Molecular mechanism by which *Djplac8-A* controls proliferation/differentiation of planarian pluripotent stem cells during regeneration

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Contents

1. Abstract

Planarians have an adult pluripotent stem cell population called neoblasts, which are the only cell source for planarian regenerative ability. It is known that neoblasts undergo transient accelerated proliferation after feeding and amputation, so-called “induced hyper-proliferation”. Although this phenomenon has been known for decades, the molecular mechanism and biological meaning behind this was unclear. Thus, I focused on induced hyper-proliferation and revealed that reduction of the expression level of a single gene, namely *Djplac8-A*, could induce hyper-proliferation after feeding or amputation.

In this thesis, I show that reduction of *Djplac8-A* expression, one of the neoblast-specific genes, led to induced hyper-proliferation after feeding, similarly to *DjP2X-A*, which was previously reported to be involved in induced hyper-proliferation after feeding. Interestingly, reduction of *Djplac8-A* expression also led to induced hyper-proliferation after amputation, and this was caused by activated JNK signaling at the post-blastema region after amputation. In addition, I found that ERK signaling was also required to induce hyper-proliferation via activation of JNK signaling. Pharmacological inhibition of JNK signaling caused failure to induce hyper-proliferation and resulted in regenerative defects. Such defects were rescued by simultaneous knockdown of *Djplac8-A* expression, suggesting that JNK-dependent suppression of *Djplac8-A* expression is indispensable for proliferation and differentiation of neoblasts during regeneration. I propose that *Djplac8-A* acts as a molecular

switch of neoblasts for entry into the active state from the steady state through induced hyper-proliferation after amputation.

2. Abbreviations

BMPs	Bone Morphogenetic Proteins
D. japonica	*Dugesia japonica*
ERK	Extracellular signal-Regulated Kinase
ESCs	embryonic stem cells
FACS	fluorescence-activated cell sorting
FGF	Fibroblast Growth Factor
GSCs	germline stem cells
HSCs	hematopoietic stem cells
HiCEP	comprehensive gene expression analysis
JNK	c-JUN N-terminal kinase
LIF	Leukaemia Inhibitory Factor
MHC	*Myosin Heavy Chain*
PSCs	pluripotent stem cells
RNAi	RNA interference
RNP	RNA-Protein
S. *mediterranea*	*Schmidtea mediterranea*
vlg	*vasa-like gene*
cNeoblasts	clonogenic neoblasts
dsRNA	double-stranded RNA
iPSCs	induced Pluripotent Stem Cells
pH3	phosphohistone H3
pJNK	phosphorylated JNK
plac8	*placenta specific gene 8*

3. Introduction

3.1 General characteristics of stem cells

Stem cells are defined by two major characteristics: 1) proliferative ability (produce two identical daughter cells) and 2) differentiative ability (transition to different cell type than itself). Thus, stem cells can perform cell proliferation and generate differentiated cells. Generally, stem cells are classified into several types depending on their differentiative ability: 1) totipotent, 2) pluripotent, 3) multipotent, 4) oligopotent, and 5) unipotent (Fig 1, Zakrzewski et al., 2019). Totipotent stem cells are the highest potential type of stem cells since a single totipotent stem cell can form a complete organism. Totipotency is a typical characteristic of plant cells because they have high dedifferentiation ability, which is going back to the undifferentiated state from a differentiated state, and a single dedifferentiated cell can differentiate into a single complete organism (Grafi et al., 2011). On the other hand, in animals, totipotentcy is observed in only in the very early stage of development, which is the fertilized egg (Bindu A and B, 2011). Pluripotent stem cells (PSCs), for example embryonic stem cells (ESCs) or induced pluripotent stem cell (iPSCs), can differentiate into all types of cells constructing individuals except for extraembryonic cells (Smith et al., 2009; Takahashi and Yamanaka, 2006). For example, ESCs can differentiate into not only all types of somatic cells, but also germ cells (Keller, 1995; Smith, 2001). Multipotent stem cells having a narrower spectrum of differentiative potentiality than that of pluripotent stem cells can differentiate into

multiple types of cells in a restricted lineage. For example, hematopoietic stem cells, which are a kind of somatic tissue stem cell, can differentiate into all types of blood cells and lymph cells (Seita and Weissman, 2010; Zakrzewski et al., 2019). Oligopotent stem cells have a narrower spectrum of differentiation than that of multipotent stem cells, thus they can differentiate into a few types of cells. For example, myeloid stem cells can differentiate into blood cells including basophils and red blood cells and so on, or lymphoid stem cells differentiate into lymphocytes, including T cells and B cells (Seita and Weissman, 2010; Zakrzewski et al, 2019). Unipotent stem cells have limited potential to differentiate into only one type of differentiated cell, for example, germline stem cells (GSCs) are a type of unipotent stem cell which differentiate into only sperm or egg (Lehmann, 2012).

Since plant cells have totipotency, as mentioned above, they can dedifferentiate, reenter the cell cycle, and differentiate into the other cell types required for regenerating tissues, organs, and even entire plants under certain conditions (Grafi et al., 2011). On the other hand, the differentiative potency of stem cells in animals becomes more limited along the developmental process and then most adult animals have a small population of stem cells with limited differentiative ability such as multipotency or unipotency (Fig. 1) (Bonnet, 2002).

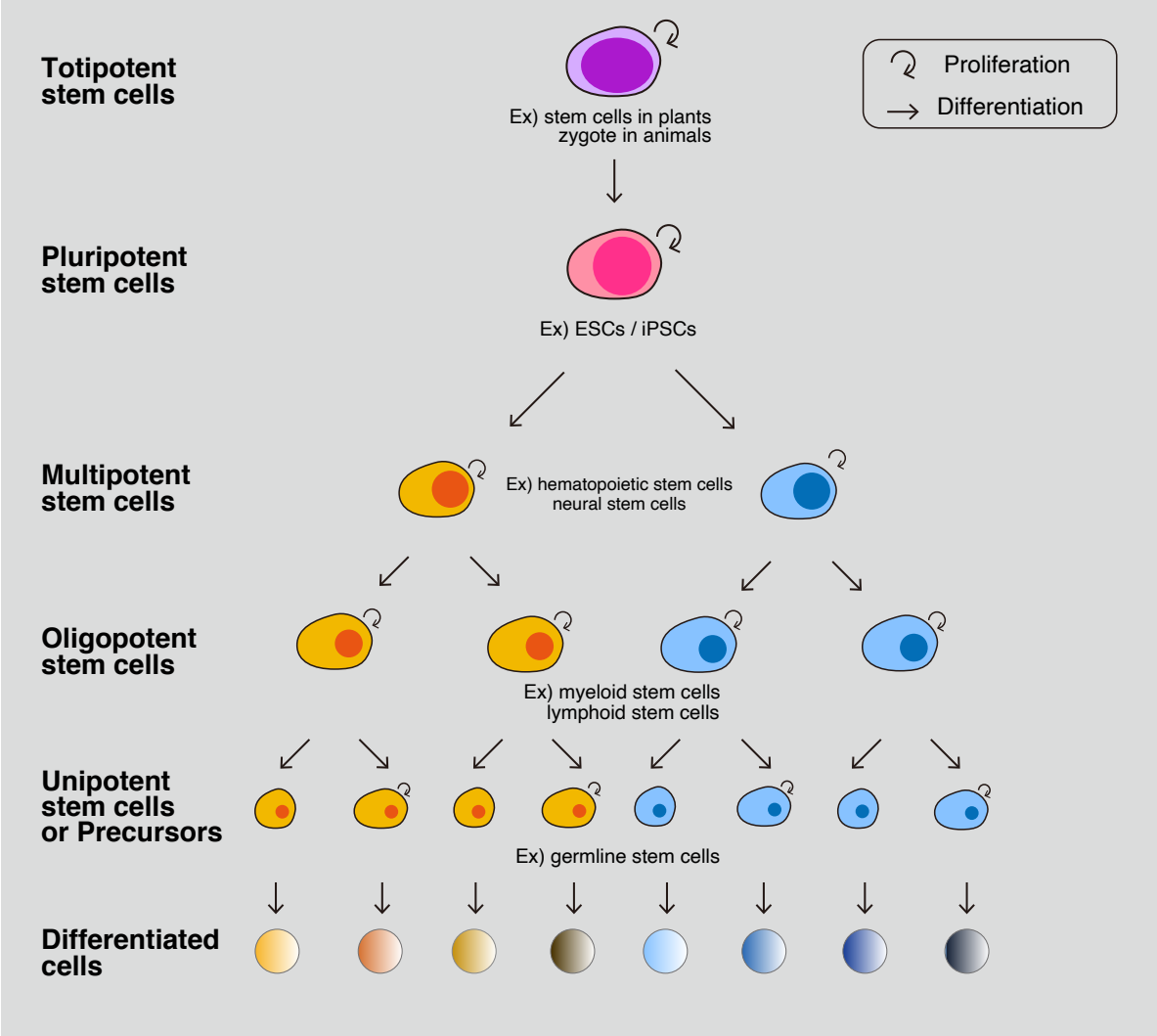


Fig 1. Hierarchy of stem cells. Proliferative ability and differentiative ability of major characteristics of stem cells. Stem cells are classified into several types depending on their differentiative ability.

3.2 Relationship between proliferation and differentiation of stem cells

Stem cells have many important roles in life phenomena, such as organogenesis during the embryonic development process, or tissue homeostasis and regeneration in adult bodies. To achieve these roles, stem cells should supply differentiated cells while an appropriate size of a population of stem cells should be kept by avoiding over-proliferation and exhaustion. For

this, for example, many tissue stem cells employ asymmetric division to produce lineage restricted progenitors (or differentiated cells) and stem cells (Inaba and Yamashita, 2012; Piccin and Morshead, 2011). Asymmetric division is regulated by intrinsic factors such as intracellular polarity or by extrinsic factors such as growth factors and/or niche (microenvironment) (Lin, 2008). For asymmetric division regulated by intrinsic factors, neuroblasts in *Drosophila* have been well studied (Inaba and Yamashita, 2012; Rolls et al., 2003). The neuroblasts divide asymmetrically to generate a daughter neuroblast and a ganglion mother cell which differentiate into neurons or glia through several subsequent cell divisions (Bowman et al., 2008). Par-3/Par-6/ atypical protein kinase K complex and PINS/Ga/MUD(NuMA) complex are localized at the apical cortex of the mother neuroblast. Par-3/Par-6/ atypical protein kinase K complex polarizes the cell and PINS/Ga/MUD(NuMA) complex orients the spindle. Then, these complexes dictate localization of fate-determinants such as Miranda, Numb at the basal side for ganglion mother cell differentiation. The spindle orientation is aligned with the apical-basal polarity axis, and thus cells at the basal side inherit the fate determinants to become a ganglion mother cell after asymmetric cell division (Knoblich, 2008) One of the typical extrinsic factors affecting asymmetric division is the niche, where is a specific area that maintain the stem cells in the undifferentiated state. For example, male GSCs of *Drosophila* divide asymmetrically in a niche-dependent way. The GSCs niche consists of hub cells at the tip of the gonad. These niche cells create a specialized area for self-

renewal by secreting ligand Upd to activate the JAK/STAT pathway and ligand Dpp to activate bone morphogenetic proteins (BMPs). Thus, part of mother GSCs which are attached to the niche (Hub cells) become daughter GSCs and another part of mother GSCs aaway from the niche become goniablasts upon division (Inaba and Yamashita, 2012). Such asymmetric division may be disadvantageous when a huge loss of the stem cell population occurs, because there is difficulty to replenish the stem cell pool by asymmetric division. For dealing with this disadvantage, stem cells can perform another type of proliferation, which is symmetric division to maintain the stem cell pool as a population (Shahriyari and Komarova, 2013).

Symmetric cell division generates two identical daughter cells (it can be two stem cells, or also two differentiated cells). For example, mouse neural stem cells symmetrically divide depending on activated Wnt signaling after injury for increasing their number (Piccin and Morshead, 2011). In contrast to this, mouse neural stem cells divide asymmetrically when Wnt signaling is absent under the normal condition (Piccin and Morshead, 2011). Also, in hematopoietic stem cells (HSCs) of mouse, asymmetric and symmetric divisions are determined depending on the CD34 mRNA expression level. To produce differentiated hematopoietic cells after injury, the expression level of CD34 mRNA is up-regulated for increasing activated-state HSCs, and then the expression level of CD34 mRNA is down regulated to quit producing differentiated cells and proliferate to maintain the HSCs population (Wilson et al., 2008).

In vitro, the decision between proliferation and differentiation of stem cells is controlled in specific conditions by certain growth factors. ES cells can proliferate indefinitely by generating only themselves when leukaemia inhibitory factor (LIF) and BMPs are added to culture medium to prevent differentiation (Chambers and Smith, 2004; Ying et al., 2003). On the other hand, fibroblast growth factor (FGF) signaling is required to exit from self-renewal state and start to differentiate (Kunath et al., 2007)..

In this way, the decisions between proliferation and differentiation are very closely connected and tightly controlled by numerous regulatory factors such as asymmetric/symmetric division or growth factors, as noted above. However, the molecular switch(es) of stem cells for determining the decision of proliferation for self-renewal versus differentiation are largely unknown. It is important to discover the molecular mechanisms keeping the balance between proliferation and differentiation in order to understand the stem cell systems in animals and plants, and to improve stem cell engineering.

3.3 Planarians

"Planarian" is usually used for describing free-living flatworms. In taxonomy, planarians are classified as Platyhelminthes (flatworms), order Tricladida. They live in both sea water and freshwater. More than 380 planarian species are reported worldwide (Tyler S, Schilling S, Hooge M, Bush L. Turbellarian taxonomic database. 2013.). In Japan and Asia,

the best-studied species of freshwater planarian is *Dugesia japonica (D. japonica)* (Fig. 2A). It is easy to find them under rocks or fallen leaves in freshwater areas such as ponds or rivers. Their body length is up to 25 mm, and they have a triangular head (Nishimura et al., 2015). Two eyes are located on the dorsal side of the head. The central nervous system, which is composed of a U-shaped brain in the head region and a pair of ventral nerve cords, is located on the ventral side inside the body (Fig. 2B; Agata et al., 1998). The intestine is composed of 3 main branches which are located along the entire body (Fig. 2C; Orii et al., 2002). Planarians can reproduce sexually and asexually depending on the breeding conditions. *Dugesia japonica* undergo sexual reproduction through sexualization from the asexual state under certain conditions, such as low temperature. However, most of them undergo asexual reproduction *via* fission. Fission consists of division at the pre- or post- pharyngeal region into two fragments when planarians reach a certain size by growth. The divided fragments become two planarians through regeneration (Agata et al., 2006; Sakurai et al., 2012).

Many species of planarian, including *D. japonica*, have extraordinary regenerative ability. When a planarian is cut into 6 fragments with a razor, wound healing begins to occur immediately after amputation. A blastema, which is composed of differentiating and differentiated cells derived from the neoblasts (planarian adult somatic pluripotent stem cells), is formed at the wound region at one day after amputation. At 3 days after amputation, eyes are regenerated, and all fragments regenerate into morphologically functionally complete

individuals within 1 week (Fig. 2D; Agata et al., 2003; Agata and Watanabe, 1999; Umesono and Agata, 2009).

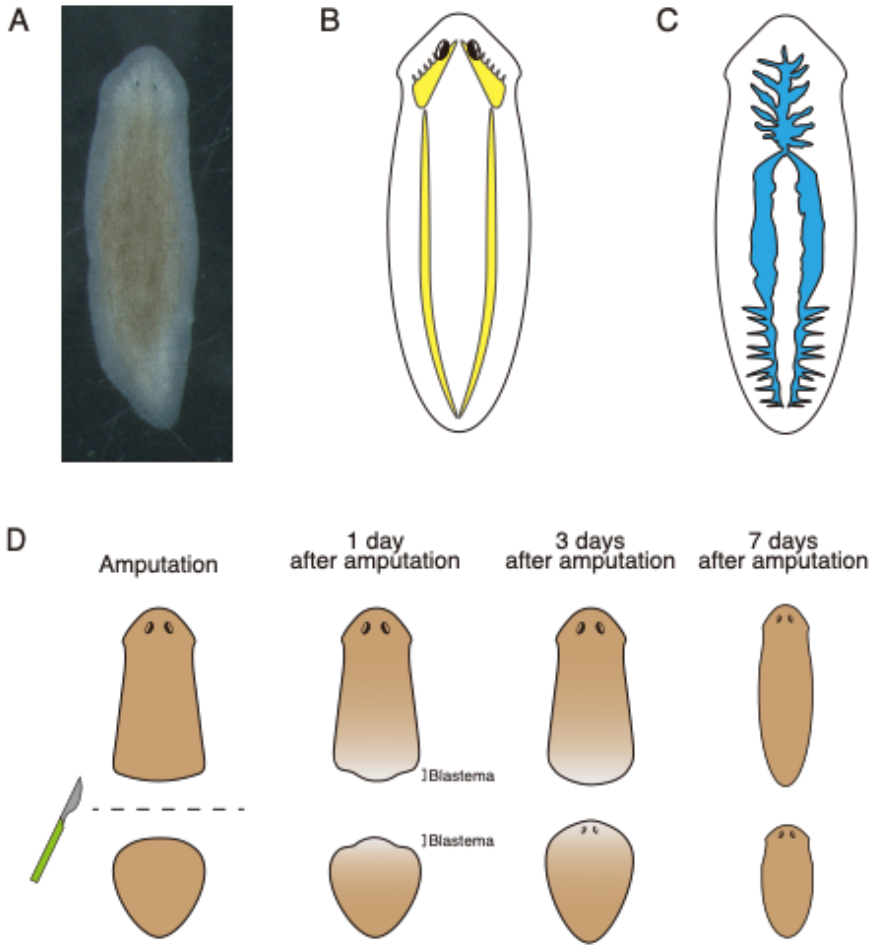


Fig 2. *D. japonica* and their regeneration (A). Freshwater planarian, *Dugesia japonica.* (B) Schematic drawing of planarian nervous system. (C) Schematic drawing of planarian intestinal duct. (D) Schematic drawings of planarian regeneration.

The regenerative ability of planarians has attracted intense interest of scientists from 18th century. T. H. Morgan favored planarians for studying regeneration (Morgan, 1898). T. H. Morgan reported that planarians could regenerate from pieces consisting of about 1/279th of

one planarian. While most early studies of planarian regeneration were observational studies about the regeneration process, the recent development of highly sophisticated experimental techniques has led to planarians becoming model animals for investigating regeneration at the molecular level.

3.4 Neoblasts: planarian adult pluripotent stem cells

Recently, planarian regeneration has been increasingly studied at the cellular and molecular levels by using highly developed biological techniques. Based on the accumulated knowledge about planarian regeneration, neoblasts, which are planarian adult somatic pluripotent stem cells, are thought to be the central cells in planarian regeneration. The neoblasts were classically classified by several morphological features through electron microscopy, namely, undifferentiated morphology with minimal cytoplasm, many free ribosomes, no endoplasmic reticulum, and chromatoid bodies which are neoblast-specific RNA-protein (RNP) granules (Morita et al., 1969; Pedersen, 1959). Neoblasts are specifically eliminated by X- or gamma-ray irradiation, followed by loss of regenerative ability, suggesting that planarians' regenerative ability is dependent on neoblasts (Wolff and Dubois, 1948).

During the past two decades, many neoblast-specific genes have been identified. First, there was a report that a gene expressed in neoblasts was a germ-cell-related gene, namely *vasa-like gene A (DjvlgA)*, in *D. japonica* (Shibata et al., 1999). After this finding, numerous

reports showed that many homolog genes of germ cell-specific or germ cell-related genes in other non-regenerative animals are expressed and function in neoblasts in *D. japonica* and a related species, *Schmidtea mediterranea* (*S. mediterranea)* (Guo et al., 2006; Hayashi et al., 2010; Reddien et al., 2005; Rossi et al., 2006; Salvetti et al., 2005; Shibata et al., 2012, 2010; Solana et al., 2009; Yoshida-Kashikawa et al., 2007). Among them, planarian *piwi* homolog family genes, the *Djpiwi* and *smedwi* families in *D. japonica* and *S. mediterranea*, respectively, are expressed specifically or predominantly in neoblasts. Especially, *DjpiwiA* (*smedwi-1* in *S. mediterranea*) has been used as a specific marker gene for neoblasts in many studies (Hayashi et al., 2010; Palakodeti et al., 2008; Reddien et al., 2005; Shibata et al., 2016; Yoshida-Kashikawa et al., 2007). *In situ* hybridization of *DjpiwiA* showed that neoblasts are localized in the mesenchymal space throughout the entire body except in the region anterior to the eyes and in the pharynx (Shibata et al., 2010). These *DjpiwiA* (or *smedwi-1*)-positive neoblasts were known to continuously proliferate (Fig. 3; Newmark and Sánchez Alvarado, 2000). Previously, neoblasts in individuals were considered to be a homogeneous population that constituted ~25% of total planarian cells (Yoshida-Kashikawa et al., 2007; Reddien et al., 2005).

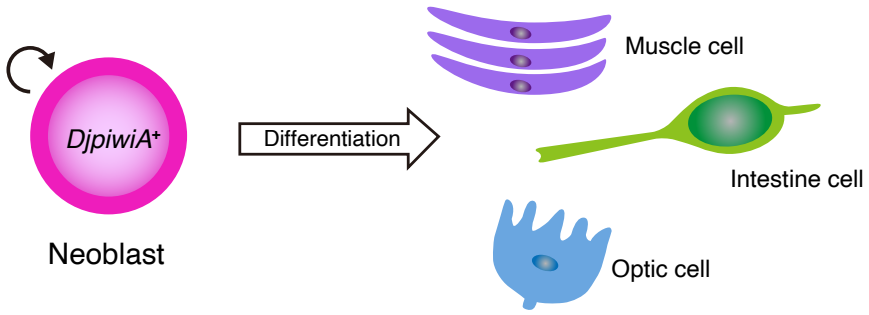


Fig. 3. Self-renewal and differentiation of neoblast. Neoblasts are the only cell population having ability to proliferate and differentiate into all types of cells in planarians. *DjpiwiA* is one of the reliable marker genes for neoblasts.

Previously, it was shown that transplantation of a cell population including neoblasts into irradiated planarians could rescue their regenerative ability, suggesting the pluripotency of neoblasts (Fig 3; Baguna et al., 1989). Establishment of a cell sorting technique using fluorescence activated cell sorting (FACS) led to further progress in the identification and purification of neoblasts. Based on comparison of the cell sorting profiles between intact and X-ray-irradiated animals, neoblasts were sorted into two fractions: X1 (S - M phase neoblasts) and X2 (G1 neoblasts and X-ray-insensitive cells) fractions (Hayashi et al., 2006). A single neoblast purified by FACS could repopulate and rescue the regenerative ability of an irradiated individual after transplantation in *S. mediterranea,* proving the pluripotency of neoblasts (Wagner et al., 2011). However, only a limited subpopulation of the neoblasts could repopulate and restore the regenerative ability when transplanted into irradiated planarians, suggesting possible heterogeneity of the differentiative ability of neoblasts, and the possibility that only a few neoblast cells might have true pluripotency. These predicted pluripotent neoblasts were

named clonogenic neoblasts (cNeoblasts) (Wagner et al., 2011). Gene expression analysis by using FACS-based single cell RT-PCR also showed heterogeneity of *DjpiwiA*-expressing neoblasts (Hayashi et al., 2010). Examination of the expression of differentiated cell marker genes, such as *myosin heavy chain A (DjMHC-A)*, which is a muscle cell-specific marker, showed that few *DjpiwiA*-expressing neoblasts were also expressing *DjMHC-A* in the X1 fraction (Hayashi et al., 2010). More recently, comprehensive gene expression analysis by single cell RNA sequencing of a considerable number of neoblasts revealed that neoblasts could be divided into 12 subpopulations according to the expression levels of *smedwi-1* and transcription factors. Among them, only one group expressing *tetraspanin* could rescue sub-lethal irradiated planarians, suggesting that *tetraspanin*+ neoblasts might be cNeoblasts (Zeng et al., 2018). Although a lot of knowledge about neoblasts has been accumulated, further study is still required to understand the regulatory mechanisms for the fundamental roles of neoblasts such as the regulation balancing proliferation *versus* differentiation.

3.5 Proliferation and differentiation of neoblasts

As mentioned above, the precise regulation of proliferation and differentiation of stem cells is a very important issue. In the case of planarians, even though neoblasts continuously proliferate, *piwi*-positive neoblasts are maintained at a certain population size (~25% of total cells) in adulthood (Hayashi et al., 2010; Newmark and Sánchez Alvarado, 2000). Based on

this, it is expected that planarians have elaborate mechanisms to regulate proliferation and differentiation. A recent report showed one example of a relationship between proliferation and differentiation of neoblasts by a cell cycle regulatory factor for arresting the cell cycle, namely *cdh1* (Sato et al., 2021). *cdh1(RNAi)* animals showed higher proliferative ability than control animals and loss of differentiative ability, suggesting that cell cycle arrest by *cdh1* is required for differentiation (Sato et al., 2021).

The proliferative rate of neoblasts can respond to external stimuli. 2 major stimuli, feeding (nutrient intake) and amputation, can accelerate proliferation of neoblasts. Transient acceleration of proliferation after feeding or amputation, named 'mitotic burst', was reported several decades ago in several planarian species (Baguñà, 1976; Saló and Baguñà, 1984). As described below, more detailed analyses of this transient acceleration of proliferation of neoblasts have been reported in *S. mediterranea* and *D. japonica.* This transient accelerated proliferation has been called several names, including mitotic burst, burst of proliferation, hyper-proliferation, etc. Thus, here I consolidate and rename this phenomenon as 'induced hyper-proliferation'. In *S. mediterranea,* it has been reported that there are 2 types of induced hyper-proliferation after amputation (Wenemoser et al., 2012). The first induced hyper-proliferation peaks by 6 hours after amputation, and is considered to be an early response to lesions caused by piercing or lesioning with/without amputation. The second induced hyper-proliferation peaks by 2 days after amputation, and is considered to be a regeneration-

dependent response caused by loss of body part(s) by amputation (Wenemoser and Reddien, 2010).

So far, although there is no report about induced hyper-proliferation after amputation in *D. japonica,* induced hyper-proliferation after feeding has been reported (Sakurai et al., 2012). Induced hyper-proliferation reached a peak by 12 hours after feeding (Sakurai et al., 2012). A neoblast-specific gene, *DjP2X-A*, which encodes an ATP-dependent ion channel, might be involved in this induced hyper-proliferation (Sakurai et al., 2012). *DjP2X-A(RNAi)* animals showed a higher fission frequency than control animals resulting from a faster growth rate. The expression level of *DjP2X-A* slightly decreases by 24 hours after feeding. Inhibition of the function of *DjP2X-A* revealed that *DjP2X-A(RNAi)* animals showed more enhanced induction of hyper-proliferation after feeding, suggesting that this might be the cause of the higher fission frequency observed in *DjP2X-A(RNAi)* animals. These results revealed a negative correlation between *DjP2X-A* and induced hyper-proliferation after feeding. In addition, *DjP2X-A(RNAi)* animals under a starved condition showed decreased mitotic activity, suggesting that *DjP2X-A* regulates the proliferation dependent on nutrient conditions (Sakurai et al., 2012). These induced hyper-proliferations observed in several planarian species are expected to be involved in supplying the differentiated cells or maintenance of the neoblast population during regeneration of body growth by feeding, but the biological meaning and molecular mechanism of these phenomena are still unclear.

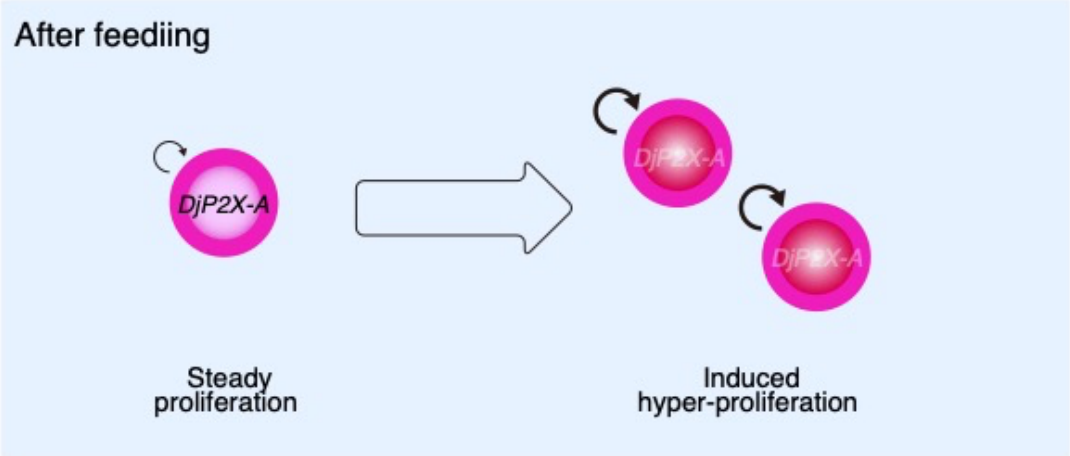


Fig 4. Expression of *DjP2X-A* of neoblasts during induced hyper-proliferation after feeding. The expression level of *DjP2X-A*, which is a neoblast-specific gene, was down-regulated after feeding.

There are 2 intracellular signaling pathways which are expected to be concerned with induced hyper-proliferation and differentiation of neoblasts after amputation, namely, the c-Jun N-terminal kinase (JNK) signaling pathway and the extracellular signal-regulated kinase (ERK) signaling pathway (Tasaki et al., 2011b, 2011a). Pharmacological inhibition of JNK signaling revealed that JNK signaling is required for normal proliferation (Tasaki et al., 2011b). After amputation, JNK signaling is strongly activated in the post-blastema region (proximal to the blastema region), where acceleration of neoblast proliferation takes place during the early stage of regeneration (Saló and Baguñà, 1984; Tasaki et al., 2011b). The other signaling pathway is ERK signaling. ERK signaling is activated in the blastema region. Pharmacological inhibition of ERK signaling caused a differentiation defect of neoblasts, suggesting that ERK signaling is required for commitment or differentiation from the proliferative state of neoblasts (Tasaki et al., 2011a). However, there is possibility that ERK signaling might also regulate

neoblast proliferation, because ERK signaling is involved in cell proliferation in many stem cell systems in other animals (Hasegawa et al., 2013). Are these signaling pathways involved in induced hyper-proliferation or not? Are there any other molecular mechanisms that regulate induced hyper-proliferation after amputation and/or after feeding? What is the biological significance of induced hyper-proliferation? The answers to these questions will be key for understanding the molecular mechanisms of the state change between proliferation and differentiation of neoblasts, which is also a key issue in stem cell biology.

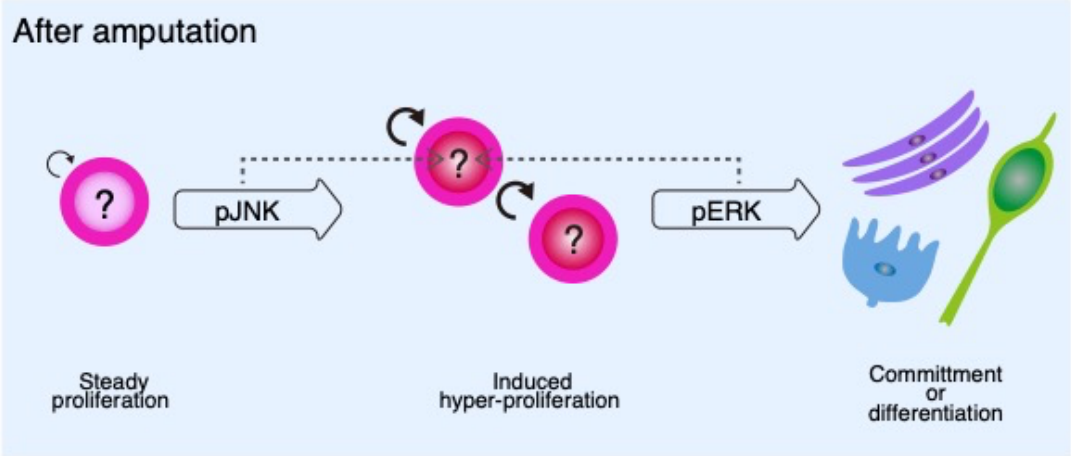


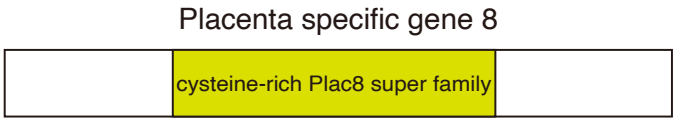
Fig 5. Unknown molecular mechanism of induced hyper-proliferation after amputation. It is known that JNK signaling is required for proliferation of neoblasts after amputation and ERK signaling is required for differentiation from neoblasts. However, the specific molecular mechanisms involving these signaling pathways for induced hyper-proliferation after amputation still remain unknown.

3.6 *Placenta specific gene 8*, a neoblast-specific gene regulating proliferation

To find the molecular mechanism related to induced hyper-proliferation, I used neoblast-specific genes identified by comprehensive gene expression analysis (HiCEP)

(Shibata et al., 2012). Among them, I focused on one of the HiCEP genes, which has homology to *placenta specific gene 8* (*plac8)*. Plac8 is small protein of about 16 kDa, and has a cysteine-rich Plac8 super family domain (Fig. 4A). A variety of functions of *plac8* in regulation of cellular activity have been reported in diverse phyla, from plants to animals (Fig. 4B; Cabreira-Cagliari et al., 2018). Depending on the investigated functions, *plac8* in various organisms is called several names, including *onzin* (as an oncogene), *cell number regulator*, and so on. (Bedell et al., 2012; Guo et al., 2010; Libault et al., 2010; Zeng et al., 2019). New consolidated nomenclature for Plac8 was proposed recently and categorized into 3 types based on domain structure (Fig 4B; Cabreira-Cagliari et al., 2018). Type I is most widely distributed in animals and plants and most of reported Plac8s belong to this type. TypeII and III exist only in plants and their functions are not well studied yet. (Cabreira-Cagliari et al., 2018). Among the various functions, a well-known function of Plac8 family proteins is regulation of proliferation in plants such as tomato or soybean (Cong et al., 2002; Guo et al., 2010; Libault et al., 2010). Considering this function, I focused on *Djplac8-A* and tested the possibility that it regulates proliferation and/or differentiation of neoblasts after feeding and/or amputation.

A



B

	Kingdom	Functions
TypeI	Animal Plant	Proliferation, Differentiation, Apoptosis, Migration, Imflammatory reaction, Heavy metal resistance, Chromatin consendation
TypeII	Plant	Ca2+ uptake
TypeIII	Plant	Unknown

Fig 6. Characteristics of Plac8 (A) Domain of placenta specific gene 8. (B) Categorization of plac8 family members and their functions.

3.7 Findings in this study

In this thesis, I revealed that JNK-dependent suppression of *Djplac8-A* expression is indispensable for induced hyper-proliferation, and that induced hyper-proliferation is required for appropriate regeneration.

First, I found that knockdown of *Djplac8-A* increased the fission frequency, suggesting that *Djplac8-A* is involved in induced hyper-proliferation after feeding, similarly to *DjP2X-A*. Next, I confirmed that induced hyper-proliferation occurred after amputation in *D. japonica*. The expression level of *Djplac8-A* was negatively correlated with induced hyper-proliferation after both feeding and amputation, suggesting that reduction of the expression of *Djplac8-A* might cause the induced hyper-proliferation. Indeed, the induced hyper-

proliferation after both feeding and amputation was accelerated by RNAi of *Djplac8-A*. In intact animals, *Djplac8-A* was specifically expressed in almost all neoblasts. I found that expression of *Djplac8-A* in neoblasts was decreased at the post blastema region after amputation, which is identical to the area of activated JNK signaling. Pharmacological inhibition of JNK signaling caused maintenance of the expression of *Djplac8-A* at the post blastema region and failure of induce hyper-proliferation, and resulted in regenerative defects. These regenerative defects were rescued by simultaneous knockdown of *Djplac8-A*. In addition, the induced hyper-proliferation was affected by inhibition of ERK signaling, suggesting the possibility that both JNK signaling and ERK signaling are involved in induced hyper-proliferation during regeneration. Taken together, these findings showed that reduction of the expression of a single gene, *Djplac8-A*, by JNK signaling is sufficient to cause induced hyper-proliferation that is required for producing differentiated cells during regeneration. Finally, I propose that *Djplac8-A* is a molecular switch of neoblasts for entry from the steady state which maintains the neoblast population into the regenerative state to supply differentiated cells during regeneration.

4. Materials and methods:

4.1 Biological samples

A clonal strain of the planarian *Dugesia japonica* (sexualizing special planarian (SSP) (2n = 16) (Shibata et al., 2012) was maintained at 24 ºC in 0.005% artificial sea water (Instant Ocean, Blacksburg, VA). Chicken liver was fed every 1 or 2 weeks to the planarians to maintain them. Planarians had been starved for at least 1 week before all experiments. Regenerating planarians used for experiments were obtained by amputation anterior or posterior to the pharynx. 3 or 5 groups of biological replicates were tested for statistical analyses.

4.2 X-ray irradiation

Animals placed on wet filter paper on ice were irradiated with 120 roentgens of X-rays using an X-ray generator (SOFTEX B-5; SOFTEX, Tokyo, Japan). 5 days after irradiation, planarians were used for experiments.

4.3 Feeding RNA interference

Double-stranded RNA was synthesized as previously described (Rouhana et al., 2013). The primers for PCR amplification were as follows:

SP6 + T7 Forward primer (for *Djplac8*)

5' GATCACTAATACGACTCACTATAGGGCAAGCTATTTAGGTGACACTATAG3'

Zap Linker + T7 Forward primer (for *DjP2X-A*)

5' GATCACTAATACGACTCACTATAGGGCTGCAGAATTCGGCACGAGG 3'

M13 Reverse primer

5' GTTTTCCCAGTCACGACGTTGTAA 3'

RNA interference (RNAi) with dsRNA was performed to knock down the target genes as follows: 25 μL of chicken liver solution (liver homogenate: culture water = 1: 1), 7 μL of 2% agarose and 7 μL of 2.0 μg/μL dsRNA were mixed and fed to 15 planarians. The mixture was frozen at -30℃ for at least 30 minutes before use. 3 successive feedings were similarly conducted at 3-day intervals after the first feeding for short-term feeding RNAi. Additional feeding was conducted after 3 successive feedings for long-term feeding RNAi. Control animals were fed with dsRNA of EGFP.

4.4 Injection RNA interference

dsRNA synthesized as described above (2 μg/μl) was injected into the intestine. 3 successive injections were performed daily after the first injection. Control animals were injected with dsRNA of EGFP.

4.5 Antibody preparation

For production of anti-DjPlac8-A antibody, peptides corresponding to 3 regions of DjPlac8-A were synthesized and injected into rabbits. Affinity-purified polyclonal antibody was obtained from the rabbit sera. All procedures were conducted by MBL (Nagoya, Japan). The amino acid sequences of the peptides were MNENKRYSNKLDYSQEC, AAEPILQQPPEYPGFPKC, and IQQPKSNTGSAREWSSGC.

4.6 Western blotting

10 planarians were dissolved in sample buffer (100 mM Tris–HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 12% β-mercaptethanol, 20% glycerol, 0.024% Bromophenol blue) and boiled for 5 min. After SDS-PAGE, samples were transferred to a Hybond P membrane, and stained with 1/800 diluted anti-DjPlac8-A antibody or 1/5000 diluted anti-α-tubulin (Sigma, T9026) as the primary antibody. Then the membrane was incubated with 1/5000 diluted secondary antibody (cytiva, RPN420). Signals were detected using SuperSignal West Dura Extended Duration Substrate (Pierce).

4.7 FACS-based single cell RT-PCR

FACS-based single cell RT-PCR **(**FBSC-PCR) was performed as previously described (Hayashi et al., 2010). The forward (FW) and reverse (RV) primer sets used were (5' to 3'):

DjG3PDH (Internal control)

FW: ACCACCAACTGTTTAGCTCCCTTAG

RV: GATGGTCCATCAACAGTCTTTTGC

Djplac8-A

FW: AAGAGCAACACAGGTAGTGCTAGGGAGTG

RV: AGAAGCACAACAACATTCACCATATCGTG

DjpiwiA

FW: CGAATCCGGGAACTGTCGTAG

RV: GGAGCCATAGGTGAAATCTCATTTG

4.8 Whole-mount immunohistochemistry

Planarians were fixed with 4% paraformaldehyde/5% methanol in 5/8 Holtfreter's solution for 30 min at room temperature after removing mucus by treatment with 2% HCl in 5/8 Holtfreter's solution. Then, samples were bleached with 6% H_2O_2 overnight at room temperature under fluorescent light. Bleached samples were treated with 50% xylene/methanol for 30 minutes at 4℃ and rinsed with 100% ethanol for 30 minutes at 4℃. Then, the samples were rehydrated through a graded ethanol series (75%, 50% and 25%) solutions in 5/8 Holtfreter's solution each for 30 minutes at 4℃. The rehydrated samples were rinsed with Triton-PBS (TPBS：2.7 mM KCl / 8.1 mM Na_2HPO_4・$12H_2O$ / 136.9 mM NaCl / 1.5 mM KH2 PO4 / 0.1% Triton X-200)

for 30 minutes at 4℃. Permeabilization was performed with 5 μg/ml Proteinase K in TPBS for 12 min at 37ºC. Next, samples were post-fixed in 4% paraformaldehyde / 5% methanol in 5/8 Holtfreter's solution for 30 minutes and rinsed with TPBS at 4℃. After blocking using 10% goat serum in TPBS, the samples were incubated with 10% goat serum in TPBS containing primary antibody overnight at 4℃. The dilution of primary antibody used was 1/800 for anti-DjPlac8-A, 1/500 for anti-DjPiwiA (Yoshida-Kashikawa et al. 2007), and 1/200 for anti-pH3 (Upstate, 06-570). The samples were washed with TPBS several times at room temperature and incubated with 10% goat serum in TPBS containing 1/1000 fluorescent-labeled secondary antibody (Alexa Fluor 594 or Alexa Fluor 488 (Molecular Probe) and 1 μg/ml Hoechst 33342 (Calbiochem) overnight at 4℃. The samples were observed with a confocal microscope (FLUOVIEW FV10i; Olympus, Tokyo, Japan) or a fluorescence stereoscopic microscope (M205FA T-RC 1; Leica, Germany).

4.9 Whole-mount *in situ* hybridization

For RNA probe synthesis, the plasmid pCR®II-TOPO containing the gene *Djplac8-A* or the plasmid pBluescript SK containing the gene *Djrunt-1* was used. The DNA linearized with Not1 at the 5' end of the target gene was used as template. Before the transcription, linearized DNA was purified using phenol/chloroform extraction and ethanol precipitation. Then the product was used as a template for antisense RNA transcription by Sp6 RNA

polymerase (Promega) or T7 RNA polymerase (Fermentas). After transcription, the probe was purified using ethanol precipitation and stored at -80℃. For fixation, bleaching, permeabilization, and post-fixation steps, the same procedures as used for whole-mount immunohistochemistry were employed. After post-fixation, the samples were soaked in hybridization solution for 1 hour at 55℃, and hybridized with Dig-labeled antisense RNA probe (which had been denatured previously for 20 minutes at 55℃) in hybridization solution for 36 hours at 55℃. Then, the samples were washed with wash solution 3 times for 30 minutes each and 3 times for 1 hour at 55℃ and rinsed with Buffer I (0.1 M maleic acid / 5x SSC / 0.1% Tween-20:pH 7.5) twice for 5 minutes each at room temperature. Then samples were treated with Buffer II (1% blocking reagent (Roche diagnostics) in Buffer I) for blocking for 30 minutes at room temperature and treated with 1/2000 alkaline phosphatase-conjugated anti-Digoxigenin antibody (Roche Diagnostics, 1093274) in BufferII overnight at 4℃. Then, the samples were rinsed with Buffer Ⅰ 6 times for 30 minutes each at room temperature and washed with TMN (0.1 M Tris-HCl / 0.1 M NaCl / 50 mM $MgCl_2$: pH 9.5) solution 2 times for 5 minutes each at room temperature. 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 for detecting signals. After the detection, TE buffer was used to stop the reaction and the samples were kept in TE buffer at 4℃.

4. 10 Whole-mount fluorescent *in situ* hybridization

All steps before detection of the signal were performed in the same way as for whole-mount *in situ* hybridization. To detect signals, a Tyramide Signaling Amplification kit (Molecular Probe TSA^{TM} Kit #12, with HRP-goat anti-rabbit IgG and Alexa Fluor 488® tyramide, Eugene, Oregon, USA) was used for color development.

4. 11 Quantitative RT- PCR

Total RNA was extracted using ISOGEN-LS (Wako) as follows: 50 µl of ISOGEN-LS was added to samples (all fragments obtained by amputation were used for regenerating samples). The samples were homogenized and then 700 µl of ISOGEN-LS was added. Then total RNA was extracted according to the manufacturer's instructions and cDNA was synthesized by using a QuantiTect Reverse Transcription Kit® according to the manufacturer's instructions (QIAGEN). The synthesized cDNA was diluted (x20) and used for gene expression analysis by quantitative (q)RT-PCR. 10 microliters 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 was analyzed using an ABI PRISM 7900 HT (Applied Biosystems). The reactions were carried out as follows : 50℃ for 2 minutes, 95℃ for 15 minutes, 50 cycles of 95℃ for 15 seconds, 60℃ for 30 seconds, 72℃ for 1 minute. The forward (FW) and reverse (RV) primer sets used were (5' to 3'):

DjG3PDH (Internal control)

FW: ACCACCAACTGTTTAGCTCCCTTAG

RV: GATGGTCCATCAACAGTCTTTTGC

pcna

FW: ACCTATCGTGTCACTGTCTTTGACCGAAAA

RV: TTCATCATCTTCGATTTTCGGAGCCAGATA

mcm2

FW: CGCTGTTGGACAAGGTCAGAAGAATGAACA

RV: CCAGAAACACAAATCTACATCTTCCAAAGG

Djplac8

FW: AAGAGCAACACAGGTAGTGCTAGGGAGTG

RV: AGAAGCACAACAACATTCACCATATCGTG

DjP2X-A

FW: GATTTCAACAATGGAATGAATTTTAGATA

RV: AAAATGTGAAACAAGTAGCAGGATCA

Djrunt-1

FW: CGGCCATCGAGTATGGTTAT

RV: ACGGCAACAATGTTTGGATT

4. 12 Chemical inhibitor treatment

JNK inhibitor SP600125 (Sigma-Aldrich, St. Louis, USA) and MAPK⁄ERK kinase (MEK) inhibitor U0126 (Cell Signaling Technology, Massachusetts, USA) were dissolved in dimethylsulfoxide (DMSO) at 100 mM and 10 mM, respectively. Amputated planarians were treated with 5 μM SP600125 from 12 hours after amputation until the indicated period of regeneration for optimal JNK inhibition, or with 25 μM SP600125 or U0126 at 4 hours after amputation for strong inhibition of JNK or ERK, respectively, for the indicated period of regeneration for each experiment.

5. Results

5.1 Knockdown of *Djplac8-A* increases fission frequency

First, I screened the neoblast-specific genes identified by comprehensive gene expression analysis, HiCEP, based on expectable conserved gene functions known in other organisms (Shibata et al., 2012). Among them, I focused on HiCEP clone number 37 gene, which has homology to *plac8*, because *plac8* has various known functions in cellular regulation, including regulation of proliferation and differentiation (Bedell et al., 2012; Guo et al., 2010; Jimenez-Preitner et al., 2011; Mao et al., 2019). Especially, many previous reports showed that the major function of *plac8* is regulation of proliferation as mentioned above (Guo and Simmons, 2011; Libault et al., 2010). Thus, I named this gene *Djplac8-A* and performed RNAi targeting *Djplac8-A* to test the possibility that this gene is involved in induced hyper-proliferation.

There are several RNAi methods using double-stranded RNA (dsRNA) to knockdown a specific gene in planarian; 1) injection RNAi, 2) soaking RNAi, and 3) feeding RNAi (short-term and long-term) (Rouhana et al., 2013; Sakurai et al., 2012). Among them, I employed a long-term RNAi method to investigate the function of *Djplac8-A.* This unique method was established in a previous study investigating a gene involved in induced hyper-proliferation after feeding, *DjP2X-A* (Fig. 4; Sakurai et al., 2012). Long-term feeding RNAi enables assessment of the proliferation status of neoblasts after feeding by counting the number of

fissions of planarians subjected to RNAi (Fig 7). For example, long-term *DjP2X-A(RNAi)* animals showed enhanced induced hyper-proliferation followed by higher fission frequency, suggesting that *DjP2X-A* is involved in neoblast proliferation after feeding (Sakurai et al., 2012).

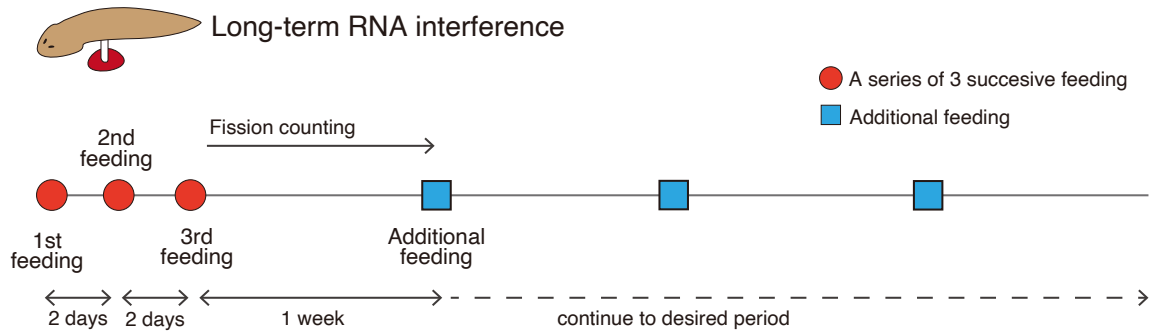


Fig 7. Time schedule of long-term feeding RNAi. During the first 1 week, a series of 3 successive feeding RNAis were conducted. After that, one feeding RNAi was conducted additionally per month. Fission was counted after a series of 3 successive feeding RNAis.

I fed planarians liver extract containing dsRNA targeting the *Djplac8-A* once per week for a month plus 3 successive feedings, as in previous reports (Rouhana et al., 2013; Sakurai et al., 2012). Interestingly, *Djplac8-A* knockdown caused increased fission frequency (Fig. 8). *Djplac8-A (RNAi)* animals underwent fission about 10 times (average), compared to about 3 times (average) for control animals during 30 days (Fig. 8). *DjPlac8-A(RNAi)* animals reproducibly showed 3 times higher fission frequency compared to the control animals (n = 10x3), which was comparable to the increase of fission rate observed in *DjP2X-A(RNAi)*

planarians (Fig 8; Sakurai et al., 2012). A question was raised by this finding, namely, do *Djplac8-A(RNAi)* animals undergo fission earlier than control animals even though they are a small size which is smaller than the size at which fission normally occurs, or do *Djplac8-A(RNAi)* animals more quickly reach the size at which fission normally occurs by growing faster? To answer this question, I measured the length of head fragments and tail fragments after fission and summed them. Both control and *Djplac8-A(RNAi)* animals underwent fission when they were over about 8~9 mm (Fig 9). These results suggest that *Djplac8-A* is involved in neoblast proliferation after feeding, resulting in a faster growth rate in *Djplac8-A(RNAi)* animals than in control animals, similarly to *DjP2X-A*. *Djplac8-A (RNAi)* animals regenerated normally after fission or artificial amputation (data not shown) indicating that normal differentiation of neoblasts appeared to occur in *Djplac8-A* knockdown animals, as it was also reported to occur in *DjP2X-A(RNAi)* planarians (Sakurai et al. 2012).

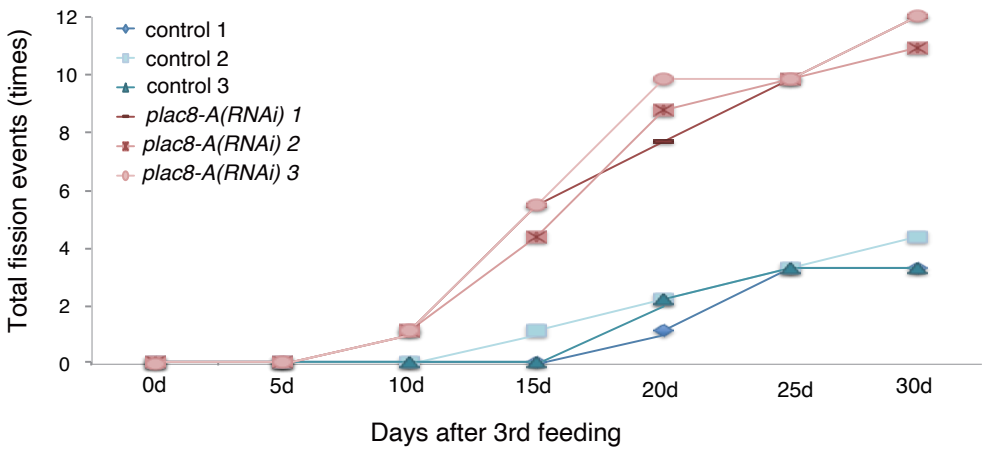


Fig. 8. Total fission events in *Djplac8-A* knockdown animals. 3 independent experiments were conducted. 10 animals (6~7mm) were analyzed for each experiment.

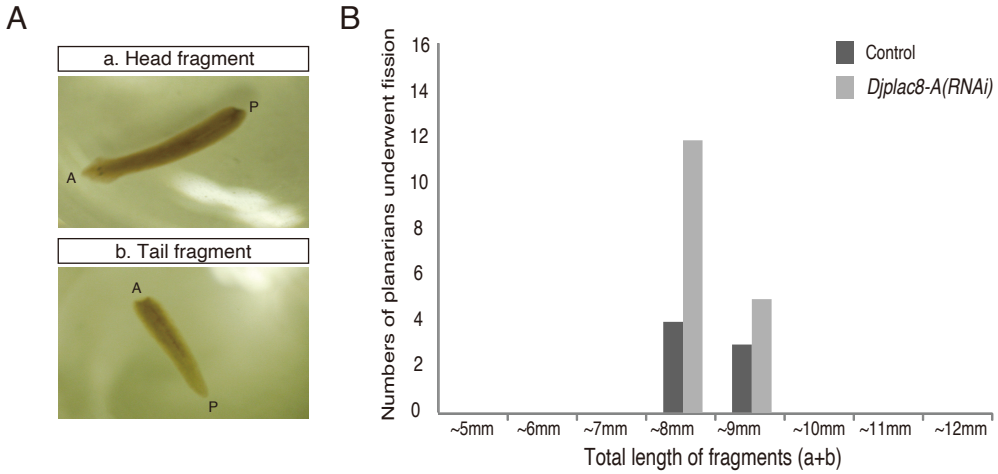


Fig. 9. Measurement of the size at which fission occurred in *Djplac8-A(RNAi)* animals. (A) Head fragment and tail fragment after fission. A indicates anterior of fragment. P indicates posterior of fragment. (B) Sum of length of head fragment and tail fragment after fission. 20 (6~7 mm) animals were analyzed for each experiment.

In addition, I conducted double-feeding RNAi of *Djplac8-A* and *DjP2X-A* for 1 month to examine whether there was an enhancement of the fission rate when these 2 genes were knocked-down. *Djplac8-A* and *DjP2X-A(RNAi)* planarians underwent fission 10 times,

compared to 3 times for control animals at 30 days, thus showing about 3 times higher fission frequency compared to the control animals (Fig, 10), which was the same as the result of single RNAi of *Djplac8-A(RNAi)* or *DjP2X-A(RNAi)*. This result indicated that there was no synergistic effect on fission events between *Djplac8-A* and *DjP2X-A* after feeding, and that *Djplac8-A* and *DjP2X-A* functions might be exerted in parallel.

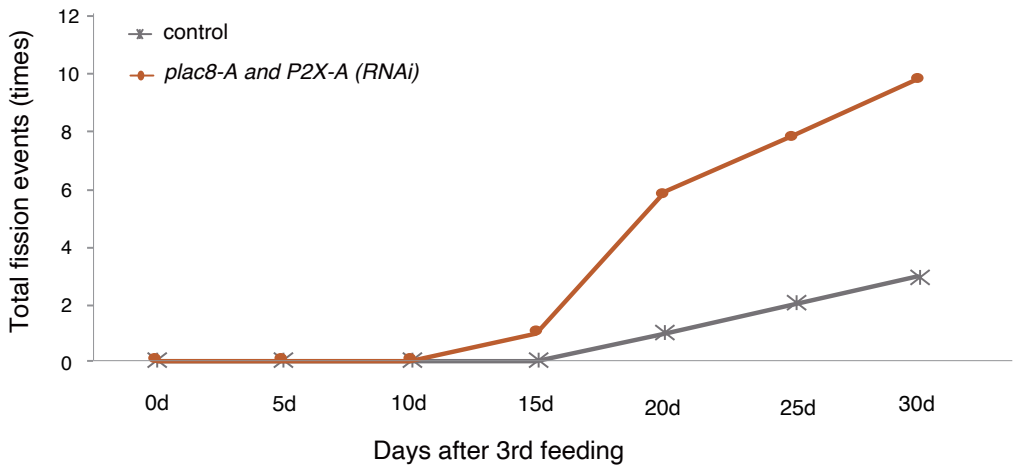


Fig. 10. Total fission events in *Djplac8-A* and *DjP2X-A* double knockdown animals. 10 animals were analyzed for each experiment.

5.2 *Djplac8-A* is expressed in most *DjpiwiA*-expressing neoblasts

In order to investigate the expression pattern of *Djplac8-A,* first I performed whole-mount *in situ* hybridization of *Djplac8-A* with or without X-ray irradiation. The expression of *Djplac8-A* showed an expression pattern typical of a neoblast-specific gene, in which *Djplac8-*

A-expressing cells were located throughout all body regions posterior to the head region except in the pharyngeal region. Indeed, the *in situ* hybridization signal of *Djplac8-A* disappeared in X-ray-irradiated planarians, in which neoblasts were eliminated (Fig. 11A). Thus, I confirmed the reported neoblast-specific expression of *Djplac8-A* (Shibata et al., 2012). This expression pattern is identical to the expression

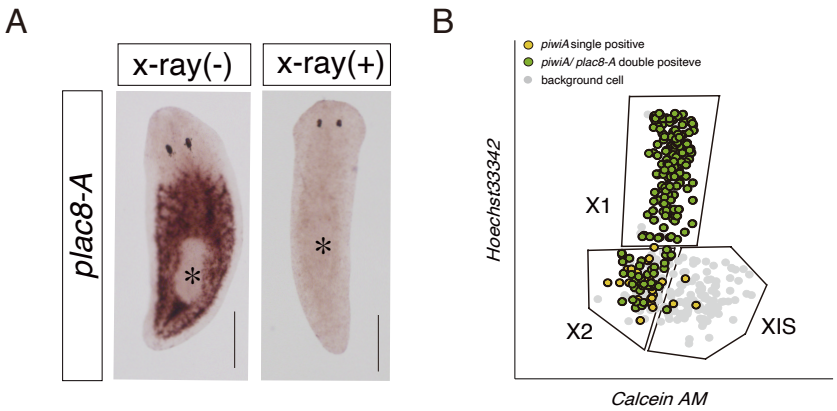


Fig. 11. Expression pattern of *Djplac8-A*. (A) Expression pattern of *Djplac8-A* shown by whole-mount in situ hybridization in intact and X-ray-irradiated animals. Scale bar is 1 mm. Asterisk indicates pharynx region. (B) Co-expression analysis of *Djplac8-A* and *DjpiwiA* by FACS-based single-cell PCR. Each circle represents a single cell in the FACS profile.

pattern of *DjpiwiA* (one of the most reliable neoblast marker genes). Among the neoblast-specific genes that showed a similar expression pattern to *DjpiwiA*, *DjP2X-A* was reported to be expressed heterogeneously in the *DjpiwiA*-positive population (in about 50% of *DjpiwiA*$^{+}$-expressing neoblasts) (Sakurai et al., 2012; Shibata et al., 2012). Next, I examined the expression of *Djplac8-A* at the single cell level in the *DjpiwiA*-positive population by FACS-based single-cell PCR (FBSC-PCR). I performed gene expression analysis on the Index Sorting

profile obtained by FBSC-PCR in intact animals. Nearly all (97% of) *Djplac8-A*-expressing cells were in the X1 fraction plus X2 fraction (231/238), which were neoblast-enriched fractions. Also, 84% of the expression of *Djplac8-A* was detected in *DjpiwiA*-expressing neoblasts (190/227) (Fig. 11B) (Hayashi et al., 2010, 2006). Thus, *Djplac8-A* was expressed throughout almost the entire *DjpiwiA*-expressing neoblasts population.

5.3 DjPlac8-A is a membrane protein

Next, I analyzed the subcellular localization of DjPlac8-A protein in the neoblasts by immunostaining using an anti-DjPlac8-A antibody that I raised. This antibody specifically recognized a 16-kDa protein in western blotting, which was coincident with the predicted size of DjPlac8-A protein (Fig. 12B). To check the specificity of the antibody, I conducted immunostaining of *Djplac8-A(RNAi)* planarians, and found that the signal disappeared in the RNAi animals (Fig. 12C), confirming the specificity of the antibody. Immunostaining using this antibody revealed that the signal was generally observed in neoblasts that were also positive for DjPiwiA protein, in accord with the result of FBSC-PCR (Fig 12. A, D; see also Yoshida-Kashikawa et al., 2007). DjPlac8-A was localized in the outermost region in the cytoplasm of the neoblasts, whereas DjPiwiA protein was observed widely throughout the cytoplasm of the neoblasts (Fig. 12D, upper panel). I also performed co-immunostaining with a planarian membrane protein, DjP2X-A, an ATP-dependent ion channel protein, since a previous report showed that DjP2X-A is localized on the cell membrane of about half of the

neoblasts (Sakurai et al., 2012). Co-localization of DjPlac8-A and DjP2X-A was observed (Fig. 12D, lower panel), strongly suggesting that DjPlac8-A is a cell membrane protein or associated with a cell membrane protein(s). This indicated that the subcellular localization of DjPlac8-A protein is in accord with the reported localization of Plac8 protein in other organisms (Guo et al., 2010).

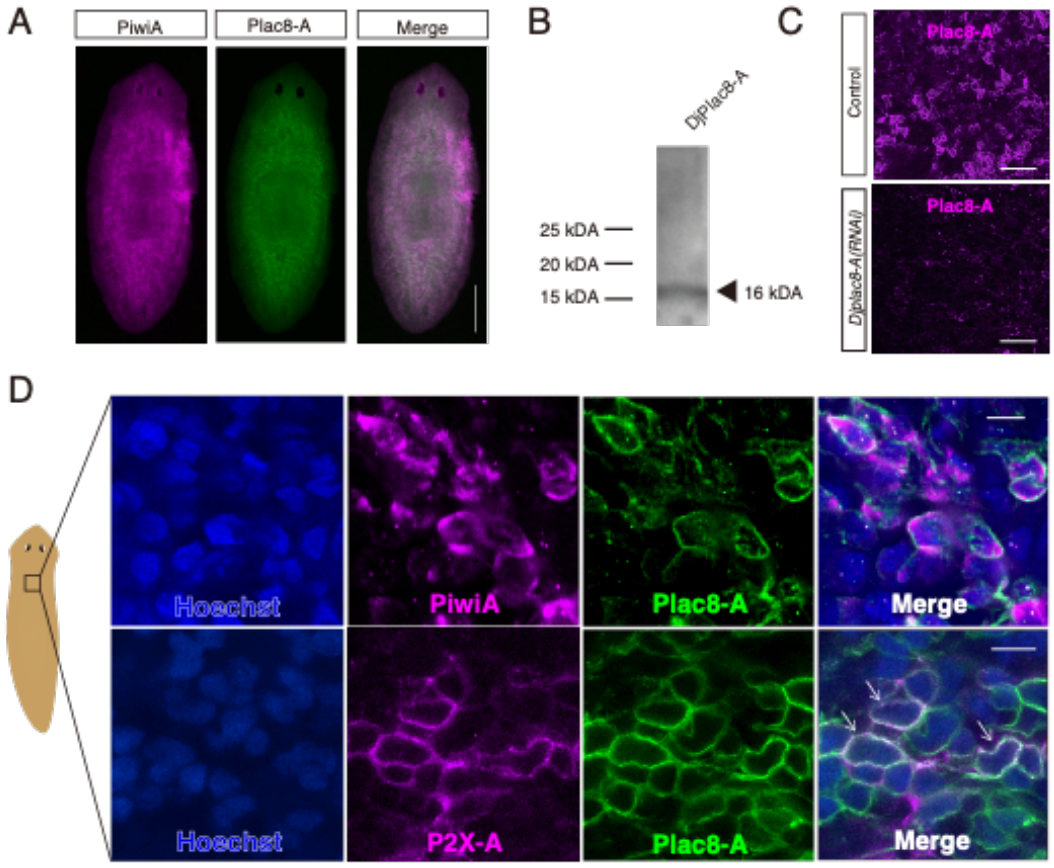


Fig. 12. Subcellular localization of DjPlac8-A. (A) Whole-mount immunohistochemistry using anti-DjPlac8-A antibody (green) and anti-PiwiA antibody (magenta) in intact animal. Scale bars, 1 mm. (B) Western blotting of intact planarians using anti-DjPlac8-A antibody. (C) Immunostaining with anti-DjPlac8-A antibody in control and Djplac8-A knockdown animals. Scale bars, 50 μm. (D) Upper panel: Immunohistochemistry using anti-DjPlac8-A antibody (green) and anti-PiwiA antibody (magenta) in intact animal. Scale bars, 10 μm. Lower panel: Immunohistochemistry using anti-DjPlac8-A antibody (green) and anti-Pi2X-A antibody (magenta) in intact animal. Arrows indicate co-localization of Djplac8-A and DjP2X-A. Scale bars, 10 μm.

Although most DjPiwiA-positive cells were also positive for DjPlac8-A, it was noteworthy that 2 subpopulations of neoblasts were negative for DjPlac8-A: *Djnanos*-positive neoblasts localized in the dorsolateral side of the body (Sato et al., 2006), and the *Djpiwi*-1-positive subpopulation localized on the dorsal longitudinal midline (Rossi et al., 2006) (Fig. 13).

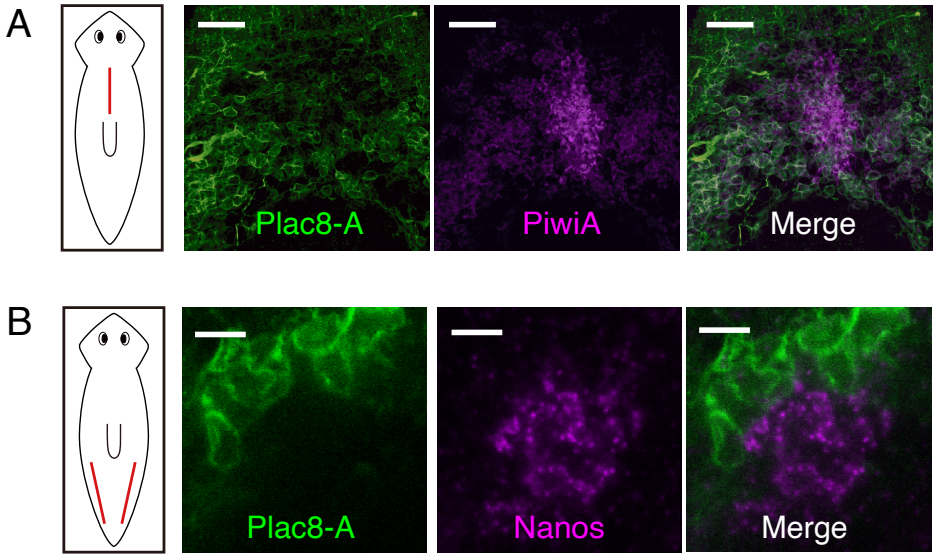


Fig. 13. Subpopulations of neoblasts did not express DjPlac8-A. (A) Red line of schematic drawing indicates midline. Scale bars, 40 μm. (B) Red lines of schematic drawing indicate regions expressing DjNanos. Scale bars, 10 μm.

plac8 family genes have a conserved domain called the Plac8 superfamily domain (Fig. 14A). In our *D. japonica* EST database (Nishimura et al., 2015; An et al., 2018), I identified 6 other genes possessing the Plac8 superfamily domain in addition to *Djplac8-A* (Fig. 14B). One of them was highly similar to *Djplac8* reported from another laboratory, which is related to immune response and development in planarian (Pang et al., 2017). Except for this *plac8*, I named the other 5 newly found homologs *Djplac8-B* to *-F,* and determined their expression

patterns: the expression patterns of *Djplac8-B* to *-F* genes showed no difference between animals with/without X-ray irradiation, suggesting that they were not expressed in the neoblasts (Fig. 14C), and therefore I focused on only *Djplac8-A* thereafter in this study.

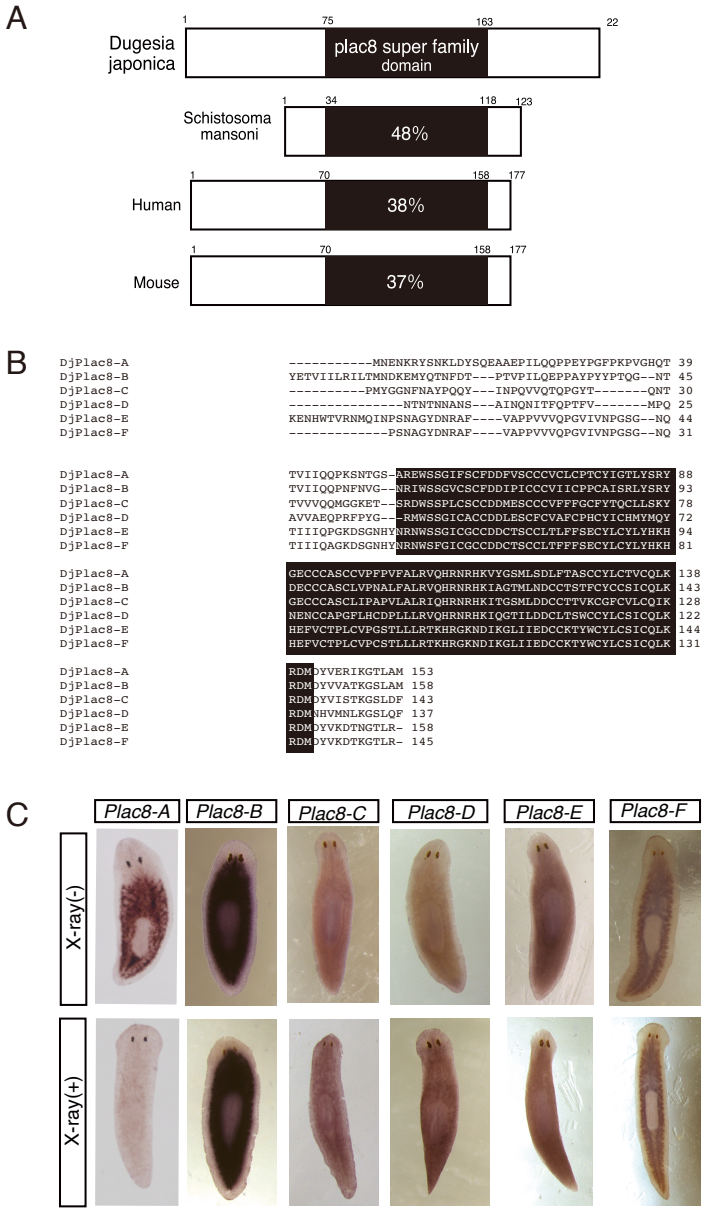


Fig. 14. *plac8* family genes in *D. japonica*. (A) Homology of Plac8 superfamily domain of DjPlac8-A with the domains of Plac8 proteins in other animals. (B) Predicted amino acid sequences of *plac8* family genes in *D. japonica*. (C) Expression pattern of *D. japonica plac8* family genes detected by whole-mount *in situ* hybridization in intact and X-ray-irradiated planarians.

5.4 *Djplac8-A* is involved in induced hyper-proliferation after feeding

First, I performed RT-PCR to examine the expression dynamics of *Djplac8-A* after feeding. The significance of differences of expression levels was evaluated by comparing the expression at each time point to that in the starved state. The expression levels of cell-proliferation marker genes such as *pcna* and *mcm2* increased soon after feeding. The expression levels of *pcna* and *mcm2* increased significantly at 12 hours after feeding, and then decreased to the steady-state level 1 week after feeding, as previously reported (Fig. 15A; see also Sakurai et al., 2012), indicating that hyper-proliferation was induced after feeding. The expression level of *Djplac8-A* was decreased at 12 hours and 24 hours after feeding, and returned to the initial steady-state level 5 days after feeding (Fig. 15B). A negative correlation between induced hyper-proliferation and gene expression after feeding was similarly observed for *DjP2X-A* (Fig. 15B; Sakurai et al., 2012), suggesting that *Djplac8-A* and *DjP2X-A* might have similar roles in induced hyper-proliferation after feeding.

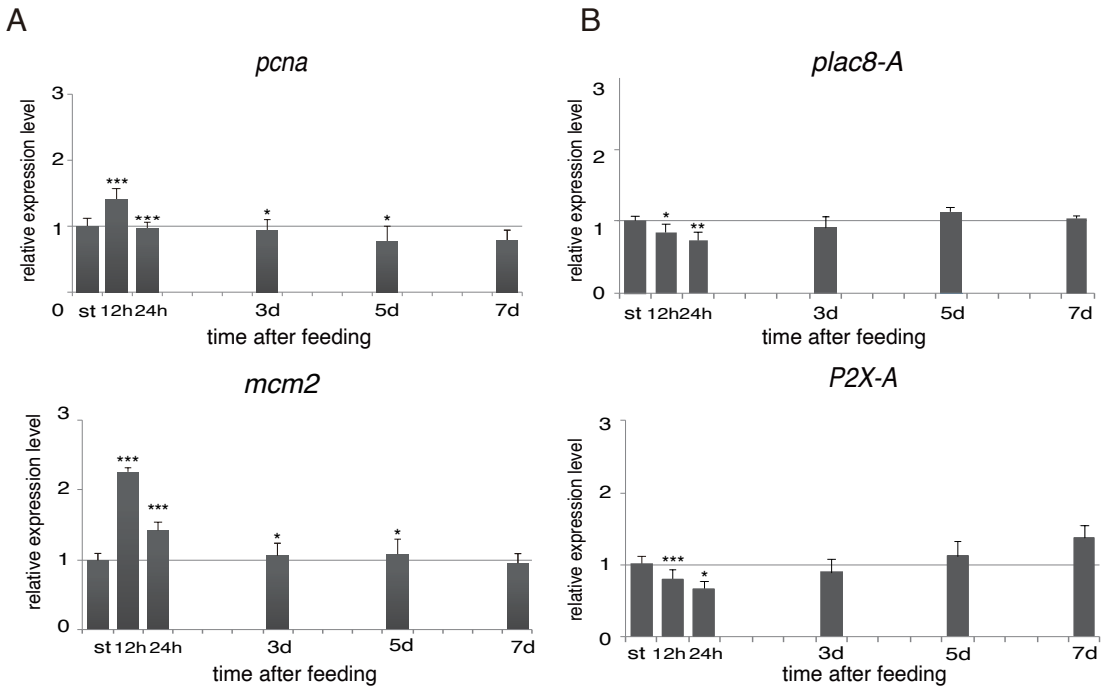


Fig. 15. Expression level of *Djplac8-A* during induced hyper-proliferation after feeding. (A) Relative expression levels of *pcna* and *mcm2* after feeding. (B) Relative expression levels of *Djplac8-A* and *DjP2X-A* after feeding. (A) and (B): Student's t-test was performed comparing expression levels between planarians and starved planarians at each time point. *** $P < 0.001$, ** $P < 0.005$, and * $P < 0.05$

As mentioned above, enhanced induced hyper-proliferation was reported after feeding in *DjP2X-A* knockdown planarians (Sakurai et al., 2012). Therefore, I investigated whether induced hyper-proliferation was enhanced in *Djplac8-A* knockdown animals after feeding. *Djplac8-A* knockdown animals showed higher expression levels of *pcna* than control animals from 12 hours to 7 days after feeding, indicating that the feeding-induced hyper-proliferation of neoblasts was enhanced by *Djplac8-A* knockdown, as it was by *DjP2X-A* knockdown (Fig. 16A). Also, after feeding, *DjP2X-A(RNAi)* animals showed an increase of M-phase neoblasts

(neoblasts positive for anti-phosphohistone H3 (pH3) immunostaining) cells at the region directly anterior to the pharynx in a previous report (Sakurai et al., 2012). I employed the same method to count pH3-positive cells as in that previous report (Sakurai et al., 2012), and found that the number of pH3-positive cells increased in *Djplac8-A(RNAi)* animals, as it did in *DjP2X-A(RNAi)* animals (Fig. 16B, C). These results indicate that *Djplac8-A* expression is negatively correlated with induced hyper-proliferation after feeding, and thus may modulate neoblast proliferation, as does *DjP2X-A* expression.

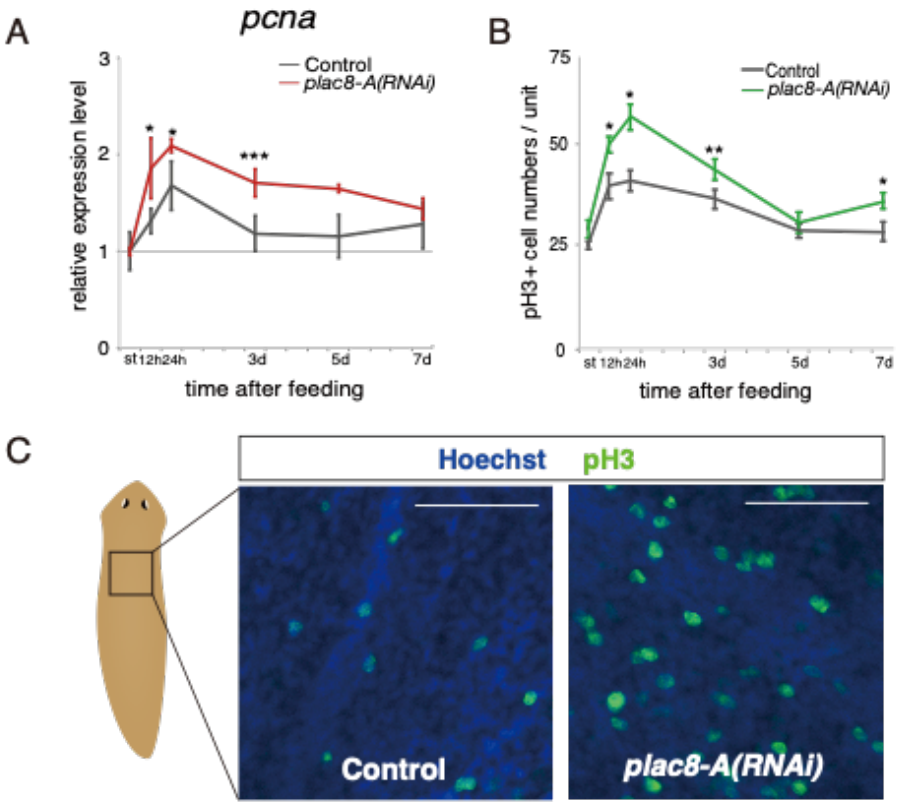


Fig. 16. Enhancement of induced hyper-proliferation after feeding in *Djplac8-A(RNAi)* animals. (A) Relative expression level of *pcna* after feeding in control animals and in *Djplac8-A(RNAi)* animals. Gene expression levels measured by qRT-PCR were analyzed in 3 biological replicates. Expression levels of genes at each time are relative to those in starved animals. (B) Quantification of the number of pH3-positive cells after feeding in control animals and *Djplac8-A(RNAi)* animals (n = 5). (A) and (B): Student's t-test was performed between control animals and *Djplac8-A(RNAi)* animals. *** $P < 0.001$, ** $P < 0.005$, and * $P < 0.05$. Error bars indicate SEM. st means starved planarians. (C) pH3-positive cells in control animals and *Djplac8-A(RNAi)* animals at 24 hours after the third feeding. Scale bar: 100 μm. Unit volume: 6.2×10^{-3} mm^3 (boxed region).

5.5 *Djplac8-A* is involved in induced hyper-proliferation after amputation

Although I showed that *Djplac8-A* might modulate the induced hyper-proliferation after feeding, similarly to *DjP2X-A*, whether these genes are also involved in induced hyper-proliferation after amputation still remained unknown. Thus, I examined whether hyper-

proliferation was induced after amputation in *D. japonica* by performing RT-PCR, because there have been no reports regarding this. The significance of differences of expression levels was evaluated by comparing the expression at each time point to that at 0 hours after amputation. The expression levels of proliferative markers were significantly increased rapidly (within 12 hours) after amputation compared to the levels at 0 hours after amputation, as they were in the case of induced hyper-proliferation after feeding, and returned to the steady-state level within 1 week (Fig. 17A). Although the patterns of the time course and level of increases or decreases of proliferative marker expression were slightly different depending on the particular experiments and proliferative markers examined, I confirmed that their expression levels certainly increased after amputation (and also after feeding), and then returned to the steady-state expression levels within 1 week (Fig 15A,17A). Thus, I concluded that proliferation is accelerated after amputation in *D. japonica,* as it is in other planarian species. In *S. mediterranea*, 2 types of induced hyper-proliferation after amputation were reported (Wenemoser and Reddien, 2010): an early response to lesions caused by piercing or lesioning with/without amputation, and a late regeneration-dependent response caused by loss of body part(s) by amputation (Wenemoser and Reddien, 2010). However, I could not detect any obvious induced hyper-proliferation in simply pierced or injured planarians without amputation in *D. japonica* (Fig. 18).

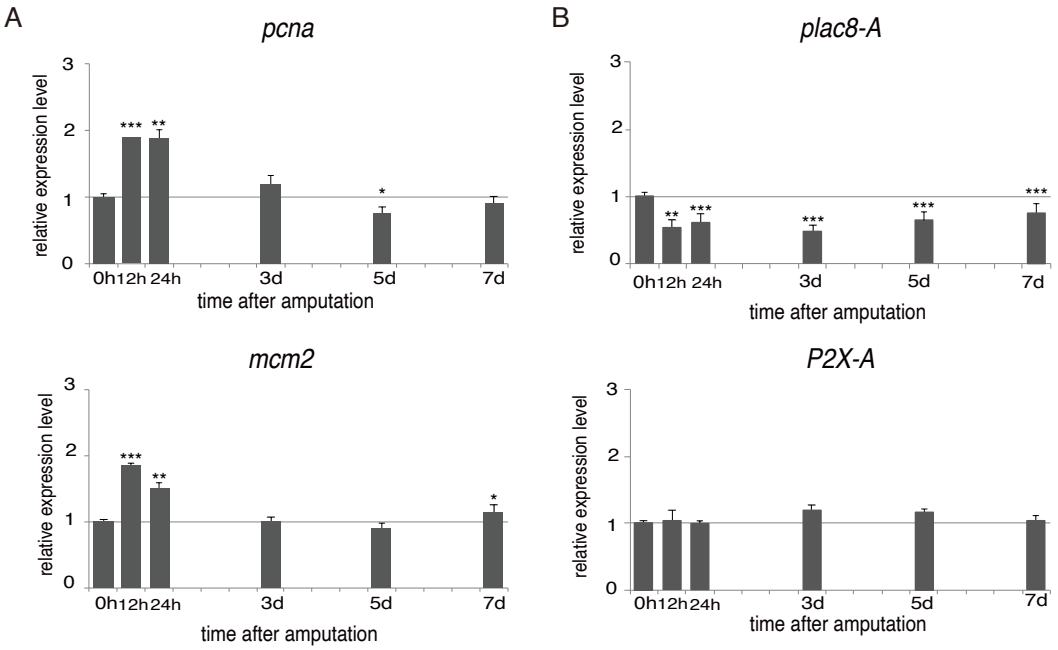


Fig. 17. Expression levels of *Djplac8-A* and *DjP2X-A* during Induced hyper-proliferation after amputation. (A) Relative expression levels of *pcna* and of *mcm2* after amputation. (B) Relative expression levels of *Djplac8-A* and of *DjP2X-A* after amputation. Student's t-test was performed by comparing expression levels between each time point and 0 hours after amputation. (A) and (B): Student's t-test was performed comparing expression levels between each time point and 0 hours after amputation. *** $P < 0.001$, ** $P < 0.005$, and * $P < 0.05$.

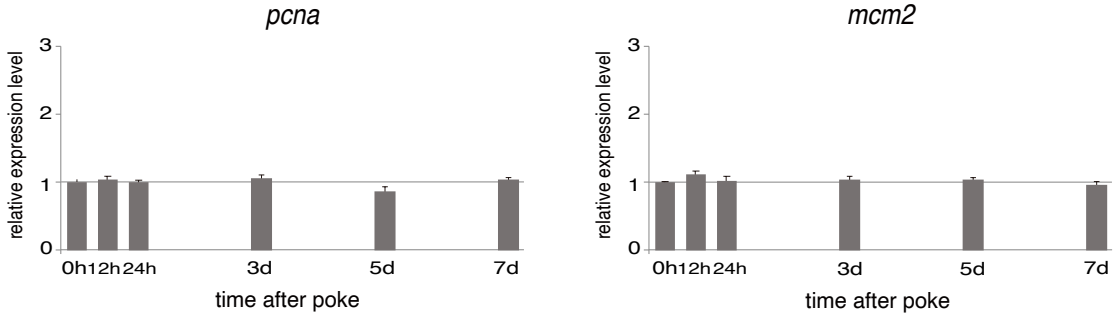


Fig. 18. Relative expression levels of proliferative markers after piercing. Relative expression levels of *pcna* and *mcm2* after piercing. Gene expression levels measured by qRT-PCR were analyzed in 3 biological replicates. Expression level of genes at each time is relative to that at 0 hour after piercing. Error bars indicate SEM.

Next, I examined the expression levels of *DjP2X-A* and *Djplac8-A* after amputation. Interestingly, the expression level of *Djplac8-A* was significantly reduced (by about 50%) within 12 hours after amputation. Down-regulation of *Djplac8-A* expression was detected during an early stage of regeneration (until 3 days after amputation), and then *Djplac8-A* expression gradually recovered to the steady-state level by 7 days after amputation. In contrast, *DjP2X-A* expression did not significantly change after amputation (Fig. 17B). The expression patterns of proliferation markers and *Djplac8-A* showed opposite trends, as observed in the case of induced hyper-proliferation after feeding, suggesting that *Djplac8-A*, but not *DjP2X-A,* is negatively correlated with the induced hyper-proliferation after amputation. To confirm this, I monitored the induced hyper-proliferation in *Djplac8-A (RNAi)* animals. For this, I employed the injection RNAi method, which is introduction of dsRNA directly into the planarian digestive duct to avoid the induction of hyper-proliferation by feeding. As expected, *Djplac8-A(RNAi)* animals showed higher expression of *pcna* than control animals at all time points examined within 1 week after amputation (Fig. 19A). The number of pH3-positive cells was also significantly increased at all the time points examined in these *Djplac8-A* knockdown animals (Fig. 19B, C). These results indicate that *Djplac8-A* exerts a negative effect on induced hyper-proliferation after amputation. I also examined whether induced hyper-proliferation was

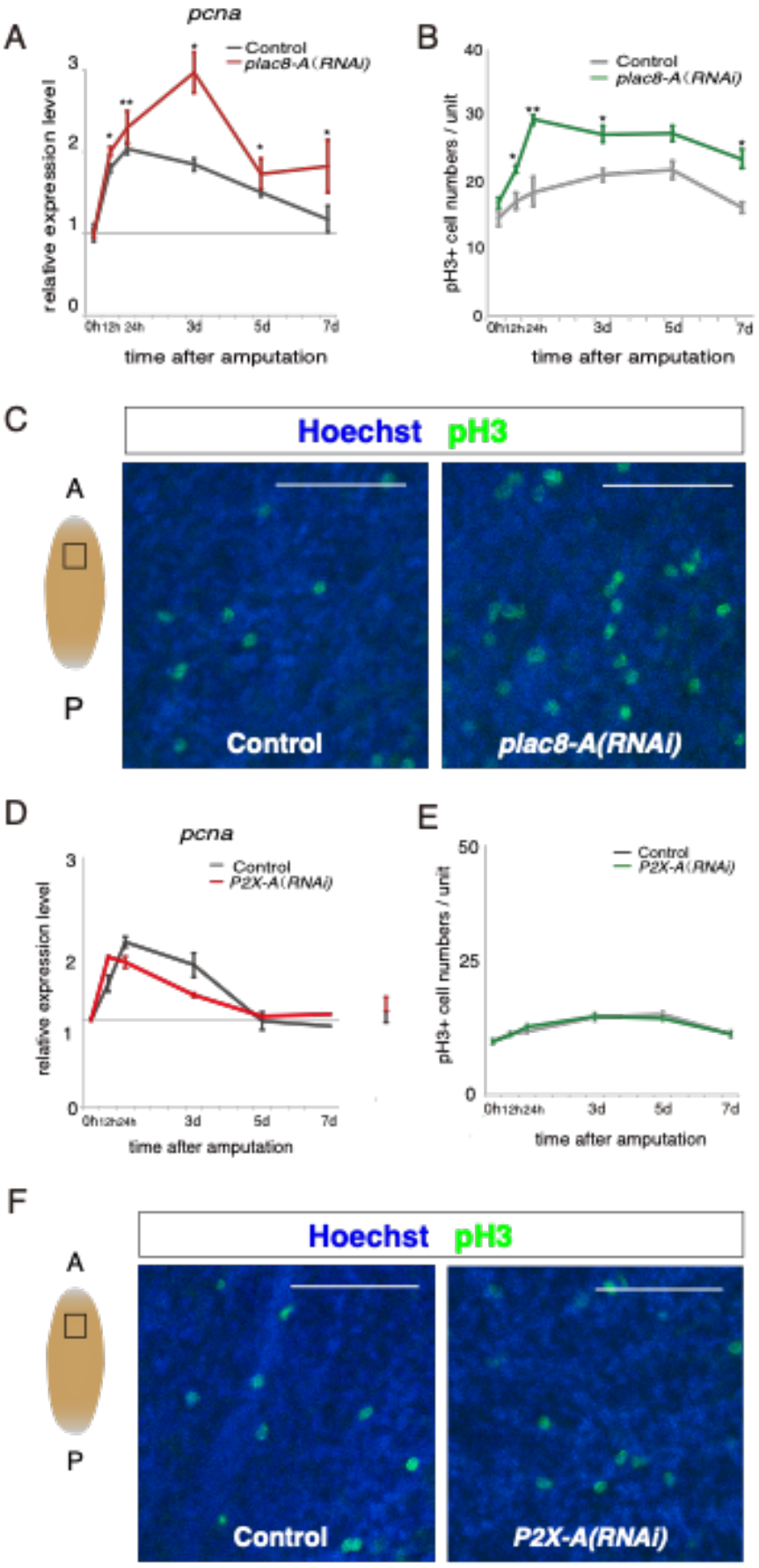

A
pcna
Control
plac8-A(RNAi)
relative expression level
0
1
2
3
0h 12h 24h
3d
5d
7d
time after amputation
B
Control
plac8-A(RNAi)
pH3+ cell numbers / unit
0
10
20
30
40
0h12h24h
3d
5d
7d
time after amputation
C
Hoechst pH3
A
P
Control
plac8-A(RNAi)
D
pcna
Control
P2X-A(RNAi)
relative expression level
0
1
2
3
0h12h24h
3d
5d
7d
time after amputation
E
Control
P2X-A(RNAi)
pH3+ cell numbers / unit
0
25
50
0h12h24h
3d
5d
7d
time after amputation
F
Hoechst pH3
A
P
Control
P2X-A(RNAi)

Fig. 19. Enhancement of induced hyper-proliferation after amputation in *Djplac8-A(RNAi)* animals, but not in *DjP2X-A(RNAi)* animals. (A) Relative expression level of *pcna* after amputation in control animals and in *Djplac8-A(RNAi)* animals. Gene expression levels measured by qRT-PCR were analyzed in 3 biological replicates. Expression level of genes at each time is relative to that at 0 hours after amputation. (B) Quantification of number of pH3-positive cells after amputation in control animals and *Djplac8-A(RNAi)* animals (n = 5). (A) and (B) Student's t-test was performed by comparison between control animals and *RNAi* animals. ** $P < 0.005$, and * $P < 0.05$. Error bars indicate SEM. (C) pH3-positive cells in control animal and *Djplac8-A(RNAi)* animal at 24 hours after amputation. Scale bars: 100 μm. Unit volume: 6.2×10^{-3} mm^3 (boxed region). (D) Relative expression level of *pcna* after amputation in control animals and *DjP2X-A(RNAi)* animals. Gene expression levels measured by qRT-PCR were analyzed in 3 biological replicates. Expression level of genes at each time is relative to that at 0 hours after amputation. (E) Quantification of number of pH3-positive cells after amputation in control animals and *DjP2X-A(RNAi)* animals (n = 5).
(D) and (E) Student's t-test was performed by comparison between control animals and *RNAi* animals. Error bars indicate SEM. (F) pH3-positive cells in control animal and *DjP2X-A(RNAi)* animal at 24 hours after amputation. Scale bars, 100 μm. Unit volume, 6.2×10^{-3} mm^3 (boxed region).

enhanced after amputation in *DjP2X-A(RNAi)* planarians, but no acceleration of induced hyper-proliferation was observed after amputation in these planarians (Fig. 19D, E, F), indicating that *DjP2X-A* is dispensable for induced hyper-proliferation after amputation. Thus, *Djplac8-A* is the only gene demonstrated thus far to be involved in induced hyper-proliferation after both feeding and amputation, and *DjP2X-A* is only involved in the induced hyper-proliferation after feeding (Fig. 20).

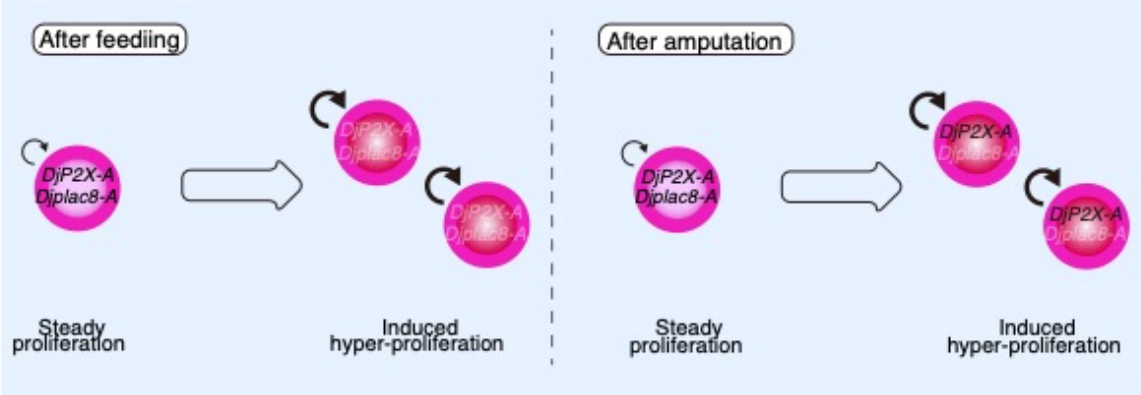


Fig. 20. Schematic drawing illustrating genes involved in induced proliferation after feeding and amputation. After feeding, expression levels of both *Djplac8-A* and *DjP2X-A* are decreased. In contrast, after amputation, only the expression level of *Djplac8-A* is decreased, not the expression level of *DjP2X-A*.

5.6 *Djplac8-A* expression disappeared from neoblasts located in the post-blastema region after amputation

I performed *in situ* hybridization to examine the spatial expression pattern of *Djplac8-A* expression during regeneration after amputation. Interestingly, I found that expression of *Djplac8-A* was drastically reduced in the post-blastema region (i.e., the regions posterior to the head blastema and anterior to the tail blastema) during regeneration at 12 hours and 24 hours after amputation (Fig. 21A). Immunostaining showed also spatial reduction of *Djplac8-A* expression at 24 hours after amputation, although neoblasts (as indicated by DjPiwiA immunostaining) were present in the post-blastema region (Fig. 21B). As regeneration proceeded, the expression of *Djplac8-A* recovered in the anterior region (except in the head) and in the posterior region, with restoration of the normal expression pattern at 7 days after

amputation (Fig. 21A). Immunostaining and western blotting also indicated that DjPlac8-A protein decreased in the post-blastema region during regeneration, whereas the DjPlac8-A level remained unchanged in the rest of the body (Fig 21. C, D, and E). Therefore, I concluded that the expression of *Djplac8-A* and its protein were transiently reduced in neoblasts located in the post-blastema region during the early stage of regeneration.

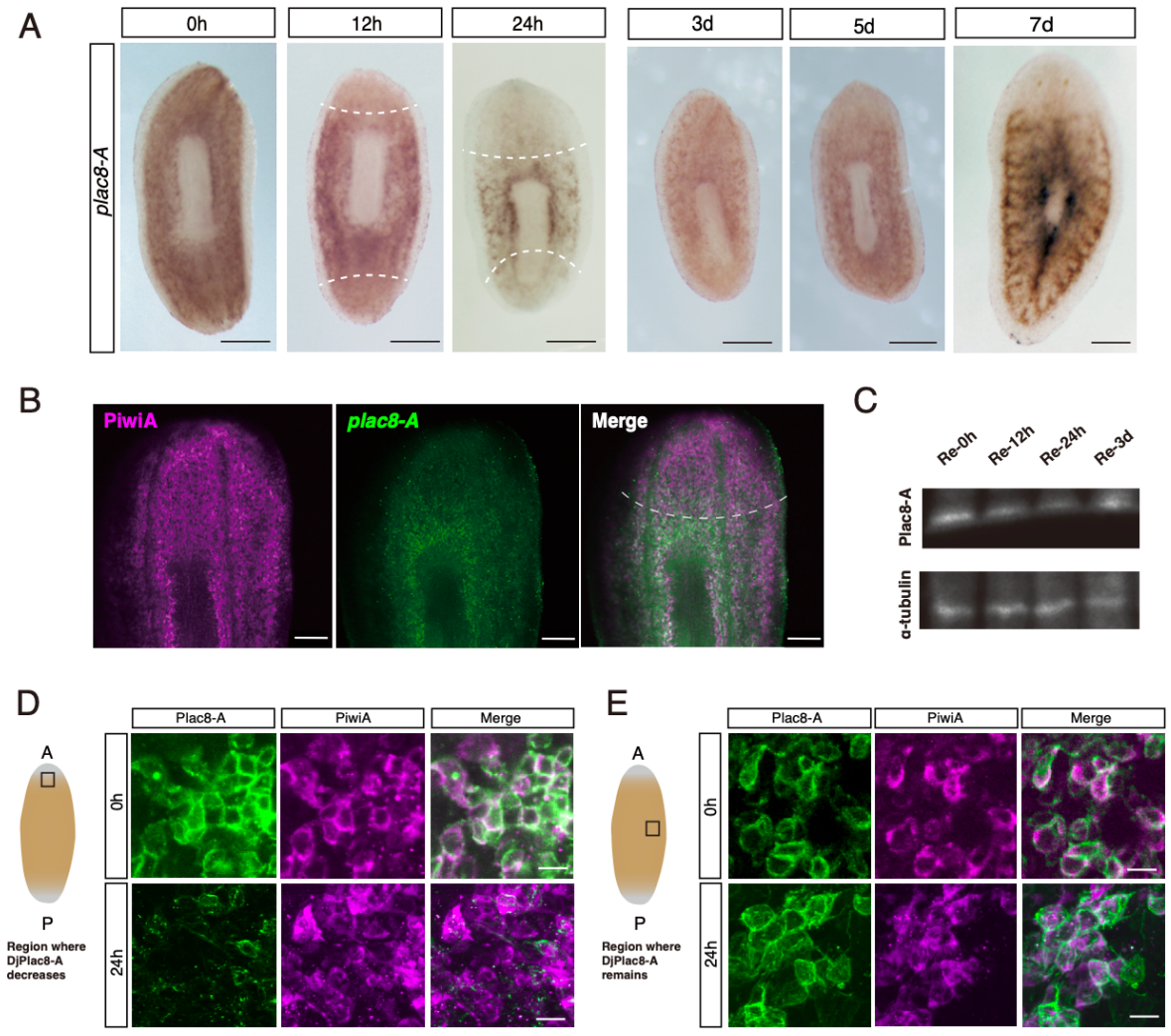


Fig. 21. Expression dynamics of Djplac8-A mRNA and protein during regeneration. (A) Expression pattern of *Djplac8-A* mRNA during regeneration determined by whole-mount *in situ* hybridization. Broken lines show boundaries between regions where the expression of *Djplac8-A* was detected and was not detected. Scale bar, 1 mm. (n = 15, 15/15) (B) Co-expression of *DjPlac8-A* and DjPiwiA immunostaining in regenerating animal. Planarians were fixed 24 hours after amputation. Broken line shows boundary between regions where expression of *Djplac8-A* was detected and was not detected. Scale bars, 150 μm. (n = 7, 7/7) (C) Western blotting of DjPlac8-A during regeneration (D) Co-immunostaining of DjPiwiA and DjPlac8-A in post-blastema region (boxed region). Planarians were fixed 24 hours after amputation. Scale bar, 10 μm. (E) Co-immunostaining of DjPiwiA and DjPlac8-A in indicated body region (boxed region). Scale bars, 10 μm. (n = 7, 7/7)

5.7 Activation of JNK signaling represses *Djplac8-A* expression in neoblasts after amputation

Previous studies showed that the post-blastema region after amputation contains many M-phase neoblasts (Saló and Baguñà, 1984; Tasaki et al., 2011b). Also, it has been reported that JNK is activated in the post-blastema region during early regeneration (Tasaki et al., 2011b). Accordingly, next I examined the possible role of JNK signaling in *Djplac8-A* expression and induced hyper-proliferation. First, I visualized the activation of JNK using anti-phosphorylated JNK (pJNK) antibody in head-regenerating planarians, and confirmed that JNK was activated in the post-blastema region (where *Djplac8-A mRNA* was decreased), as reported (Fig. 22A; Tasaki et al., 2011b). Then, to test whether JNK signaling can indeed regulate the expression of *Djplac8-A* in this region during regeneration, I examined the expression of *Djplac8-A* in planarians treated with 25 μM SP600125, which is a JNK inhibitor, after amputation. A previous report showed that treatment with 25 μM SP600125 caused loss of almost all mitotic neoblasts by blocking the entry into M-phase of the cell cycle (Tasaki et al., 2011b). In control animals, expression of *Djplac8-A* decreased in the post-blastema region at 24 hours after amputation (Fig. 22B, upper panel), but in SP600125-treated planarians, *Djplac8-A* was still expressed in neoblasts there (Fig. 22B, lower panel). I then measured the expression level of *Djplac8* after amputation in SP00125-treated planarians by qRT-PCR. In the inhibitor-treated animals, the level of expression of *pcna* was not increased. Furthermore,

the level of expression of *Djplac8-A* was not changed in SP600125-treated planarians after amputation, in contrast to the decrease of the expression level of *Djplac8-A* in control amputated animals (Fig. 22C). Taken together, these findings led me to conclude that activation of JNK signaling contributes to the induced hyper-proliferation via repression of *Djplac8-A* expression in neoblasts located in the post-blastema region after amputation.

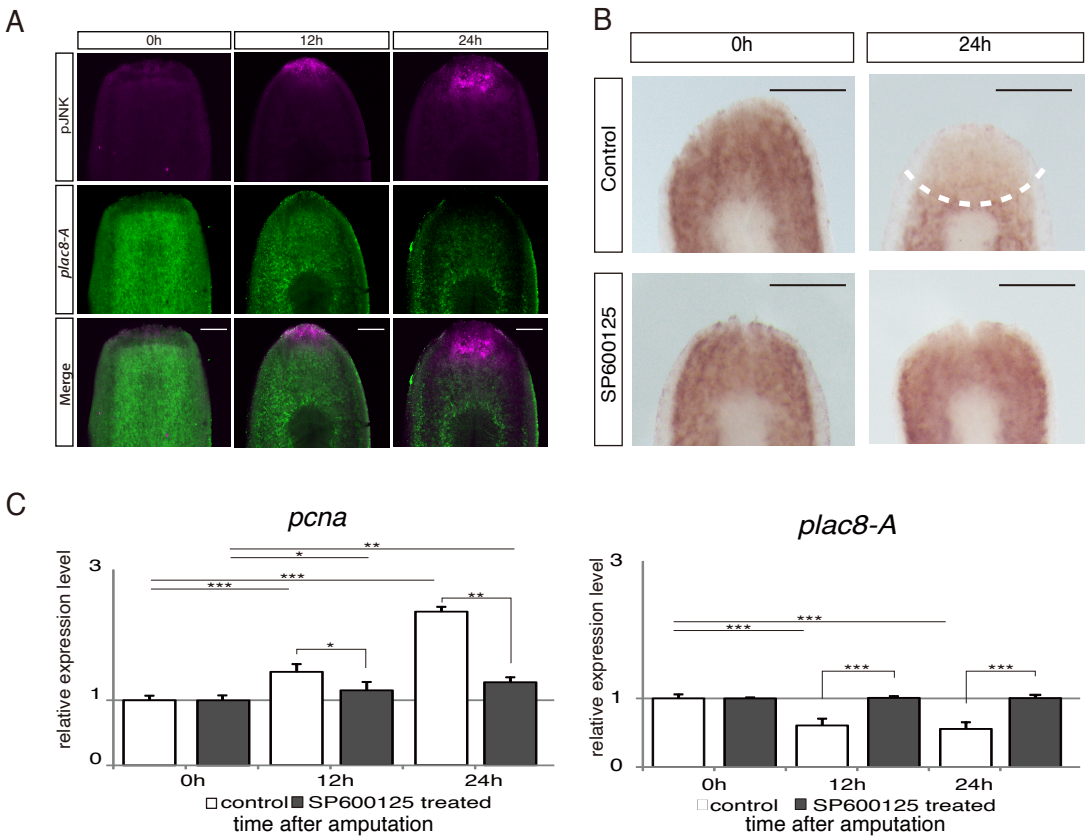


Fig. 22. Relationship between expression of *Djplac8-A* and JNK signaling. (A) Expression of *Djplac8-A* and activation of JNK during early regeneration. Signals for *Djplac8-A* (green) were detected by *in situ* hybridization, and signals for activation of JNK were detected by immunohistochemistry using anti-phosphorylated JNK antibody (magenta). Scale bars, 150 μm. (n = 7, 7/7) (B) Expression pattern of *Djplac8-A* at 24 hours after amputation in control animal and SP600125-treated animal. Broken line shows boundary between regions where expression of *Djplac8-A* was detected and not detected. Scale bars, 1 mm. (n = 10, 10/10: DMSO-treated planarians showed reduction of *Djplac8-A* expression at 24 hours after amputation in post-blastema region. 9/10 SP600125-treated planarians showed no expression change of *Djplac8-A* at 24 hours after amputation in post-blastema region.) (C) Relative expression levels of *pcna* and *Djplac8-A* in control animals and SP600125-treated animals after amputation. Gene expression levels measured by qRT-PCR were analyzed from 3 biological replicates. Expression level of genes at each time is relative to that at 0 hours after amputation. Student's t-test was performed by comparison between control animals and inhibitor-treated animals or comparison between each time point and 0 hours. *** $P < 0.001$, ** $P < 0.005$, and * $P < 0.05$. Error bars indicate SEM.

Next, I tested the possibility that ERK signaling affects the expression of *Djplac8-A* and the induced hyper-proliferation of neoblasts, since the ERK signaling pathway is also known to be one of the major signaling pathways for promoting the proliferation of stem cells, such as spermatogonial stem cells (Hasegawa et al., 2013), although the role of ERK signaling was reported to promote the differentiation of the neoblasts in *D. japonica* (Tasaki et al., 2011a). I checked the induced hyper-proliferation of neoblasts and the expression of *Djplac8-A* in regenerating animals treated with U0126, a MEK-specific inhibitor that specifically blocks ERK signaling (Favata et al., 1998; Tasaki et al., 2011a). Planarians were amputated and treated with 25 μM U0126, which blocks the neoblasts' differentiation (Tasaki et al., 2011a; Umesono et al., 2013). First, I monitored induced hyper-proliferation by detecting the expression level of *pcna*, and found relatively weaker elevation of *pcna* expression in the U0126-treated animals compared to the increase of *pcna* expression in the amputated control animals (Fig. 23A). The expression level of *Djplac8-A* was not changed by U0126 treatment after amputation (Fig. 23A). These results raised the question of whether ERK signaling can directly affect the expression of *Djplac8-A*, or whether ERK signaling affected *Djplac8-A* expression via a signaling cascade through JNK phosphorylation. To answer this, I monitored the activation of JNK in U0126-treated planarians by immunostaining with anti-pJNK antibody, which revealed that the signal of pJNK was greatly reduced in the post-blastema region in U0126-treated planarians (Fig. 23B). Taken together, these findings suggest that U0126 induced failure of the

activation of JNK signaling, resulting in a low level of induced hyper-proliferation in planarians after amputation. Thus, induced hyper-proliferation after amputation might require both JNK signaling and ERK signaling.

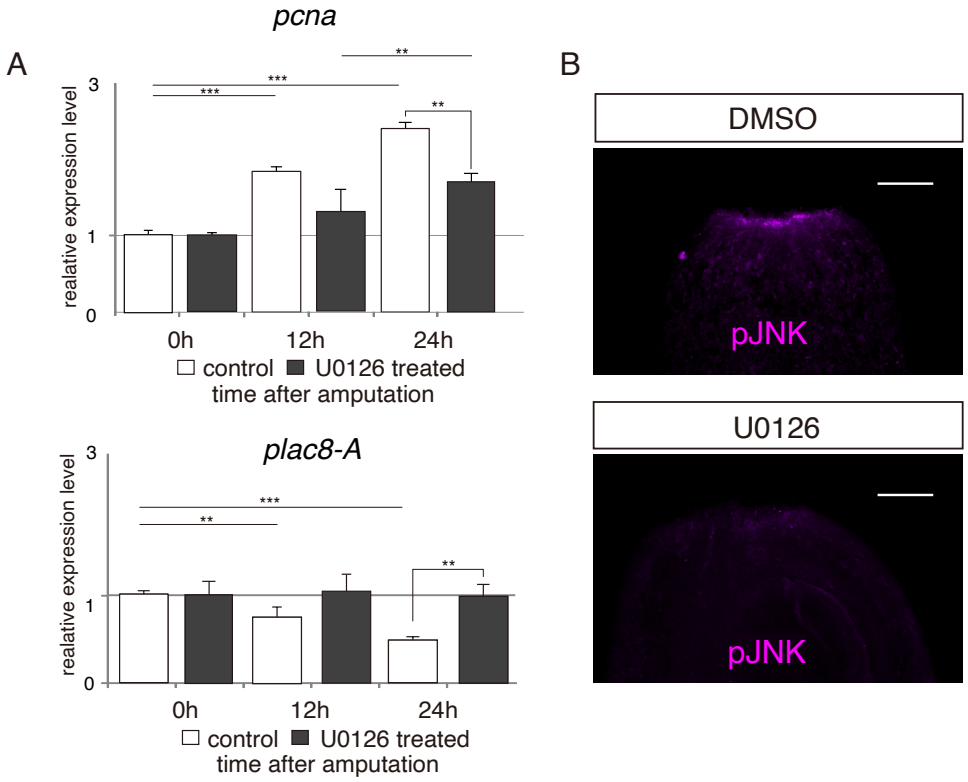


Fig. 23. Relationship between expression of *Djplac8-A* and ERK signaling. (A) Relative expression levels of *pcna* and *Djplac8-A* in control animals and U0126-treated animals after amputation. Student's t-test was performed by comparison between control animals and inhibitor-treated animals or comparison between each time point and 0 hours. *** $P < 0.001$, ** $P < 0.005$. Gene expression levels measured by qRT-PCR were analyzed from 3 biological replicates. Expression level of genes at each time is relative to that at 0 hours after amputation. Error bars indicate SEM. (B) JNK activation detected by immunohistochemistry in control animal and U0126-treated animal. Planarians were fixed 9 hours after amputation. Scale bars, 150 μm. (n = 7, 5/7)

5.8 Induced hyper-proliferation is required for progression of neoblast differentiation

Next, to elucidate the biological consequences of the induced hyper-proliferation of neoblasts after amputation, I investigated whether inhibition of this induced hyper-proliferation affected regeneration. First, I examined the effect of 25 μM SP600125 by treating planarians with 25 μM SP600125 after amputation and allowing them to regenerate. Planarians treated with 25 μM SP600125 throughout regeneration showed severe regenerative failure. At 1 week after amputation, planarians treated continuously with 25 μM SP600125 after amputation showed head and tail regression or died. This regenerative failure was in accord with but more severe than the reported defects when the inhibitor treatment was delayed, namely, incomplete head or tail structure regeneration in planarians treated with 25 μM SP600125 during only a limited time (from after 12 h after amputation until 3 days after amputation) (Tasaki et al., 2011b). That previous report showed that treatment with 25 μM SP600125 after amputation caused failure of regeneration by blocking wound healing in addition to blocking progression of neoblasts' cell cycle from S- to M-phase in almost all neoblasts, as mentioned above (Tasaki et al., 2011b). Therefore, I tested lower concentrations of SP600125, and found that the optimal concentration of SP600125 that enabled normal proliferation by allowing entry into M-phase to some extent, but did not induce hyper-proliferation, was 5 μM. At this optimal concentration, planarians regenerated partially, showing regenerative defects as indicated by the incidence of

cyclopia and eye-absent planarians (Fig. 24A). Then I tested whether this partial regenerative defect could be abrogated by RNAi of *Djplac8-A*. For this, we amputated and treated planarians with 5 μM SP600125 after a series of injection RNAi treatments against *Djplac8-A*, and then observed the regeneration of these planarians at 1 week after amputation. Most of the control animals (injection of dsRNA of *EGFP*) with inhibitor treatment (70.8% of planarians) failed to regenerate normally: 13.4% of control animals were dead and 57.4% of them showed cyclopia or no eyes (Fig. 24A, B). In contrast, there was no death of *Djplac8-A(RNAi)* animals treated with the SP600125. *Djplac8-A(RNAi)* animals treated with the SP600125 all survived and underwent regeneration. 40% of *Djplac8-A(RNAi)* animals treated with the SP600125 showed normal eye regeneration, suggesting that they had higher regenerative ability than control animals (Fig. 24B). The ratios of eye-absent animals and cyclops were also decreased compared to those in control animals (Fig. 24B). I also examined the regeneration of visual neurons by performing immunostaining of DjArrestin. Most SP600125-treated *Djplac8-a(RNAi)* animals (86% of planarians; 18/21) completely regenerated the visual neurons, whereas SP600125-treated control animals failed to regenerate these neurons (Fig. 24C). Thus, attenuation of *Djplac8-A* expression by RNAi could rescue the regenerative defects caused by inhibition of JNK signaling.

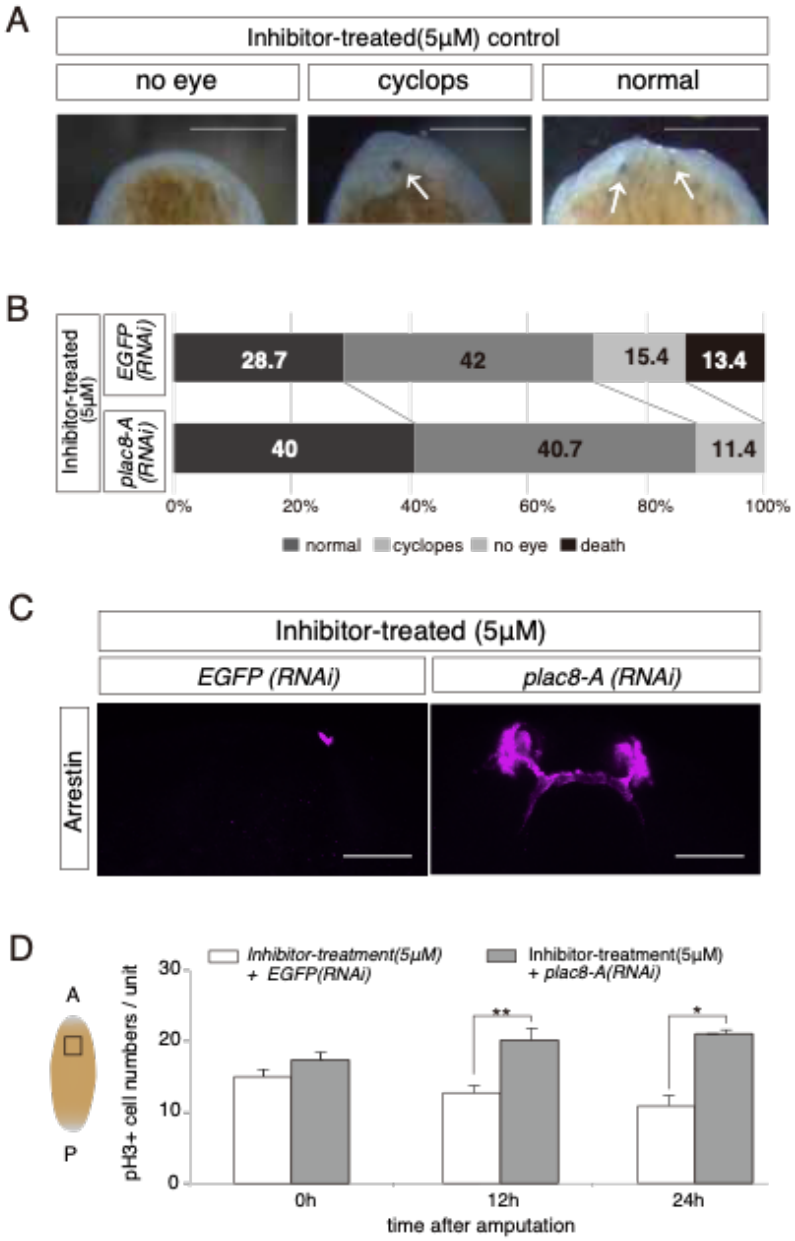


Fig. 24. Regeneration defects in SP600125-treated animals by knockdown of *Djplac8-A*. (A) Images showing eye regeneration in planarians with SP600125-treatment. Arrows indicate regenerated eyes. Scale bars; 1mm (B) Percentages of animals with defect in eye regeneration after combination of SP600125-treatment and knockdown of *EGFP* and *Djplac8-A*. The percentages were calculated using 3 groups of biological replicates. Each group included 15 planarians. (C) Regeneration of visual neurons detected by immunohistochemistry using anti-Arrestin antibody in planarians with combination of SP600125-treatment and knockdown of *EGFP* and *Djplac8-A* (left: n = 21, 18/21 right: n = 21, 18/21). Scale bars, 50 μm. (D) Quantification of the number of pH3-positive cells in planarians with combination of SP600125-treatment and knockdown of *EGFP* and *Djplac8-A* (n = 5). Student's t-test was performed by comparison between inhibitor-treated *EGFP(RNAi)* animals and inhibitor-treated *Djplac8-A(RNAi)* animals. ** $P < 0.005$, * $P < 0.05$.

I then tested whether these rescues by RNAi against *Djplac8-A* in SP600125-treated animals are associated with enhanced cell cycling of the neoblasts, like the induced hyper-proliferation caused by reduction of *Djplac8-A* expression in normal planarians. Control SP600125-treated(5 μM) animals did not show induced hyper-proliferation after amputation, although they maintained their basal rate of mitosis (Fig. 24D). In contrast, SP600125-treated(5 μM) *Djplac8-A(RNAi)* animals showed an increase of mitotic cell number relative to that in control animals, comparable to the amputation-induced increase of mitotic cell number in normal planarians. That is, RNAi of *Djplac8-A* could restore the induced hyper-proliferation in SP600125-treated animals after amputation. These results suggested that reduction of *Djplac8-A* expression alone is sufficient to cause the induced hyper-proliferation after amputation, and this induced hyper-proliferation might play an important role in regeneration by promoting the progression of neoblast differentiation.

To test the importance of induced hyper-proliferation in the cell differentiation of neoblasts, I checked the expression of *runt-1* in SP600125-treated planarians. It was reported that *runt-1* is expressed in a certain population in the neoblast population located in the post-blastema region, and is involved in the commitment of neuronal and eye cells during head regeneration in *S. mediterranea* (Wenemoser et al., 2012). First, I confirmed the expression pattern of *runt-1* in *D. japonica.* Expression of *Djrunt-1* was rapidly increased at 3 hours after amputation and continued to increase during early regeneration (Fig. 25A). This elevation of

Djrunt-1 expression after amputation was attenuated by X-ray irradiation (Fig. 25A). Also, *in situ* hybridization showed that *Djrunt-1* was expressed in the post-blastema region in early regeneration, and this expression was weakened by X-ray irradiation (Fig. 25B). Thus, I confirmed that *Djrunt-1* was expressed in the neoblasts localized in the post-blastema region during regeneration, as reported for *runt-1* in *S. mediterranea* (Wenemoser et al., 2012). Finally, I tested the relationships among activation of JNK, reduction of *Djplac8-A* expression, and expression of *Djrunt-1* by a combinatory experiment using SP600125 and *Djplac8-A(RNAi)*. When planarians were treated with 5 μM SP600125 after amputation, *Djrunt-1* expression was slightly increased, but not to its normal expression level at 12 hours after amputation (Fig. 25C). However, RNAi of *Djplac8-A* in the inhibitor-treated animals could significantly restore *Djrunt-1* expression during early regeneration (Fig. 25D). Taken together, these results suggested that the induced hyper-proliferation caused by inhibition of *Djplac8-A* expression as a result of activation of JNK signaling is crucial for commitment of the neoblasts after amputation.

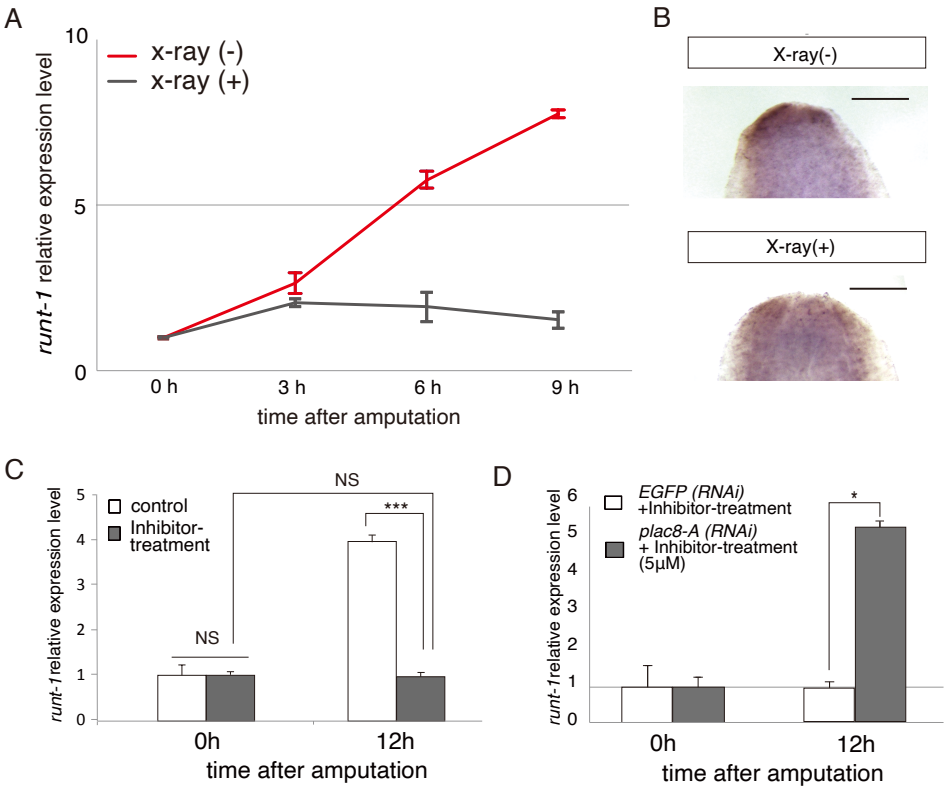


Fig. 25. Decrease of the expression level of *Djplac8-A* is required to increase of the expression level of *Djrunt-1*. (A) Relative expression level of *runt-1* after amputation. Gene expression levels were analyzed by qRT-PCR from 3 biological replicates. Error bars indicate SEM. (B) Expression pattern of *Djrunt-1* in intact and X-ray-irradiated animals determined by whole-mount *in situ* hybridization. Planarians were fixed at 9 hours after amputation. Scale bar, 100 μm. (n = 10, 10/10). (C) Relative expression level analysis of *Djrunt-1* at 0 hours and 12 hours after amputation in control animals and SP600125-treated animals. Student's t-test was performed by comparison between control animals and inhibitor-treated animals. *** $P < 0.001$. (D) Relative expression level of *Djrunt-1* at 0 hours and 12 hours after amputation in planarians with SP600125-treatment (optimal) with/without knockdown of *Djplac8-A*. Student's t-test was performed by comparison between inhibitor-treated *EGFP(RNAi)* animals and inhibitor-treated *Djplac8-A(RNAi)* animals. * < 0.05, *** $P < 0.001$. (C) and (D) Gene expression levels were analyzed by qRT-PCR from 3 biological replicates. Expression level of genes at each time is relative to that at 0 hours after amputation. Error bars indicate SEM.

6. Discussion

6.1 Induced hyper-proliferation as “conserved stem cell behavior” in planarians

Analysis of the dynamics of adult stem cell system *in vivo* is difficult in mammals because they are few in number and it is hard to access them. However, the high regenerative ability of planarians and their neoblasts (which are the only resource for regeneration) provide advantages for inverstigating the adult stem cell system. The large population of neoblasts make it easy to observe them *in vivo*, and regeneration using neoblasts give us a simple paradigm for investigating the fundamental roles, proliferation, and differentiation of stem cells. Especially, induced hyper-proliferation is a specific phenomenon in planarians that is useful for investigating the dynamics of proliferation in response to an external stimulus. Induced hyper-proliferation has been known for decades to occur after feeding or amputation in various species of planarians, for example*, Dugesia tigrina* and *S. mediterranea*(Baguñà, 1976; Saló and Baguñà, 1984; Wenemoser and Reddien, 2010). During the induced hyper-proliferation, mitotic cells (M-phase cells) are rapidly increased, and thereafter gradually return to the steady-state level within about 1 week (Saló and Baguñà, 1984). Induced hyper-proliferation in response to amputation or feeding, in which neoblasts might supply differentiated cells during regeneration or growth, seems to be a common event across planarian species. In the case of *S. mediterranea*, induced hyper-proliferation after amputation occurs with two phases. The first phase occurs as a result of simple wounding such as stabbing, and the second phase occurs as

a result of complete amputation of the bodies (Wenemoser and Reddien, 2010). However, in *D. japonica,* I found that amputation could accelerate neoblasts' proliferation, but simple wounding without regeneration, such as stabbing, could not evoke such acceleration, which is relevant to the first mitotic phase in *S. mediterranea* (Fig. 18). Thus, the regulation of stem cell responses to wounding seems to differ among planarian species.

6.2 Predictable molecular pathway of induced hyper-proliferation after feeding

Based on the increase of fission frequency, enhanced induced hyper-proliferation in *Djplac8-A(RNAi)* and *DjP2X-A(RNAi)* animals and expression patterns of *Djplac8-A* and *DjP2X-A* after feeding, all of these results suggest that these two genes have a similar function in the regulation of induced hyper-proliferation after feeding (Fig. 8, 9, 15; Sakurai et al., 2012). Furthermore, double *Djplac8-A and DjP2X-A(RNAi)* animals showed not a synergistic effect, but the same fission frequency rate as single RNAi animals, strongly suggesting that these two genes might function in parallel or in the same pathway (Fig. 10, 26). It remains necessary to investigate the relationship between *Djplac8-A* and *DjP2X-A* in the molecular pathway of regulation of neoblast proliferation, for example, whether one of these genes is upstream to the other. Measuring the expression level of *Djplac8-A* in *DjP2X-A(RNAi)* animals (or vice versa) might be helpful for identifying the molecular pathway.

After feeding, nutrient inflow might increase the extracellular ATP concentration. *P2X* is a membrane receptor of ATP (Di Virgilio and Adinolfi, 2017). Many reports have been shown that stimulation of P2X changes the permeability of ions, resulting in regulation of proliferation in various cells (Baricordi et al., 1999; Harada et al., 2000; Ohtani et al., 2011). Especially in several types of cancer cells, *P2X7* and ATP play a crucial role in proliferation regulation, and thus *P2X7* has been investigated as a target of cancer treatment (Di Virgilio et al., 2009). By referring to P2X functions in other organisms, possible molecular function(s) of DjP2X-A can be predicted. Restoration of the DjP2X-A protein level may increase the ion influx during induced hyper-proliferation after feeding to suppress hyper-proliferation, and as a result steady state proliferation might be restored. However, in *DjP2X-A(RNAi)* animals, neoblasts might not be able to restore the steady state after induced hyper-proliferation because of their failure to increase the DjP2X-A level, resulting in their faster growth and increased fission frequency (Sakurai et al., 2012). One of the interesting studies showing a relationship between P2X and proliferation showed that P2X regulates not only proliferation of pancreatic beta-cells but also their secretion of insulin, which is one of major anabolic hormones (Ohtani et al., 2011). In *S. mediterranea*, it has been reported that insulin-like peptide modulates neoblast proliferation depending on nutrient conditions (Miller and Newmark, 2012). These reports suggest the possibility that DjP2X-A influences insulin-like peptide secretion for regulating neoblasts' proliferation after feeding.

In addition to insulin-like peptide, mTOR signaling, which is a conserved signaling pathway that relates nutrient intake to cell proliferation in many types of cells, might be involved in induced hyper-proliferation after feeding (Gokhale and Shingleton. 2015). It has been reported that mTOR signaling regulates the neoblast population depending on the nutrient condition (Iglesias et al., 2019). Interaction between *plac8* and the AKT/mTOR pathway was reported in nasopharyneal carcinoma cells, which are a type of cancer cells in the neck (Huang et al., 2020). *plac8* inhibits macroautophagy and cell proliferation through the AKT/mTOR pathway. Macroautophagy is one of the processes by which cells digest their organelles, and which has been shown to regulate responses to nutritional stress and tumorigenesis (Huang et al., 2020; Lahiri et al., 2019). My findings support the speculation that down-regulated *plac8* can activate mTOR signaling, and then activated mTOR signaling mediates between the nutritional state and proliferation. Investigation of the relationships of *DjP2X-A*, *Djplac8-A*, and possible signaling pathway(s) involved in induced hyper-proliferation after feeding will be required to identify more detailed molecular mechanisms of the induction of hyper-proliferation after feeding (Fig. 26).

Furthermore, a recent report showed that the expression level of *blitzschnell(bls)* is decreased after feeding in *S. mediterranea*, similarly to the levels of of *DjP2X-A* and *Djplac8-A* (Pascual-Carreras et al., 2020). RNAi of *bls* caused a similar phenotype, namely increased cell number and body size, to the phenotypes of *DjP2X-A(RNAi)* and *Djplac8-A(RNAi)* animals.

bls regulates the proliferation/apoptosis ratio through mTOR signaling, resulting in a change of body size (Pascual-Carreras et al., 2020). However, *bls* is expressed in differentiated secretory cells, in contrast to *Djplac8-A* and *DjP2X-A*, which are specific to neoblasts, suggesting that induced hyper-proliferation might be regulated by not only neoblasts but also differentiated cells. Further studies of the molecular mechanisms of induced hyper-proliferation after feeding will lead to a better understanding of the regulatory systems of neoblasts depending on nutritional conditions. In addition, since cell number regulation is directly related to planarian body size (Takeda et al., 2009), it will be helpful for understanding how constant body size is maintained by planarians during their long life.

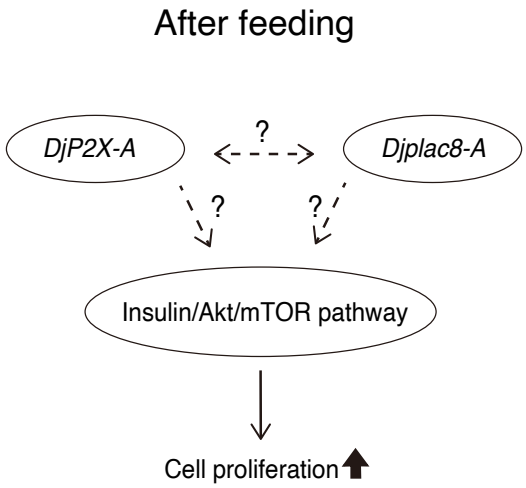


Fig. 26. Schematic drawing of possible molecular pathway involved in induced hyper-proliferation after feeding. Investigation of the relationship between candidate molecules regulating induced hyper-proliferation is required for unraveling the molecular pathway for induced hyper-proliferation after feeding.

6.3 Predicted molecular function of DjPlac8-A protein in induced hyper-proliferation

A recent report showed that one of the *plac8* homologs in the planarians *D. japonica* is related to immune response and development (Pang et al., 2017). Here, we identified 6 additional *plac8* homologs in *D. japonica*, and showed that, among them, *Djplac8-A* is the only neoblast-specific gene that is involved in neoblast proliferation. *plac8* has multiple functions in diverse phyla, from plants to animals, as mentioned above. Thus, *plac8* has been called several names, including *cell number regulator, onzin*, and so on, depending on its function and known related genes. Newly consolidated nomenclature for Plac8 was proposed recently(Cabreira-Cagliari et al., 2018). In plants, most studies showed that *plac8* homologs regulate cell proliferation negatively and control the weight and size of fruits in tomato, maize, and so on (Guo et al., 2010; Guo and Simmons, 2011; Libault et al., 2010). On the other hand, diverse functions of *plac8* in animals, including regulation of cell differentiation, proliferation, apoptosis, and migration have been reported (Bedell et al., 2012; Li et al., 2006; Mao et al., 2019). Here, I revealed that *Djplac8-A* is involved in induced hyper-proliferation of neoblasts via a decrease of its expression level after both amputation and feeding. This supports the notion that *plac8* and its function in regulating cell proliferation and/or differentiation are conserved in many organisms across diverse phyla.

How does DjPlac8-A regulate proliferation of neoblasts in planarian? Since common features of Plac proteins between plants and planarians are that Plac8 is a negative regulator of mitotic activity, and is a membrane-associated protein (Frary et al., 2000; Fig. 12), it is possible to speculate about the detailed molecular function of DjPlac8-A by referring to Plac8 of plants. Because most of the Plac8 in plants is localized as a transmembrane protein, it is considered to affect mitotic activity indirectly (Cong and Tanksley, 2006; Li and He, 2015; Libault et al., 2010). For example, Plac8 regulates cell proliferation negatively by interacting with casein kinase II (CKII)β subunit in tomato and soybean. Modified CKIIβ after interacting with Plac8 translocates from the cytosol to the nucleus and activates the CKIIα subunit, which represses the cell cycle (Cong and Tanksley, 2006; Libault et al., 2010). In addition, another report showed a negative correlation between organ size and *plac8* expression level (Li and He, 2015). In this case, Plac8 interacts with AG2, which is an agamous-like MADS domain protein localized at the cell membrane. Then, modified AG2 translocates to the nucleus, and binds to the CArG-box in the Cyclin promotor, resulting in enhanced repression of the cell cycle. However, after a decrease of the Plac8 protein level, AG2 is released without modification from the membrane, resulting in weakened repression of the cell cycle (Li and He, 2015). These results obtained in plants suggest that absence of Plac8 leads to loss of a cell cycle repressor, and consequently promotes cell proliferation (Fig. 27). Therefore, investigating the molecules

that interact with DjPlac8-A in future studies will be important for deeply understanding the cell cycle control by DjPlac8-A.

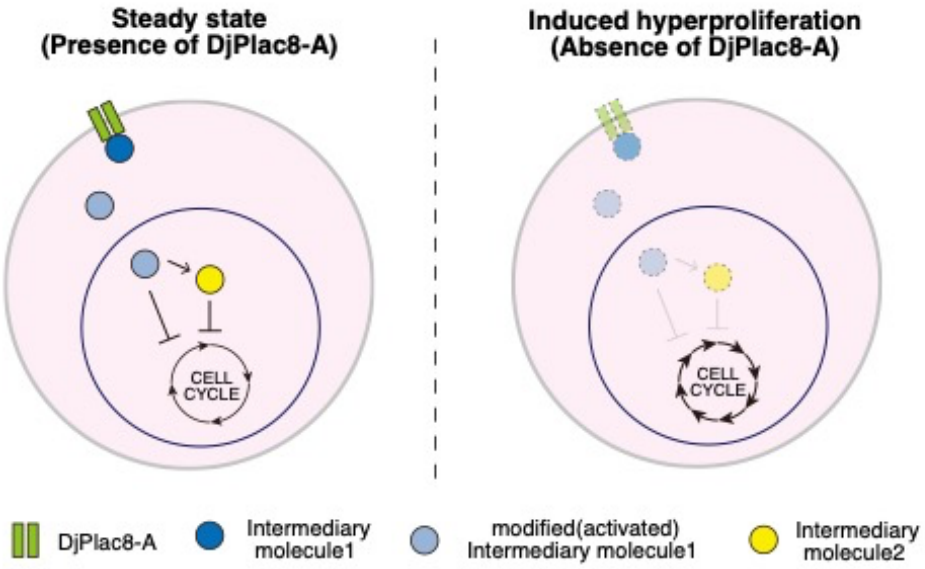


Fig. 27. Schematic drawing showing predicted molecular function of DjPlac8-A protein in induced hyper-proliferation. In the steady state, in which DjPlac8-A is present, the molecular pathway for suppressing the cell cycle can function. However, during induced hyper-proliferation, in which DjPlac8-A is absent, this molecular pathway cannot function, resulting in promotion of the cell cycle. Examples of intermediary molecule 1 suggested by referring to previous reports could be CKIIβ or AG2. Examples of intermediary molecule 2 suggested by referring to previous reports could be CKIIα or CArG-box at the Cyclin promotor.

Translocation of the subcellular localization of Plac8 protein from the cell membrane is also commonly observed in vertebrates. For example, in mice, Plac8 is known to translocate from the cell membrane to the nucleus, and to bind there to the promoter of a transcription factor, C/EBPβ, induce its transcription, and consequently promote the differentiation of brown

adipocytes (Jimenez-Preitner et al., 2011). I could not observe obvious translocation of DjPlac8-A during regeneration in this study. However, producing DjPlac8-A-GFP transgenic animals might enable us to show more clearly the DjPlac8-A localization pattern in the neoblasts during regeneration in the future. This study revealed that induced hyper-proliferation of neoblasts is important not only for neoblasts' expansion but also for supplying differentiated cells for normal regeneration. The biological role of induced hyper-proliferation in cellular differentiation during planarian regeneration is discussed below.

6.4 JNK and ERK signaling in the induced hyper-proliferation

I showed that activated JNK signaling down-regulates the expression of *Djplac8-A* in the neoblasts located in the post-blastema region after amputation. Treatment of animals with an appropriate concentration of SP600125 that maintained the expression of *Djplac8-A* resulted in failure of induced hyper-proliferation, indicating that JNK signaling elicited induced hyper-proliferation in the neoblast pool after amputation (Fig. 28, 29). In addition, I confirmed that ERK signaling also impacts the expression of *Djplac8-A* after amputation by cooperating with JNK signaling. ERK signaling is known to be important for differentiation of the neoblasts (Tasaki et al., 2011a). At a high concentration of ERK inhibitor, U0126, *de novo* differentiation of somatic cells from the neoblasts was disrupted (Tasaki et al., 2011b). During regeneration in *D. japonica*, neoblasts supply all types of differentiated cells, including cells that secrete

growth factors (Hayashi et al., 2011; Yazawa et al., 2009). This leads us to hypothesize that after amputation, newly differentiated cells are also required for maintaining activated JNK signaling during regeneration, and this activated JNK signaling might regulate the expression level of *Djplac8-A* during regeneration (Fig. 28, 29). It seems likely that U0126 treatment blocked the supply of the cells needed to maintain the activation of JNK signaling after amputation, and consequently the reduction of *Djplac8-A* expression and induced hyper-proliferation were prevented. Thus, I propose that both JNK and ERK signaling might be required to maintain the induced hyper-proliferation during regeneration.

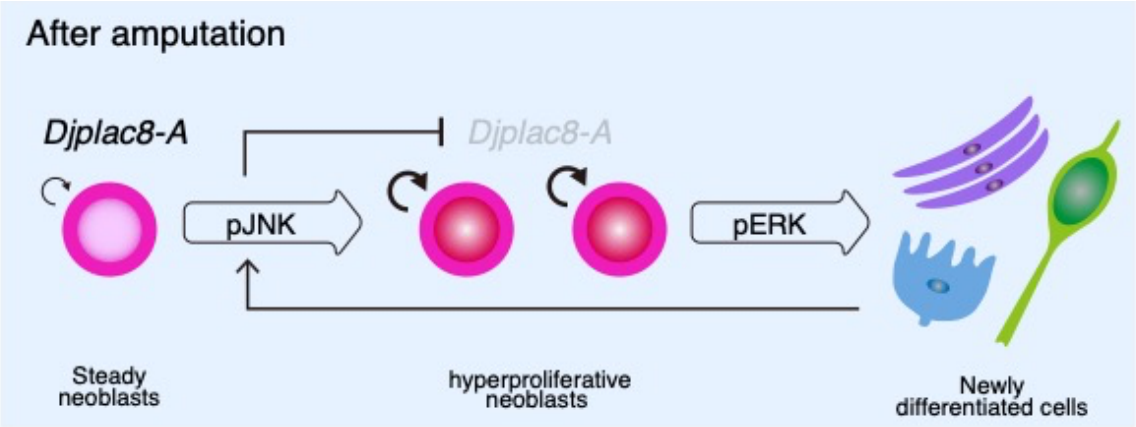


Fig. 28. Signaling pathways involved in induced hyper-proliferation after amputation. Induced hyper-proliferation requires both JNK signaling and ERK signaling. After amputation, activated JNK signaling inhibits *Djplac8-A* and thereby induces hyper-proliferation, and ERK signaling is required for differentiation of cells from neoblasts. Newly differentiated cells might be required to maintain the JNK signaling.

6.5 *Djplac8-A* is a switch for transition of the cellular state of neoblasts during induced hyper-proliferation for regeneration

So far, induced hyper-proliferation has been thought to be a unique feature of neoblasts for enlarging their population at the onset of the regeneration process (Wenemoser and Reddien, 2010). However, my results strongly suggest that induced hyper-proliferation is an indispensable event for rapid and proper supply of differentiated cells during regeneration. Losing body part(s) leads immediately to various kinds of reactions, including wound healing and rearrangement of body polarity in the remaining tissues (Gurley et al., 2010; Kato et al., 2001). Induced hyper-proliferation is regarded as the initial reaction of neoblasts during regeneration. Here I revealed a molecular mechanism involving transition of the cellular state through induced hyper-proliferation. I propose that *Djplac8-A* acts as a switch to shift the state of neoblasts from steady state to active state in early regeneration (Fig. 29).

Djplac8-A is expressed in almost all *piwiA*-expressing neoblasts, except in the *Djnanos*$^{+}$ neoblasts subpopulation and the *Djpiwi-1*$^{+}$ neoblasts subpopulation (Fig. 13). Thus, decreasing the *Djplac8-A* expression can induce a qualitative state change in almost the whole stem cell pool. Although the above two DjPlac8-A^{-} populations should be further characterized, *Djnanos*$^{+}$ neoblasts are considered to be germline-specified stem cells, not somatic cell-specified stem cells, based on previous reports showing a relationship between *nanos* and germline differentiation (Sato et al., 2006; Wang et al., 2007). It is interesting that a simple

mechanism of regulation by one gene (*Djplac8-A*) can activate almost the whole neoblast population, which consists of several subsets with molecular heterogeneity. Considering this, I can expect that *Djplac8-A* is quite far up-stream in the molecular pathway of neoblast regulation and thus can influence a variety of consequences, including proliferation and differentiation. The subcellular localization of DjPlac8-A at the cell membrane (or membrane associated) might also support this expectation, because membrane proteins might rapidly sense the extracellular environmental change in response to amputation (and also in response to feeding) (Figs. 12, 27). Therefore, suppression of *Djplac8-A* might be a rapid strategy to regulate the participation of as many, and as varied, neoblasts as possible for regeneration. A recent report showed a close relationship between cell cycle regulation by factors such as Cyclin and CDKs and proliferation or differentiation of stem cells (Neganova et al., 2009). Especially, it was reported that specification or differentiation of human ESCs occurred during unusual cell cycling such as increasing cell division or expanded G_1 phase because ESCs have a short G1 phase, which has been thought to provide a limited time window during which they accept the differentiation signals (Liu et al., 2019; Neganova et al., 2009). It is basically unknown which phase of the cell cycle is the time for the fate decision for differentiation from stemness in neoblasts. Previous reports showed the possibilities that initialization of differentiation might occur in G2 phase in *D .japonica* and specification for specific lineages occurs in S phase in *S. mediterranea* (Hayashi et al., 2010; Van Wolfswinkel et al., 2014).

Recent studies have characterized the fate decision of neoblasts in more detail. Cell cycle arrest by *cdh1* for exiting to G0 is required for *D. japonica* neoblasts to differentiate (Sato et al., 2021). Fate-specific transcription factors are highly expressed in S/G2/M phases in contrast to their lower expression in G1 phase, suggesting that the fate decision occurs in S/G2/M phase in *S. mediterranea* (Raz et al., 2021). Therefore, a qualitative change of the neoblasts' state through cell cycle control by the *Djplac8-A*-pathway could be another clue for disclosing the details of the fate decision of neoblasts.

I revealed that a decrease of *Djplac8-A* expression is required to induce expression of a transcription factor for differentiation (Fig. 25). This suggested that a qualitative state change to an active state of neoblasts by decreasing *Djplac8-A* expression is indispensable for normal regeneration. In mouse, activated HSCs are increased with high expression level of CD34 mRNA to produce differentiated hematopoietic cells by symmetric division after injury (Wilson et al., 2008). Likewise, the active state of *Djplac8-A*-negative neoblasts is essential for producing a sufficient differentiated cell supply by promoting differentiation. In addition, the expression level of *Djplac8-A* might be related to symmetric/asymmetric division of neoblasts, similarly to that of HSCs. It is known that EGF signaling is involved in regulating symmetric/asymmetric division of neoblasts and that through asymmetric division, specified neoblasts produce progeny and themselves retain potency (Lei et al., 2016; Raz et al., 2021). My finding that a decrease of *Djplac8-A* expression can promote differentiation suggests that

it is possible that *Djplac8-A*-negative cells become progeny, while neoblasts that restore the *Djplac8-A* expression (*Djplac8-A*-positive cells) might become daughter neoblasts with the potency to differentiate during asymmetric division.

In future studies, it will also be important to examine the function of *Djplac8-A* in the steady state or the return to the steady state from the active state after regeneration through recovery of the expression level of *Djplac8-A*. My results suggested that *Djplac8-A* represses acceleration of the cell cycle in the steady state. Thus, recovery of the expression of *Djplac8-A* at the end of regeneration is important for precisely balancing the differentiated cell supply and demand in order to avoid abnormal regeneration (Fig. 28). Understanding this kind of mechanism will be helpful in the medical field, including for applications of stem cell therapy. Transplantation of stem cells in the activated state would seem to be useful for increasing the efficiency of stem cell-based therapy, because activated stem cells would proliferate rapidly *in vivo* and respond faster to environmental stimuli without needing to undergo the process of *in vivo* activation. Therefore, in-depth studies of the molecular mechanisms regulating heterogeneous stem cell populations will be important for not only stem cell research but also the medical field.

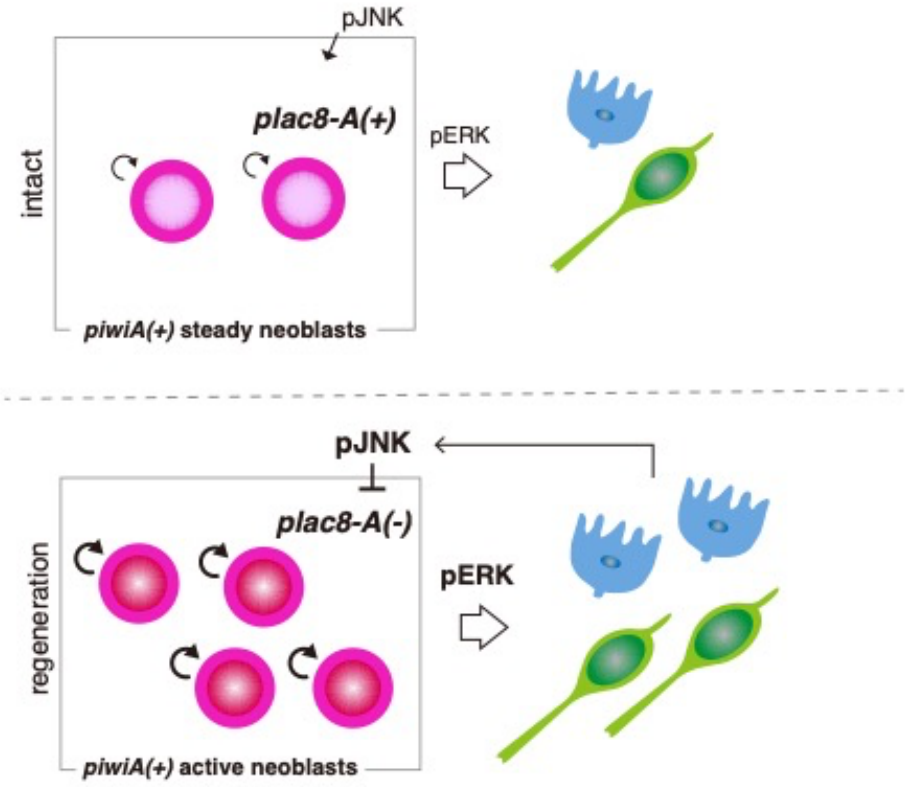


Fig. 29. Schematic drawing showing acquisition of active state of neoblasts via control of *Djplac8-A* expression. Molecular mechanism involving transition of the cellular state of neoblasts through induced hyper-proliferation during regeneration.

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