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Review Article

Cellular and molecular dissection of pluripotent adult somatic stem cells in planarians

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Freshwater planarians, Plathelminthes, have been an intriguing model animal of regeneration studies for more than 100 years. Their robust regenerative ability is one of asexual reproductive capacity, in which complete animals develop from tiny body fragments within a week. Pluripotent adult somatic stem cells, called neoblasts, assure this regenerative ability. Neoblasts give rise to not only all types of somatic cells, but also germline cells. During the last decade, several experimental techniques for the analysis of planarian neoblasts at the molecular level, such as *in situ* hybridization, RNAi and fluorescence activated cell sorting, have been established. Moreover, information about genes involved in maintenance and differentiation of neoblasts has been accumulated. One of the molecular features of neoblasts is the expression of many RNA regulators, which are involved in germline development in other animals, such as *vasa* and *piwi* family genes. In this review, we introduce physiological and molecular features of the neoblast, and discuss how germline genes regulate planarian neoblasts and what differences exist between neoblasts and germline cells.

Key words: adult somatic stem cell, germline-specific genes, neoblast, planarian, pluripotency.

Introduction

Adult somatic stem cells (ASCs) are maintained by self-renewal and differentiate into function-specific cells in order to replace dead and injured cells in various tissues. Due to their scientific appeal and potential medical application, stem cells in mammalian tissues are the best documented ASCs. However, ASCs exist in many species belonging to various phyla (Sánchez Alvarado & Kang 2005; Agata *et al.* 2006; Bosch 2008). Whereas the fundamental cellular and molecular bases of these ASCs are still poorly understood, the accumulation of knowledge about ASCs from diverse species may give us a good opportunity not only to understand the common principles of stem cells, but also guide their use in regenerative medicine.

Freshwater planarians, turbellarians belonging to phylum Plathelminthes, are known to possess ASCs called neoblasts (Baguñà 1981). *Dugesia japonica* is a common planarian species in Japan. It measures up to 2 cm in size and has clearly defined organs such as a well-organized brain with different types of neurons, a pharynx for feeding and excretion in the central portion of its body, and an intestine composed of three main branches (Fig. 1; Umesono *et al.* 1997; Agata *et al.* 1998; Kobayashi *et al.* 1998; Umesono *et al.* 1999; Cebria *et al.* 2002b,c; Nishimura *et al.* 2007a,b; Umesono & Agata 2009). Despite their complex body structure, planarians have robust regenerative ability, in other words, asexual reproduction ability. Almost any small fragment from anywhere behind the eyes of the planarian body can regenerate and develop into a complete animal, with a functional brain, within a week after amputation (Agata *et al.* 2003; Rossi *et al.* 2008; Umesono & Agata 2009). Planarians' remarkable ability to regenerate is strictly dependent on their neoblasts (Baguñà *et al.* 1989; Agata & Watanabe 1999; Newmark & Sánchez Alvarado 2002; Salò & Baguñà 2002; Agata *et al.* 2003, 2006; Reddien & Sánchez Alvarado 2004; Sánchez Alvarado 2006, 2007; Rossi *et al.* 2008). During regeneration, the neoblasts give rise to all types of cells, not only somatic but also germline cells (Sato *et al.* 2006; Handberg-Thorsager & Salò

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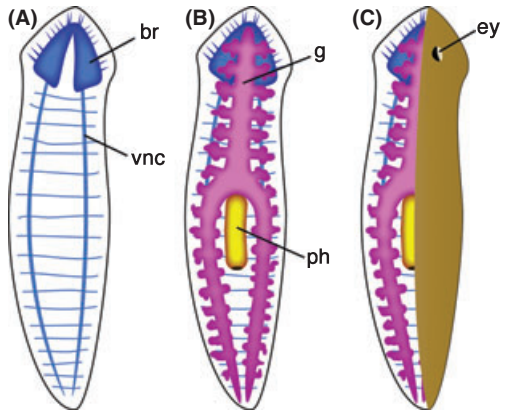

Fig. 1. Schematic illustration of main organs of *Dugesia japonica*. (A) Nervous system. A brain (br) exists in the anterior region of the ventral side. A pair of ventral nerve cords (vnc) extends from the brain to the tail tip. (B) Digestive organ. A pharynx (ph) exists in the central portion of the body and acts as a mouth and anus. A gut (g) occupies almost the entire mesenchymal space and delivers nutrition instead of a blood circulatory system. (C) A pair of eyes (ey) is observed in the head region of the dorsal side.

2007; Wang *et al.* 2007). Neoblasts also supply all cells in intact individuals during the 'wear and tear' of tissue homeostasis (Newmark & Sánchez Alvarado 2000).

Initially, neoblasts were defined by their morphological features, based on electron microscopy (Pedersen 1959; Morita 1967; Hori 1992). Ten years ago, we identified *DjvlgA* (*vasa-like gene A*), the first planarian gene identified with neoblast-enriched expression (Shibata *et al.* 1999). Since then, a considerable number of genes have been shown to be expressed in the neoblasts and to function in neoblast regulation in several planarian species (Salvetti *et al.* 2000; Ogawa *et al.* 2002; Sánchez Alvarado *et al.* 2002; Orii *et al.* 2005; Salvetti *et al.* 2005; Reddien *et al.* 2005a,b; Guo *et al.* 2006; Sato *et al.* 2006; Handberg-Thorsager & Salò 2007; Oviedo & Levin 2007; Rossi *et al.* 2007; Wang *et al.* 2007; Yoshida-Kashikawa *et al.* 2007; Palakodeti *et al.* 2008; Solana *et al.*, 2009). Furthermore, the establishment of a stem cell purification method, based on fluorescence activated cell sorting, has enabled us to analyze the cellular aspects of neoblasts in greater detail (Hayashi *et al.* 2006; Higuchi *et al.* 2007). Thus, much knowledge about the cellular and molecular bases of neoblasts has been accumulated during the past decade. Here, we introduce what is currently known about the cellular and molecular features of the neoblasts, and discuss the molecular mechanisms that regulate and maintain the pluripotency of neoblasts.

Morphological and physical features of the neoblasts

In the planarian, neoblasts (~30% of total cells) can be observed throughout the entire mesenchymal space of the body with the exception of the pharyngeal region and the region anterior to the eyes (Fig. 1; Baguñà *et al.* 1989; Newmark & Sánchez Alvarado 2000). This distribution pattern corresponds to the regenerative ability of *D. japonica*, in which complete regeneration can be achieved from body fragments anywhere posterior to the eyes except for the pharynx.

Neoblasts are the only cell population possessing continual proliferative ability in planarians. Newmark & Sánchez Alvarado (2000) were able to visualize mitotic cells using BrdU in a related species, *Schmidtea mediterranea*. Only neoblasts incorporate BrdU immediately after treatment, and almost all neoblasts incorporate BrdU within 2 days of injection (Kang & Sánchez Alvarado 2009). Some BrdU-incorporating cells are also visualized with anti-phosphorylated histone H3 antibody (pH3), an indicator of mitotic (M-phase) cells (Newmark & Sánchez Alvarado 2000). *D. japonica* neoblasts also incorporate BrdU specifically, and can be labeled with anti-pH3 antibodies (Fig. 2A–D; Inoue *et al.* 2007; Yoshida-Kashikawa *et al.* 2007; Higuchi *et al.* 2008). Genes related to the process of DNA replication such as *minichromosome maintenance 2* homologue, *DjMCM2*, or *proliferating cell nuclear antigen*, *Djpcna*, are also specifically expressed in the neoblasts (Salvetti *et al.* 2000; Orii *et al.* 2005). The cell cycle of neoblasts is accelerated by feeding or amputation of the animals (Baguñà 1974, 1975; Kang & Sánchez Alvarado 2009), which probably indicates the expeditious supplying of cells that accompanies regeneration and growth of an individual.

Classically, electron microscopic observations revealed the typical morphological features of neoblasts. Neoblasts are approximately 10 μm in size, of round or ovoid shape, and show a high nucleus/cytoplasm ratio (Fig. 2E). No obvious organelles other than free ribosomes and mitochondria are observed in their scanty cytoplasm (Pedersen 1959). An unambiguous structural feature of neoblasts is cytoplasmic chromatoid bodies. Chromatoid bodies are observed as are round, electron-dense structures that are not surrounded by a membrane (Fig. 2E; Morita 1967; Coward 1974; Hori 1982). These structures show sensitivity to Ribonuclease A and actinomycin-D treatment (Hori 1982; Auladell *et al.* 1993), indicating that these structures are ribonucleoprotein (RNP) complexes. The size and number of chromatoid bodies decreases during the neoblast cell differentiation process (Hori

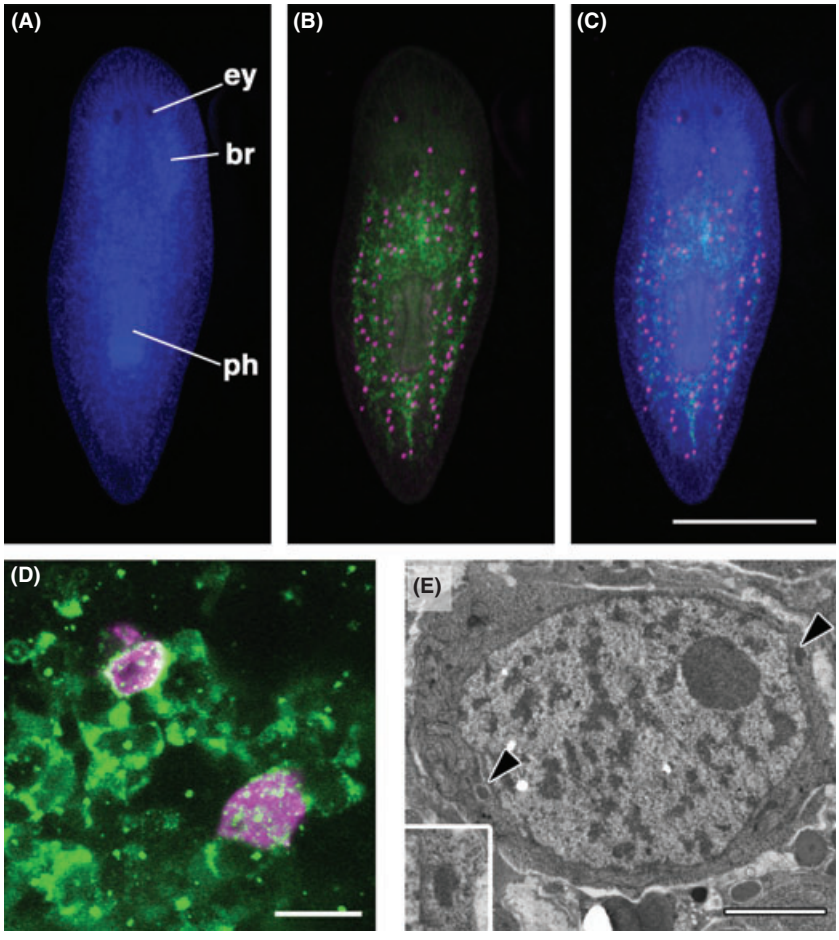

Fig. 2. Distribution pattern and morphology of neoblasts in the planarian *Dugesia japonica*. (A) Hoechst staining (blue) shows the main organs. A pair of eyes (ey) and a brain (br) exist in the head region. The pharynx (ph) occupies the central portion of the body. (B) Expression of DjPiwiA (green) and anti-phospho-Histone H3 antibody staining (magenta) indicates the distribution pattern of neoblasts. Neoblasts are localized throughout the mesenchymal space of the entire body except the head and pharyngeal region. (C) Merged image of A and B. Scale bar is 500 μm. (D) Anti-phospho-Histone H3 antibody staining (magenta) indicates the mitotic ability of neoblasts, which are identified by expression of *DjpiwiA* (green). Scale bar is 10 μm. (E) Ultrastructure of the neoblast observed by transmission electron microscopy. Arrowheads indicate chromatoid bodies in the cytoplasm of neoblasts. Higher-magnification view of the chromatoid body is shown in the box. Scale bar is 2 μm.

1982, 1997), suggesting that these structures may be involved in neoblast maintenance.

Pluripotency of the neoblasts

Although there is no definitive experimental evidence for the pluripotency of neoblasts, which could be proved by clonal cell culture or single cell transplantation, multiple experiments support the notion of the pluripotency of the neoblasts. Planarians irradiated with the appropriate dose of X- or gamma-rays lose their regenerative ability due to the specific elimination of neoblasts (Wolff & Dubois 1948). Planarians with an irradiated anterior half of the body can regenerate a head due to migration of neoblasts from a non-irradiated posterior part (Wolff & Dubois 1948). A population of stem cells, isolated via size fractionation, can restore the regenerative ability of irradiated planarians (Baguñà *et al.*; Kobayashi *et al.* 2008). Furthermore, planarian regeneration proceeds in an intercalative, position-dependent manner (Agata *et al.* 2003), and superfluous body structures, such as a brain or pharynx, are induced when a head fragment is transplanted into the tail region of planarians (Kobayashi *et al.* 1999). Finally, when a small piece of the middle region of the body is grafted at the original position but in the dorsoventral-reversed orientation, ectopic structures such as a head or tail are frequently formed at the grafted position (Kato *et al.* 1999, 2001). These observations and the fact that almost any tiny fragment of the planarian body can regenerate an entire organism (Morgan 1898) show not only that the neoblasts can respond to changes of their environment, but also that neoblasts can give rise to all types of cells.

Heterogeneity of the neoblasts

We developed a fluorescence activated cell sorting (FACS) method for cells of *D. japonica*, based on Hoechst staining for DNA content and Calcein-AM staining for cell size (Hayashi *et al.* 2006). Comparison of cell sorting profiles of intact and X-ray irradiated planarians revealed two different X-ray sensitive cell fractions: X1 and X2 (Fig. 3A,B). The X1 fraction is composed of S to M-phase neoblasts, as confirmed by higher DNA

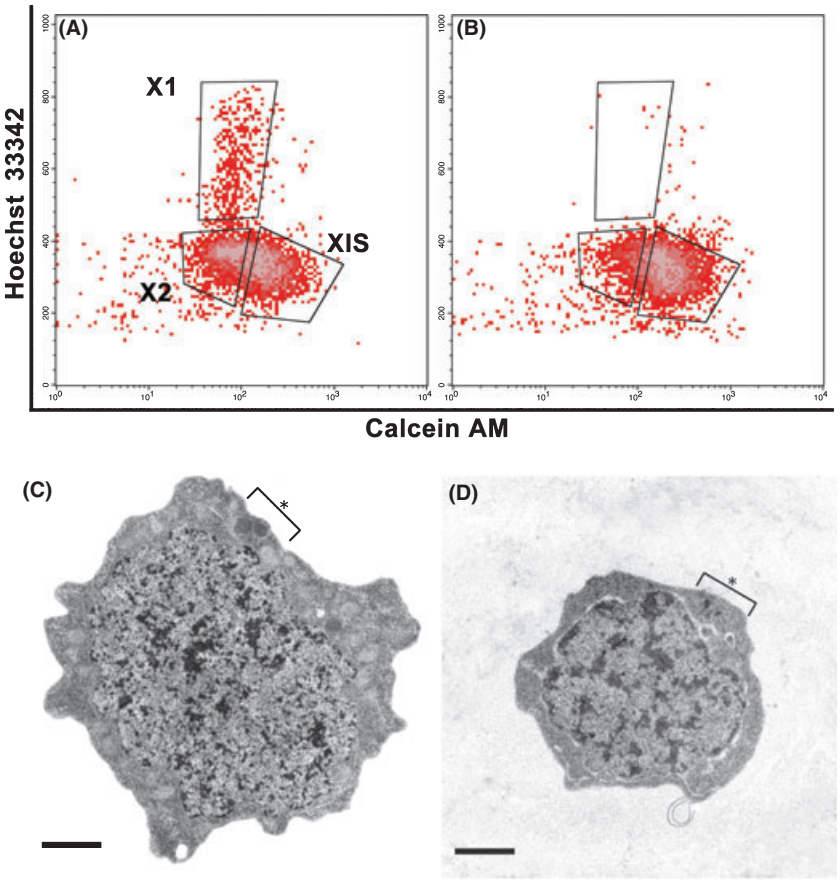

Fig. 3. Cell sorting profiles of intact and X-ray-irradiated planarians by fluorescence activated cell sorting (FACS) identifies two types neoblasts with different morphologies. (A) Cell sorting profile of intact planarians. Cells are separated into three fractions, X1, X2 and XIS (B) Cell sorting profile of X-ray-irradiated planarians. The majority of cells in the X1 fraction and about half of the cells in the X2 fraction disappear, identifying these cells as X-ray-sensitive neoblasts. (C) Morphology of Type 1 cells (typical neoblasts) in the X1 fraction (Higuchi *et al.* 2007). These cells have scanty cytoplasm, chromatoid bodies and nuclei rich in euchromatin. (D) Morphology of Type 2 cells preferentially observed in the X2 fraction. Chromatoid bodies and scanty cytoplasm are also observed, but developed heterochromatin is observed in the nuclei of Type 2 cells. Type 2 cells are smaller than Type 1 cells. Scale bar is 1 μm. Asterisk indicates location of some of the chromatoid bodies. (C and D) are modified from Higuchi *et al.* (2007).

content and gene expression profiles (Fig. 3A; Hayashi *et al.* 2006). About half of the cells sorted into the X2 fraction are G1 neoblasts, and the remaining half of the X2 fraction and most of the X-insensitive fraction (XIS) are differentiated cells showing tolerance to irradiation (Fig. 3A,B).

Detailed analysis of cells collected by FACS revealed heterogeneity in the neoblast population (Higuchi *et al.* 2007). Cells were categorized into one of three types according to their morphological features: stem cell, differentiating cell, or differentiated cell. Surprisingly, a considerable number of differentiating cells were observed in the X1 fraction (17% of X1 cells; Higuchi *et al.* 2007). Neoblasts are thought to stop proliferation after commitment, but this finding indicates that some differentiating cells possess the ability to divide. Heterogeneity among morphologically classified stem cells was also reported, and stem cells classified as ''Type B'', which here we rename ''Type 2'' cells, exist in the X2 fraction (Fig. 3D; Higuchi et al. 2007), and stem cells smaller in size than typical neoblasts (originally ''Type A'' stem cells, which we rename ''Type 1'' stem cells, here). Type 2 cells also have a smaller number of chromatoid bodies, and contain more tightly condensed heterochromatin (Fig. 3C,D). It is possible to consider Type 2 stem cells as typical neoblasts in a different phase of the cell cycle from Type 1 neoblasts (Gurley & Sánchez Alvarado 2008). However, the majority of stem cells in the X2 fraction are definitely Type 1 stem cells, which suggests that Type 2 cells may belong to a distinctly new class of planarian stem cells.

Recently, the existence of a highly dormant state of hematopoietic stem cells has been reported (Wilson *et al.* 2008). In many cases, true stem cells have shown a state of slow cell cycle progression (Fuchs 2009), suggesting that Type 2 stem cells may be slow-cycling stem cells. Type 2 cells may also be descendents of Type 1 stem cells on their way to commitment, which is comparable to reported observations in *S. mediterranea* (Eisenhoffer *et al.* 2008). Gene expression analysis in *D. japonica* has also revealed heterogeneity of the neoblasts (Rossi *et al.* 2006, 2007; Sato *et al.* 2006; Salvetti *et al.* 2009). It seems that, after all, the planarian stem cell system is a more complex system than the homogenous cell population of the neoblasts they were previously thought likely to be.

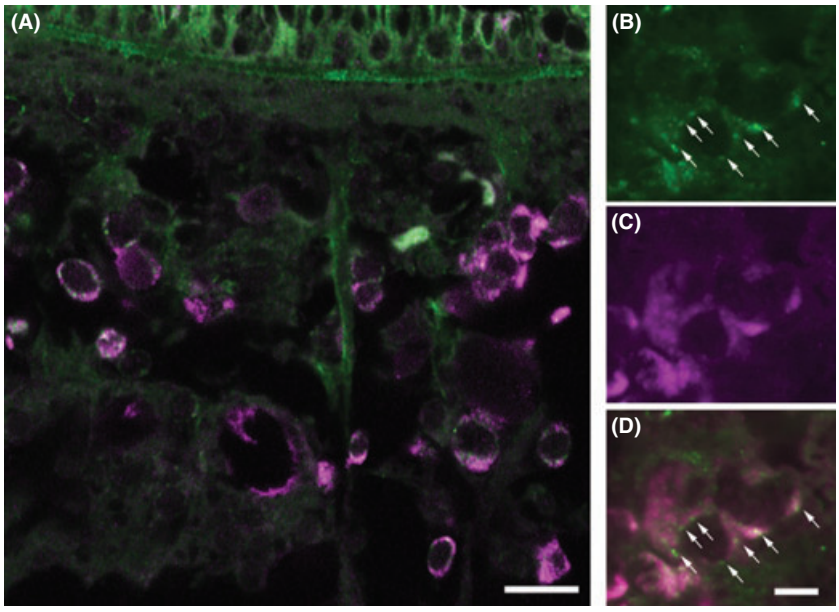

Fig. 4. Subcellular localization of DjCBC-1 and DjPiwiA protein in neoblasts. (A) Double immunohistochemistry using anti-DjCBC-1 antibody (green) and anti-DjPiwiA antibody (magenta). Both proteins are detected in the cytoplasm of neoblasts. Scale bar is 10 μm. (B) Subscellular localization of DjCBC-1 protein. Punctate signals (arrows) are observed in the cytoplasm of the neoblast. (C) Subcellular distribution of DjPiwiA protein. DjPiwiA protein is observed widely diffused in the cytoplasm of neoblasts. (D) Merged image of B and C. DjCBC-1 and DjPiwiA protein localization does not completely overlap. Scale bar is 5 μm.

Molecular features of the neoblasts

Components of the chromatoid bodies

As mentioned above, a distinguishable characteristic of the neoblasts is the presence of chromatoid bodies. The morphological features of chromatoid bodies in planarian ASCs resemble those of well-documented RNP granules in the cytoplasm of germ cells of several animals (Seydoux & Braun 2006), implying commonality of the protein and RNA components between such cytoplasmic structures. Thus far, only two chromatoid body protein components have been identified in planarian neoblasts: DjCBC-1 and SpolTud-1 (Yoshida-Kashikawa *et al.* 2007; Solana *et al.* 2009). Both of these proteins are homologues of components of germline granules in other organisms. We identified *chromatoid body component-1* (*Djcbc-1*), which is a gene in *D. japonica* that codes for a protein about 70% identical to members of the RCK/p54/Me31b/Dhh1p family of DEAD box RNA helicases (Yoshida-Kashikawa *et al.* 2007). DjCBC-1 protein is observed in the chromatoid bodies (Fig. 4A,B). Expression of *tudor* homologues in planarian neoblasts has been reported by us and others (Fig. 5A; Yoshida-Kashikawa *et al.* 2007; Solana *et al.* 2009). A Tudor-domain-containing protein from *Schmidtea polychroa* was also shown to localize to chromatoid bodies, and to be required for long-term stem cell self-renewal (Solana *et al.* 2009). Another component is *nanos* mRNA, which is detected in chromatoid bodies of germline precursor cells described below (Sato *et al.* 2006).

Piwi and interacting small RNAs in the neoblasts

The Piwi sub-family of Argonaute proteins, and small non-coding Piwi-interacting RNAs (piRNA) are essential for germline development, germline stem cell renewal, epigenetic regulation, and repression of transposable elements (Cox *et al.* 1998; Aravin *et al.* 2007; Girard & Hannon 2007; Yin & Lin 2007; Siomi *et al.* 2008; Malone & Hannon 2009). Piwi-deficient animals show severe defects in germline stem cell maintenance and germ cell differentiation (Lin & Spradling 1997; Cox *et al.* 1998; Kuramochi-Miyagawa *et al.* 2004; Carmell *et al.* 2007). *piwi* homologues in planarians, *Smedwi-1*, *-2* and *-3* (or *DjpiwiA*, *B*, and *C*, respectively), are exclusively or preferentially expressed in neoblasts (Fig. 2A; Reddien *et al.* 2005b; Rossi *et al.* 2007; Yoshida-Kashikawa *et al.* 2007; Palakodeti *et al.* 2008; Hayashi *et al.* in this issue), and are essential for precise cell differentiation and/or stem cell maintenance (Reddien *et al.* 2005b; Palakodeti *et al.* 2008). Comprehensive sequence analysis revealed enrichment of piRNAs in neoblasts of *S. mediterranea*, of which 32% mapped to transposons (Friedländer *et al.* 2009), suggesting that Piwis prevent transposable element activity in the neoblasts of planarians. Piwi family proteins localize to germline granules in fly and mouse (Seydoux & Braun 2006; Kotaja & Sassone-Corsi 2007). Although both DjCBC-1 and DjPiwiA protein are present in the neoblasts (Fig. 4A), DjPiwiA does not seem to co-localize with DjCBC-1 in chromatoid bodies in *D. japonica* (Fig. 4B–D).

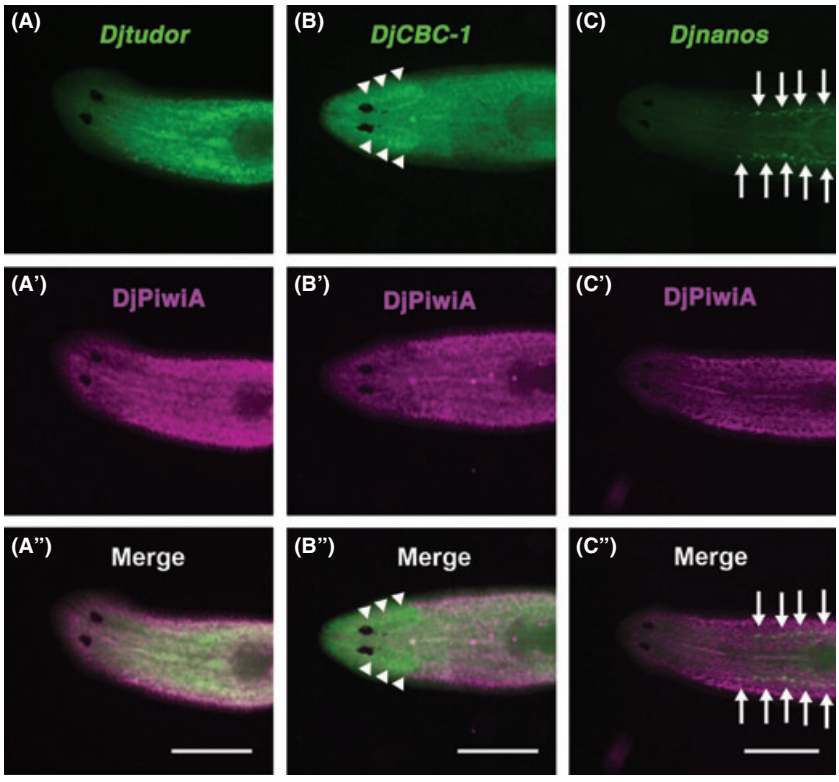

Fig. 5. Expression pattern of Type A genes, Type B genes and *Djnos*. (A) Expression pattern of Type A gene, *Djtudor* (green). Expression of Type A genes is only detected in neoblasts. (A') Anti-DjPiwiA antibody staining in the same sample (magenta). (A'') Merged image of A and A'. Almost of all of the neoblasts co-express DjPiwiA and *Djtudor*. (B) Expression pattern of Type B gene, *Djcbc-1*. Arrowheads indicate brain. (B') Anti-DjPiwiA antibody staining in the same sample. (B'') Merged image of B and B'. Type B genes' expression is detected in both neoblasts and brain cells. (C) Expression pattern of *nanos* in asexual planarians. Dorso-lateral cell cluster expressing *Djnos* (arrows). (C') Anti-DjPiwiA antibody staining in the same sample. (C'') Merged image of C and C'. Only a subpopulation of neoblasts express *Djnos*. Bars, 500 μm.

Expression of germline-specific genes in the planarian ASCs

As mentioned in the Introduction, the first gene shown to be expressed in planarian neoblasts was *vasa-like gene A. DjvlgA* codes for proteins with sequence similarity to Vasa, a DEAD box RNA helicase component of germ granules in other animals (Lasko & Ashburner 1988; Fujiwara *et al.* 1994; Yoon *et al.* 1997; Shibata *et al.* 1999). *DjvlgA* and *DjvlgB* are expressed in mature testes and ovaries of sexual planarians. *DjvlgA*, however, is also expressed in neoblasts and differentiating blastema cells, suggesting that *DjvlgA* may be involved in commitment and differentiation of neoblasts (Shibata *et al.* 1999; Newmark & Sánchez Alvarado 2002). In fact, *DjvlgA* and *DjvlgB* belong to the *PL10/DDX3* subfamily of helicases, which is a subfamily of DEAD box proteins, closely related to *vasa* (Mochizuki *et al.* 2001). An actual member of the *vasa* subfamily of DEAD box proteins has been identified in the planarian *Dugesia dorotocephala* (Mochizuki *et al.* 2001), and its orthologue is also present in our *D. japonica* expressed sequence tag (EST) database.

Homologues of several proteins involved in post-transcriptional regulation in the germline of different organisms have since been shown to be relevant to planarian neoblasts. *Djpum*, a member of the PUF (Pum and FBF)-domain family of proteins required for the maintenance and function of germline cells in flies and nematodes (Lin & Spradling 1997; Parisi & Lin 1999; Crittenden *et al.* 2002; Wickens *et al.* 2002), is expressed in planarian neoblasts and brain neurons (Salvetti *et al.* 2005). RNAi-mediated knock-down of *Djpum* leads to loss of regeneration and a reduction of neoblast number in *D. japonica* (Salvetti *et al.* 2005). Notably, DjCBC-1 homologue Dhh1p interacts physically with Pumilio homologue Mpt5p in yeast extracts (Goldstrohm *et al.* 2006), which implies that DjPum may also be associated with chromatoid bodies. Similar results with regard to phenotype and expression pattern were observed for *bruli* in *S. mediterranea* (Guo *et al.* 2006). *Smed-bruli* is a member of the Bruno-like family of proteins, which includes *bruno*, an RNA-binding protein that represses translation of *osk* mRNA in *Drosophila* germline cells (Webster *et al.* 1997; Nakamura *et al.* 2004).

Other RNP component homologue genes in the neoblasts

Genes shown to be components of dense ribonucleoprotein structures such as mammalian chromatoid bodies, stress granules and processing bodies (P bodies) are expressed in the neoblasts. These ribonucleoprotein

Table 1. Classification of RNA-binding proteins in planarians according to their expression patterns

Homology	Predicted localization	Published gene name or clone ID in planarians	Subcellular localization in planarians	References	E-value (closest Dm or Hs)
Neoblasts (Type A)					
TIA1 cytotoxic granule associated protein (*Homo sapiens*)	SG PB	00412_HN (Dj)		Kedersha *et al.* 1999, 2005	4e-26 HsTIA1
Y-Box factor protein (*Aplysia californica*)	SB	03689_HH (Dj)		Nakamura *et al.* 2001	1e-22 DmYps
Translation eIF4E protein (*A. californica*)	SG PB PG	03978_HH (Dj)		rev Eulalio *et al.* 2007	2e-39 HsEIF4E
Tudor protein (*Xenopus laevis*)	CB PG	05895_HH Spoltud-1 (Sp)	CB	Solana *et al.* 2009; Hosokawa *et al.* 2007; Boswell & Mahowald 1985	3e-05 Dm*tud*
Seawi (*Strongylocentrotus purpuratus*)	CB PG	DjPiwiA (Dj) Smedwi1 (Sm)	Cytosol	This review; Yoshida-Kashikawa *et al.* 2007; Reddien *et al.* 2005a,b; Harris & Macdonald 2001; Kotaja *et al.* 2006; Houwing *et al.* 2007; rev Eulalio *et al.* 2007; rev Kotaja & Sassone-Corsi 2007	2e-68 HsPIWIL1
Piwi (*Botryllus primigenus*)	CB PG	Smedwi2 (Sm)	?	Reddien *et al.* 2005a,b; Palakodeti *et al.* 2008; Harris & Macdonald 2001; Kotaja *et al.* 2006; Houwing *et al.* 2007; rev Eulalio *et al.* 2007; rev Kotaja & Sassone-Corsi 2007	3e-69 HsPIWIL1
Neoblasts + other cells (Type B)					
Translation initiation factor 3 90 kDa subunit (eIF3 p90) (*Schizosaccharomyces pombe*)	SG	00218_HN (Dj)		Kedersha *et al.* 2002	3e-17 HseIF3B
me31B protein (*Drosophila melanogaster*)	PB SG PG SB	DjCBC-1⁄(Dj)	CB	Yoshida-Kashikawa *et al.* 2007; rev Eulalio *et al.* 2007	1e-157 Dm*me31b*
PL10 (*Danio rerio*)	SG PG CB	DjvlgA (Dj) SpolvlgA (Sp)	?	Shibata *et al.* 1999; Solana *et al.* 2009; Ming-Chih Lai *et al.* 2008	1e-161 HsDDX3X
Putative RNA helicase (DEAD box) protein (*D. rerio*)	SG PG CB	02217_HH (Dj)		Ming-Chih Lai *et al.* 2008	5e-70 HsDDX3X
DEAD (Asp-Glu-Ala-Asp) box polypeptide 48 protein (*D. rerio*)	NG, nucleus	05792_HH (Dj)		Giorgi *et al.* 2007	0.0 HsEIF4A3
Fragile X mental retardation protein 1 (*X. laevis*)	SG PB NG	06718_HH (Dj)		Antar *et al.* 2005; Barbee *et al.* 2006; Kwak *et al.* 2008	9e-49 HsFXR1
Putative pumilio (*Schistosoma mansoni*)	SG PG	DjPum (Dj)	?	Salvetti *et al.* 2005; Vessey *et al.* 2006;A Noble *et al.* 2008	2e-130 Dm*pum*
Bruno-3 (*Tribolium castaneum*)	CB	Bruli (Sm)	Cytosol	Guo *et al.* 2006; Snee & Macdonald 2004	9e-48 Dm*aret*

Table 1. (Continued)

Homology	Predicted localization	Published gene name or clone ID in planarians	Subcellular localization in planarians	References	E-value (closest Dm or Hs)
HIWI (*H. sapiens*)	CB PG	Smedwi3 (Sm)	?	Rossi *et al.* 2007; Harris & Macdonald 2001; Kotaja *et al.* 2006; Houwing *et al.* 2007; rev Eulalio *et al.* 2007; rev Kotaja & Sassone-Corsi 2007	2e-162 PIWIL1
Subpopulation of neoblasts					
Putative nanos (*S. mansoni*)	PG	Djnos (Dj) Smed-nanos/Smednos (Sm)	? (*nanos* mRNA in CB)	Sato *et al.* 2006; Wang *et al.* 2007; Jaruzelska *et al.*, 2003	1e-17 HsNANOS1
PIWI (*B. primigenus*)	CB PG	Djpiwi-1 (Dj)	?	Rossi *et al.* 2006; Harris & Macdonald 2001; Kotaja *et al.* 2006; Houwing *et al.* 2007; rev Eulalio *et al.* 2007; rev Kotaja & Sassone-Corsi 2007	9e-71 HsPIWIL1

Closest homologe name is (Hs) for H. sapiens or (Dm) for Drosophila melanogaster, followed by NCBI Entrez gene official symbol.
CB, chromatoid bodies or nuage; PB, processing bodies; SG, stress granules; NG, neuronal granules; PG, polar granules or germ-cell granules; SB, sponge bodies.
Table modified from Yoshida-Kashikawa *et al.* 2007.

complexes are implicated in different modes of post-transcriptional regulation of gene expression, such as mRNA decay, mRNA storage and translational repression (Eulalio *et al.* 2007; Besse & Ephrussi 2008; Balagopal & Parker 2009). In order to consider the composition and function of chromatoid bodies in planarians, we have categorized genes encoding planarian RNA-binding proteins into Type A, Type B, or subpopulation of neoblasts (Table 1). Type A means neoblast-specific genes (Fig. 5A). Among proteins encoded by Type A genes, TIA is a stress granule and P body component involved in translational repression and stress granule formation (Forch *et al.* 2000; Kedersha *et al.* 2000; Dixon *et al.* 2003). This gene is also known to regulate alternative splicing of apoptosis-promoting factor Fas mRNA (Izquierdo *et al.* 2005). Interestingly, neoblast death seems to occur in an apoptotic manner not only under stress conditions, such as irradiation or depletion of vital factors, but also in intact planarians (Hwang *et al.* 2004; Salvetti *et al.* 2005). The *TIA* expression in the neoblasts suggests that stress granules could exist in neoblasts under stress conditions. Type B genes, some of which are required for neoblast maintenance, are expressed in both the stem cells and brain neurons (Fig. 5B; Salvetti *et al.* 2005; Guo *et al.* 2006; Yoshida-Kashikawa *et al.* 2007), indicating that those two types of cells have similar post-transcriptional regulation mechanisms. Interestingly, not all chromatoid bodies contain DjCBC-1 (categorized as Type B) (Fig. 5B and Table 1 Yoshida-Kashikawa *et al.* 2007), which suggests heterogeneity of chromatoid bodies. One possible reason for this heterogeneity may be an association of the chromatoid bodies with other kinds of RNP granules.

miRNA in the neoblasts

Another class of small non-coding RNA that is robustly expressed in planarians is microRNAs (miRNAs), which are message-specific regulators of gene expression (Palakodeti *et al.* 2006; Friedländer *et al.* 2009; González-Estévez *et al.* 2009; Lu *et al.* 2009). Large-scale profiling by two different laboratories identified 10 *S. mediterranea* miRNAs whose expression was enriched in neoblasts, five of which were classified as enriched by both groups (Friedländer *et al.* 2009; Lu *et al.* 2009). Interestingly, the function of chromatoid body components during mouse spermiogenesis can be divided into repression of retrotransposons or miRNA processing, by the fact that Miwi and Mili are necessary for retrotransposon silencing, whereas Tudor domain protein Tdrd6 is required for proper expression of miRNAs (Vasileva *et al.* 2009; Wang *et al.* 2009). Something similar could be occurring in

the chromatoid bodies of planarian neoblasts, although DjPiwiA is not localized in the chromatoid bodies.

Germline cell specification from neoblasts by nanos

Chromatoid bodies are also detected in planarian germline cells (Teshirogi & Ishida 1987). Sexual-state planarians develop mature testes located dorsolaterally, and a pair of ovaries in the ventral side of the neck region, both of which are completely absent in asexual planarians (Newmark *et al.* 2008). The expression of some neoblast components has been confirmed in germline cells of sexual planarians (Shibata *et al.* 1999; Newmark *et al.* 2008; Solana *et al.* 2009), suggesting a parallel program for regulation of gene expression among neoblast and germline cells in planarians as well.

What regulates the emergence of the mature germline? Which genes are responsible for germline cell specification? These questions were partially answered with the identification of *nanos*, a CCHC zinc-finger protein, which plays a crucial role in germline cell establishment in early embryogenesis, and in germline stem cell maintenance and differentiation, by inhibiting translation of specific mRNAs (Forbes & Lehmann 1998; Asaoka-Taguchi *et al.* 1999; Kraemer *et al.* 1999; Wang & Lin 2004; Kobayashi 2005; Nakamura & Seydoux 2008). In planarians, *nanos* (or *nos*) is specifically expressed in sparmatogonia and oogonia of sexual planarians, as well as in germline precursor cells in asexual animals (Fig. 5C; Sato *et al.* 2006; Wang *et al.* 2007). Depletion of *nanos* by RNAi in *S. mediterranea* causes clear loss of testis, ovaries and germ cell precursors, but not neoblasts. The co-expression of several neoblast marker genes and *nanos* in asexual planarians indicates that *nanos*-expressing cells are a subpopulation of neoblasts (Table 1; Wang *et al.* 2007). These cells differentiate into mature gonads during epigenetic sexual specification in planarians, suggesting that *nanos*-positive cells in asexual individuals are germline precursor cells (Sato *et al.* 2006). Neoblasts show constant cell division, even in intact animals (Newmark & Sánchez Alvarado 2000; Kang & Sánchez Alvarado 2009), whereas germline precursors seem to be arrested at the G2 phase, analogous to Nos-regulated presumptive germline precursor cells (PGCs) in *Drosophila* (Asaoka-Taguchi *et al.* 1999; Sato *et al.* 2006).

Conclusions regarding ''molecular features of the neoblasts''

A pattern is emerging, in which homologues of post-transcriptional regulators in the germline and early development of other organisms (Wickens *et al.* 2000; Seydoux & Braun 2006) are expressed in the neoblast and/or required to maintain the stemness and viability of neoblasts. Thus, neoblast chromatoid bodies are likely to be involved in post-transcriptional regulation of mRNA, although further studies will be needed to determine their function in detail (Fig. 6A). The mentioned reports indicate that molecular mechanisms common to planarian pluripotent somatic stem cells and germline cells are required for neoblast maintenance: strong post-transcriptional regulation by several RNA-binding proteins and miRNAs, and the assurance of genomic stability required for pluripotency or totipotency by inactivation of transposable elements by Piwi and piRNAs (Fig. 6A).

Generally, the most fundamental difference between somatic and germline stem cells is the inability of

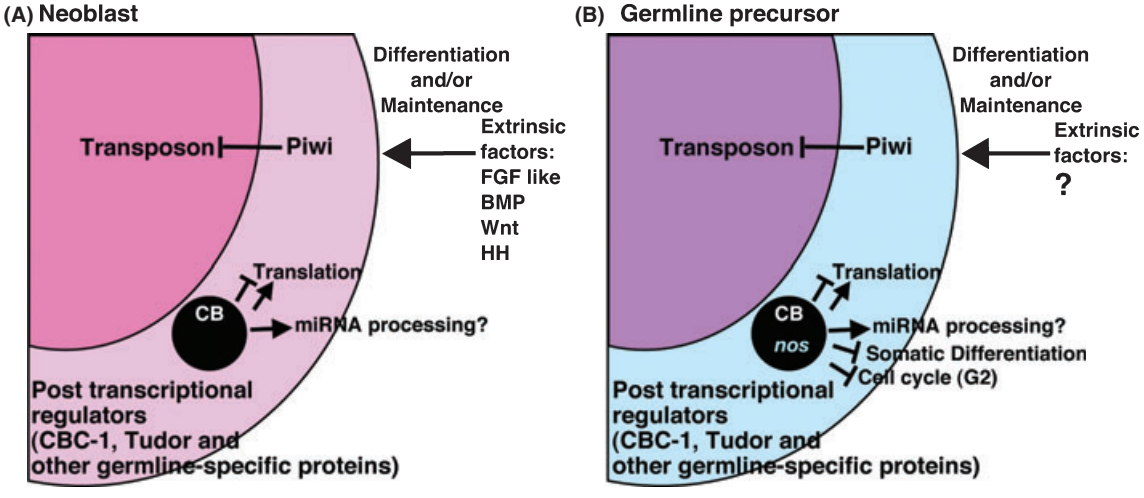

Fig. 6. Model of molecular mechanisms that regulate (A) neoblasts and (B) germline precursor cells.

germline stem cells to differentiate into somatic cells, in order to assure progeny via sexual reproduction. In *Drosophila*, *nanos*-deficient PGCs adopt both somatic and germline fate (Hayashi *et al.* 2004), which indicates that *nanos* is crucial for preventing the adoption of somatic fate by germline precursors, and that PGCs have innate multipotency. Based on this, neoblasts maintain pluripotency and therefore can give rise to all types of cells for both asexual and sexual reproduction. Thus, neoblasts possess molecular mechanisms akin to those in germline cells, but germline precursors must maintain their germline-restricted fate until sexualization (Fig. 6B). It is likely that *nanos* is involved in inhibition of the cell cycle, apoptosis, and differentiation into the somatic state in asexual planarians as well as *Drosophila* PGCs (Hayashi *et al.* 2004; Sato *et al.* 2007). The molecular mechanisms for specification of germline precursors in planarians, apart from the involvement of *nanos*, are still unclear. Comprehensive gene analysis of germline-specific genes, however, will probably identify genes that play a role upstream, downstream, or with *nanos* (Zayas *et al.* 2005; Newmark *et al.* 2008). Better understanding of the process of epigenetic germline specification in planarians should shed light on vertebrate gametogenesis and the evolution of germline cells (Extavour 2007). Thus, planarian stem cells will also provide a good opportunity to understand not only evolutionary aspects of pluripotent stem cells and germline cells, but also the RNA world in the stem cells.

Signaling molecules involved in neoblast regulation

Recently, it has been shown that differentiation of the neoblasts seems likely to be regulated by several signaling molecules. RNAi-silencing of *β-catenin*, which is known to be involved in wnt signaling in other animals, in *S. mediterranea* leads to ectopic head formation in the tail region (Gurley *et al.* 2008; Petersen & Reddien 2008; Adell *et al.* 2009). Bone morphogenetic protein (BMP) is also required for proper regeneration and maintenance of the dorsoventral axis in planarians (Molina *et al.* 2007; Orii & Watanabe 2007; Reddien *et al.* 2007). *nou-darake* (ndk) encodes a fibroblast growth factor receptor (*FGFR*) like protein, which lacks an intracellular domain (Cebria *et al.* 2002a). After knockdown of *ndk*, ectopic brains are formed beyond the head region (Cebria *et al.* 2002a). Triple knockdown of *ndk* and two FGF receptors expressed in the neoblasts of *D. japonica*, *DjFGFR1* and *DJFGFR2*, (Ogawa *et al.* 2002) inhibits ectopic brain formation, suggesting that FGF-like signaling is involved in brain cell differentiation (Cebria *et al.* 2002a; Umesono & Agata 2009). Also, hedgehog (Shh) signaling controls A-P patterning (Yazawa *et al.* in press). All of these facts suggest that several signaling molecules direct the proper differentiation of the neoblasts (Fig. 5A). Interestingly, signaling molecules involved in these pathways are also involved in the regulation of ASCs or germline stem cells in other model organisms. Bone morphogenetic protein (BMP) and Shh are required for germline stem cell maintenance and differentiation in flies. Wnt signaling is required for hair follicle stem cell differentiation in mammals, and FGF for differentiation of embryonic stem (ES) cells (Wong *et al.* 2005; Kunath *et al.* 2007; Nishikawa & Osawa 2007; Ying *et al.* 2008). These connections indicate that the molecular basis for the regulation of pluripotency or multipotency is probably conserved in species among various phyla.

Conclusions and future prospects

The expression of genes that are exclusive to the germline in vertebrates is also observed in pluripotent or multipotent ASCs in basal metazoans and some bilaterians, archeocytes of Porifera, interstitial cells of Cnidaria, neoblasts of Annelida, and stem cells of parasitic Arthropoda (Mochizuki *et al.* 2000, 2001; Shukalyuk *et al.* 2007; Sugio *et al.* 2008; Funayama *et al.* unpubl. data, 2010). As is the case with planarians, these organisms perform asexual reproduction using pluripotent or mulitipotent ASCs, which suggests that the use of ''germline-specific genes'' by somatic stem cells may be a way to achieve asexual reproduction (Agata *et al.* 2006). Furthermore, these basal metazoans produce germline cells from ASCs, which implies that ASCs might have been the evolutionary origin of germline cells (Extavour 2007). Colonial ascidians belonging to Urochordata can also reproduce asexually; however, their multipotent ASCs don't seem to express ''germline-specific genes'' (Sunanaga *et al.* 2006, 2007, 2008). One explanation for this could be the suggested separation of somatic and germline stem cells in colonial ascidians (Laird *et al.* 2005). Thus, basic research about ASCs in diverse species of various phyla provides a great opportunity to get insight into cell contributions to reproduction, as well as the origin and evolution of germline cells. Comparison of molecular signatures of ASCs in these animals and ASCs in relatively higher metazoans, such as vertebrates, will reveal the common molecular bases of stem cells.

In addition to the natural advantages of planarians, recent technical and bioinformatics advances in planarian stem cell research have made planarians the model organism of choice for studies of stem cell

biology *in vivo* (Sánchez Alvarado & Kang 2005; Rossi *et al.* 2008) and epigenetic specification of germ cells (Newmark *et al.* 2008). As reviewed here, planarians are also intriguing animals for research on RNA regulation and cytoplasmic ribonucleoprotein structures.

Many fundamental issues remain to be resolved. In 1990, Professor K. Watanabe established clonal strains, gifu iruma (GI) and sexualizing special planarian (SSP), from single *D. japonica* individuals. For approximately 20 years, both strains have been asexually maintained in the laboratory. However, we still don't know how neoblasts maintain pluripotency and genomic stability over time despite their continuous and rapid division (Kang & Sánchez Alvarado 2009). How planarians maintain a constant number of the neoblasts (~30%) in their body is also unclear. Whether there is a stem cell ''niche'', which is known to be an important place or group of cells for the regulation of stem cells in other animals, is not known in planarians. We tried to identify neuronal stem cells in planarians by analysis of *musashi* homologues, but there was no clear evidence of their existence (Higuchi *et al.* 2008). In fact, whether there are any lineage-committed adult stem cells in planarians, apart from germline precursors, is still unclear. Understanding the complexity and biological-state flexibility of neoblasts in planarians will advance our knowledge of stem cells in general, and the application of ES or iPS cells in the medical field.

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