated overnight at RT on a rotating platform (120 rpm) in 500μl of primary antibodies diluted in PBSTB. A large proportion of the primary antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa; see Table 1 for more information). The planarians were rinsed once and then washed extensively in PBSTB for 6–8 h at RT. The specimens were incubated overnight in the appropriate secondary antibodies diluted in PBSTB [1:400 Alexa 488 anti-mouse, 1:500 Alexa 488 anti-goat (Molecular Probes, Eugene, OR); 1:500 CY3 anti-guinea pig, 1:1000 CY3 anti-rabbit, and 1:500 CY5 anti-rat (Amersham, Piscataway, NJ)]. After incubation, the specimens were washed as described above, mounted in Vectashield (Vector Laboratories, Burlingame, CA), and observed under epifluorescence using a Zeiss Axiovert S-100 microscope. Animals displaying antibody cross-reactivity were further analyzed with a Leica TCS NT or Olympus Fluoview 300 confocal microscope.

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