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Heterogeneity of planarian stem cells in the S/G2/M phase

SÖREN MORITZ[1], FRANZISKA STÖCKLE[1], CLAUDIA ORTMEIER[1], HENNING SCHMITZ[1], GUSTAVO RODRÍGUEZ-ESTEBAN[2], GÖRAN KEY[3] and LUCA GENTILE*[,1]

[1]*Planarian Stem Cell Laboratory, Max Planck Institute for Molecular Biomedicine, Münster, Germany,* [2]*University of Barcelona, Department of Genetics, IBUB, Barcelona, Spain and* [3]*Max Planck Institute for Molecular Biomedicine, Münster, Germany*

ABSTRACT The planarian adult stem cell (pASC) population has a specific molecular signature and can be easily visualized and isolated by flow cytometry. However, the lack of antibodies against specific surface markers for planarian cells prevents a deeper analysis of specific cell populations. Here, if we describe the results of the immunoscreening of pASC plasma membrane proteins (PMPs). A novel papain-based method for planarian cell dissociation enabling both high yield and improved cell viability was used to generate single cell preparations for PMP purification. PMPs were used for intraperitoneal immunization of mice and thus about 1000 hybridoma clones were generated and screened. Supernatants collected from the hybridoma clones were first screened by ELISA and then by live immuno-staining. About half of these supernatants stained all the planarian cells, whereas the other half specifically labeled a subfraction thereof. A detailed analysis of two hybridoma supernatants revealed that large subfractions of the X1, X2 and Xin populations differentially express specific membrane markers. Quantitative PCR data disclosed a correlation between the immunostaining results and the expression of markers of the early and late progeny, also for those pASCs in the S/G2/M phase of the cell cycle (X1 population). Thus, about two thirds of the cycling pASCs showed a specific membrane signature coupled with the expression of markers hitherto considered to be restricted to differentiating, post-mitotic progeny. In summary, a library of 66 monoclonal antibodies against planarian PMPs was generated. The analysis of two of the clones generated revealed that a subset of cells of the X1 population expresses early and late progeny markers, which might indicate that these cells are committed while still proliferating. The findings demonstrate the usefulness of our PMP antibody library for planarian research.

KEY WORDS: *stem cell heterogeneity, cell surface marker, mAb library, planarian*

Introduction

Planarians are the true masters of regeneration. Their remarkable ability to regenerate an entire animal from virtually any small body fragment (Sanchez Alvarado *et al.*, 2002; Handberg-Thorsager *et al.*, 2008) is unparalleled among both vertebrates and invertebrates. Planarian regeneration is based on planarian adult stem cells (pASCs) also named neoblasts. Experiments in the late 80s suggested that neoblasts behave pluripotent as population (Baguña, 1989), but only recently has it been elegantly shown by single cell transplantation into irradiated hosts that at least some (clonogenic) neoblasts are indeed pluripotent (Wagner *et al.*, 2011). Purification of pASCs by FACS (fluorescence-activated cell sorting) is possible by staining isolated planarian cells for nuclear content (Hoechst 33342) and cytoplasmic size (Calcein AM). Since pASCs are the only proliferating cells in the asexual strains of *Schmidtea mediterranea,* irradiation efficiently ablates them. This allowed identifying one irradiation-insensitive (Xin) and two irradiation-sensitive (X1 and X2) cell populations (Reddien *et al.*, 2005; Hayashi *et al.*, 2006). While the X2 population is partially irradiation-sensitive and

Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; FACS, flourescence-activated cell sorting; pASC, planarian adult stem cell; PMP, pASC plasma membrane proteins.

***Address correspondence to:** Luca Gentile. Planarian Stem Cell Laboratory, Max Planck Institute for Molecular Biomedicine, von-Esmarch-Str. 54, 48149 Münster, Germany. Tel: +49-251-8346-870. Fax: +49-251-8346-900. e-mail: luca.gentile@mpi-muenster.mpg.de

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hence heterogeneous by definition, the X1 population appears to be rather homogeneous, as many genes required for proper pASCs function are expressed by the entire stem cell population (reviewed in Gentile *et al.*, 2011). However, there is some evidence for heterogeneity of the X1 population at both the ultrastructural (Higuchi *et al.*, 2007) and the transcript levels (Eisenhoffer *et al.*, 2008; Hayashi *et al.*, 2010; Pearson and Sanchez Alvarado, 2010; Scimone *et al.*, 2010).

Isolation of specific cell types – including stem cells – could be achieved using antibodies specific to cell surface epitopes. One example are the long-term self-renewing human hematopoietic stem cells, which are defined by a remarkably long set of cell surface markers (Lin-/CD34+/CD38-/CD90+/CD45RA-; Notta *et al.*, 2010). Compared to other model organisms, available antibodies against planarian cells are scarce and directed against intracellular epitopes. Moreover, none has been described to be specific for pASCs so far (Bueno *et al.*, 1997). It is conceivable that different planarian cell types also differ in their cell surface epitopes, which is why we opted for generating a library of mouse monoclonal antibodies against the planarian membrane proteome. In order to produce such a library, we needed to ensure efficient and reliable isolation of large amounts of planarian cells. Papain (Papaya peptidase I) is a cysteine protease with a broad specificity, which preferentially hydrolyzes hydrophobic and aromatic residues (Kimmel and Smith, 1954), which is often used for the dissociation of neural tissues (Moritz *et al.*, 2008).

With this work, we raised the first library of mouse monoclonal antibodies directed against the plasma membrane proteome of planarian cells. The majority of these antibodies recognized surface epitopes of all or portions of the planarian cells. Among them, two antibodies (6-9.2 and 8-22.2) identified subfractions of the X1 population that we further characterized at the transcript level. All the stem cell markers tested did not reveal any difference in their expression compared to the negative counterparts, while the markers specific for both early and late progeny (Eisenhoffer *et al.*, 2008) were unequivocally upregulated. This finding was not related to a specific phase of the cell cycle, thus we hypothesized a correlation with the early commitment of proliferating stem cells. Further experiments are needed to substantiate this hypothesis, in order to establish when and how the planarian stem cells become committed and to elucidate the role of the early and late progeny genes, whose function in planarians is not known yet. All the genes mentioned in this paper are planarian genes, thus the prefix 'Smed-' is omitted.

Results and Discussion

Planarian cell dissociation with papain improves both yield and cell viability

In order to attain a sufficient amount of plasma membrane proteins (PMPs) for the downstream immunization, an efficient method for the dissociation of intact animals into a single cell suspension is required. In recent years, various methods have been used to dissociate planarian tissues – mechanical (Fernandez-Taboada *et al.*, 2010), trypsin-based (Reddien *et al.*, 2005; Hayashi *et al.*, 2006) and collagenase-based dissociation (Wagner *et al.*, 2011). Papain is a gentle protease that has been successfully used to isolate cells with rather complex morphologies, such as visual neurons from adult rats (Huettner and Baughman, 1986). Thus, the effectiveness of the papain-based planarian cell dissociation was compared to the trypsin-based (according to Hayashi *et al.*, 2010) and the mechanical dissociation. To quantify the efficiency of the dissociation protocols in terms of cell yield and viability, the animals were weighed before dissociation. Dissociated cells were stained with Hoechst 33342 and Calcein AM (H/C) and propidium iodide (PI) and analyzed by flow cytometry. The gating logic of our flow cytometric analysis is depicted in Fig. 1A. Papain-based dissociation yielded $4.2 \times 10^5 \pm 8.3 \times 10^4$ cells/mg, trypsin-based dissociation $1.3 \times 10^5 \pm 6.7 \times 10^4$ cells/mg and mechanical dissociation $3.3 \times 10^5 \pm 4.2 \times 10^4$ cells/mg (Fig. 1B). Both papain-based and mechanical dissociation yielded significantly more cells per milligram of animal weight than trypsin-based dissociation ($p \leq 0.01$). Papain-based dissociation yielded 62.2 ± 11.1% of live cells on total events, whereas mechanical dissociation yielded 49.1 ± 14.6% and trypsin-based dissociation 38.7 ± 2.1% of live cells (Fig. 1C). Interestingly, the percentage of live cells in the samples dissociated with papain is significantly higher compared to those dissociated with trypsin ($p \leq 0.01$; Fig. 1C). Also the percentage of single cells in the cell suspension is significantly higher for papain-based dissociation compared to both trypsin-based ($p \leq 0.01$, Fig. 1C) and mechanical dissociation ($p \leq 0.05$, Fig. 1C). Concerning the planarian FACS populations defined by H/C staining, a similar proportion of X1 and X2 cells was found in the papain-based and mechanical, but not in the trypsin-based preparation. ($p \leq 0.05$; Fig. 1C). Also, the percentage of the Xin cells obtained by using papain-based dissociation (13.5 ± 5.5%) was much higher than the one obtained with trypsin-based dissociation (5.2 ± 0.5%; $p \leq 0.01$; Fig. 1C).

As our immunization strategy required a large amount of planarian single cells for the isolation of immunogens, we aimed to optimize the dissociation protocol. Previously, we utilized mechanical dissociation (Fernandez-Taboada *et al.*, 2010), which produces a good yield of viable cells, but is time consuming and laborious. We therefore tested whether papain could be a suitable protease for the dissociation of planarian cells. All the dissociation methods tested yielded a cell suspension suitable for flow cytometry. However, the number of cells isolated per milligram of animal weight varied greatly among the methods, with papain-based dissociation being the most efficient one. The low efficiency of trypsin-based dissociation was in part due to the stickiness of the tissue fragments after incubation with the protease. Another reason is that many fragments could not be completely dissociated. This is also the case using mechanical and, to a lesser degree, papain-based dissociation. Nevertheless, the poor results obtained with trypsin-based dissociation might also be attributed to the fact that the protocol was optimized on another planarian species, *Dugesia japonica*. Flow cytometric analysis revealed that the cell suspension obtained using papain contains less debris (events with sub-G1 DNA content) and more viable cells compared to the other methods tested. Papain-based cell dissociation also performs best when the relative proportion of the planarian cell populations is taken into account. Our data suggest that Xin cells are particularly sensitive to both mechanical and trypsin-based dissociation. This may be due to the incomplete dissociation and/or the lysis of the large differentiated cells and implies that papain-based dissociation reflects the total planarian cell population more accurately.

In summary, we could convincingly show that our papain protocol

is a dissociation method that combines high yield, improved cell viability and a better representation of the planarian cell populations with ease of use. With our papain protocol it is possible to generate hundreds of millions planarian cells for any downstream purpose within approximately 1.5 hours. On the other hand, the dissociation of very small samples such as dissected blastemata is also possible without significant loss of cells.

Generation of antibodies against planarian plasma membrane proteins

We aimed to generate antibodies directed against cell surface epitopes of planarian cells, with a focus on antibodies specific to pASCs or subpopulations thereof. In a preliminary attempt, we immunized mice using sorted neoblasts of the X1 fraction. Although various antibodies – including cell type-specific ones – were generated, all of them (n=23) were directed against intracellular epitopes (data not shown).

We therefore changed our strategy and immunized the mice using purified PMPs. The workflow for the generation of the antibody library is depicted schematically in Fig. 2A. Purified planarian PMPs were injected intraperitoneally into BALB/C mice and, after four immunizations, immune sera (IS) were taken and tested by Western blotting. Equal amounts of planarian PMPs and whole cell lysate were loaded and probed with two IS from different mice (3018 and 3019). Several distinct bands ranging from 20 to 150 kDa were detected in the lanes where PMPs were loaded, whereas we found no signal in the whole cell lysate lanes (Fig. 2B). The absence of detectable signals in whole cell lysate is probably due to the low abundance of membrane proteins in the preparation. A control IS (from a non-immunized BALB/C mouse) did not reveal any signals. Although both mice showed comparable immune reactions, mouse 3018 displayed stronger signals, especially in the high molecular weight range (Fig. 2B). The IS were also tested by immunocytochemistry (ICC) on isolated live planarian cells. Immune sera from both the immunized mice showed membrane staining (Fig. 2C) but, similar to the Western blot, IS 3018 showed a stronger signal. Interestingly, IS 3018 produced signals for both the IgM and IgG antibody isotypes, while IS 3019 predominantly showed signals for the IgG isotype. Considering the strength and quality of the immune reaction observed, we therefore decided to use mouse 3018 for the generation of the hybridoma library.

In total, 960 hybridomas were picked from the cloning plates and expanded. The hybridoma supernatants were tested for immunoreactivity against PMPs by ELISA. Eighty-nine hybridoma supernatants reacted with PMPs and 76 hybridoma clones could be expanded. To investigate whether the antibodies could successfully label planarian cells, ICC was performed. Of 76 super-

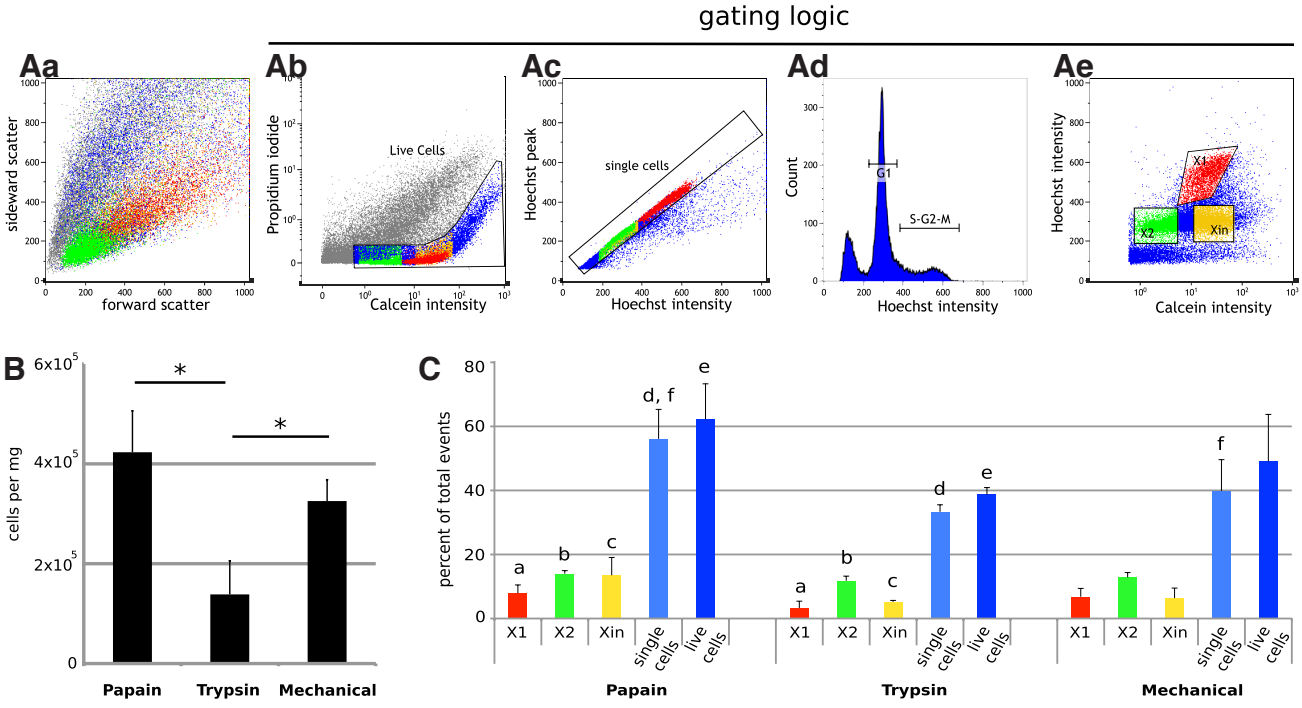

Fig. 1. Papain-based dissociation of planarian cells is highly efficient. *Planarians were dissociated using papain or trypsin as protease or by mechanical dissociation and stained with Hoechst 33342 / Calcein AM / Propidium iodide. Forward scatter versus sideward scatter plot of total events of our flow cytometric analysis is shown.* **(Aa)** *The events were triggered on Hoechst staining and gated for live cells (Calcein$^+$ / Propidium iodide$^-$;* **(Ab)***), then for single cells (Hoechst^{intensity} versus Hoechst^{peak};* **(Ac)***). The Hoechst histogram for live single cells reveals different nuclear contents of the planarian cells, which correspond – for the majority of the cells – to different phases of the cell cycle (G1 = 2n; 2n < S-G2-M ≤ 4n;* **(Ad)***). The dot plot in which Calcein intensity is plotted against Hoechst intensity allows the identification of the planarian FACS populations X1 (red), X2 (green) and Xin (yellow)* **(Ae)***. The different fractions are mapped back with their respective colors to the plots shown in* **(Aa-c)***. The animals were weighed before dissociation and the yield of each dissociation method was expressed as cells per milligram of animal* **(B)***; n = 4. The cell suspensions obtained with either method were analyzed by flow cytometry* **(C)***; n = 4. The number of the events that correspond to each gate (X1, X2, Xin, single cells and live cells) is expressed as percentage of total events. a, b, f: $p \leq 0.05$; *, c, d e: $p \leq 0.01$.*

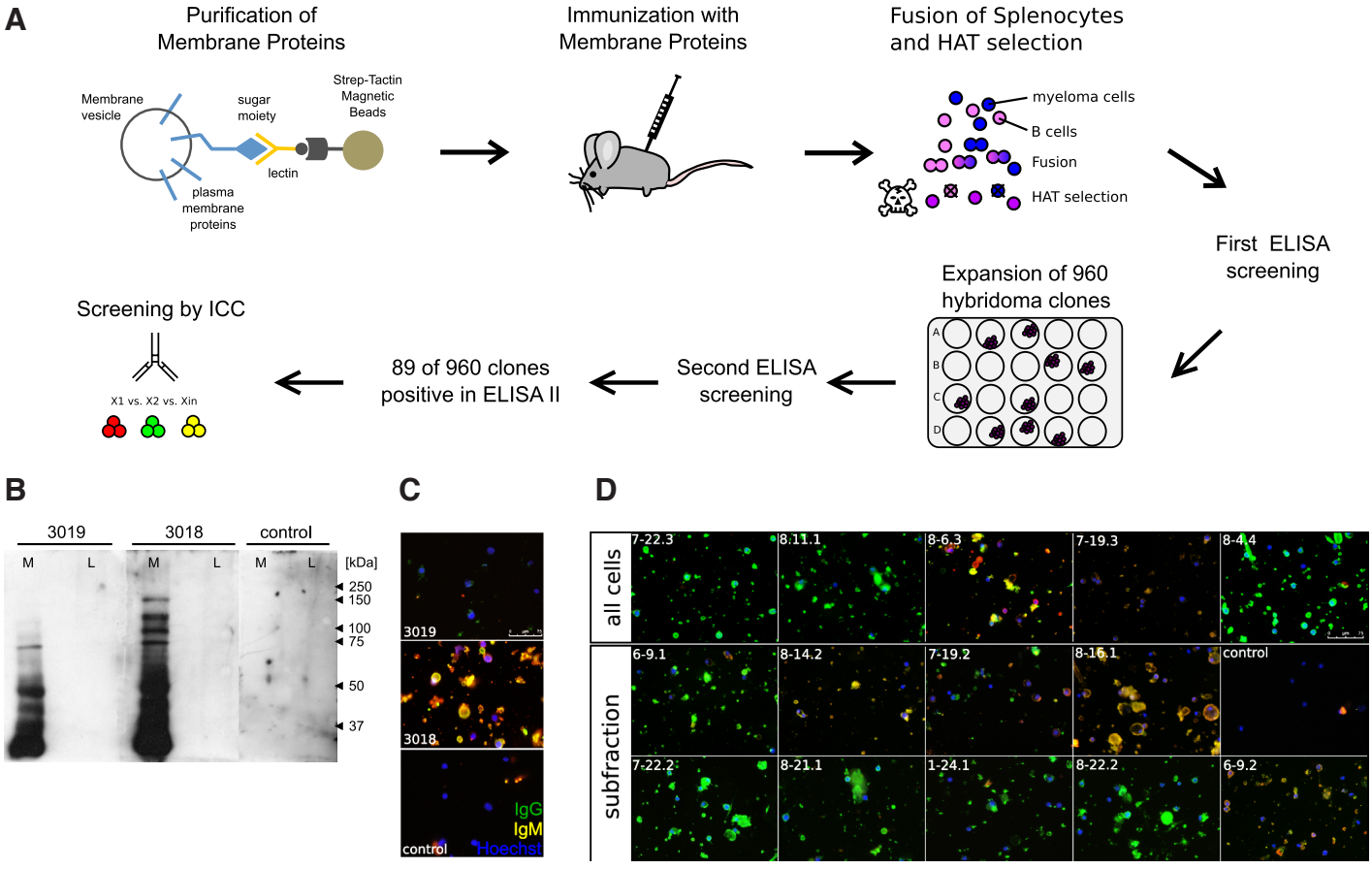

Fig. 2. Generation and screening of the mouse anti-planarian cell surface protein library of monoclonal antibodies. *Workflow followed for generating and screening the monoclonal antibody (mAb) library* **(A)**. *Planarian plasma membrane proteins (PMPs) were isolated using the Qproteome plasma membrane kit. Two mice were immunized four times with 50 μg of purified PMPs. Mouse 3018 was sacrificed and splenocytes were isolated. After PEG-assisted fusion with myeloma cells and HAT selection of the hybridomas, supernatants were screened by ELISA for reactivity against planarian PMPs. After expansion, clones were screened by a second round of ELISA. Eighty-nine hybridomas were found to produce mAbs with high affinity for PMPs. Planarian PMPs and whole cell lysates were loaded in equal amounts and separated by SDS-PAGE. The proteins were blotted onto PVDF membrane and probed with immune sera from mice 3019 and 3018 and control serum from a non-immunized mouse* **(B)**. *Hybridoma clones were screened by immunocytochemistry on planarian cells* **(C)**. *Signals for mouse-IgGs are shown in green, signals for mouse-IgMs are shown in yellow and nuclei were counterstained with Hoechst 33342 (shown in blue). A panel of antibodies that reacted with planarian live cells is shown* **(D)**. *The upper row (all cells) depicts hybridomas producing antibodies that immunostained virtually every planarian cells. The lower rows (subfractions) depict hybridomas whose antibodies immunostained a subfraction of total planarian cells. Scale bars: 75 μm; M, PMP; L, NP-40 whole cell lysate.*

natants tested, 51 showed reactivity against live planarian cells or subfractions thereof. In Fig. 2D a panel of hybridoma supernatants is presented that either recognize all planarian cells (upper panel) or subfractions of them (lower panel). Control mouse IgGs did not produce any staining (center-right). We also tested the hybridoma library on fixed and permeabilized cells. Sixty-six supernatants reacted to all planarian cells or subfractions thereof, indicating that 15 hybridomas solely recognized intracellular epitopes, whereas many of the extracellular epitopes seem to be resistant to paraformaldehyde fixation (data not shown).

In summary, PMPs were purified from planarian cells dissociated with papain and used for the immunization of two mice. The immunization succeeded, as shown by Western blotting and immunostaining and mouse 3018 was chosen to generate the antibody library according to the higher intensity and the overall better quality of the immunoreaction. The 3018 library consists of 66 hybridoma clones, 51 of which produce antibodies reacting against all live planarian cells (n=26) or subpopulations of them (n =25), while the remaining 15 antibodies detect intracellular epitopes. The ratio of antibodies suitable for live cell immunostaining (77%) is relatively high, and underscores the high efficiency of our approach to generate cell surface-specific antibodies.

Some of the antibodies generated are specific for subsets of the planarian FACS populations

To further investigate our antibody library, we selected four clones (1-24.1, 6-9.2, 7-22.2 and 8-22.2) that reacted with subfractions of live planarian cells. In order to establish which cell population reacted with the respective antibody, planarian cells were sorted according to H/C/PI staining and immunostained with the hybridoma supernatants (Fig. 3 A-D). In each fraction, the percentage of cells positive for each of the 4 antibodies was determined. Hybridoma clone 1-24.1 (IgG1, κ) did not stain any X1 cells, but 11 ± 2% of the X2 cells and 32 ± 8% of the Xin cells (Fig. 3D'), hybridoma

clone 6-9.2 (IgM, κ) stained 45 ± 11% of the X1 cells, 35 ± 8% of the X2 cells and 36 ± 14% of the Xin cells (Fig. 3C'), hybridoma clone 7-22.2 (IgG1, κ) stained 84 ± 4% of the X1 cells, 94 ± 8% of the X2 cells and 84 ± 5% of the Xin cells (Fig. 3B') and hybridoma clone 8-22.2 (IgG1, κ) stained 76 ± 6% of the X1 cells, 42 ± 4% of the X2 cells and 64 ± 3% of the Xin cells (Fig. 3A').

Determining the percentage of the sorted cells that were positive for each of the 4 antibodies, we found that hybridoma clone 1-24.1 did not stain any cells in the X1 fraction, a few cells in the X2 and approximately 30% of the cells in the Xin fraction. Although clone 1-24.1 does not recognize the stem cells, it fulfilled our expectations to find antibodies specific for subpopulations of differentiated cells. This could either be used to sort a specific differentiated cell type or to exclude it from a planarian cell suspension (negative selection). Eventually, none of the four clones analyzed displayed an immunostaining pattern compatible with a pure population of stem cells (high in X1, low in X2 and none in Xin); however, antibodies 6-9.2, 7-22.2 and 8-22.2 immunostained a different percentage of any given FACS populations (Fig. 3), and therefore we thought it unlikely that the three antibodies recognized the same cell surface antigen. It was equally unlikely that each of the antibodies labeled a specific cell type, because the added percentages of the cells positive for each antibody in each population largely exceeded 100%. Although we expected heterogeneity in the X2 fraction, which is a mixture of stem cells in the G1 phase, differentiating and differentiated cells (Higuchi *et al.*, 2007; Eisenhoffer *et al.*, 2008), and in the Xin fraction, which contains various types of large differentiating and differentiated cells, such a heterogeneity of the X1 fraction – as revealed by cell surface marker expression – was not anticipated. Thus, we further investigated the planarian cell fractions positive and negative for the respective antibodies using quantitative PCR (qPCR).

Antibodies 6-9.2 and 8-22.2 revealed a subset of X1 cells expressing early and late progeny genes

In order to examine more closely the heterogeneity of the FACS populations as revealed by antibodies 6-9.2, 8-22.2 and, to a minor degree, 7-22.2, we assessed the expression signature of the cell subfractions by qPCR. We quantified the expression of several genes, such as markers of stem, differentiating and differentiated cells.. After incubation with 6-9.2, 8-22.2 or 7-22.2 supernatants followed by appropriate secondary antibodies, live cells were sorted in accordance with the intensity of the immune signal. Normal mouse IgGs were used to set the gates in such a way as to have less than 4.0% of positive cells for each of the FACS fractions. Presumably, these few positive cells were the consequence of unspecific antibody binding (Fig. 4 A-C). Immunostaining with 6-9.2 produced a bimodal distribution of the signal for both the X1 and Xin populations, with negative and positive peaks. The signal of the $X2^{6\text{-}9.2+}$ cells is more broadly dispersed. Within the X1 population 58.1 ± 2.2% of the cells were $6\text{-}9.2^{+}$, while 30.1 ± 1.3% were $6\text{-}9.2^{-}$ (Fig. 4A'). In the X2 population 40.6 ± 2.6% of the cells were $6\text{-}9.2^{+}$ and 46.4 ± 2.6% were $6\text{-}9.2^{-}$ (Fig. 4B'), while in the Xin population 51.3 ± 1.9% of the cells were $6\text{-}9.2^{+}$ and 31.9 ± 1.9% were $6\text{-}9.2^{-}$ (Fig. 4C'). Remarkably, both the 6-9.2 positive and negative subsets of cells plot back to the H/C gate without revealing any peculiar distribution related to the cell cycle (Fig. S1).

For each subfraction, the expression of *Piwi1* (*Piwil1*, stem cell marker; Reddien *et al.*, 2005), NB.32.1g, *Agat1* (*Gatm*), *Cyp1a1* (early and late progeny markers; Eisenhoffer *et al.*, 2008) and *Myhc* (T-mus, myocytes and differentiated muscle cell marker; Cebrià *et al.*, 1996) was assessed. In agreement with published data (Reddien *et al.*, 2005; Eisenhoffer *et al.*, 2008), *Piwi1* was expressed at high levels in the X1 population and at low and very low levels in the X2 and Xin populations, respectively. No differ-

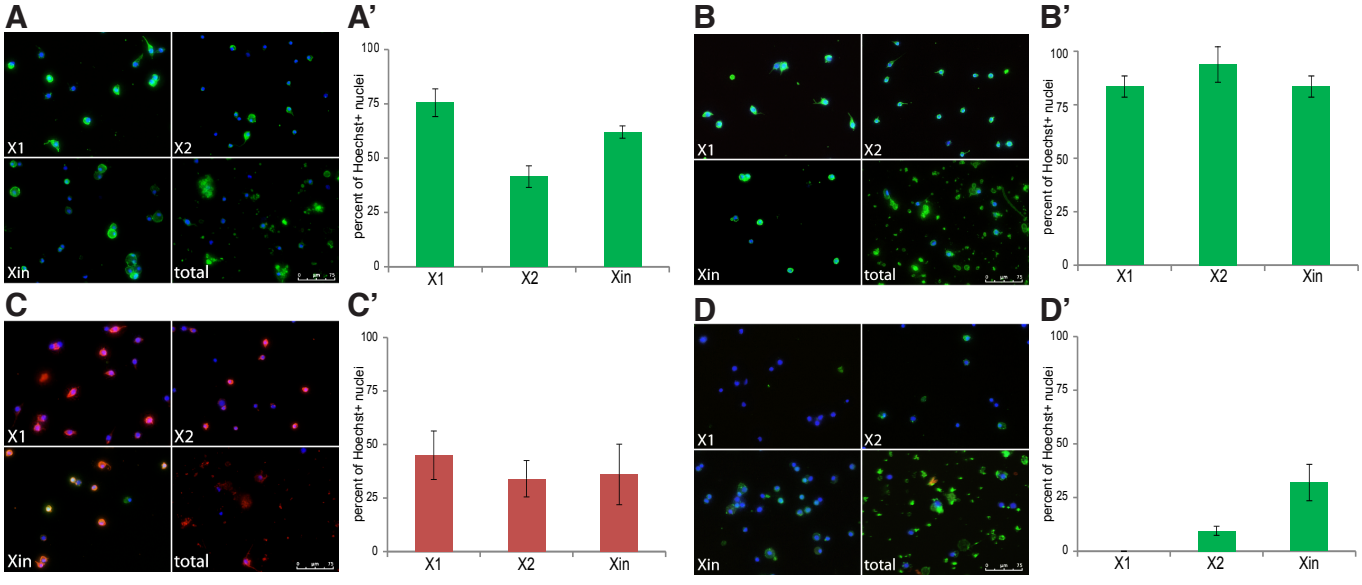

Fig. 3. Immunocytochemistry of selected antibodies on X1, X2 and Xin FACS populations of planarian cells. *Total planarian cells were stained with Hoechst 33342 and CalceinAM and sorted according to the FACS gating depicted in Fig. 1A. Cells were plated on plastic dishes and live-immunostained with the supernatants of the hybridomas 8-22.2* **(A)**, *7-22.2* **(B)**, *6-9.2* **(C)** *and 1-24.1* **(D)** *and fixed. Photomicrographs of immunostained sorted planarian cells of the X1, X2 and Xin fractions as well as total planarian cells are shown. Signals for mouse-IgGs are shown in green, signals for mouse-IgMs are shown in yellow and nuclei were counterstained with Hoechst 33342 (shown in blue). The number of immunostained cells is expressed as percentage of positive nuclei per total nuclei* **(A'-D')**. *ICC data are presented as Mean ± SD of 2 independent experiments; scale bar: 75 μm.*

ences were found between the 6-9.2$^+$ and 6-9.2$^-$ subfractions within each population (Fig. 4D). *Bruli* (*Celf3*; Guo *et al.*, 2006), *SmB* (*Snrpb*; Fernandez-Taboada *et al.*, 2010) and *cyclin B* (*Ccnb1*; Reddien *et al.*, 2005) were also investigated, and none of them displayed a differential expression between the cells in the positive and negative subfractions (Fig. S2). Also the expression of *Myhc* did not show any significant differences, with the exception of the X1 fraction, where 6-9.2$^+$ cells showed a 3-fold upregulation compared to the 6-9.2$^-$ cells ($p \leq 0.05$, Fig. 4E). Remarkably, the expression of *Myhc* in some X1 cells was also recently reported in *D. japonica* (Hayashi *et al.*, 2010). Interestingly, all markers of progeny cells tested (NB.32.1g, *Agat1*, *Cyp1a1*) were found upregulated in all the 6-9.2$^+$ subfractions. The early progeny marker NB.32.1g was expressed at moderate/high levels in X2$^+$ and Xin$^+$ cells, and at low level in X1$^+$ cells. Comparing positive to negative subfractions, NB.32.1g was upregulated about 16-fold in the X1$^+$ cells ($p \leq 0.0001$), 2-fold in the X2$^+$ cells ($p \leq 0.0001$) and 4-fold in the Xin$^+$ cells ($p \leq 0.0001$, Fig. 4F). *Agat1*, a late progeny marker, was also moderately upregulated in all the 6-9.2$^+$ subfractions, namely 7-fold in the X1$^+$ ($p \leq 0.0001$), 2.5-fold in the X2$^+$ ($p \leq 0.05$) and 5-fold in the Xin$^+$ subfraction ($p \leq 0.05$; Fig. 4G). A similar albeit not statistically significant trend was observed for the other late progeny marker, *Cyp1a1* (Fig. S3A).

We repeated the qPCR on positive and negative subfractions for the antibodies 8-22.2 and 7-22.2. Although in the comparison with 6-9.2 the percentage of 8-22.2 positive cells in each fraction was different (Fig. S4 A'-C'), a striking similarity was found when qPCR data were compared. In fact, X1$^{6\text{-}9.2+}$ and X1$^{8\text{-}22.2+}$ cells are indistinguishable according to the expression of NB.32.1g and *Agat1*, ($p=0.3640$ and $p=0.5179$, respectively; Fig. 4 D-G and Fig. S4 D-G). Considering also that the immune signal intensity distributions for the two antibodies are similarly shaped, (cf. Fig. 4A'-C' with Fig. S4 A'-C'), we cannot exclude that the divergence found in the cell number might result from intrinsic differences between the two antibodies – such as antibody isotypes or affinity to the epitope – rather than from the recognition of a different antigen.

The scenario changes radically when the 7-22.2 antibody is considered. The signal intensity distribution was bimodal for all the FACS fractions (Fig. S5 A'-C'). However, for the X1 fraction we observed a large proportion of cells staining positive for the antibody, leaving few cells in the negative gate (Fig. S5A'). These data correlate very well with our ICC data (Fig. 3B'), while for the other two populations – X2 and Xin – we found that the positive cells were fewer than those counted in ICC. As for the 6-9.2 and 8-22.2 immunostainings, 7-22.2 positive and negative subfractions of both the X1 and Xin population also showed a similar expression of *Piwi1*, while X2$^+$ cells expressed the stem cell marker at a level doubling that of their negative counterpart ($p \leq 0.05$; Fig. S5D). On the other hand, *Myhc* is expressed at very low levels in all the negative subfractions, whereas it is upregulated 4-, 6- and 14-fold in X1$^+$, X2$^+$ and Xin$^+$ subfractions, respectively ($p \leq 0.05$; Fig. S5E). Once more, significant differences were also found between positive and negative subfractions in the expression of both early and late progeny markers. However, contrary to what we observed for both 6-9.2 and 8-22.2 antibodies, NB.32.1g, *Agat1* and *Cyp1a1* were found upregulated in the negative subfractions of the 7-22.2 immunostained cells. Specifically, NB.32.1g was upregulated 5- and 7-fold in X2$^-$ and Xin$^-$ cells, respectively ($p \leq 0.0001$; Fig S4F),

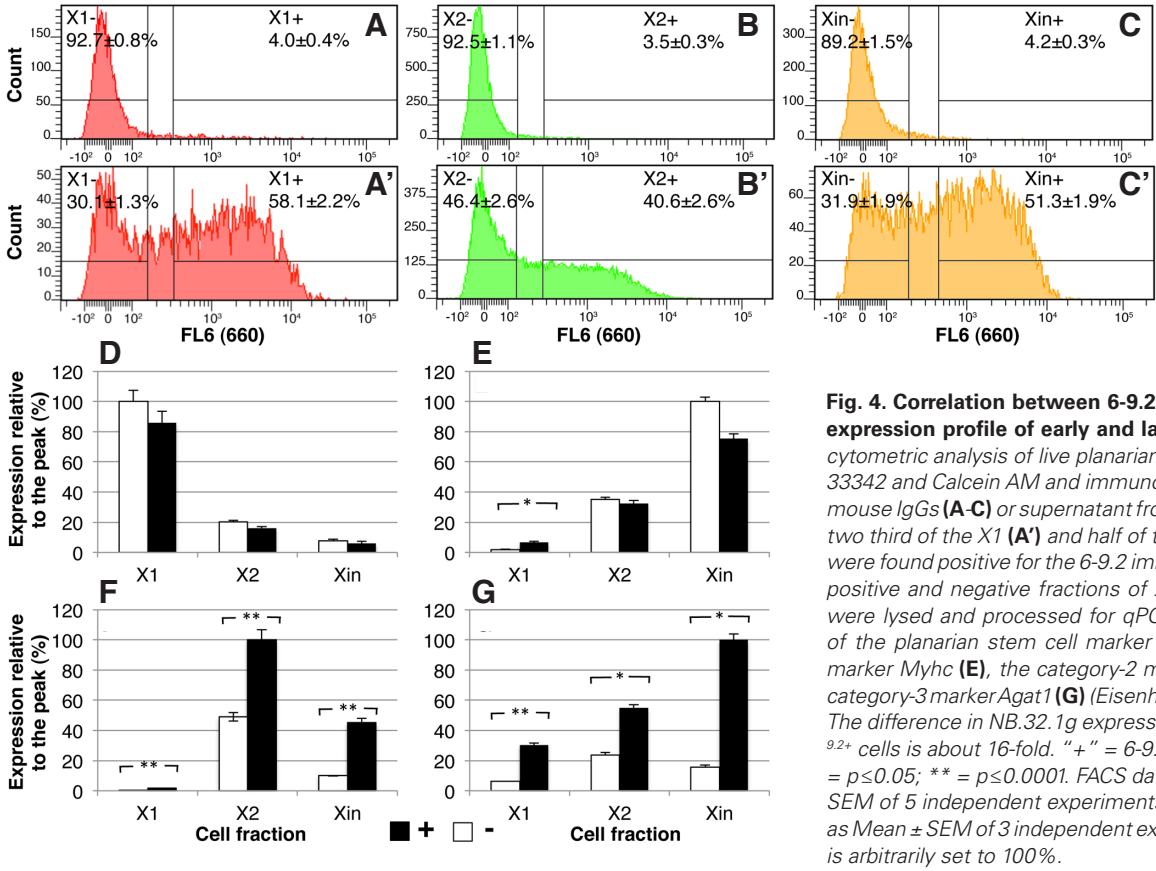

Fig. 4. Correlation between 6-9.2 immunostaining and the expression profile of early and late progeny markers. *Flow cytometric analysis of live planarian cells stained with Hoechst 33342 and Calcein AM and immunostained with either generic mouse IgGs* **(A-C)** *or supernatant from clone 6-9.2* **(A'-C')**. *About two third of the X1* **(A')** *and half of the X2* **(B')** *and Xin* **(C')** *cells were found positive for the 6-9.2 immunostaining. After sorting, positive and negative fractions of X1, X2 and Xin populations were lysed and processed for qPCR analysis. The expression of the planarian stem cell marker Piwi1* **(D)**, *the muscle cell marker Myhc* **(E)**, *the category-2 marker NB.32.1g* **(F)** *and the category-3 marker Agat1* **(G)** *(Eisenhoffer* et al., *2008) are shown. The difference in NB.32.1g expression between X1$^{6\text{-}9.2\text{-}}$ and X1$^{6\text{-}9.2+}$ cells is about 16-fold. "+" = 6-9.2$^+$ cells; "-" = 6-9.2$^-$ cells; * = $p \leq 0.05$; ** = $p \leq 0.0001$. FACS data are presented as Mean ± SEM of 5 independent experiments; qPCR data are presented as Mean ± SEM of 3 independent experiments; peak expression is arbitrarily set to 100%.*

while *Agat1* was upregulated 5-, 4- and 6-fold in the $X1^-$, $X2^-$ and Xin^- subfractions, respectively ($p \leq 0.01$, $p \leq 0.0001$ and $p \leq 0.0001$; Fig. S5G). Expression of NB.32.1g did not differ between $X1^+$ and $X1^-$ subfractions, while *Cyp1a1* was significantly upregulated only in the Xin^- fraction (Fig. S3C).

In an attempt to better understand the molecular features of the subfractions of cells labeled by some antibodies of the 3018 library, we assessed the expression of markers specific for stem, differentiating and differentiated cells in the subsets of cells defined by 6-9.2, 7-22.2 or 8-22.2 immunostaining. Interestingly, we never found any difference in the expression levels of the stem cell markers tested within the same FACS population, independently of the antibody used (Fig. 4D; Fig. S2, S4D, S5D). This suggests that none of the antibodies is specific for antigens solely expressed by the pASCs. However, all the cells positive for either the 6-9.2 or 8-22.2 antibody were highly enriched for the category-2 marker NB.32.1g, with a clear upregulation in the $X1^{6\text{-}9.2+}$ and the $X1^{8\text{-}22.2+}$ cells. A statistically significant upregulation was also observed for the category-3 marker *Agat1*. Regardless of whether 6-9.2 and 8-22.2 antibodies recognize the same antigen or not, they do define subfractions of the X1 population, as does the antibody 7-22.2. The percentage of $6\text{-}9.2^+$ cells in the X1 population is quite high (about 60%), but the expression of both NB.32.1g and *Agat1* is relatively low, when compared to both X2 and Xin populations. According to published results (Eisenhoffer *et al*., 2008; Table S2), only a small proportion of X1 cells expresses the progeny markers; specifically, the category-2 marker NB.21.11e and the category-3 marker *Agat1* are expressed by 0.7% and 2.4% of the X1 cells, respectively. It could be assumed that the category-2 marker that we considered (NB.32.1g) would behave in a similar way. Therefore, only a small portion of the $X1^{6\text{-}9.2+}$ cells expresses the progeny markers at high level, while the majority of the $X1^{6\text{-}9.2+}$ cells do not. These cells might express other progeny markers, either known or unknown, or represent a primed state of the stem cell, in which changes in the chromatin state are a prerequisite for changes in gene expression. This is consistent with the percentage of X1 cells expressing the chromatin remodeling protein CHD4, involved in, and essential for stem cell differentiation (Scimone *et al*., 2010). This gene is required for the differentiation of *Agat1*-expressing cells; hence its upregulation in early committed X1 cells could act as a trigger for the expression of the progeny markers and the effective differentiation of the stem cells.

Concluding remarks

The data presented in this paper indicate that: i) we successfully setup a papain-based dissociation protocol that improves both yield and cell viability compared to other published methods; ii) we established a library of 66 mouse monoclonal antibodies against planarian plasma membrane proteins, 51 of which show membrane-specific immunostaining of live cells; iii) approximately half of these antibodies recognize subpopulations of whole planarian cells and at least three of them (6-9.2, 7-22.2 and 8-22.2) recognize subfractions of the three FACS populations, demonstrating that the stem cells in S/G2/M phase (X1 cells) are heterogeneous at the level of surface markers; iv) the heterogeneity of the X1 population is not related to the progression throughout the cell cycle, since $X1^{6\text{-}9.2+}$ and $X1^{8\text{-}22.2+}$ do not cluster differently from $X1^{6\text{-}9.2-}$ and $X1^{8\text{-}22.2-}$ in relation to the Hoechst signal intensity; v) the integration of live-immunostaining FACS data and qPCR data revealed the existence of at least two discrete subpopulations of X1 cells, both expressing all the stem cell markers tested at the same level: one (about two third of the X1 population) is made up of $6\text{-}9.2(8\text{-}22.2)^+$/NB.32.1g$^+$/*Agat1*$^+$/*Myhc*$^+$ cells and one (about one third of X1 population) is made up of $6\text{-}9.2(8\text{-}22.2)^-$/NB.32.1g$^-$/*Agat1*$^-$/*Myhc*$^-$ cells, which probably consists of uncommitted self-renewing stem cells. Whether the $6\text{-}9.2^+$ (or $8\text{-}22.2^+$) cells in the X1 population are committed to differentiate (and therefore no longer self-renewing) can only be determined by putting their actual pluripotency to the test and by comparing the clonogenic efficiency of single $6\text{-}9.2^+$ or $6\text{-}9.2^-$ cells engrafted into a lethally irradiated host.

Materials and Methods

Species and maintenance

The animals (clonal line BCN10) used for this study were maintained as previously described (Fernandez-Taboada *et al*., 2010). Animals used for the experiments were starved for at least one week.

Cell dissociation

To determine the amount of cells isolated per milligram of live animal, the animals were weighted before dissociation. To this end, four 8 to 10 mm long animals were placed onto a glass slide and residual planarian water was completely removed using a KimWipe tissue. Unless differently stated, chemicals are from Sigma-Aldrich, Germany. Prior to dissociation the animals were incubated 1 minute in 2% L-cysteine hydrochloride pH 7.4 to remove the mucus. Mechanical and trypsin-based dissociation were performed as previously described (Fernandez-Taboada *et al*., 2010; Hayashi *et al*., 2010). For papain-based dissociation the animals were cut into small pieces on a glass slide. The pieces were then transferred using wide bore-hole 1000G tips (Art-tips, MβP, USA) into 1.5 ml reaction tubes using 250 μl CMFH (2.5 mM NaH_2PO_4 $2H_20$; 14.3 mM NaCl; 10.2 mM KCl; 9.4 mM $NaHCO_3$; 15 mM Hepes; 0.1% BSA; 0.5% Glucose; pH 7.2). Then 250 μl of 2 x papain solution (30U/ml Papain (Worthington Biochemical Corp., USA); 2 mM L-Cysteine in CMFH) was added and the reaction was incubated for 1 hour at 25°C. After adding 500 μl of 2 x STOP solution (1mg/ml chicken ovomucoid; 40μg/ml DNaseI (Worthington Biochemical Corp., USA) in CMFH) the pieces were gently triturated by pipetting. The cells were then washed once, resuspended in CMFH and counted using a hemocytometer.

Purification of planarian plasma membrane proteins

The purification of planarian plasma membrane proteins (PMPs) was performed using the Qproteome™ Plasma Membrane Protein Kit (Qiagen, Germany), according to the manufacturer's instructions. Because planarian cells are smaller than mammalian cells, $5x10^7$ cells were used for each preparation. Briefly, planarians were dissociated into a single cell suspension using papain. Cells were incubated in hypotonic buffer and subsequently mechanically disrupted using a 27-gauge needle. Large organelles (nuclei, mitochondria) were removed from the lysate by centrifugation. The PMPs were isolated from the supernatant by adding lectin-based ligands coupled to avidin, which were then bound to streptactin-coupled magnetic beads. The beads were subsequently separated by applying a strong magnetic field (Magnetic Particle Concentrator MPC-S, Dynal, Germany).

Immunization with plasma membrane proteins

Prior to immunization, isolated PMPs were mixed 1:1 with Adjuvant MM (GERBU, Germany). For each immunization approx. 20 μg of PMPs were used for intraperitoneal injection. After four rounds of immunization, immune sera were collected for testing.

Production of the antibody library

To produce the antibody library, mouse 3018 was sacrificed and the spleen

isolated and dissociated. Splenocytes were mixed 5:1 with myeloma cells P3-X63-Ag8 (ATCC, USA). Cell fusion was performed in the presence of polyethylene glycol as described previously (PEG 1500 - Serva, Heidelberg, Germany; Key *et al.*, 1993). The hybridoma cells were seeded into 8 cloning plates (cloning plate 24 well, 16 compartments/well, Greiner Bio-one, Germany) and selected in HAT (hypoxanthine, aminopterine, thymidine) medium for about 10 days. Hybridoma supernatants were screened for immunoreactivity against planarian PMPs by ELISA.

ELISA

Planarian PMPs were diluted to a concentration of 1μg/ml in PBS. Every well of a PVC microtiter plate was coated with 50μl of the diluted membrane proteins and incubated for 1 hour at RT or overnight at 4°C. Coating solution was removed and unspecific binding sites were blocked by adding 1% BSA in PBS for 30 minutes at RT. In each well, 50 μl of the respective hybridoma supernatant was added and incubated for 1 hour at RT. After rinsing with PBS for three times, PMP-coated plates were incubated with 50 μl of anti-mouse IgG- (Fc specific, 1:20000) and anti-mouse IgM-HRP-conjugated (1:1000) in 1% BSA in PBS for 1 hour at RT. After incubation with the secondary antibodies, PMP-coated plates were washed three times with PBS. For detection, 50 μl of substrate solution containing 60 ng/ml 3,3′,5,5′-tetramethylbenzidine (TMB) and 0.006% H_2O_2 in 0.1 M citrate buffer, pH 6.0 was dispensed in each well. After sufficient color development, 50 μl 2 M H_2SO_4 was added to the wells to stop the reaction. Absorbance was detected at 450nm with a plate reader (Synergy MX, Biotek Instruments, Germany).

Flow cytometry and cell sorting

Staining of planarian cells for flow cytometry was performed as previously described (Fernandez-Taboada *et al.*, 2010). Data were acquired using a Gallios flow cytometer (Beckmann Coulter, Germany). For live staining of planarian cells with the antibodies of the library, the cell suspension was incubated 90 minutes at RT with Hoechst 33342 and CalceinAM under agitation. Then, the respective hybridoma supernatant was added (1:4 in CMFH) and the cells were incubated for 20 additional minutes at RT under agitation. Subsequently, the cells were stained with rabbit anti-mouse Alexa Fluor 647 (1:1000 in CMFH) for 15 minutes. After filtering the cell suspension through a 30 μm nylon mesh, propidium iodide was added at a concentration of 1 μg/ml for dead cells exclusion. Fluorescence activated cell sorting was performed using a BD FACSAria II (Becton Dickinson, Germany).

Immunocytochemistry of planarian cells

Planarian cells were plated onto cell culture dishes coated with poly-ornithine (10 μg/ml in water). The cells were allowed to adhere to the plates for 2 hours at RT or overnight at 4°C. Subsequently, the cells were live stained with the respective hybridoma supernatant diluted 1:1 in CMFH for 15 minutes at RT. After wash in CMFH and fixation with 4% paraformaldehyde for 15 minutes, the cells were incubated with goat anti-mouse IgG Alexa Fluor 488 (1:500, Invitrogen, Germany), goat anti-mouse IgM Alexa Fluor 568 (1:500, Invitrogen, Germany) and 1 μg/ml Hoechst 33342 (Invitrogen, Germany) in PBS containing 1% BSA and 5% normal goat serum (Sigma-Aldrich, USA) for 30 minutes at RT. Imaging was performed using a Leica AF6000 inverted fluorescence microscope (Leica, Germany). To stain permeabilized planarian cells, the cells were fixed with 4% paraformaldehyde and permeabilized with PBS containing 0.3% Triton X-100 and 5% normal goat serum. Subsequently, the cells were immunostained as described above.

Quantitative realtime PCR

Reverse transcription and qPCR were performed as previously described (Fernandez-Taboada *et al.*, 2010). Briefly, 2 x 10^4 cells from each FACS subpopulation were collected and RNA was extracted using RNA XS columns (Machery & Nagel, Germany) according to the manufacturer's instructions. After reverse transcription with random hexamer primers, qPCR was performed using Taqman chemistry. Gapdh (mk4.002051.00) served as endogenous control. Relative quantification of gene expression was calculated using the ΔΔCt method, with calibration on the peak expression. Probes and primers are listed in supplemental Table 1. For each condition, three technical replicates for each of the three biological replicates were performed.

Western blotting

Planarians cells were lysed in 20 mM Tris-HCl pH 8.0; 150 mM NaCl; 1% Nonidet P-40; 10% Glycerol; 2 mM EDTA; 1x complete protease inhibitor (referred to as NP-40 lysis buffer). The lysate was homogenized through a 27 Gauge needle and cleared from cellular debris by centrifugation. Whole cell lysate and purified planarian membrane proteins were each mixed 1:1 with 2x Laemmli buffer (126 mM Tris-HCl pH 6.8; 20% Glycerol; 4% SDS; 10% β-Mercaptoethanol; 0.02% Bromphenol blue.) and denatured for 5 minutes at 99°C. The samples were separated by SDS-PAGE and transferred onto a PVDF membrane (Immobilon, Millipore, Germany). The membrane was blocked in PBS containing 0.1% Tween 20 and 5% non-fat dry milk powder (blocking solution; Bio-Rad, Germany) and probed with the immune sera 1:2000 in blocking solution. As secondary antibodies, goat anti-mouse IgM- and goat anti-mouse IgG-HRP conjugated 1:10.000 in blocking solution were used. Immunodetection was performed with ECL Plus Western Blotting Detection Reagents (Amersham Bioscience, Germany).

Statistical analysis

Prism 5.0 (Graphpad, USA) was used for the statistical analysis. Percentages of cells obtained by the different dissociation protocols and of the immunostained cells are expressed as Mean ± SD; The percentages of live-immunostained (FACS) cells and gene expression data are expressed as Mean ± SEM. Two-tailed t-test was used to compare side-by-side FACS and qPCR datasets. One-way ANOVA was used to weight the variance of the qPCR data from the 6-9.2 against 8-22.2 subfractions and to compare the cell dissociation methods (with Dunnet post-hoc test).

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References

BAGUÑA, J., SALO, E., AULADELL, C. (1989). Regeneration and pattern formation in planarians. III. Evidence that neoblasts are totipotent stem cells and the cells. *Development* 107: 77-86.

BUENO, D., BAGUNA, J. and ROMERO, R. (1997). Cell-, tissue-, and position-specific monoclonal antibodies against the planarian Dugesia (Girardia) tigrina. *Histochem Cell Biol* 107: 139-149.

CEBRIÀ, F., VISPO, M., BUENO, D., CARRANZA, S., NEWMARK, P. and ROMERO, R. (1996). Myosin heavy chain gene in Dugesia (G.) tigrina: a tool for studying muscle regeneration in planarians. *Int J Dev Biol* 40: S177-S178.

EISENHOFFER, G.T., KANG, H. and SANCHEZ ALVARADO, A. (2008). Molecular analysis of stem cells and their descendants during cell turnover and regeneration in the planarian Schmidtea mediterranea. *Cell Stem Cell* 3: 327-339.

FERNANDEZ-TABOADA, E., MORITZ, S., ZEUSCHNER, D., STEHLING, M., SCHOLER, H.R., SALO, E. and GENTILE, L. (2010). Smed-SmB, a member of the LSm protein superfamily, is essential for chromatoid body organization and planarian stem cell proliferation. *Development* 137: 1055-1065.

GENTILE, L., CEBRIA, F. and BARTSCHERER, K. (2011). The planarian flatworm: an *in vivo* model for stem cell biology and nervous system regeneration. *Dis Model Mech* 4: 12-19.

GUO, T., PETERS, A.H. and NEWMARK, P.A. (2006). A Bruno-like gene is required for stem cell maintenance in planarians. *Dev Cell* 11: 159-169.

HANDBERG-THORSAGER, M., FERNANDEZ, E. and SALO, E. (2008). Stem cells and regeneration in planarians. *Front Biosci* 13: 6374-6394.

HAYASHI, T., ASAMI, M., HIGUCHI, S., SHIBATA, N. and AGATA, K. (2006). Isolation of planarian X-ray-sensitive stem cells by fluorescence-activated cell sorting. *Dev*

Growth Differ 48: 371-380.

HAYASHI, T., SHIBATA, N., OKUMURA, R., KUDOME, T., NISHIMURA, O., TARUI, H. and AGATA, K. (2010). Single-cell gene profiling of planarian stem cells using fluorescent activated cell sorting and its "index sorting" function for stem cell research. *Dev Growth Differ* 52: 131-144.

HIGUCHI, S., HAYASHI, T., HORI, I., SHIBATA, N., SAKAMOTO, H. and AGATA, K. (2007). Characterization and categorization of fluorescence activated cell sorted planarian stem cells by ultrastructural analysis. *Dev Growth Differ* 49: 571-581.

HUETTNER, J.E. and BAUGHMAN, R.W. (1986). Primary culture of identified neurons from the visual cortex of postnatal rats. *J Neurosci* 6: 3044-3060.

KEY, G., BECKER, M.H., BARON, B., DUCHROW, M., SCHLUTER, C., FLAD, H.D. and GERDES, J. (1993). New Ki-67-equivalent murine monoclonal antibodies (MIB 1-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. *Lab Invest* 68: 629-636.

KIMMEL, J.R. and SMITH, E.L. (1954). Crystalline papain. I. Preparation, specificity, and activation. *J Biol Chem* 207: 515-531.

MORITZ, S., LEHMANN, S., FAISSNER, A. and VON HOLST, A. (2008). An induction gene trap screen in neural stem cells reveals an instructive function of the niche and identifies the splicing regulator sam68 as a tenascin-C-regulated target gene. *Stem Cells* 26: 2321-2331.

NOTTA, F., DOULATOV, S. and DICK, J.E. (2010). Engraftment of human hematopoietic stem cells is more efficient in female NOD/SCID/IL-2Rgc-null recipients. *Blood* 115: 3704-3707.

PEARSON, B.J. and SANCHEZ ALVARADO, A. (2010). A planarian p53 homolog regulates proliferation and self-renewal in adult stem cell lineages. *Development* 137: 213-221.

REDDIEN, P.W., OVIEDO, N.J., JENNINGS, J.R., JENKIN, J.C. and SANCHEZ ALVARADO, A. (2005). SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. *Science* 310: 1327-1330.

SANCHEZ ALVARADO, A., NEWMARK, P.A., ROBB, S.M. and JUSTE, R. (2002). The Schmidtea mediterranea database as a molecular resource for studying platyhelminthes, stem cells and regeneration. *Development* 129: 5659-5665.

SCIMONE, M.L., MEISEL, J. and REDDIEN, P.W. (2010). The Mi-2-like Smed-CHD4 gene is required for stem cell differentiation in the planarian Schmidtea mediterranea. *Development* 137: 1231-1241.

WAGNER, D.E., WANG, I.E. and REDDIEN, P.W. (2011). Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. *Science* 332: 811-816.

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