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The planarian P2X homolog in the regulation of asexual reproduction

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ABSTRACT The growth in size of freshwater planarians in response to nutrient intake is limited by the eventual separation of tail and body fragments in a process called fission. The resulting tail fragment regenerates the entire body as an artificially amputated tail fragment would do, and the body fragment regenerates a tail, resulting in two whole planarians. This regenerative ability is supported by pluripotent somatic stem cells, called neoblasts, which are distributed throughout almost the entire body of the planarian. Neoblasts are the only planarian cells with the ability to continuously proliferate and give rise to all types of cells during regeneration, asexual reproduction, homeostasis, and growth. In order to investigate the molecular characteristics of neoblasts, we conducted an extensive search for neoblast-specific genes using the High Coverage Expression Profiling (HiCEP) method, and tested the function of the resulting candidates by RNAi. Disruption of the expression of one candidate gene, *DjP2X-A* (*Dugesia japonica* membrane protein P2X homologue), resulted in a unique phenotype. *DjP2X-A* RNAi leads to an increase of fission events upon feeding. We confirmed by immunohistochemistry that DjP2X-A is a membrane protein, and elucidated its role in regulating neoblast proliferation, thereby explaining its unique phenotype. We found that DjP2X-A decreases the burst of neoblast proliferation that normally occurs after feeding. We also found that DjP2X-A is required for normal proliferation in starved animals. We propose that DjP2X-A modulates stem cell proliferation in response to the nutritional condition.

KEY WORDS: *planarian, fission, P2X, pluripotent stem cell*

Introduction

Reproduction is a fundamental feature of the survival of an organism. Germ cell-mediated sexual reproduction is commonly employed among multicellular animals. However, asexual reproduction mediated by somatic multi- or pluripotent stem cells is also observed in many invertebrate species (Agata *et al.,* 2006). Although the regulation of sexual reproduction via germ cells has been well studied, our understanding of the cellular and molecular mechanisms underlying asexual reproduction in multicellular organisms remains unclear.

The freshwater planarian *Dugesia japonica* possesses robust regenerative ability that allows for regeneration of a complete functional individual from almost any fragment produced by artificial amputation. *D. japonica*'s regenerative capabilities are employed during asexual reproduction under natural conditions. Planarians can grow or de-grow in response to nutrient uptake, while maintaining their normal body proportions, by modulating the number of cells composing their bodies (Baguñá and Romeo, 1981; Oviedo *et al.*, 2003; Takeda *et al.,* 2009). Upon feeding, asexual *D. japonica* propagates by spontaneously dividing its body into two fragments posterior to the pharynx in a process called "fission" (Agata *et al.,* 2006; Takeda *et al.,* 2009). Both fragments regenerate and develop into complete functional planarians via a process like that which occurs after artificial amputation, and thus planarians propagate asexually.

In some invertebrates, such as hydra, colonial tunicates, and planarians, it has been proposed that the somatic stem cell system

Abbreviations used in this paper: DjP2X-A, *Dugesia japonica* P2X homologue; HiCEP, high coverage expression profiling; P2X, type 2, subtype X ionotropic purinergic receptor.

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has an important role in the asexual reproduction (Agata *et al.,* 2006). Among these animals, planarians have been best studied at the cellular and molecular level regarding the relationship between somatic stem cells and artificial regeneration (Agata *et al.,* 2006; Reddien *et al.,* 2005; Shibata *et al.,* 2010). Planarian somatic stem cells, called "neoblasts", are unique among planarian cells in that they are able to proliferate and show sensitivity to X- and gamma-ray irradiation (rev. in Shibata *et al.,* 2010). Neoblasts also show pluripotency, meaning that they can give rise to all types of cells, including germline cells. When planarians are irradiated with X- or gamma-rays, the neoblasts are specifically eliminated and consequently of the animals lose regenerative ability (Wolff and Dubois, 1948). Transplantation of a single neoblast can restore the regeneration in irradiated planarians, demonstrating both the pluripotency of neoblasts and their indispensability for planarian regeneration (Wagner *et al.,* 2011). Recently, a requirement for JNK (c-Jun N-terminal kinase) signaling in the cell cycle progression of the neoblasts in *D. japonica* was reported (Tasaki *et al.,* 2011). Prevention of cell proliferation by U600125, a JNK inhibitor, blocks regeneration, indicating that proper cell proliferation of the neoblasts is necessary for regenerative ability.

Recent efforts of planarian researchers have unveiled a part of the molecular signature of the neoblasts and the function of different components of this "signature". Notably, it has been reported that many genes encoding for RNA-binding proteins, for example *piwi/ago* family genes, and genes related to DNA replication, such as *pcna* or *MCM* (*Proliferating cell nuclear antigen* and *Minichromosome maintenance*, respectively) genes, are expressed and function in the neoblasts of *D. japonica* (reviewed by Shibata *et al.,* 2010; Rouhana *et al.,* 2010). RNA interference (RNAi) experiments have revealed that some of the RNA-binding protein genes are involved in neoblast maintenance, proliferation or differentiation, and knockdown of these genes' expression results in regenerative defects (Rouhana *et al.,* 2010). Although the proper functioning of neoblasts is important for planarian regenerative ability, we still do not know the relationship between the neoblasts and fission, or the molecular mechanism of fission.

Comprehensive gene expression analyses provide abundant information about the molecular signature of cells or tissues. In order to define "stemness" in terms of the molecular signature of the neoblasts, we carried out comprehensive gene expression analysis in *D. japonica* by the HiCEP (High Coverage Expression Profiling) method (Shibata *et al.,* 2012). HiCEP is a method of gene expression analysis based on AFLP (amplified length polymorphism) that enables us to detect and compare genome-wide gene expression between different experimental samples (Fukumura *et al.,* 2003). Using HiCEP, we compared gene expression among intact planarians, X-ray-irradiated planarians, and a neoblast-enriched fraction collected by FACS (fluorescence activated cell sorting), and thereby found 42 genes that are specifically expressed in X-ray-sensitive cells, mainly the neoblasts (Shibata *et al.,* 2012). Among them, we found that a *P2X* homolog, named *DjP2X-A,* is expressed in the neoblasts. P2X is a family of P2 receptors that bind extracellular ATP, resulting in increased cation conductance for many kinds of ions (Roberts *et al.,* 2006). In vertebrates, it is known that P2X is widely functional throughout the body, including in neural cells and stem cells. It regulates a great variety of cellular functions, such as cell growth, apoptosis, cytokine release, cell proliferation and the development of cancer (Roberts *et al.,* 2006).

Here, we report that continued feeding of *DjP2X-A* double-stranded RNA (dsRNA) increased the fission frequency to about three fold-higher than that of control animals. We also found that DjP2X-A is a membrane protein involved in slowing down of the rate of proliferation of the neoblasts after feeding. Finally, we found that DjP2X-A is required for normal cell proliferation under poor nutrient conditions. From these results, we propose that DjP2X-A modulates neoblast proliferation in response to nutritional status, and that increased neoblast proliferation is one of the causes of fission.

Results

DjP2X-A *encodes a neoblast-specific protein in planarians*

DjP2X-A was identified as a neoblast-specific gene through comprehensive gene expression analysis as described above (Methods; Shibata *et al.,* 2012). Whole-mount *in situ* hybridization analysis showed that this gene is expressed in a typical neoblast-specific expression pattern in intact animals, in which cells expressing *DjP2X-A* were observed throughout the body in the mesenchymal space posterior to the eyes (Fig.1A). *DjP2X-A* expression was not detected in animals depleted of neoblasts by X-ray irradiation (Fig. 1B), indicating that *DjP2X-A* is specifically expressed in the neoblasts. During regeneration, *DjP2X-A*-positive cells were not observed in the blastema region, indicating that *DjP2X-A* was expressed exclusively in undifferentiated neoblasts, and was not expressed in their differentiating progeny located in the blastema (Fig. 1 C-H). Faint expression of *DjP2X-A* was detected in the post-blastema region, where actively proliferating neoblasts are accumulated early in regeneration (Fig. 1 D,E).

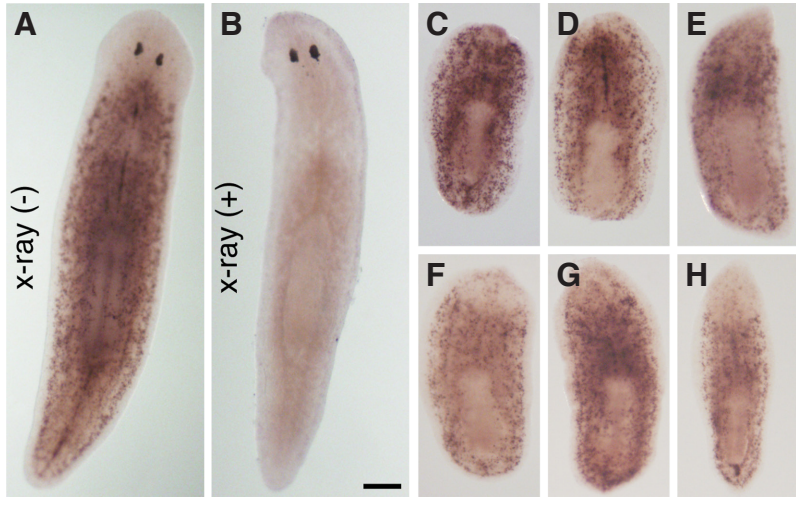

Fig. 1. Distribution pattern of *DjP2X-A* mRNA expressing cells in intact and regenerating planarians. (A) *Expression of* DjP2X-A *mRNA in an intact animal detected by whole-mount* in situ *hybridization. Cells expressing* DjP2X-A *were observed throughout the body except in the head region.* **(B)** *Expression of* DjP2X-A *in X-ray-irradiated planarians. The expression completely disappeared. Expression of* DjP2X-A *in regenerating planarians at* **(C)** *12 hr,* **(D)** *1 day,* **(E)** *2 days,* **(F)** *3 days,* **(G)** *4 days and* **(H)** *5 days after amputation. Scale bar: 500 μm.*

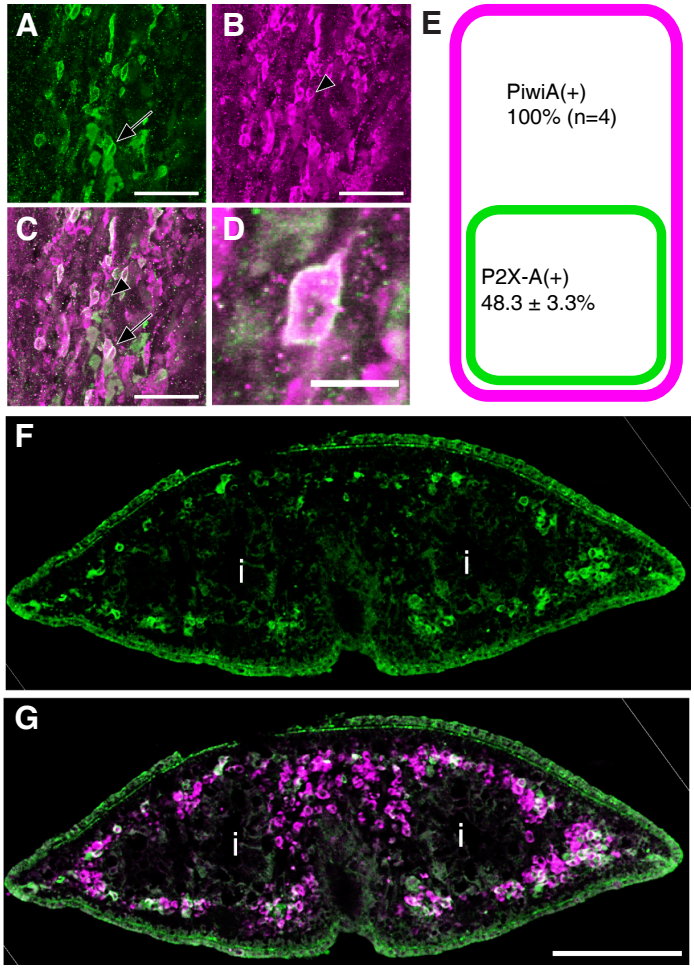

Fig. 2. Detailed observation of DjP2X-A protein-positive cells. (A) *Cells stained with anti-DjP2X-A antibody.* **(B)** *Cells stained with anti-DjPiwiA antibody.* **(C)** *Merged view of (A) and (B). Arrow indicates DjP2X-A/DjPiwiA-double-positive cell, and arrowhead indicates DjPiwiA-positive/DjP2X-A-negative cell. Scale bar: 50 μm.* **(D)** *Higher magnification of DjP2X-A/DjPiwiA-double-positive cell. DjP2X-A was localized on the cell surface. Scale bar: 10 μm.* **(E)** *Quantification of DjP2X-A-positive cell population among DjPiwiA-positive cells. The number of cells was counted in the region in front of the pharynx using confocal microscopy (n=4). The fraction of DjP2X-A-positive cells was 48.3 ± 3.3%.* **(F)** *Immuno-histochemistry of a transverse section anterior to the pharynx using anti-DjP2X-A antibody.* **(G)** *Merge of signals from anti-DjP2X-A (green) and DjPiwiA (magenta) antibody staining on the transverse section. Dorsal is to the top. i: intestine. Scale bar: 200 μm.*

Heterogeneous expression of DjP2X-A protein in the neoblast population

Next we performed simultaneous whole-mount immunohistochemistry using anti-DjP2X-A antibody and the neoblast marker anti-DjPiwiA antibody (Shibata *et al.,* 2010). By detailed observation of DjP2X-A-positive cells, we found heterogeneous expression of DjP2X-A protein in the neoblast population (Fig. 2). We found neoblasts expressing both DjPiwiA and DjP2X-A (Fig. 2 A,B,C, arrow), and neoblasts expressing only DjPiwiA but not DjP2X-A (Fig. 2 A,B,C, arrowhead). High magnification analysis revealed that DjP2X-A protein is localized on the cell surface, enclosing the DjPiwiA signal uniformly localized throughout the cytoplasm of neoblasts (Fig. 2D). These results indicate that DjP2X-A is localized at the membrane of neoblasts, like members of the P2X family proteins expressed in cells in other animals.

We counted the number of DjP2X-A-positive cells among DjPiwiA-positive cells, and found that about 48 % of DjPiwiA-positive cells (i.e., neoblasts) were also positive for DjP2X-A (Fig. 2E). This result is consistent with the observations made by single cell PCR analysis, in which about half of cells expressing *DjpiwiA* mRNA were positive for *DjP2X-A* mRNA (Shibata *et al.,* 2012). Section immunohistochemistry using both anti-DjP2X-A antibody and anti-DjPiwiA antibody revealed that cells positive for both DjP2X-A and DjPiwiA were localized in the outermost layer of the mesenchymal space, near the epidermis, whereas many DjPiwiA-single-positive neoblasts were observed in a more internal region surrounding the intestine (Fig. 2 F,G).

RNAi of DjP2X-A *increases planarian fission frequency*

In order to elucidate the function of DjP2X-A, we conducted RNAi experiments. We employed a feeding RNAi method, in which dsRNA is introduced into planarians by mixing it with their food. We fed planarians dsRNA-containing liver three times in 1 week, and then amputated the planarians into three parts (anterior and posterior to the pharynx). The amputated fragments showed no defects and seemed to be able to regenerate normally within a week (data not shown).

Next, we performed long-term feeding of dsRNA without amputation. Planarians were fed dsRNA-containing food once per week for a month, after the third feeding of the initial week described above (taken as 0 day of RNAi). Surprisingly, we found that *DjP2X-A(RNAi)* planarians underwent fission more frequently than control animals after the initial RNAi series (Fig. 3A). We counted the number of fission events in both control and *DjP2X-A(RNAi)* animals for the entire length of the experiment (n=30 each). *DjP2X-A(RNAi)* planarians underwent fission about 28 times (2.57-fold higher than control), compared to about 10 times for control animals (Fig. 3A and Table 1). We performed the same experiment and included groups of X-ray-irradiated planarians. Five days after the initial week of RNAi feedings, control and *DjP2X-A(RNAi)* planarians were exposed to X-rays in order to eliminate their neoblasts. We found that neither control nor *DjP2X-A(RNAi)* X-ray-irradiated planarians ever fissioned, and they eventually died within 14 days after irradiation (Table 1). In contrast, non-irradiated *DjP2X-A(RNAi)* planarians underwent fission 14 times, compared to 5 times for control animals, indicating that the increase in fission observed after *DjP2X-A* RNAi occurs in a neoblast-dependent manner (Table1).

The increase in fission events observed in *DjP2X-A(RNAi)* did not result in smaller animals. We measured the fragments

TABLE 1

TOTAL NUMBER OF FISSION TIMES AFTER RNAi

	exp.1		exp. 2		exp. 3	
	Total # of fission times	X-ray	Total # of fission times	X-ray	Total # of fission times	X-ray
control	7	-	5	-	0 (dead within 2weeks)	+
P2X(RNAi)	18	-	14	-	0 (dead within 2weeks)	+
cont. : P2X(RNAi)	1 : 2.57		1 : 2.8			

21 days after 3rd feeding

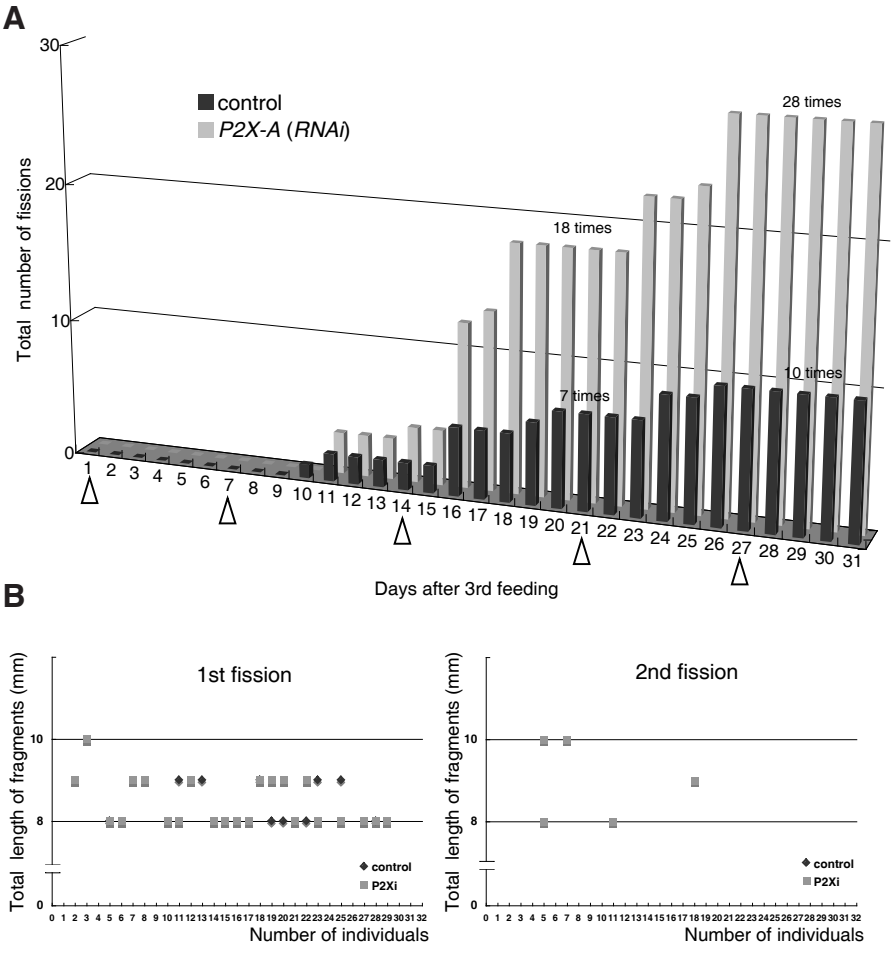

Fig. 3. Total fission events and size of planarians during the feeding RNAi. (A) *After feeding food containing dsRNA three times in 1 week, planarians were fed food containing dsRNA every week (arrowheads), and their fission events were counted. A total of 30 (6 mm) animals were evaluated.* **(B)** *Total length of the fragments of planarians derived from fission. Anterior and posterior fragments from a single planarian were measured and the sum of the two lengths was plotted.*

produced by fission in control and *DjP2X-A* RNAi animals. In both the control and *DjP2X-A(RNAi)* animals, the average length of the fragments after fission was over 8 mm (8-10 mm), even in the case of fragments produced by a second fission (Fig. 3B). These results indicated that the increase in fission events did not occur at the expense of size, and suggested that the growth of the planarians was promoted by *DjP2X-A* RNAi.

DjP2X-A expression negatively correlates with proliferative features

Previously, it was reported that the number of neoblasts in the M-phase of the cell cycle increases transiently after feeding (Baguñà 1974; Kang and Sánchez Alvarado, 2009). This transient acceleration of neoblast proliferation is called the “mitotic burst”. This mitotic burst is composed of two phases. The first phase peaks 12 to 24 hours after feeding, and the second wave 3 to 5 days after feeding. The animals return to a steady mitotic state 7 days after feeding, regardless of their body length (Baguñà 1974).

We observed that fission events tended to occur 2 days after every feeding (Fig. 3), and thus we speculated that DjP2X-A might be working by regulating the mitotic burst that occurs after feeding. To examine the relationship between DjP2X-A and cell proliferation, we analyzed the feeding-induced changes of two proliferative cell marker genes and *DjP2X-A*, by quantitative RT-PCR (qRT-PCR). We used *Djpcna* and *DjMCM2* as proliferative cell marker genes, which are mainly expressed in the S-phase of the cell cycle in neoblasts (Hayashi *et al.,* 2010). The expression level of *Djpcna* rapidly increased and peaked 12-24 hours after feeding, stayed above the steady state level until the third day after feeding, and reached a steady state level within 7 days (Fig. 4A). We also obtained similar results in the case of *DiMCM2* (Fig. 4A and Fig. 5A). The transient increase in the expression levels of these DNA replication-related genes coincided with the mitotic burst observed by immunofluorescence with antibodies against the M-phase marker phospho-histone H3 (pH3) and shown in previous reports (Fig 4 A,B; Baguñà 1974; Kang and Sánchez Alvarado, 2009; Newmark and Sánchez Alvarado, 2000). Thus, like the M-phase marker pH3, the expression of proliferative cell marker genes *Djpcna* and *DjMCM2* can be used to monitor increases in the mitotic cycling.

In contrast to the expression dynamics of proliferative cell markers, *DjP2X-A* expression declined slightly 12-24 hours after feeding. A slight increase in *DjP2X-A* expression was observed 3 days after feeding. *DjP2X-A* expression continued to rise and seemed to be at its highest level 7 days after feeding (Fig. 4A). The expression pattern of *DjP2X-A* appeared to be opposite to that of proliferative cell markers, suggesting a negative correlation between *DjP2X-A* expression and the post-feeding mitotic burst.

In order to examine whether the localization or number of DjP2X-A-positive cells changed in response to feeding, we performed detailed immunohistochemistry on sections using anti-DjP2X-A antibody. No obvious change of the distribution of DjP2X-A-positive cells was observed (data not shown), and no significant change of the number of DjP2X-A-positive cells was detected after feeding (Fig. 4D). Double immunohistochemistry using anti-DjP2X-A and anti-pH3 antibodies revealed that there were not only pH3/DjP2X-A-double-positive cells (Fig. 4C arrowhead), but also pH3-single positive and DjP2X-A-single positive cells (Fig. 4C). However, during the mitotic burst, the population of DjP2X-A/pH3-double-positive cells increased gradually. The proportion of DjP2X-A-positive cells among pH3-positive cells was approximately 30% before feeding (Fig. 4E). Seven days after feeding, the proportion increased and reached approximately 56% (Fig. 4E; $p < 0.05$), suggesting that DjP2X-A is involved in controlling cell proliferation after feeding.

DjP2X-A is involved in repressing post-feeding mitotic bursts

To elucidate the relationship between cell proliferation and DjP2X-A, we performed qRT-PCR analyses of proliferative cell markers

in recently fed *DjP2X-A(RNAi)* animals. We fed planarians food containing control or *DjP2X-A* dsRNA three times, and compared the expression of proliferative cell markers at several times after feeding with the expression just before feeding. We found that *DjP2X-A(RNAi)* animals showed higher *DjMCM2* expression levels than control animals, especially 1 to 7 days after feeding (Fig. 5 A,B). Similar results were obtained in analyses of *Djpcna* expression (data not shown), suggesting that proliferation, and not just *DjMCM2* expression, was higher in *DjP2X-A(RNAi)* than in control animals. Histological analysis using anti-pH3 antibody also showed that the number of pH3-positive cells was higher in *DjP2X-A(RNAi)* animals than in control animals 5 days after feeding (Fig. 5 C,D). These results show that DjP2X-A function is negatively associated with the mitotic burst after feeding.

Based on all these results, we propose that DjP2X-A negatively regulates the mitotic acceleration that occurs after feeding. Normally, neoblast proliferation is accelerated by feeding, and then decelerated by DjP2X-A after feeding. *DjP2X-A(RNAi)* planarians seem to be unable to slow down the accelerated proliferation that occurs in response to feeding. The maintenance of the accelerated rate of neoblast proliferation ultimately leads to the increased fission frequency observed in *DjP2X-A(RNAi)*.

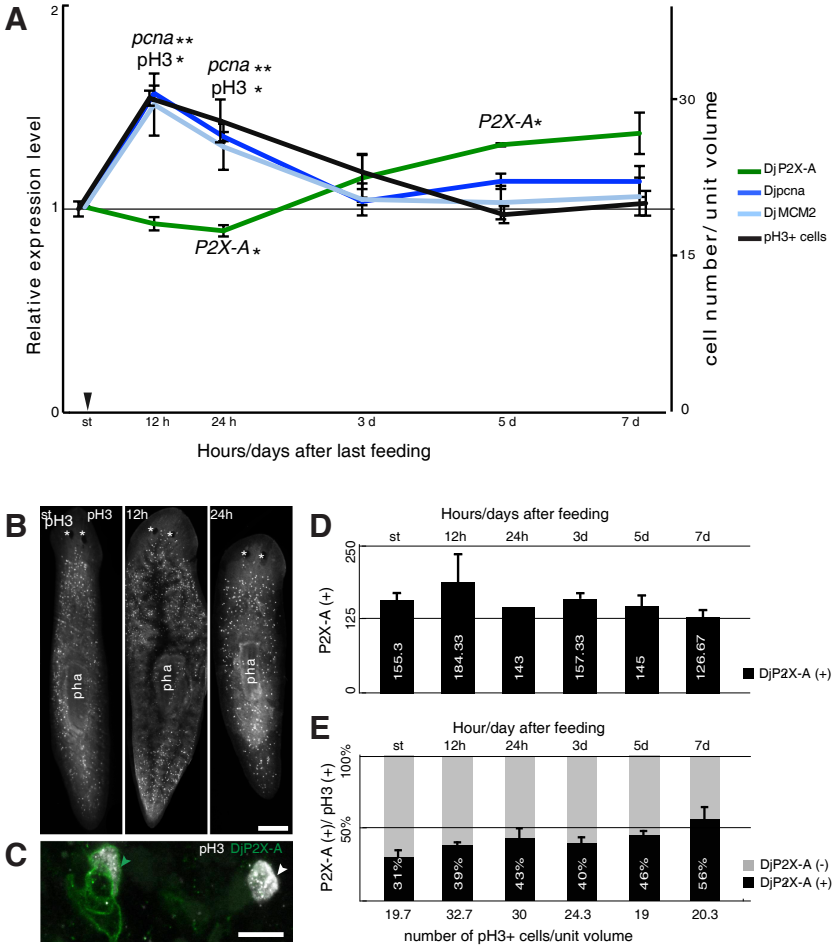

Fig. 4. Gene expression analyses and histological analyses of DjP2X-A after feeding. **(A)** *Gene expression level analysis by qRT-PCR from three independent experiments. The value at each time is expressed relative to that in starved animals. st: starved. ** p<0.005. * p<0.05* **(B)** *Whole-mount immuno-histochemistry using anti-pH3 antibody after feeding. Asterisk: eye. Scale bar: 500 μm.* **(C)** *Higher magnification view of whole-mount immuno-histochemistry using anti-pH3 antibody and anti-DjP2X-A antibody. Arrowheads indicate anti-DjP2X-A/ anti-pH3 double-positive cells. Scale bar: 50 μm.* **(D)** *Counting of DjP2X-A-positive cells in front of the pharyngeal region after feeding (n=3). Unit volume: 1.1 x 10^{-2} mm^3.* **(E)** *Quantification of DjP2X-A-positive cells among the pH3-positive cells in front of the pharyngeal region after feeding (n=3). Unit volume: 1.1 x 10^{-2} mm^3.*

DjP2X-A functions in activation of the cell cycle in starved animals

The above experiments clearly showed that DjP2X-A functions in controlling neoblast proliferation in response to feeding. We wondered whether DjP2X-A also regulates neoblast proliferation under starved conditions, during which mitotic bursts are not observed. We approached this question by disrupting DjP2X-A function by injection of dsRNA, a method that does not involve feeding and therefore does not induce a mitotic burst.

Surprisingly, *DjP2X-A(RNAi)* animals showed lower proliferative activity than control animals. The *Djpcna* expression level in *DjP2X-A(RNAi)* planarians was approximately 40% of that seen in control animals (Fig. 6A). This result agreed with histological analyses using BrdU labeling. The number of BrdU-labeled cells was significantly lower in *DjP2X-A(RNAi)* planarians when compared to control animals, whereas pH3-positive cells appeared to be only slightly decreased (Fig. 6 B,C). This decrease of BrdU labeling indicated that DjP2X-A is required to maintain the proliferative activity of neoblasts under starved conditions. Additionally, depletion of *DjP2X-A* by dsRNA injection did not have an effect on regeneration (data not shown), suggesting that *DjP2X-A* is not critical for cell differentiation.

Finally, we fed planarians that had previously undergone control or *DjP2X-A* dsRNA injection, and analyzed *Djpcna* expression at various times thereafter. Before feeding, the *Djpcna* expression level in *DjP2X-A(RNAi)* planarians was 40% of the level seen in the control animals. However, the expression of *Djpcna* in *DjP2X-A(RNAi)* planarians increased after feeding and ultimately became comparable to that seen in control animals by the seventh day after feeding (Fig. 6D).

Based on these results, we propose that DjP2X-A functions as a bidirectional cell-cycle modulator depending on nutrient availability: DjP2X-A works as a positive regulator under starved conditions and a negative regulator under eutrophic conditions.

Discussion

Here, we demonstrated heterogeneous expression and functions of DjP2X-A in the neoblasts of *D. japonica.* The extracellular ATP-binding region and cysteine residues needed to form disulfide bonds are conserved in the DjP2X-A amino acid sequence, suggesting that this protein can bind to extracellular ATP and work as an ionotropic receptor, like its homologs in other animals. The localization of the DjP2X-A protein on the membrane of neoblasts also supports this notion. It has been reported that there are seven human P2X family genes, and that their expression

is widely distributed throughout the entire human body. Each of them forms hetero-trimers to work as ionotropic receptors (Mio *et al.,* 2009). We also found five additional P2X family genes in *D. japonica* (Fig. S1); however, none of them were expressed in the neoblasts (Sakurai, unpublished data), suggesting that DjP2X-A might form homo-trimers on the neoblast membrane.

It is known that micro-environmental factors, such as growth factors or peptides, can regulate the cell proliferation and differentiation of stem cells. Recent findings have shown that various growth factors are involved in specifying the body polarity of planarians, such as the antero-posterior and dorsal-ventral axes. Hh (Hedgehog) and Wnt family members induce the differentiation of posterior cells from neoblasts (Gurley *et al.,* 2008; Yazawa *et al.,* 2009). BMP (Bone morphogenetic protein) is involved in dorsal cell specification of neoblasts (Molina *et al.,* 2007). Although it is clear that these factors can be expected to influence the cell fate via interactions with factors present in the cell membrane of neoblasts, the implications for cell proliferation of the neoblasts are still unclear. Here, we report the possibility that small molecules expected to exist in the mesenchymal space, such as ATP and ions, control cell proliferation via factors present in the membrane of neoblasts. This might be an important finding for understanding the relationship between two fundamental behaviors: the cell proliferation and differentiation of stem cells.

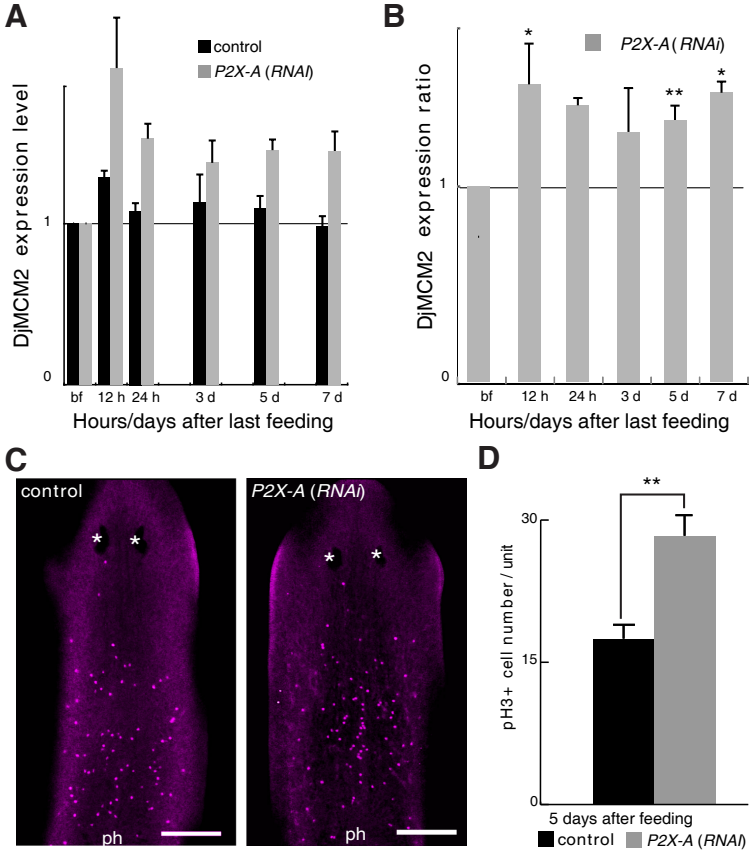

Fig. 5. Cell cycle defects observed after feeding *DjP2X-A* dsRNA. (A) *and* **(B)** *Expression level transition of* Djpcna *after third dsRNA feeding.* **(A)** *Relative expression level of* DjMCM2 *after* DjP2X(RNAi) *by qRT-PCR (n=4). Starved animals (st) were used to obtain the relative value. Both* DjP2X-A(RNAi) *and control animals were assayed after the third feeding.* **(B)** *Expression level of* DjMCM2 *in* DjP2X(RNAi) *animals relative to that in control animals. ** p<0.05. * p<0.01.* **(C)** *Images showing pH3-positive cells in both* DjP2X-A(RNAi) *and control animals at 5 days after the third feeding of dsRNA. Dorsal view. pha: pharynx. asterisk: eye. Scale bar: 250 μm.* **(D)** *Quantification of the number of pH3-positive cells (n=3). * p<0.05. Unit volume: 1.1 x 10^{-2} mm^3.*

DjP2X-A is involved in transient acceleration of neoblast proliferation after feeding

It has been reported that neoblasts show transient acceleration of proliferation after feeding, and return to a steady state of proliferation within a week (Baguñá, 1974). This transition could be monitored by quantification of the expression levels of *Djpcna* and *DjMCM2* in the present study (Fig. 4A), or by counting the number of pH3-positive cells (Kang and Sánchez Alvarado, 2009). We found that the expression level of *DjP2X-A* showed a negative correlation with the expression of cell proliferation markers: the *DjP2X-A* expression level was down-regulated when cell proliferation was accelerated, and up-regulated when the acceleration of cell proliferation subsequently decreased (Fig. 4A). This negative correlation was also observed during early regeneration (Fig. 1 C,D,E), suggesting that cue molecules that regulate the first mitotic burst might down-regulate expression of *DjP2X-A*. Furthermore, the number of DjP2X-A/pH3 double-positive cells increased from 24 hours to 7 days after feeding (Fig. 4E). From these results, we speculate that DjP2X-A is involved in the accelerated proliferation of neoblasts after feeding and is regulated by signals similar to those causing this acceleration. Furthermore, no obvious change of the number of DjP2X-A-positive cells was observed after feeding, suggesting that increased *DjP2X-A* expression by the neoblasts might occur after feeding.

We discovered that *DjP2X-A(RNAi)* animals showed a higher level of *DjMCM2* expression and increased number of mitotic cells compared to control animals after feeding (Fig. 5). These increases caused by RNAi were not observed during the initial acceleration of proliferation. This suggests that the function of DjP2X-A as an anti-proliferator is not always active, but rather works to slow the acceleration of proliferation resulting from some stimuli (such as food intake) to a normal steady state. Indeed, it has been reported that the P2X negatively regulates cell proliferation by regulating the Ca2+ current in human mesenchymal stem cells (Coppi *et al.,* 2007). We also observed that *DjP2X-A* expression was negatively correlated with the acceleration of proliferation during regeneration. Rat P2X7 has been reported to be involved in the differentiation of rat astrocytes in which ERK1 and 2 have been activated (Panenka *et al.,* 2001). *DjP2X-A(RNAi)* animals did not show obvious differences in the regeneration compared to control planarians. This result rules out the possibility the DjP2X-A functions in differentiation. Based on these results, we conclude that expression of DjP2X-A is up-regulated after the acceleration of proliferation, and that DjP2X-A then works to slow down proliferation.

DjP2X-A as a cell-cycle activity modulator

Although our data revealed that DjP2X-A works to negatively regulate cell proliferation after feeding, we also observed that *DjP2X-A(RNAi)* animals under starved conditions in injection RNAi experiments showed lowered

proliferative activity (Fig. 6). In murine microglia, P2X acts as a bifunctional receptor; for example, it can behave either as a death-inducing or a growth-promoting receptor depending on the level of its activation (Adinolfi *et al.,* 2005; Bianco *et al.,* 2006). Accordingly, we speculate that DjP2X-A changes its function in cell proliferation according to the cellular environment of the neoblasts: it works as a positive regulator in a starved environment and a negative regulator in a eutrophic environment.

How does DjP2X-A change its function in regulating proliferation according to the environment? It can be predicted that differences of extracellular ATP concentration lead to changes in the permeability of ions by affecting the activity of DjP2X-A (Fig. 7). In many types of cells in mammals, stimulation of P2X7 by ATP affects the permeability of ions into the cells, which in turn affects various cell behaviors, including cell proliferation (Adinolfi *et al.,* 2005). Accumulating evidence shows that in human neural stem cells, ions from various types of ion channels regulate proliferation (Yasuda & Adams 2010). Thus, the accelerated cell proliferation of the stem cells that occurs after feeding might be suppressed by higher concentrations of ions via DjP2X-A (Fig. 7A). In contrast, the extracellular ATP concentration is probably low in starved animals, and therefore DjP2X-A passes only a small amount of ions, which seems likely to be an appropriate amount of ions to keep the proliferation of the neoblasts at the steady state (Fig 7B). Thus, DjP2X-A might have bimodal and opposing functions affecting the proliferation of the neoblasts according to nutrient conditions.

What kind of molecular mechanism could induce acceleration of cell proliferation after feeding? Because the expression level of *DjP2X-A* was decreased during bursts of proliferation, and the neoblasts around the intestine did not express DjP2X-A, the molecular mechanism regulating cell cycle acceleration is also anticipated to regulate the expression of *DjP2X-A*. One possible candidate mechanism involves the mTOR pathway, which is known to have an important role in proliferation through regulating metabolic activity in various animals, including invertebrate animals such as *Drosophila Melanogaster* and *Caenorhabditis elegans* (Hafen 2004; Stiles *et al.,* 2004). It was reported that P2X7 transfected into HEK cells is downregulated by PIP2, which is upstream of the mTOR pathway (Zhao *et al.,* 2007). It has been reported that planarians have IGFR, AKT and PTEN homolog genes, which are involved in the mTOR pathway (Ogawa *et al.,* 2002; Oviedo *et al.,* 2008). Smed-Akt acts as a positive regulator of neoblast proliferation, and its expression is down-regulated by Smed-PTEN as well as the PI3K-Akt-TOR pathway in mammalian stem cells, suggesting that this pathway might accelerate neoblast proliferation after feeding in planarians (Sabatini 2006; Oviedo *et al.,* 2008). Thus, it would be of interest to investigate the expression of *DjP2X-A* in planarians with a disrupted mTOR pathway.

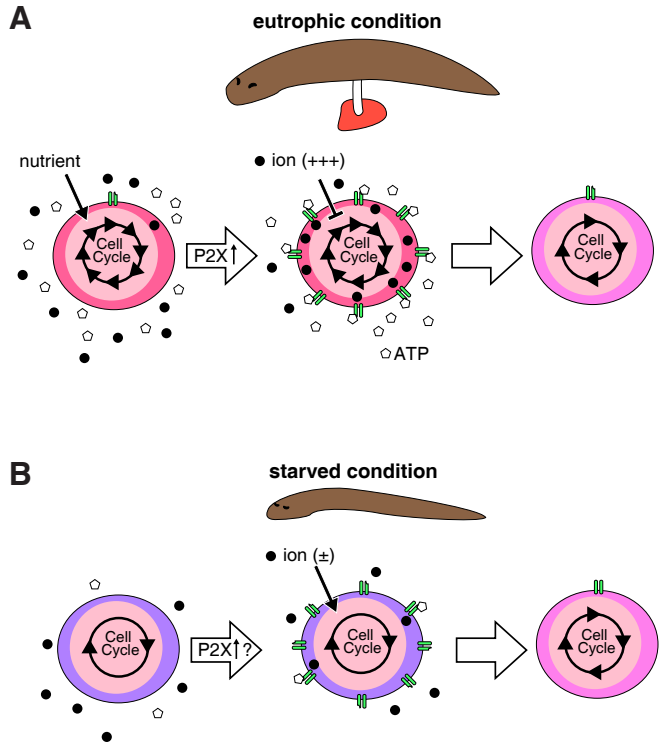

Fig. 7. A schematic illustration of predicted DjP2X-A function. (A) *Eutrophic condition and* **(B)** *starved condition.*

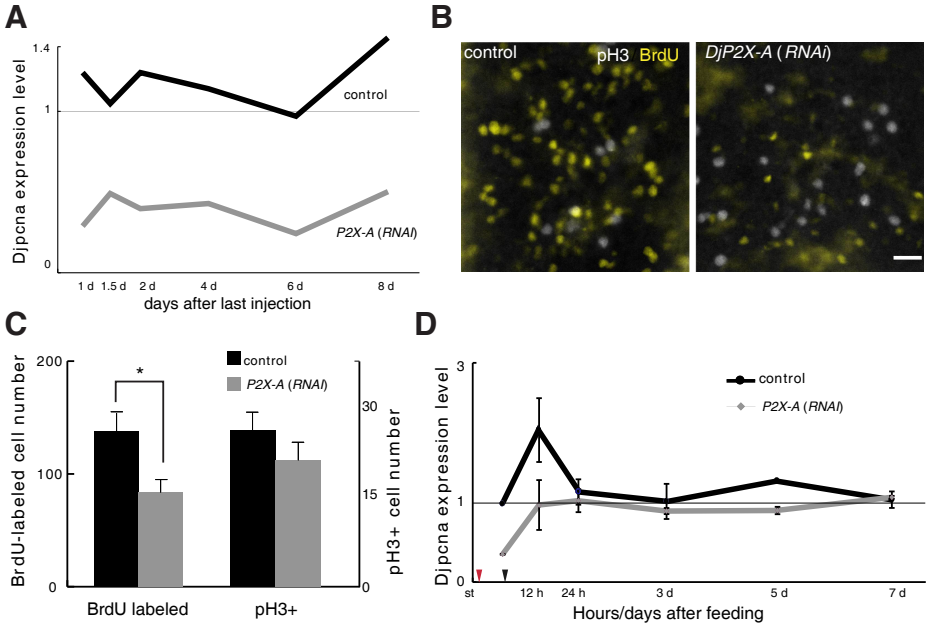

Fig. 6. *DjP2X-A* (RNAi) defects in starved condition. (A) *Expression level of* Djpcna *in animals subjected to dsRNA injection. Red arrowhead indicates time of third dsRNA injection.* **(B,C)** *Quantification of pH3-positive cells and of BrdU-labeled cells in both* DjP2X-A(RNAi) *and control animals at 1 day after the third dsRNA injection (n=3). Dorsal view. Unit volume: 1.1 x 10^{-2} mm^3. * p<0.05. Scale bar: 75 μm.* **(D)** *Expression level transition of* Djpcna *after dsRNA injection and feeding. Red arrowhead indicates time of the third dsRNA injection. Black arrowhead indicates time of feeding.*

DjP2X-A *RNAi increases planarian fission frequency*

Although the precise mechanism of planarian fission is not known, it is known that fission occurs in a manner dependent

on body length; in other words, dependent on the growth of body size. The first phenotype observed in *DjP2X-A(RNAi)* was increased fission frequency (Fig. 3). Indeed, *DjP2X-A(RNAi)* animals produced by feeding dsRNA had a higher rate of proliferation than control animals. The differentiation of the neoblasts seemed to be normal. The planarian body size is directly proportional to the total cell number (Takeda *et al.,* 2009). Therefore, we speculate that *DjP2X-A(RNAi)* animals might have an increase of total body cells and tend to grow faster than control animals. Actually, we measured and summed the length of both fragments just after fission, and observed that all of the planarians that underwent fission were over 8 mm long (Fig. 3B). This body length may be the threshold for fission, and our results strongly suggest that *DjP2X-A(RNAi)* animals grow and reach this "threshold" faster than control animals.

Overall, this study has given a glimpse into the relationship between the regulation of proliferation by DjP2X-A and fission. In addition, it has shown that the bidirectional function of DjP2X-A appears to contribute to the mechanism of the maintenance of the stem cell proliferation. More detailed analyses of how the acceleration and deceleration of the cell cycle are regulated by the microenvironment will give new insights into molecular and cellular mechanisms of not only asexual reproduction, but also stem cell dynamics.

Materials and Methods

Biological samples

A clonal strain of planarian *Dugesia japonica*, sexualizing special planarian (SSP) (2n=16), was cultured at 23°C in water sterilized by autoclaving. Chicken liver was fed to planarians once a week or once every 2 weeks. Animals had been starved for at least 1 week before all experiments.

X-ray irradiation

Animals placed on wet filter paper on ice were irradiated with 160 Roentgen of X-rays using an X-ray generator (SOFTEX B-5; SOFTEX, Tokyo, Japan).

Feeding RNA interference

AMEGAscript Kit (Ambion) was used for double-stranded RNA (dsRNA) synthesis. The linearized DNA with a T7 or T3 promoter site was used as a template for antisense or sense RNA transcription. Antisense or sense RNA was transcribed for 3 hours at 37°C. After RNA transcription, RNAs were treated with DNAse TURBO to remove the template DNA, and the RNA transcripts were mixed and extracted with phenol/chloroform. Mixed RNA transcripts were denatured for 20 minutes at 65°C and annealed for 40 minutes at 37°C. The resultant dsRNA was precipitated with ethanol and dissolved in water at 2.0 μg/μL.

dsRNA was used to knock down the target genes by RNAi. Twenty-five microliters of chicken liver solution, 5 μL of 2% agarose and 5 μL of 2 μg/μL dsRNA were mixed and presented to a group of 15 planarians. This food solution was prepared in small aliquots (about 35 μL) and frozen at -30°C for storage. Two successive feedings were conducted at 3-day intervals after the 1st feeding. Control animals were fed dsRNA of EGFP.

Injection RNA interference

RNA interference was also performed by injecting dsRNA directly, prepared as described above, into the planarian intestine. Two successive injections were conducted at intervals of one day after the first injection. Control animals were injected with dsRNA of EGFP.

Whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization was performed as described previously (Umesono *et al.,* 1997). The plasmid pBluescript SK containing the gene of interest was used as the template for RNA probe synthesis. The plasmid was linearized with NotI at the 5' region of the target genes. The linearized DNA was used as a template for antisense RNA transcription by T7 RNA polymerase (Fermentas). Antisense RNA probe was labeled with digoxigenin (Dig) according to the manufacturer's instructions (Roche Diagnostics).

Animals were treated with 2% HCl in 5/8 Holtfreter's solution for 5 minutes to clear epidermal mucus, and fixed in 4% paraformaldehyde/5% methanol in 5/8 Holtfreter's solution for 1 hour at 4°C. Then, the samples were bleached with 5% H_2O_2 in methanol overnight at room temperature under fluorescent light. Bleached samples were treated with 1:1 xylene:ethanol solution for 30 minutes at room temperature and rinsed in 100% ethanol for 30 minutes at 4°C. Then, the samples were rehydrated through a graded ethanol series (75%, 50% and 25%) in 5/8 Holtfreter's solution for 30 minutes each at 4°C. The rehydrated samples were rinsed in TPBS for 30 minutes at 4°C and permeabilized by 2 μg/ml proteinase K in TPBS treatment for 7 minutes at 37°C. Then, the samples were post-fixed in 4% paraformaldehyde / 5% methanol in 5/8 Holtfreter's solution for 30 minutes at 4°C and rinsed in TPBS twice each for 5 minutes at 4°C. Next, the samples were soaked in prehybridization solution for 1 hour at 55°C, and incubated with Dig-labeled antisense RNA probes (probes were denatured previously for 20 minutes at 65°C) in hybridization solution for 36 hours at 55°C. Then, the samples were washed in wash solution 8 times for 30 minutes each at 55°C and rinsed in Buffer I, 2 times for 5 minutes each at room temperature. The rinsed samples were treated with Buffer II for blocking for 1 hour at room temperature and treated with 1/2000 alkaline phosphatase-conjugated anti- Digoxigenin antibody (Roche Diagnostics) in Buffer I overnight at 4°C. Samples were rinsed in Buffer I four times for 30 minutes each at room temperature and washed in TMN solution two times for 5 minutes each at room temperature. In order to detect color signals, a mixture of 3.5 μg/ml 5-bromo-4-chloro-3-indolyl phosphatase (Roche Diagnostics) and 1.8 μg/ml 4-nitro blue tetrazolium chloride (Roche Diagnostics) in TMN solution was used. After the detection, TE buffer was used to stop the reaction and the samples were stored in TE buffer at 4°C.

Antibody

A cDNA fragment encoding DjP2X-A was amplified by PCR with primers 5'-TTTGGATCCAAACTCAATGGTATTGGGATGACG-3' and 5'-TTTAAGCTTGAATAGATCTCTGTATTCTTTGG-3' using plasmid DNA as a template, and digested with *BamHI* and *HindIII*. The digested fragments were sub-cloned into pQE30 vector (QIAGEN). Recombinant 6xHis-DjP2X-A protein was purified and used as antigen. Mouse monoclonal antibodies for DjP2X-A were generated by MBL (Nagoya, Japan).

Whole-mount immunohistochemistry

The sample animals were treated with 2% HCl in 5/8 Holtfreter's solution for 5 minutes to clear epidermal mucus, and fixed in Carnoy's solution (60% ethanol / 30% chloroform / 10% acetic acid) for 2 hours at 4°C. Then, the samples were bleached with 5% H_2O_2 in methanol overnight at room temperature under fluorescent light. Bleached samples were treated with 1:1 xylene:ethanol solution for 30 minutes at room temperature and rinsed in 100% ethanol for 30 minutes at 4°C. The samples were rehydrated through a graded ethanol series (75%, 50% and 25%) for 30 minutes each at 4°C. The rehydrated samples were rinsed in TPBS for 30 minutes at 4°C and permeabilized with 2 μg/ml proteinase K in TPBS treatment for 7 minutes at 37°C. Then, the samples were post-fixed in 4% paraformaldehyde/5% methanol in 5/8 Holtfreter's solution for 30 minutes at 4°C and rinsed in TPBS twice at 4°C for 5 minutes each. For blocking, the samples were soaked in 10% goat serum in TPBS for 30 minutes at room temperature. After blocking, the samples were incubated in 10% goat serum in TPBS containing 1/1000 diluted primary antibody overnight at 4°C. The samples were rinsed in TPBS five times for 30 minutes each at room temperature, and incubated in 10% goat serum in TPBS containing 1/1000 fluorescent-labeled goat secondary antibody (Alexa Fluor 594 or Alexa Fluor 488 (Molecular Probes)) and 1/1000 Hoechst33342 (Calbiochem) overnight at 4°C. Finally,

the samples were rinsed in TPBS four times for 30 minutes each at room temperature, and mounted with Fluorescent Mounting Medium (Dako).

BrdU incorporation and detection

Bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich) was dissolved at 10 mg/ml in water and injected into the planarian intestine. The injected animals were incubated at 23°C in culture water. The animals were then treated with 2% HCl in 5/8 Holtfreter's solution for 5 minutes to clear epidermal mucus, and fixed in 4% paraformaldehyde / 5% methanol in 5/8 Holtfreter's solution for 1 hour at 4°C. Immunohistochemistry was performed as described above. After incubation with secondary antibody, samples were post-fixed in 4% paraformaldehyde/5% methanol in 5/8 Holtfreter's solution for 15 minutes at 4°C and rinsed in TPBS twice at 4°C for 5 minutes. Samples were then rinsed twice with TPBS for 30 minutes each, and treated with 2N HCl for 30 minutes at room temperature. After they were rinsed two times with TPBS for 30 minutes each at room temperature, the samples were blocked in 10% goat serum in TPBS for 30 minutes at room temperature. After blocking, the samples were incubated in 10% goat serum in TPBS containing 1/25 diluted anti-BrdU antibody (Becton, Dickinson) overnight at 4°C. The next day, the samples were rinsed in TPBS four times for 30 minutes each at room temperature, and BrdU signals were detected using a TSA labeling Kit No. 2 (Molecular Probes).

Section immunohistochemistry

The sample animals were fixed in Carnoy's solution (60% ethanol / 30% chloroform / 10% acetic acid) for 2 hours at 4°C. The fixed samples were dehydrated with ethanol and xylene solutions of gradually increasing concentration. Then the dehydrated samples were embedded in paraffin and sectioned at 10 μm. Paraffin was removed from the sections with xylene and a series of ethanol solutions of gradually decreasing concentration. Then the sections were washed in TPBS and autoclaved in 10 mM citric acid buffer (pH 6.0) for 10 minutes at 120°C and washed in TPBS. For blocking, the sections were soaked in 10% goat serum in TPBS for 30 minutes at room temperature. After blocking, the sections were incubated in 10% goat serum in TPBS containing 1/1000 primary antibody overnight at 4°C. The next day, the sections were rinsed in TPBS five times for 30 minutes each at room temperature, and incubated in 10% goat serum in TPBS containing 1/1000 diluted fluorescent-labeled goat secondary antibody (Alexa Fluor 594 / Alexa Fluor 488 (Molecular probe)) overnight at 4°C. The sections were rinsed in TPBS three times for 10 minutes each at room temperature, and mounted in Fluorescent Mounting Medium (Dako).

Quantitative real-time PCR analysis

Total RNA was extracted by using ISOGEN-LS (Wako) following the manufacturer's protocol. cDNA was synthesized using a QuantiTect Reverse Transcription Kit® according to the manufacturer's instructions (QIAGEN). The synthesized cDNA was appropriately diluted, and used for gene expression analysis by quantitative real-time PCR. Reactions were carried out in 10 μl of real-time PCR mixture containing 1x QuantiTect SYBR green PCR master mix (QIAGEN), 0.3 μM gene-specific forward/reverse primers and 1 μL of diluted cDNA template by incubation in an ABI PRISM 7900 HT (Applied Biosystems). The reactions were carried out as follows: 50°C for 2 minutes, 95°C for 15 minutes, 50 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 1 minute.

The forward and reverse primer sets for the investigated genes are listed below (5' to 3').

G3PDH
FW: ACCACCAACTGTTTAGCTCCCTTAG
RV: GATGGTCCATCAACAGTCTTTTGC

Djpcna
FW: ACCTATCGTGTCACTGTCTTTGACCGAAAA
RV: TTCATCATCTTCGATTTTCGGAGCCAGATA

DjMCM2
FW: CGCTGTTGGACAAGGTCAGAAGAATGAACA
RV: CCAGAAACACAAATCTACATCTTCCAAAGG

DjP2X-A
FW: GATTTCAACAATGGAATGAATTTTAGATA
RV: AAAATGTGAAACAAGTAGCAGGATCA

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