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Master Thesis

渦蟲 DDX6 核糖核酸解旋酶 DjCBC-1 之功能研究

Functional study of the planarian RNA helicase DDX6, DjCBC-1

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國立臺灣大學碩士學位論文
口試委員會審定書

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Functional study of the planarian RNA helicase DDX6, DjCBC-1

本論文係王嬿涵君（R03B21012）在國立臺灣大學生命科學系完成之碩士學位論文，於民國 105 年 7 月 11 日承下列考試委員審查通過及口試及格，特此證明

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誌謝

碩士班的時光說長不長，然而經歷的一切卻也不是三言兩語就能夠道盡的。在我短暫的研究生涯中，遇到非常多貴人。首先，萬分感謝朱家瑩老師願意指導實驗經驗不足的我，同時給予很大的實驗自由度，讓我在兩年之內熟習許多實驗技術，從幾乎零經驗到可以獨立進行實驗。另外，不論口頭報告或是論文書寫，老師都不厭其煩地與我討論架構，並且花費寶貴的時間幫我修正錯誤，讓我學習如何有邏輯地描述研究成果。老師讓我在研究所階段學到許多受用一生的能力。

修習郭典翰老師開設的課程讓我更加瞭解實驗原理，培養了實驗失敗時自行優化實驗條件以得到更佳實驗結果的能力。蘇迪勒風災生科館停電時，郭老師大方出借小型培養箱，讓我的實驗動物在炎炎夏日得以生存。而兩次所上進度報告皆有幸能得到陳示國老師的提點，陳老師更不吝出借實驗室的付費軟體，讓我能快速分析行為實驗結果，著實給予我很大的幫助。除此之外，兩位老師還在百忙之中抽空擔任我的口試委員，帶給我許多新的想法和建議，真心感謝兩位老師。

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摘要

再生是生物體更新或修復受損的細胞和組織，藉以維持正常生理功能與生存的方式。扁形動物渦蟲綱具有強大的再生能力，其體內存在大量多功能性成體幹細胞（neoblasts），能夠增生並分化成所有類型的細胞。因此，維持成體幹細胞增生與分化的平衡，對於渦蟲的生理恆定和再生過程都極為重要。DDX6 蛋白屬於 DEAD-box RNA 解旋酶家族，真核生物中的 DDX6 同源蛋白不論在序列或功能上都具有高度保守性，並參與多項細胞生理機制。最近的研究更指出，DDX6 能夠協同微核糖核酸（microRNA）影響哺乳類神經幹細胞分化，亦可透過降解 mRNA 和抑制轉譯的方式調控哺乳類上皮幹細胞的增生與分化。然而，對於渦蟲 DDX6 同源蛋白 DjCBC-1 是否能調控成體幹細胞增生與分化至今仍尚未明瞭。先前研究顯示，*Djcbc-1* 表現在成體幹細胞、神經和生殖細胞當中，而抑制 *Djcbc-1* 的表現量會限制渦蟲眼點的再生。本研究進一步證實 *Djcbc-1* 高量表現於再生組織，並發現 *Djcbc-1* 的表現與否能顯著地影響處於再生期的渦蟲之存活率。除此之外，抑制 *Djcbc-1* 的表現量也會改變渦蟲腦部結構的完整性，並且大幅減弱渦蟲的負趨光性。我的研究結果顯示，*Djcbc-1* 參與渦蟲個體的生理恆定、再生過程、以及負趨光性的調控。

關鍵字：

DEAD-box 核糖核酸解旋酶、DDX6、DjCBC-1、組織恆定性、再生、渦蟲

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Abstract

Regeneration is the process of renewal and regrowth of damaged tissue to maintain the normal physiological functions for surviving. Planarian has a robust ability of regeneration. Regeneration in planarians requires neoblasts, the pluripotent somatic stem cells, which can proliferate and differentiate into all different cell types. Regulation of neoblasts proliferation and differentiation is critical for planarians homeostasis and regeneration. DDX6, a member of the DEAD-box protein family, is highly conserved among most eukaryotes. DDX6 homologs play multiple roles in many biological processes. Recent studies showed that DDX6 regulates the differentiation of mammalian neural stem cells by microRNAs, as well as regulates the proliferation and the differentiation of mammalian epidermal stem cells by mRNA degradation and translational repression. However, it is unclear that whether DjCBC-1, the DDX6 ortholog in planarians, also regulates the proliferation and differentiation of neoblasts. Previous studies showed that *Djcbc-1* is expressed in neoblasts, neurons, and germline cells. In addition, depletion of *Djcbc-1* limited the formation of photoreceptors. Here, I further demonstrated that *Djcbc-1* is highly expressed in the regenerating tissue, and is essential for planarian survival at regeneration status. Besides, depletion of *Djcbc-1* perturbed the homeostasis of brain structure and reduced the negative phototaxis in planarians. Collectively, these results suggest that *Djcbc-1* participates in tissue homeostasis, regeneration, and the regulation of negative phototaxis in planarians.

Keywords:

DEAD-box helicase 6, DDX6, DjCBC-1, homeostasis, regeneration, planarian

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1. Introduction

1.1 Regeneration in planarians

Regeneration is the process of renewing and regrowing damaged tissues or organs for maintaining the normal physiological functions that are essential for the survival of organisms. Many kinds of animals have been used as the model organisms to study regeneration. For example, sea stars can regenerate arms after injury (Ramsay et al., 2001); zebrafish can regenerate hearts and fins after resection (Akimenko et al., 1995; Poss et al., 2002); and salamander can regenerate new limbs after damage (Kumar and Brockes, 2012). One of the well-known model animals is planarian, which has a robust ability of regeneration. Planarian can regrowth their missing body part (Wenemoser et al., 2012), and each body fragment can regenerate into an individual. It was described more than hundred years ago that the minimal size of a planarian fragment for completing successful regeneration was 1/279th of the volume of the intact planarian (Morgan, 1901), and this size of planarian tissue contains about 10000 cells (Sanchez Alvarado, 2004). Unlike most of the regenerative animals, amputated at any orientation, planarian can fully regenerate into a completely normal form. Besides, a planarian is capable of regenerating a new head and central nervous system (CNS) after being injured. It takes 7 days for a planarian to regenerate a new head (Slack, 2011). With this astonishing power of regeneration, planarian has been wildly used as a model system for regeneration studies. Although various species of planarians have been identified, *Schmidtea mediterranea* (Smed) has been used as the model species in many research groups in America and Europe. On the other hand, researchers in Asia selected *Dugesia japonica* (Dj) for their study. Here we use *Dugesia japonica* in the following studies.

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1.2 Neoblasts in planarian regeneration and homeostasis

Regeneration in planarians requires the somatic stem cells, known as the neoblasts, which are small in size (5-10 μm) and abundant in planarian (Baguna and Slack, 1981). Neoblasts is also the source of homeostatic tissue turnover (Gonzalez-Estevez et al., 2012; Tu et al., 2012; Zhu et al., 2015). There are three remarkable features of neoblasts. First, neoblasts are pluripotent stem cells. It can not only proliferate in a self-renewal manner but also differentiate into all types of different cells (Reddien, 2013). Second, because of the high rate of cell division, the undifferentiated neoblasts is featured with high nucleo-cytoplasmic (N/C) ratio (Alie et al., 2011). Third, neoblasts can rapidly proliferate and are highly sensitive to γ-ray and x-ray irradiation (Guedelhoefer and Sanchez Alvarado, 2012; Wagner et al., 2011).

When planarian gets injured or damaged, the gene expression level is accurately regulated during planarian regeneration (Wenemoser et al., 2012; Wurtzel et al., 2015). The process of regeneration starts with wound healing (Reddien and Sanchez Alvarado, 2004). Next, the apoptotic rate near the wound site raises and this triggers the proliferation of neoblasts. Neoblasts will migrate toward the wound site, proliferate to increase the cell population size, and differentiate into early progeny cells in the regenerating tissue. Early progeny cells then differentiate into late progeny cells, which in turn become different types of differentiated cells to finish the regeneration process (Gentile et al., 2011).

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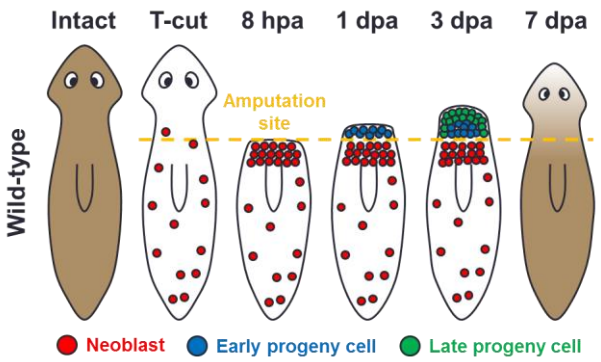
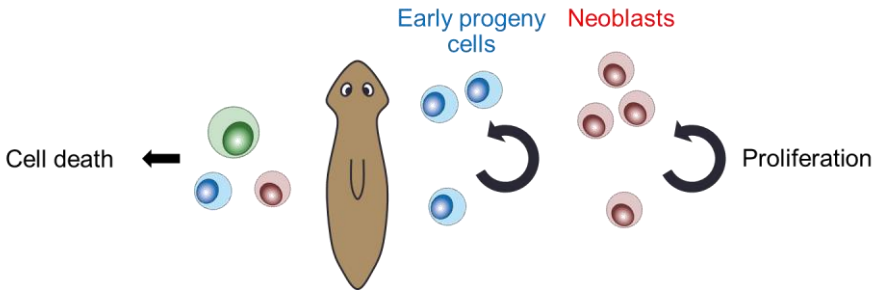


Time course and cells in planarian regeneration

The long-term process of homeostasis depend on the balance between cell proliferation and cell death, and it may be seen as macroscopic of regeneration (Tu et al., 2012). Because of their sensitivity to γ-irradiation, neoblasts, early progeny cells and late progeny cells can be eliminated by exposure to γ-irradiation (Eisenhoffer et al., 2008). After neoblasts exhaust, planarian will be dead soon due to that no new cells are supplied for tissue renewal. Therefore, the regulation of neoblasts proliferation and differentiation is critical for both of planarian regeneration and homeostasis.



The balance between cell proliferation and cell death

1.3 Nervous systems and the behaviors of planarians

Planarian possesses a central nervous system (CNS) with an evolutionarily primitive brain structure (Inoue et al., 2015). The planarian brain consists of a pair of cephalic ganglia in the head arranged in a U-shape (Agata et al., 1998). Planarian brain contains

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various types of neurons, including photoreceptor neurons, chemosensory neurons, thermosensory neurons and so on (Roberts-Galbraith and Newmark, 2015).

The visual organs of planarian consist of a pair of eyespots that are located on the dorsal side and both eyespots are connected to the brain. The eyespots contain two cell types: pigment cells and photoreceptor neurons. The pigment cells are responsible for capturing the light. The photoreceptor neurons are in charge of transforming the photons into signals and transmitting signals toward the brain. The photoreceptor neurons are bipolar, one of their rhabdomeres press close into a pigmented cup structure, and the other innervation to the brain. The neural processes origination from the left and right photoreceptors cross each other at the optic chiasm, which integrates inputs from both sides of a planarian, on the dorsal-medial side of the cephalic ganglia. (Agata et al., 1998; Paskin et al., 2014).

Multiple types of sensory neurons assist planarian to detect many kinds of stimuli from the external environment, so that planarian is capable of responding to the stimuli with a variety of behaviors. For example, planarian moves away from light but moves towards food. A recent study showed that planarian can not only respond to distinct signals respectively but also can integrate more than one external stimuli to make a decision (Inoue et al., 2015).

Normal planarian shows several types of behaviors. To avoid predators in the wild, planarian shows negative phototaxis. A recent study has shown that light of different wavelengths can cause different responses. Most planarians avoid short-wavelength light such as blue light and ultraviolet (UV) but almost exhibit no response to the long-wavelength light such as infrared (IR) (Paskin et al., 2014). Therefore, the short wavelength light is a strong stimulation for planarian. In addition to negative phototaxis, planarian also displays negative thermotaxis, negative thigmotaxis, and chemotaxis.

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Planarian is tended to stay at the place with lower temperature and smooth surface. Besides, planarian is attracted by food materials such as chicken liver and yolk (Inoue et al., 2015). In additions, planarian also has spontaneous behaviors of wigwag movement that lead them to an apparent wall preference (Akiyama et al., 2015).

Planarian provides a model to examine the integration capability of the brain by simple behavioral experiments. Furthermore, it was reported that planarian CNS complete functional regeneration within a week (Roberts-Galbraith and Newmark, 2015), and technically whether a planarian shows a normal behavior pattern can be a valuable experimental leverage for evaluating if a planarian has completed functional regeneration in an experimental condition.

1.4 DEAD-box helicase 6 (DDX6)

Many genes contribute to balancing neoblasts proliferation and differentiation. These genes are regulated at many different levels. One important process that regulates gene expression is post-transcriptional regulation. DEAD-box protein family widely participates in this process (Linder and Jankowsky, 2011).

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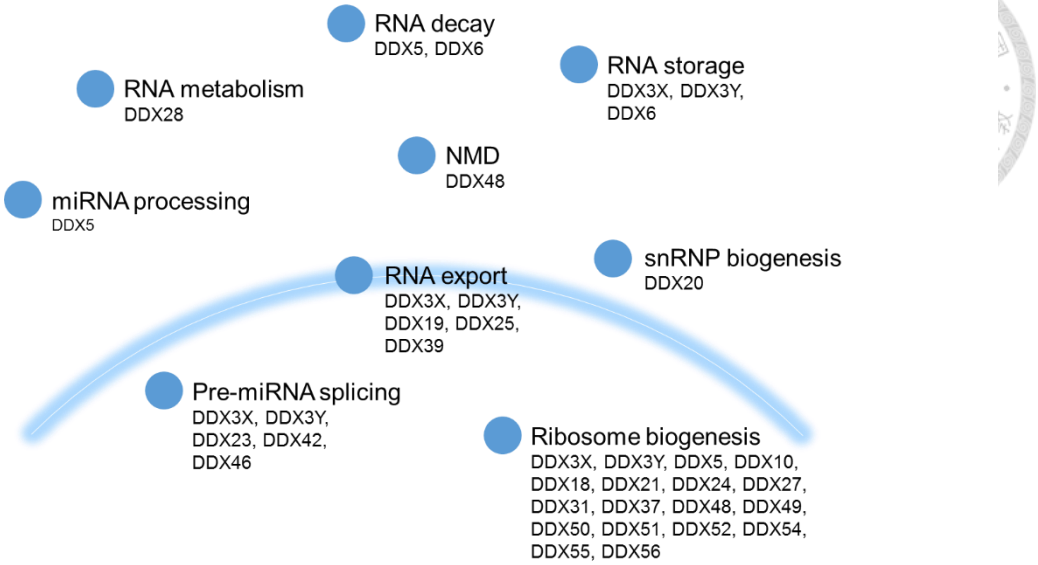
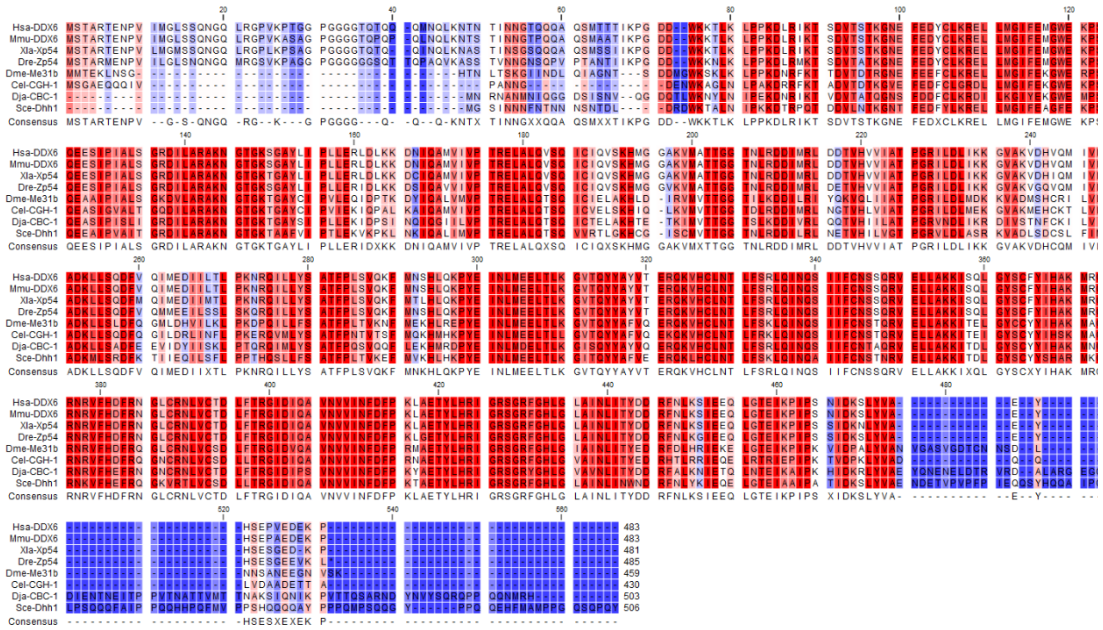


DEAD-box RNA helicases in post-transcriptional gene regulation

Among these DEAD-box RNA-binding proteins, I focus on the DEAD-box RNA helicase 6 (DDX6). DDX6 is highly conserved among most eukaryotes.



DDX6 is highly conserved between eukaryotes

Studies on the orthologs of DDX6 in various species showed that DDX6 is involved in the regulation of gene expression at post-transcriptional level in yeast, *Xenopus*, and

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human. In yeast, DDX6 functions in translational repression and mRNA decapping (Carroll et al., 2011; Coller and Parker, 2005). In *Xenopus* oocytes, DDX6 is involved in translational repression and RNA degradation (Minshall and Standart, 2004; Waghray et al., 2015; Weston and Sommerville, 2006). In mammalian cells, DDX6 mediates translational repression induced by microRNAs (Chu and Rana, 2006). One of the special features of DDX6 is that it promotes microRNA-mediated gene silencing by interacting with mRNA decapping complex (Jonas and Izaurralde, 2015; Mathys et al., 2014).

Specifically, recent studies in mammalian cells have shown that DDX6 regulates differentiation of neural stem cells by ways of microRNAs (Nicklas et al., 2015), as well as the proliferation of stem cells and differentiation of epidermal progeny cells by mRNA degradation and translational repression (Wang et al., 2015). However, it is unclear that whether the DDX6 ortholog in planarians also regulates neoblasts proliferation and differentiation.

1.5 DjCBC-1, the DDX6 ortholog in *Dugesia japonica*

The DDX6 ortholog in *Dugesia japonica* is called chromatoid body component 1 (DjCBC-1). DjCBC-1 is localized at the chromatoid body (Yoshida-Kashikawa et al., 2007). The chromatoid body is one of the characteristic morphological features of neoblasts; it is electron-dense and localized in the cytoplasm (Kashima et al., 2016; Yoshida-Kashikawa et al., 2007). Besides, the chromatoid body is the place where the RNA was processed and the RNA-associated proteins are converged (Hay and Coward, 1975). The chromatoid body is composed with noncoding RNAs such as miRNA, piRNA, and lncRNA (Meikar et al., 2014), as well as the RNA-associated proteins such as Tudor, Piwi, and Vasa (Chuma and Nakano, 2013).

A recent study revealed that chromatoid bodies are a heterogeneous population of

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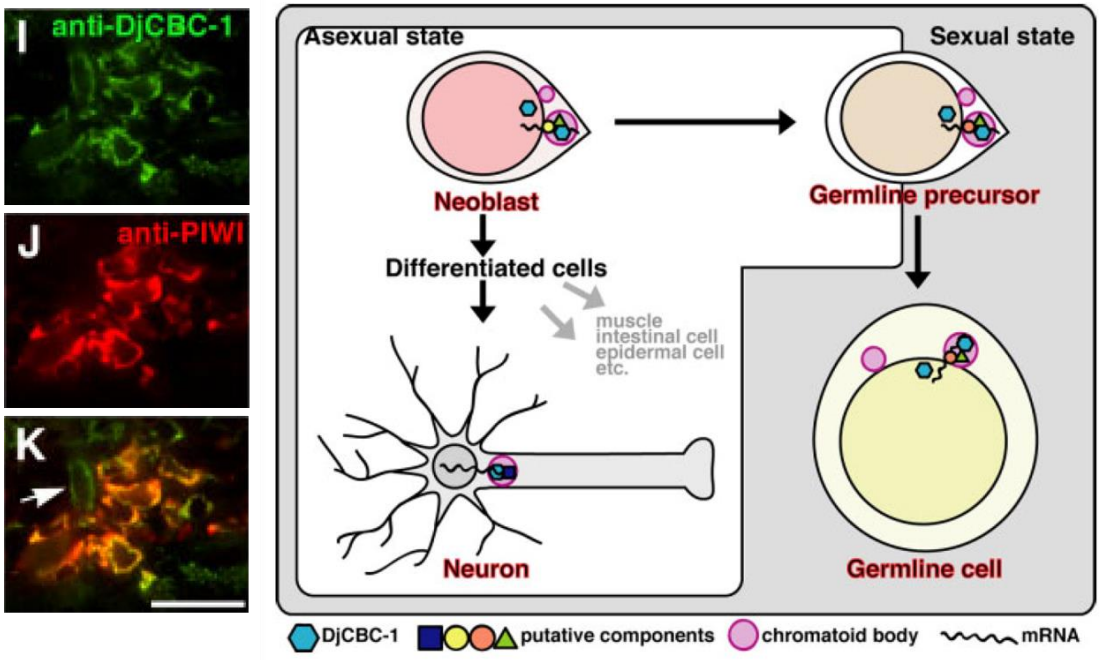
bodies. There are at least two kinds of chromatoid bodies in planarian. These two are independent of each other. One class specifically contains DjPIWI-C, and it may be involved in piRNA processing and transposable elements repression (Kashima et al., 2016). The other class contains DjCBC-1, and the function of these chromatoid bodies remains largely unknown (Yoshida-Kashikawa et al., 2007). However, since not all chromatoid bodies can be tagged with DjCBC-1, we consider that the name DjCBC-1 may not reflect the actual situation, but we will temporarily keep the original name of this gene to avoid confusion.



Localization of DjCBC-1 in planarian

(Yoshida-Kashikawa et al., 2007)

Double immunostaining of DjCBC-1 and the neoblasts marker DjPIWI-A in the previous study suggested that DjCBC-1 not be only expressed in neoblasts, but also in differentiated cells and germline cells (Yoshida-Kashikawa et al., 2007). It has also been reported that depletion of *Djcbc-1* reduced regeneration to a limited extent and resulted in abnormal photoreceptor organization, such as a cyclopia phenotype. By comparing the

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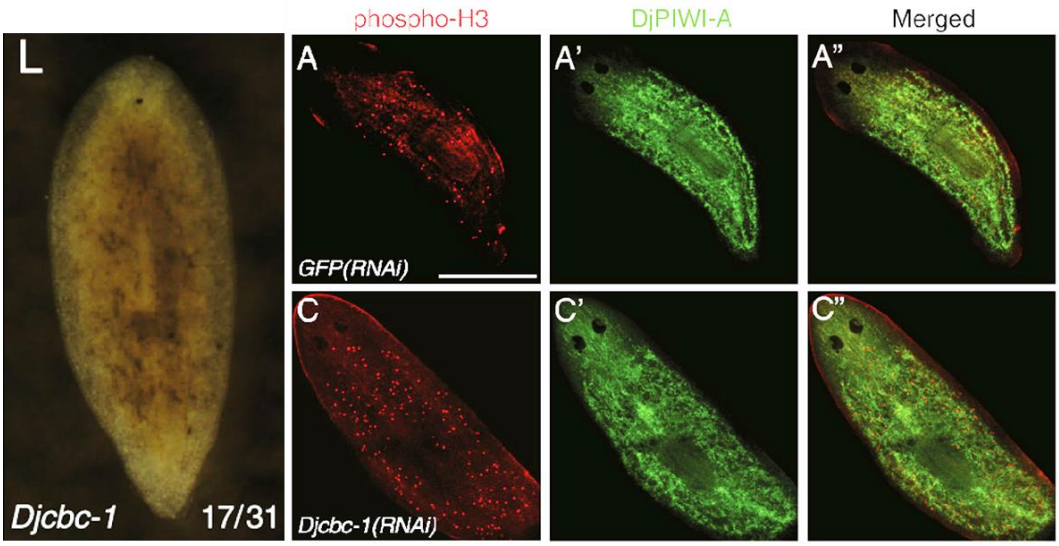
distribution of all neoblasts and the mitotically active neoblasts with anti-DjPIWI-A and anti-phospho-Histone 3 (H3p) immunostaining, it was revealed that depletion of *Djcbc-1* did not affect the expression level of DjPIWI-A but increased the mitotically active neoblasts. It suggested that DjCBC-1 is not necessary for neoblasts maintenance but may play some roles in neoblasts differentiation processes during regeneration (Rouhana et al., 2010). However, the details to link DjCBC-1 functions in regeneration and neoblasts differentiation processes are largely unknown.



Effects of *Djcbc-1* depletion

(Rouhana et al., 2010)

In this study, I will dissect the function of DjCBC-1 in planarian with two specific aims:

Aim 1. To analyze the function of DjCBC-1 in planarian homeostasis and regeneration.

Aim 2. To identify the role of DjCBC-1 in planarian CNS.

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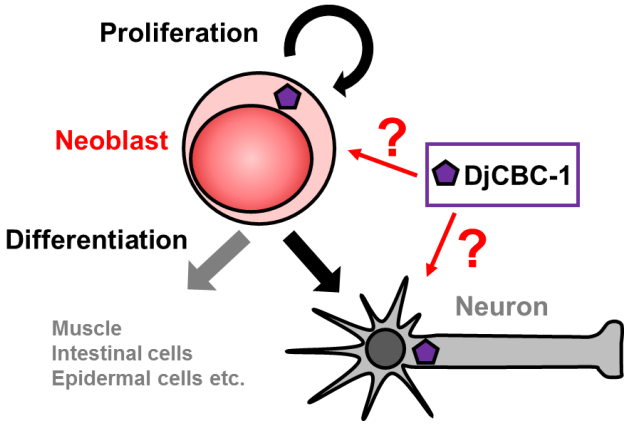

Possible function of DjCBC-1 in planarian

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2. Materials and Methods

2.1 Animals

In our lab, we used *Dugesia japonica*, one of fresh water planarian, as a model animal. The planarian was captured in Jiaoxi Township, northeastern of Taiwan. The population size of planarian was maintained by irregular capture and regular amputation. After being captured, the planarian was maintained in the lab at least one month for the experiment. The planarian was kept in instant ocean water (0.05g/L Instant Ocean Sea Salts in double-distilled water) at 18ºC in the dark. The fresh instant ocean water was replaced at least twice times a week. The planarian was fed with chicken liver at least once a week, and was starved at least three days for follow-up experiment.

2.2 Synthesis and purification of digoxigenin (DIG)-labeled RNA probes

The partial or complete sequences (about 500-1500 base pairs) of target genes that used as the templates of in vitro transcription were cloned into TA vector by PCR with cDNA of *Dugesia japonica*. The following primer pairs were used:

Djcbc-1	Forward_0001:	5’ ATGAATAGAAACGCCAATATG 3’
	Reverse_1515:	5’ TCAATGTCGCATATTCTGC 3’
Djpcna	Forward_0039:	5’ ATGTTTGAAGCCAAGCTTGTTAAAG 3’
	Reverse_0818:	5’ TCATTCATCATCTTCAATTTTTGGC 3’
Djprog-1	Forward_0089:	5’ CGAAAATTAATCAAAAAAATGAAATTGC 3’
	Reverse_0784:	5’ CAAATCAATAACATATTTTCACTTTTAC 3’
Djprog-2	Forward_0084:	5’ CTGTAAAATGCATGTTTGTTCCTC 3’
	Reverse_0581:	5’ TTAACGAGATTGTTTACATTCTGCC 3’

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Djopsin	Forward_0729:	5’ CGTGAAAGCCGTTAGAGTTC 3’
	Reverse_1001:	5’ AAGGGATTGTACATAGCTGATG 3’
Djarrestin	Forward_0041:	5’ CTTGGAAATAGAGATTTCTTTGATG 3’
	Reverse_1255:	5’ AGATTTCAAATATGATTTTCCGTGC 3’
Djchat	Forward_0291:	5’ AATTGGGTTGCCGATTTATG 3’
	Reverse_0970:	5’ AGAGCGCAATTAACATTATCTCTTATT 3’
Djpc-2	Forward_0925:	5’ TGAAACAATGCGTGCAATTGTAGAGG 3’
	Reverse_2680:	5’ GCATCGGTATCTATCTCGAGTATC 3’
Djsyt	Forward_0032:	5’ GTTGGATATATTTCATTGTTGTGTG 3’
	Reverse_1608:	5’ CATACTACTCTCAAATAGCCAATAG 3’
Djsnap-25	Forward_0055:	5’ GGAAGGCGATCTTAATAATGAACC 3’
	Reverse_1054:	5’ CATCATAAGGAAGAAGTATGACGC 3’

The orientation of cDNA inserts in TA vectors sense or anti-sense was verified by PCR with a combination of target genes primers and M13 forward (-20) or M13 reverse (-24) sequencing primers. Anti-sense was used as generating (DIG)-labeled RNA probes for WISH while sense was used as a control for confirming the expression pattern of anti-sense probes was authentic. The single-strand RNA probe was synthesized by in vitro transcription with T7 RNA polymerase, reacting for 3 hours at 37ºC. The DNA template was digested by DNase I at 37 ºC for 15-30 minutes. Then the reaction was stopped by 0.5M EDTA (pH=8.0), and the RNA was precipitated by adding 4M Lithium Bromide (LiCl) and 100% ethanol and store at -20ºC overnight or -80 ºC for 1 hour. After that, RNA was washed by 75% ethanol and then dissolved by 20 μL of nuclease-free H_2O. Formation of RNA synthesis was confirmed by running electrophoresis in a 1.0% agarose gel in 0.5% TAE.

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2.3 Whole-mount *in situ* hybridization (WISH)

The protocol of whole-mount in situ hybridization was similar as reported (Pearson et al., 2009). The Planarian with 2 to 7 mm in length was treated with 5% N-acetyl cysteine (in PBS) for 3 to 5 minutes, and then fixed with 4% formaldehyde (in PBS) for at least 20 minutes (do not over 45 minutes). After washed with 0.3% PBSTx (0.3% Triton-X100 in 1x PBS), planarian was rinsed with 50% methanol (in 0.3% PBSTx) for 5 minutes, and then 100% methanol for 10 minutes. After that, planarian was stored at -20ºC for at least 1 hour for dehydration. The planarian was general bleached with 6% H_2O_2 (in 100% methanol) under direct light, overnight. In this case, the pigment of planarian body will eliminate but the eyespots pigment will remain. The planarian was seriously bleached with 1% formamide and 6% H_2O_2 (in 0.3% PBSTx) under direct light for 1.5 to 2 hours (do not over 2 hours). In this situation, the eyespots pigment will be eliminated. After eliminating pigment, the planarian will be washed with 100% methanol for 5 minutes and 50% methanol (in 0.3% PBSTx) for 10 minutes. Then rinsed with 0.3% PBSTx for 10 minutes and replaced 0.3% PBSTx by proteinase K solution (2 μg/mL proteinase K in 0.3% PBSTx with 0.1% SDS) for 20 minutes. The planarian was fixed with 4% formaldehyde (in PBS) again for 10 minutes and was followed by 2 times 0.3% PBSTx wash. The planarian was placed in 50% pre-hybridization solution (50% formamide, 5x SSC, 1% Tween-20 in 0.3% PBSTx) for 10 minutes, and was then incubated in 100% pre-hybridization solution (50% formamide, 5x SSC, 1% Tween-20, and 1 mg/ml yeast torula RNA in DEPC-ddH_2O) at 56ºC for 2 hours. Probe hybridization was performed in hybridization solution (50% formamide, 10% dextran sulfate, 5x SSC, 1% Tween-20, and 1 mg/ml yeast torula RNA) with the DIG-labeled RNA probe (200-400 ng per μL) at 56ºC for at least 16 hours. The planarian was washed in a series of 50% pre-hybridization solution (in 2x SSC), 2x SSC (in ddH_2O with 0.1% Triton-X), and 0.2x

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SSC (in ddH_2O with 0.001%) at 56 ºC, each solution twice times and 30 minutes for each time. The planarian was washed with MABT (100 mM pH 7.5 maleic acid, 150 mM NaCl, and 0.1% Tween-20) for 10 minutes twice times after the planarian returned to room temperature. The planarian was incubated in blocking solution (5% goat serum in MABT) for at least 1 hours, then incubated in antibody solution (anti-DIG-AP in blocking solution, 1:4000) at 4ºC, overnight. The planarian was followed by at least 6 times washed with MABT for 20 minutes per time. Development buffer was initiated by adding 0.45% NBT and 0.35% BCIP mixture after AP buffer (10% 1M Tris-Cl, 2% 5M NaCl, 5% 1M $MgCl_2$, 0.1% Tween-20 in ddH_2O) incubation. After color development, the planarian was fixed with 4% formaldehyde (in PBS) again for 10 minutes and was followed by 0.3% PBSTx wash. 100% EtOH was used for eliminating the background signal. The planarian was rinsed with 50% EtOH (in 0.3% PBSTx) for 5 minutes, then was washed with 0.3% PBSTx. The planarian was soaked in 80% glycerol (in 0.3% PBSTx) at 4ºC before mounting.

2.4 Gamma (γ)-irradiation

The sample was explored by 90Gy γ-irradiation at National Taiwan University, College of Medicine. Then the planarian was fixed at 2 day post irradiation (dpi), 4 dpi and 7 dpi either perform whole-mount *in situ* hybridization or RT-qPCR experiment, or recorded the survival rate. The results of RT-qPCR and survival rate were statistic by Student's t-test and Log-rank (Mantel-Cox) test, respectively.

2.5 RNA isolation and cDNA preparation

Total RNA was extracted by TRIZOL reagent (Invitrogen). General sample collect

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3 intact planarians for RNA isolation. To enhance the efficiency of extracting total RNA, 0.5μl glycogen (Roche) was added to the FACS sample. The cDNA was synthesized by using a QuantiTect Reverse Transcription Kit (QIAGEN). The synthesized cDNAs were diluted and used for gene expression analysis by RT-qPCR.

2.6 Quantitative reverse-transcription PCR (RT-qPCR)

The RT-qPCR was implemented by SYBR® Green qPCR Kits (Bio-rad) and machine (Bio-rad). Run program as following: 1. 98 ºC for 3mins 2. 98 ºC 10secs 3. Anneal at 60 ºC for 30sec 4. Back to step 2 and run 40 cycles. *Djbeta-tubulin* (*Djβ-tubulin*) was used as internal control for normalization. The results were analyzed by using Student's t-test. The following primers were used in the amplification reaction:

Djβ-tubulin	Forward:	5' TGTCAGTGAACAATTTACTGCC 3'
	Reverse:	5' GTTGATATTCACTAACCAAATCATTC 3'
Djcbc-1	Forward:	5' GTACAGAACTTGCTAAGCACACTG 3'
	Reverse:	5' GACCAGGAGTGGCCAAAATAATG 3'
Djpiwi-A	Forward:	5' CGCTAATCCAAATCCGGGAAC 3'
	Reverse:	5' GGAGCCATAGGAGAAATCTCATTTG 3'
Djpcna	Forward:	5' GTGAGGCTATCACTATTACTGTAG 3'
	Reverse:	5' GGTTCAGTCATTTCAATGGTTACG 3'
Djprog-1	Forward:	5' GCGATCCGTTGTATGCGAAATTG 3'
	Reverse:	5' TCAACAATGCTCTGCATTCTTTGTTTG 3'
Djprog-2	Forward:	5' TGCGAGTTTCCAGAAGAGAGC 3'
	Reverse:	5' GCAGCATCACAATCAGCATCG 3'
Djmcp	Forward:	5' CTGCGGGATTAGCATTTGGTC 3'
	Reverse:	5' CAACGCCTGCAATTCCTTCTG 3'

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Djagat-3	Forward:	5’ CTAAATCTCTTGGCATAGCAGC 3’
	Reverse:	5’ GACCAGAAGCTCAAGATTTCGG 3’
Djopsin	Forward:	5’ GAAAATGGCACAGAAAATGAATGC 3’
	Reverse:	5’ TTATCGCATAAGGGGTCCATG 3’
Djarrestin	Forward:	5’ CTGAAATTGGATGAAGAAACTGC 3’
	Reverse:	5’ AGATTTCAAATATGATTTTCCGTGC 3’
Djchat	Forward:	5’ AACGACATCAGCTGACAGACC 3’
	Reverse:	5’ CCCATTTGCTACGAGGAAGAG 3’
Djndk	Forward:	5’ CGATCAGTATTCAGTTCTCAGTG 3’
	Reverse:	5’ GGTATGGATTAGCATTATTGAATTGTG 3’
Djpc-2	Forward:	5’ TGCAACCAATGATGGAGAAACTG 3’
	Reverse:	5’ AGGGTGCATTGCCCATATAAATC 3’
Djsyt	Forward:	5’ GGCATTGGTCGGACATGTTG 3’
	Reverse:	5’ TTTCCGGCATTTCTTGGAGG 3’
Djsnap-25	Forward:	5’ AAGAAGAAGCGGGCAAAGACA 3’
	Reverse:	5’ TTGAACGCCCATTCCGTTT 3’

2.7 Fluorescence-activated cell sorting (FACS)

The protocol of fluorescence-activated cell sorting was modified from previous studies (Hayashi and Agata, 2012; Hayashi et al., 2006; Resch et al., 2012). The detail steps were described as followed. 5-20 planarians with 8 to 15 mm in length that discarded instant ocean water and rinsed with calcium- and magnesium-free solution (CMF-B: 2.56 mM $NaH_2PO_4 \cdot H_2O$, 10.21 mM KCl, 14.28 mM NaCl, 9.42 mM NaHCO3, and 1% BSA) were amputated into 3-5 fragments on the ice. After eliminating CMF-B, the fragments were cut into as smaller pieces as possible. The samples were re-suspended with CMF-B

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and transferred into an eppendorf, and then treated with 0.25% (w/v) trypsin in CMF for 5 minutes at room temperature to digest samples. Samples were completely dissociated into single cells by gentle pipetting more than 60 times. Pelleted the sample by centrifugation at 1500 g for 5 min, removed supernatant and re-suspended the sample in CMF-B, repeated these 3 steps twice. After that, the samples were filtered through a 70-μm-pore size cell strainer (BD) and then a 20-μm nylon net filter (Millipore) to remove tissue fragments. The single-cell suspensions were incubated with 18 μg/mL Hoechst 33342 (Sigma) and 0.5 μg/mL calcein-AM (Sigma) for at least 2 hours (best 4 hours) at 20ºC. A flow cytometric analysis used a FACS Aria III cell sorter (BD). The percentage of cell populations were analyzed by FlowJo and statistic by Student's t-test.

2.8 DsRNA-mediated RNA interference (RNAi) experiment

The *Djcbc-1* DNA sequence (1,515 base pairs) was cloned by PCR with cDNA of *Dugesia japonica*. The partial YFP sequence (717 base pairs) used as a control was cloned by PCR with the pEYFP-C1 vector. The following primers were used:

Djcbc-1	Forward_0001:	5' ATGAATAGAAACGCCAATATG 3'
	Reverse_1515:	5' TCAATGTCGCATATTCTGC 3'
YFP	Forward:	5' ATGGTGAGCAAGGGCG 3'
	Reverse:	5' ACTTGTACAGCTCGTCC 3'

The templates for in vitro transcription were generated by PCR that using target sequence primers conjugated with T7 promoter sequence: 5' TAATACGACTCACTATA GGG '3. The following primers were used:

T7-*Djcbc-1*	Forward:	5' TAATACGACTCACTATAGGGATGAATAGAA ACGCC 3'
	Reverse:	5' TAATACGACTCACTATAGGGTCAATGTCGC

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		ATATTC 3’
T7-YFP	Forward:	5’ TAATACGACTCACTATAGGGATGGTGAGCA AGGGC 3’
	Reverse:	5’ TAATACGACTCACTATAGGGACTTGTACAG CTCGTCC 3’

The double-strand RNA was synthesized by in vitro transcription with T7 polymerase reacting for 5 hours or overnight at 37ºC. Other procedures were the same as the protocol of synthesis and purification of digoxigenin (DIG)-labeled RNA probes. The steps of RNAi experiment was similar as reported (Newmark et al., 2003; Rouhana et al., 2013). The artificial food that total volume 16 μL was mixed with 4 μg dsRNA in 8 μL chicken liver, 4 μL 2% low-melting agarose gel, and 1 μL red food color. This artificial food mix was placed at 4ºC to solidify. The planarians were fed with food mix at room temperature every three or four days. RT-qPCR of the target gene was used to check the efficiency of RNAi. The results were statistic by Student’s t-test.

2.9 Photophobia assay

Photophobia assay was performed as described (Paskin et al., 2014; Pearson et al., 2009). The container of the test was a rectangular shaped box that had sides of lengths 11.2 cm, 3.2 cm, and 3 cm, lying along the x-, y-, and z-axes, respectively. The container was laid on a sheet of white cardboard which containing lines for dividing the box into four equal quadrants of zones (2.8 cm × 3.3 cm) that named as Q1 to Q4. The daylight fluorescent lamp tube with the switch was used as light source and placed nearby the Q1, creating the light-to-dark gradient from Q1 to Q4. The video recorder was set above the testing container at a fixed distance of about 40 cm. The testing container was filled to a depth of 0.5 cm with planarian water for each trial. A planarian was placed into Q1 in the

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dark before the light switched on. At 2 minutes post the light switched on, the location of planarian was recorded. Each trial was repeated 3 times. A planarian was allowed to rest before the next trial. The results were statistic by ANOVA and Bonferroni's multiple comparisons test.

2.10 Motility assay

Motility assay can examine the moving ability of planarian. The container was 9 cm petri dish that laid on a sheet of white cardboard. Planarian was initially placed at the center of the dish and recorded for at least 2 mins. Then used behavior tracking software ANY-maze™ to analysis the distance that planarian moved during 2 mins from the video. The results were statistic by Student's t-test.

2.11 Immunofluorescence (IF) staining with phospho-Histone 3 (H3p) (Ser10) or SYNORF1 (Synapsin) antibody

The Planarian with 2 to 7 mm in length was treated with 5% N-acetyl cysteine (in PBS) for 3 to 5 minutes, and then fixed with 4% formaldehyde (in PBS) for at least 2 hours. After rinsed with 0.3% PBSTx (0.3% Triton-X100 in 1x PBS), planarian was bleached with 6% H_2O_2 (in 1x PBS) under direct light, overnight. After eliminating pigment, planarian was performed 2 times wash with 0.3% PBSTx for 5 minutes. The planarian was incubated in blocking solution (10% goat serum in 0.3% PBSTx) for at least 1-2 hours, then incubated in antibody solution at room temperature, overnight. The antibody solution of phospho-Histone 3 (H3p) was 1:1000 while SYNORF1 (Synapsin) was 1:100 in blocking solution. The planarian was followed by at least 12 times washed with 0.3% PBSTx for 20 minutes per time, and then was incubated in secondary antibody

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solution at 4ºC, overnight. The secondary antibody for H3p was anti-rabbit-Alexa 488 (1:1000) while Synapsin was anti-mouse-Alexa 488 (1:1000) in 0.3% PBSTx. The planarian was followed by at least 6 times washed with 0.3% PBSTx for 20 minutes per time, and then stained with Hoechst 33342 (18 μg/mL Hoechst 33342 1:1000 in 0.3% PBSTx) for at least 30 minutes (best overnight). The planarian was soaked in 80% glycerol (in 0.3% PBSTx) at 4ºC before mounting. If the efficiency of Hoechst staining was unacceptable, the planarian was mounting in 100% glycerol with Hoechst 33342 (18 μg/mL Hoechst 33342 1:1000 in 100% glycerol).

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3. Results

3.1 *Djcbc-1* was highly expressed in brain and neoblasts of planarian

To confirm the expression pattern of *Djcbc-1* in intact planarians (Rouhana et al., 2010; Yoshida-Kashikawa et al., 2007), I performed the whole mount in situ hybridization (WISH) and analyzed the expression pattern of *Djcbc-1*. *Djcbc-1* was detected in the brain, dorsal midline, and lateral lines of planarian (Fig 1A). *Djpiwi-A*, which is highly expressed in the dorsal midline, was used as the marker for neoblasts. Because both *Djcbc-1* and *Djpiwi-A* were highly expressed in the dorsal midline, it suggested that *Djcbc-1* is also expressed in neoblasts.

To further confirm the expression of *Djcbc-1* in neoblast, I examined the level of *Djcbc-1* in neoblast-eliminated planarians. If *Djcbc-1* is specifically expressed in neoblast, I expect to see the down-regulation of *Djcbc-1* after elimination of neoblast by γ-irradiation. The lineages of cells differentiated from neoblasts are illustrated in Fig1B, and the marker genes for each category of cells are labeled as well (Fig 1B). Because γ-irradiation abolished the neoblasts supply for further differentiation, it could be observed that the expression levels of marker genes for neoblasts and progeny cells declined at 2, 4, 7 days post γ-irradiation (dpi) (Fig 2A). The level of *Djcbc-1* after γ-irradiation was examined by RT-qPCR (Fig 2B) and WISH (Fig 2C) at various time points post γ-irradiation (90 Gy). The results showed that expression level of *Djcbc-1* was also sensitive to γ-irradiation (90 Gy), indicated that *Djcbc-1* was expressed in both neoblasts and progeny cells. Interestingly, the expression pattern of *Djcbc-1* in the brain (differentiated cells), which is less sensitive to γ-irradiation (90 Gy), could still be noticed (Fig 2C, the right panel).

To directly examine the expression of *Djcbc-1* in neoblasts, I used fluorescence-

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activated cell sorting (FACS) to isolate the neoblasts, as well as the other two lineages of planarian cells (Fig 3A). Because *Djcbc-1* is also highly expressed in brain (Fig 1A, Fig 2C), I removed the head fragment before making single cells suspension for FACS to specifically monitor the level of *Djcbc-1* in neoblasts from the trunk. The proliferating cells with duplicated DNA content locate on the upper half of Fig 3A, due to the increased amounts of Hoechst Blue-A signals. These replicating cells are defined as X1 population (shown in red), with the majority of mitotic neoblasts. The remaining cells are divided into two populations by the Hoechst Red-A signal that represents the cell size. Smaller cells, including the non-mitotic neoblasts and early progeny cells, are defined as X2 population (shown in blue), while Xins population refer to late progeny cells and differentiated cells (shown in green). The RT-qPCR results of marker genes for neoblasts, early progeny cells, and late progeny cells showed that I have successfully separated different cell populations by FACS (Fig 3B). The RT-qPCR for *Djcbc-1* also showed the higher levels of *Djcbc-1* in isolated X1 and X2 populations (Fig 3C). These results suggested that *Djcbc-1* was highly expressed in CNS, neoblasts, and early progeny cells.

3.2 *Djcbc-1* was highly expressed in the regenerating tissue of planarian

Since *Djcbc-1* was highly expressed in neoblasts and neoblasts are enriched in regenerating tissue, I next questioned whether the expression of *Djcbc-1* is increased in the regeneration process of planarian. To examine if the level of *Djcbc-1* was induced during regeneration, I did the time course experiments of WISH after amputation. The WISH for *Djcbc-1* during regeneration at 1, 2, 3 days post amputation (dpa) showed that the *Djcbc-1* level in regenerating tissues was elevated at 1 dpa to 3 dpa (Fig 4A). The RT-

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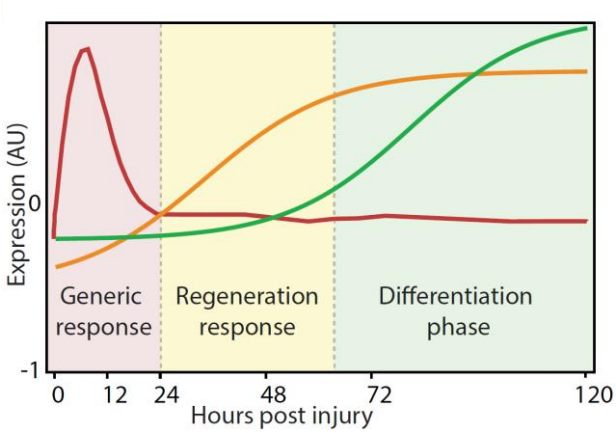
qPCR analysis for regenerating tissue at 3dpa also showed the up-regulated level of *Djcbc-1* in head regeneration (Fig 4B) and tail regeneration (Fig 4C). These results showed that *Djcbc-1* was highly expressed in the regenerating tissue. Because many regeneration essential genes, such as *Djpiwi-A* or *Djpcna,* are highly expressed in the regenerating tissue, the expression of *Djcbc-1* in regenerating tissue suggested that *Djcbc-1* may play some roles in planarian regeneration. On the other hand, since the expression level of *Djcbc-1* was raised at 1 dpa to 3 dpa, it indicated that *Djcbc-1* may not only be involved in regeneration but also in differentiation and generic response, which were related to wound response (Wurtzel et al., 2015).



A temporal model of wound response and initiation of planarian regeneration (Wurtzel et al., 2015)

3.3 *Djcbc-1* was essential for the survival of newly regenerated planarian

I have observed that *Djcbc-1* was highly expressed in the regenerating tissue, while it is still unclear whether *Djcbc-1* is involved in planarian regeneration. To characterize the function of *Djcbc-1*, I utilized the dsRNA-mediated RNA interference (RNAi) to specifically deplete *Djcbc-1*. Planarians were fed with 4 μg dsRNA of *Djcbc-1* mixed with chicken liver and 2% low melt agarose every three or four days. Planarians fed with

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dsRNA for YFP were used as a control. After at least 3 round of dsRNA feeding, I collect the total RNA and analyzed the knockdown effect by RT-qPCR. The average efficiency for *Djcbc-1* RNAi was about 80% (Fig 5A). The WISH results also showed a high efficiency of *Djcbc-1* RNAi (Fig 5B).

Though the previous study has described that depletion of *Djcbc-1* did not result in an abnormal phenotype (Kashima et al., 2016; Rouhana et al., 2010; Yoshida-Kashikawa et al., 2007), I observed that newly regenerated planarians were more sensitive to depletion of *Djcbc-1*, even resulted in death. To test if the timing or planarian size is critical for *Djcbc-1* RNAi induced phenotypes, I examine the effect of *Djcbc-1* RNAi in planarian with different size after regeneration. I choose two sizes of planarian and started treating with *Djcbc-1* RNAi at 9 dpa (Fig 6A and 6B), 12 dpa (Fig 6C and 6D), or 20 dpa (Fig 6E and 6F). Both survival rate (Fig 6A, 6C, and 6E) and median survival (Fig 6B, 6D, and 6F) records showed that small planarian (length < 5mm) is more sensitive to the depletion of *Djcbc-1*. Because the efficiencies of *Djcbc-1* RNAi for all groups reached more than 60% (Fig 6G), results from Figure 6 indicated that the difference of survival rates between each group was not caused by the variation of RNAi efficiencies. It also suggested that the size of the planarian affected the sensitivity planarian to the treatment of *Djcbc-1* RNAi. Furthermore, I observed that the timing of starting RNAi after amputation also contributed to the sensitivity of *Djcbc-1* RNAi in planarian. For the planarian with the same size (< 5mm or > 5mm), both the survival rate (Fig 7A and 7C) and median survival (Fig 7B and 7D) records that planarian is more sensitive to depletion of *Djcbc-1* at the earlier stage of regeneration. Therefore, I used the 12 dpa planarian with small (< 5 mm) size (Fig 8A) for detail examination of the survival after *Djcbc-1* RNAi. Depletion of *Djcbc-1* in newly regenerated (12 dpa) planarians results in the lesion and fragmentation phenotype before death (Fig 8B). The quantitative record for phenotypes

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(Fig 8C) and survival rate (Fig 8D) in newly regenerated planarian showed that all of the planarians died after long-term treated with *Djcbc-1* RNAi. According to this observation, I then chose the experimental condition where the planarian was initially treated with *Djcbc-1* RNAi at 12-14 dpa for the following experiments in this thesis. Also, the animals were treated with *Djcbc-1* RNAi for more than six rounds. In this case, planarians could survive for further experiments, and the effects of *Djcbc-1* RNAi were easy to be observed.

3.4 Depletion of *Djcbc-1* delayed the eyespots formation during planarian head regeneration

To test if depletion of *Djcbc-1* affects planarian regeneration, I amputated these *Djcbc-1*-depleted planarians and monitored their regeneration phenotypes. Various phenotypes for eyespots formation were observed during regeneration in both control and *Djcbc-1* RNAi planarian (Fig 9A). I quantified the number of planarian with each phenotype at 4 dpa, 7 dpa, and 10 dpa. The quantitative results showed that depletion of *Djcbc-1* delayed the eyespots formation in both trunk (Fig 9B) and tail (Fig 9C) fragments after amputation. This result is consistent with the previous study, which showed that depletion of *Djcbc-1* limited the formation of photoreceptors (Rouhana et al., 2010).

3.5 The neoblasts population was not declined after *Djcbc-1* RNAi

Because the important function of *Djcbc-1* orthologs in regulating gene expression has been reported, I speculated that depletion of *Djcbc-1* disturbs the gene expression in neoblasts or progeny cells. As a result, I did the WISH (Fig 10A) and RT-qPCR (Fig 10B) for cell type marker genes to examine if depletion of *Djcbc-1* alters the cell population composition. Surprisingly, the expression patterns of marker genes of neoblasts and

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progeny cells was not affected after *Djcbc-1* RNAi (Fig 10A). Meanwhile, the RT-qPCR results showed that depletion of *Djcbc-1* did not significantly influence the expression levels of important marker genes *Djpiwi-A* (for neoblasts), *Djprog-1* and *Djprog-2* (for early progeny cells), and *Djagat-3* (for late progeny cells) (Fig 10B). However, depletion of *Djcbc-1* affected the expression levels of *Djpcna* (for neoblasts) and *Djmcp* (for late progeny cells) (Fig 10B). These results suggested that depletion of *Djcbc-1* may disturb the balance of gene expression in neoblasts and thwart the process of regeneration.

Furthermore, I did FACS to monitor the population sizes of various cell types in control (Fig 11A) or *Djcbc-1* RNAi (Fig 11B) planarians. The results showed that depletion of *Djcbc-1* significant raised the population size of X1 (Fig 11C, P=0.001) while the population size of X2 (Fig 11D, P=0.630) and X1+X2 (Fig 11E, P=0.382) were not significantly changed. The ratio of X1 in the X1+X2 population (mitotic and non-mitotic neoblasts) was increased after *Djcbc-1* RNAi (Fig 11F). Besides, the immunofluorescence staining (Fig 12A) and the quantification results (Fig 12B) showed that the density of $H3p^+$ cells, which are known as mitotic cells, were raised after *Djcbc-1* RNAi. These results showed that depletion of *Djcbc-1* increased the mitotic cells of planarians, which is similar to what has been observed in previous study (Rouhana et al., 2010). In conclusion, my results showed that depletion of *Djcbc-1* delayed the eyespots formation and increased the mitotic cells of planarians. It suggested that depletion of *Djcbc-1* did not block the cell division of neoblasts, but may affect gene expression profiles in neoblasts and the differentiation process, especially for the process of eyespots formation.

3.6 Depletion of *Djcbc-1* may affect the activity of neoblasts

To examine if *Djcbc-1* is involved in neoblasts maintenance and activity, we quantify

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the survival of planarian under irradiation with control or *Djcbc-1* RNAi. Percent survival results showed a significant difference (**P< 0.01) between the control and *Djcbc-1* RNAi planarians with a 5 Gy γ-irradiation (Fig 13A). It suggested that *Djcbc-1*-depleted planarians were more sensitive to non-lethal dose γ-irradiation (5 Gy). Consequently, although the depletion of *Djcbc-1* increased the mitotic neoblasts of planarians (Fig 11C, 11F and 12B), the newly divided neoblasts in this condition might lose some properties and were impaired with non-lethal dose γ-irradiation. Taken together, our data suggested that depletion of *Djcbc-1* did not decrease the cell population of neoblasts, but reduce the activity of neoblasts. Therefore, the newly regenerated planarians could not survive after *Djcbc-1* RNAi.

In addition, percent survival of planarian with lethal dose γ-irradiation (90Gy) showed a significant difference (****P< 0.0001) between the control and *Djcbc-1* RNAi planarians (Fig 13B). It suggested that *Djcbc-1*-depleted planarians were more sensitive to lethal dose γ-irradiation (90 Gy). In this case, most of neoblasts were dead at 0 to 1 dpi, but the survival rates were still different between control and *Djcbc-1* RNAi planarians. It indicated that *Djcbc-1* may play important roles in lineages and differentiated somatic cell. One of the possible function of *Djcbc-1* is to participate in the stress response, e.g. γ-irradiation.

3.7 Depletion of *Djcbc-1* perturbed the brain of planarian during homeostasis

My results as shown in the previous experiments suggested that *Djcbc-1* was participating in eyespots formation of planarian (Fig 9) without the decrease of neoblasts population (Fig 10, 11 and 12). However, it is still not clear that if *Djcbc-1* RNAi affected

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some specific genes and resulted in the reduction activity of neoblasts (Fig 13) and the abnormal phenotypes. To clarify the possible role of *Djcbc-1*, I further examined the expression levels and patterns for those genes that are specifically expressed at eyespots or brain. Two different groups of *Djcbc-1*-depleted planarian were analyzed in this experiment for examining the function of *Djcbc-1* in homeostasis or in regeneration. For the effect of *Djcbc-1* in homeostasis, I treated the planarian with more than 6 rounds of RNAi, named as the long-term *Djcbc-1*-depleted planarian (Fig 14 and 15). For the effect of *Djcbc-1* in newly regenerated eyes, I monitored the expression of eyespot- and brain-specific genes in *Djcbc-1*-depleted planarian after regeneration (Fig 17 and 18).

The schematic diagram of experimental conditions of long-term *Djcbc-1*-depleted planarians used in the following experiments is shown in Fig 14A. After a long-term *Djcbc-1* RNAi depletion of *Djcbc-1* did not change the expression levels of *Djopsin* and *Djarrestin* (Fig 14B), the two essential genes for eyespots formation and function (Dong et al., 2012). Consistently, the WISH results showed that depletion of *Djcbc-1* did not affect the expression patterns of *Djopsin* and *Djarrestin* (Fig 14C). I also used several genes, including *Djchat, Djndk, Djsyt, Djsnap25* and *Djpc2*, as the markers for various subtypes of neurons in planarian brian. Depletion of *Djcbc-1* did not significantly change the expression levels of *Djchat* (P=0.149) and *Djndk* (P=0.706), but increased the expression level of *Djpc-2* (*P<0.05) (Fig 15A). Surprisingly, depletion of *Djcbc-1* raised the level of *Djsyt* (**P<0.01) but did not significantly change the level of *Djsnap-25* (P=0.064) (Fig 15A), while both *Djsyt* and *Djsnap-25* are essential for negative phototaxis (Takano et al., 2007). Although the differences of expression levels for *Djpc-2 and Djsyt* were statistical significant between the control and *Djcbc-1*-depleted planarians, all the differences were less than 25%. It needs further experiments to determine whether this change is sufficient to cause significant biological difference.

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Though the expression levels (Fig 14B) and patterns (Fig 14C) of eyespots genes were not affected in *Djcbc-1*-depleted planarian, the WISH (Fig 15B) and IF (Fig 15C) results showed that depletion of *Djcbc-1* perturbed the expression patterns of brain marker genes in planarian with long-term *Djcbc-1* RNAi. The expression pattern of *Djchat* was diffused at the branch of the brain, and the pattern for *Djsyt* was also disordered in *Djcbc-1*-depleted planarians (Fig 15B). In addition, the expression of Synapsin was disappeared in long-term *Djcbc-1*-depleted planarians (Fig 15C). I further quantified the expression levels of eyespot- or brain-specific genes in FACS-isolated differentiated cells by RT-qPCR. The results showed that depletion of *Djcbc-1* decreased the expression levels of *Djchat, Djndk*, and *Djpc-2* especially in Xins population (Fig 16). It indicated that *Djcbc-1* may participate in the process of differentiation from early progeny cells to late progeny cells and differentiated cells.

3.8 Depletion of *Djcbc-1* impaired the brain regeneration of planarian

To dissect the function of *Djcbc-1* in the process of differentiation during regeneration, I also examined the expression of eyespots- and brain-specific genes in *Djcbc-1*-depleted planarian during regeneration at 10-13 dpa. Fig 17A shows the schematic diagram and the experimental conditions of *Djcbc-1*-depleted planarians during regeneration for the following experiments. The RT-qPCR results showed that the expression levels of eyespots marker genes, e.g. *Djopsin* and *Djarrestin*, were not significantly changed in *Djcbc-1*-depleted planarians during regeneration (Fig 17B). The WISH results also showed that depletion of *Djcbc-1* did not affect the expression patterns of *Djopsin* and *Djarrestin* in planarian during regeneration (Fig 17C). The RT-qPCR results of brain marker genes showed that the expression levels of brain marker genes were not significantly changed in *Djcbc-1*-depleted planarians during regeneration (Fig

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18A). However, the WISH (Fig 18B) and IF (Fig 18C) results showed that depletion of *Djcbc-1* perturbed the expression patterns of several brain marker genes during regeneration. In *Djcbc-1*-depleted planarian, the expression patterns of *Djchat* was diffused at the branch of the brain, the level of *Djsyt* was decreased (Fig 18B) and Synapsin was disappeared (Fig 18C). These results were similar to that of planarian with long-term *Djcbc-1* RNAi (Fig 15B and 15C). In addition, the ratio of planarian with abnormal phenotype in *Djcbc-1*-depleted planarians was slightly raised as compared to that with long-term RNAi (Fig 15B, 15C, 18B and 18C). These results suggested that *Djcbc-1* may not be only involved in the process of differentiation, but also participate in planarian regeneration.

3.9 Depletion of *Djcbc-1* abolished the negative phototaxis of planarian

Although *Djcbc-1* RNAi delayed the eyespots formation of planarian, most of the *Djcbc-1*-depleted planarian could regenerate two eyespots at 10 dpa (Fig 9B and 9C). I further examined the eyespots and brain specific genes and found that the expression patterns of *Djchat*, *Djsyt*, and Synapsin were altered after *Djcbc-1* RNAi (Fig 15 and 18). These results indicated that the newly formed eyespots may not be fully developed, and depletion of *Djcbc-1* may perturb the integration capability of the brain. In this situation, I wonder if the newly formed eyespots in *Djcbc-1*-depleted planarians were functional for light sensing. Therefore, I performed the photophobia assay and monitored the negative phototaxis behavior of planarians to examine whether the eyespots of *Djcbc-1*-depleted planarian had normal function. The schematic diagram of experimental design is shown in Fig 19A. Planarian was initially placed at Q1 in dark, and the numbers of planarian in each zone (Q1-Q4) were recorded at 2 minutes after exposuring to light from the Q1 side. The gradient from light to dark was formed from Q1 to Q4. Normal and control planarian

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showed negative phototaxis and tended to move away from Q1 rapidly and stayed in Q4 (Fig 19A and 19B). The quantitative results of photophobia assay showed that depletion of *Djcbc-1* interfered the negative phototaxis in long-term *Djcbc-1*-depleted (RNAi more than 6 rounds) planarian (Fig 19C and 19D) and the *Djcbc-1*-depleted planarian after regeneration at 11 dpa (Fig 19E and 19F). It suggested that although most of the planarian treated with *Djcbc-1* RNAi could complete eyespots regeneration at 10 dpa (Fig 9B and 9C), the function of eyespots in *Djcbc-1*-depleted planarian was limited or impaired as comparing to the normal planarian.

To confirm that the defect of negative phototaxis behavior in *Djcbc-1*-depleted planarian did not result from the loss of mobility for escaping from light stimuli, I next did the movement assay to examine the moving ability of *Djcbc-1*-depleted planarian. I quantified the mean speed of moving for each planarian in control or *Djcbc-1* RNAi worms. No significant difference has been showed in the moving speed of control and *Djcbc-1* RNAi planarian during homeostasis (Fig 20A, P=0.502) or after regeneration at 11 dpa (Fig 20C, P=0.111). It suggested that the moving ability of planarian was not influenced by depletion of *Djcbc-1*. In addition, the percentage of planarians moving away from the stimulus of blue light or mechanical stimuli (touch) showed that, depletion of *Djcbc-1* did not affect the behavior of escaping from strong stimulation in long-term *Djcbc-1* RNAi or newly regenerated planarians. Collectively, it revealed that depletion of *Djcbc-1* indeed reduced the negative phototaxis behavior of planarian without affecting the motility.

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4. Discussion

4.1 Summary and significance

Previous study has shown that DjCBC-1 is expressed in neoblasts, neurons, and germline cells (Yoshida-Kashikawa et al., 2007). Here, we utilized the combination of WISH, γ-irradiation, RT-qPCR, and FACS to confirm the expression of *Djcbc-1* (Fig 1, 2 and 3). Furthermore, we demonstrated that *Djcbc-1* is also highly expressed in regenerating tissue (Fig 4). Our results conclude that *Djcbc-1* is highly expressed in neoblasts (stem cells), brain (CNS), germline cells, and regenerating tissue of planarian. Remarkably, we discovered that depletion of *Djcbc-1* in newly regenerated planarians causes obviously abnormal phenotype (Fig 6, 7 and 8), which has not been observed and described in previously reports. (Kashima et al., 2016; Rouhana et al., 2010; Yoshida-Kashikawa et al., 2007).

One study has shown that depletion of *Djcbc-1* limited the formation of photoreceptors in the trunk fragment of the planarian that was transverse amputated at pre- and post-pharyngeal regions before regenerating for a week (Rouhana et al., 2010). Here we further defined the phenotypes of incomplete eyespot formation by quantifying various phenotypes of *Djcbc-1*-depleted trunk and tail fragments at 4 dpa, 7 dpa, and 10 dpa (Fig 9B and 9C). The results showed that depletion of *Djcbc-1* delayed the eyespots formation in both trunk and tail fragments during planarian head regeneration. However, even the head regeneration is delayed, most of *Djcbc-1*-depleted planarians were able to completely regenerate two eyespots at 10 dpa. Since we used feeding RNAi method to knockdown *Djcbc-1* in planarian, it is possible that loss of food-uptake ability in newly amputated fragments may reduce the RNAi efficiency. Therefore, the phenotype of incomplete eyespots regeneration in *Djcbc-1* depleted worms may be rescued at 10 dpa,

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when the *Djcbc-1* level was restored. To overcome this issue, we will consider replacing our RNAi method with dsRNA-injection and re-analyzing the effect of long-term RNAi for *Djcbc-1* in the future.

We hypothesize that the *Djcbc-1*-depletion induced deficiency of regeneration is a result of reducing the neoblasts population in planarian (Guo et al., 2006; Rouhana et al., 2010; Salvetti et al., 2005; Solana et al., 2009). We therefore examined if depletion of *Djcbc-1* alters the neoblasts numbers and distribution by various approaches. Surprisingly, we found that depletion of *Djcbc-1* did not affect the expression of marker genes for neoblasts, early or late progeny cells (Fig 10A), and even increased the expression levels of *Djpcna* and *Djmcp* (Fig 10B). In our FACS and IF results, which showed that depletion of *Djcbc-1* rises the mitotic cell numbers of planarian (Fig 11C, 11F, and 12), was similar to that of previously study by using double-IF with anti-DjPIWI-A and anti-H3p (Rouhana et al., 2010). However, we found that planarian has become more sensitive to γ-irradiation (5 Gy) after depletion of *Djcbc-1* (Fig 13). It indicates that even though the number of mitotic neoblasts is increased after depletion of *Djcbc-1* (Fig 11C, 11F, and 12), these newly divided neoblasts may not exhibit a similar property as compared to the wild-type neoblasts that are resistant to low-dose irradiation. Collectively, depletion of *Djcbc-1* did not block the cell division of neoblasts, but may alter the property or sub-populations of neoblasts, and then affect the differentiation process, especially the process of eyespots formation.

To investigate the potential target genes of DjCBC-1 in regulating the regeneration process, we examined the expression levels and patterns of certain genes that are specifically expressed at eyespots or CNS in *Djcbc-1*-depleted planarian during homeostasis (Fig 14 and 15) and regeneration (Fig 17 and 18). We found that depletion of *Djcbc-1* alters the expression patterns of *Djchat* and *Djsyt*, and perturbs the

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morphology of brain (CNS) that labeled by a synaptic marker, Synapsin. Among them, *Djsyt* is essential for negative phototaxis (Takano et al., 2007). Our data was not consistent with previously study, which had described that *Djcbc-1*-depleted planarian was able to complete the regeneration with normal CNS morphology (Yoshida-Kashikawa et al., 2007). We also performed the photophobia assay to quantify and analyze the influences of normal eyespots function of planarian after *Djcbc-1* RNAi, and observed that depletion of *Djcbc-1* impaired the negative phototaxis behavior of planarian (Fig 19). In addition, results of motility and stimulating-runaway assay further confirm that the defect of negative phototaxis behavior in *Djcbc-1*-depleted planarian was not the consequence of losing moving ability after touching or blue-ray stimulation (Fig 20). Collectively, these results indicate that *Djcbc-1* may not only participate in the differentiation process of planarian regeneration, but also required for negative phototaxis by affecting certain subtypes of brain neurons.

In conclusion, DjCBC-1 is potentially involved in the maintenance of functional active neoblasts and differentiation of neurons (Fig 21A). In maintaining the homeostasis, DjCBC-1 is critical for the survival of newly regenerated planarian (Fig 21B). On the other hand, DjCBC-1 plays an important role in eyespots and brain formation during regeneration (Fig 21C), and is required for the negative phototaxis behavior of planarian (Fig 21D). Our results provide evidences to show the multiple aspects of DjCBC-1 functions in planarian differentiation and homeostasis.

4.2 Depletion of *Djcbc-1* in newly regenerated planarians results in abnormal phenotype, even leads to death

Our lab normally use the intact planarian captured from wild and kept in the lab for

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at least one month. After that, we maintain the population of planarian by regular amputation, and do the RNAi or WISH experiments after they have fully regenerated. In this condition, we did not observe any lethal or abnormal phenotype in *Djcbc-1*-depleted planarian. We speculate that two possibilities for this result: The depletion of *Djcbc-1* is not potent enough to eliminate enough amount of *Djcbc-1* protein for inducing phenotype, or there are some other functionally redundant proteins that can substitute *Djcbc-1*.

Accidentally, I once used the planarians that was regenerating at 9 dpa for the RNAi experiment, and noticed that the unexpected abnormal phenotype started to appear after 3 or 4 rounds of *Djcbc-1* RNAi. We next analyzed the effect of *Djcbc-1* depletion at various time points after amputation or in planarian with different sizes. Our results concluded that *Djcbc-1* is critical for planarian survival at regeneration status: the planarians with in an earlier stage of regeneration, as well as with a smaller size, are more sensitive to *Djcbc-1* RNAi (Fig 6 and 7).

It is generally considered that planarian is able to complete regeneration within 7-14 days when the eyespots are formed and planarian exhibit chemotaxis or negative phototaxis. However, according to our results, we speculate that the internal regeneration processing may take longer than what the morphology shows. The balance between cell proliferation and cell death may not reach the homeostasis on day 7-14 after amputation (Tu et al., 2012). At this window of timing, the newly proliferated neoblasts or progeny cells are still under an unstable circumstance, in which any disturbance on the gene expression profile may lead to a strong effect on determine the fate of cells. Therefore, depletion of *Djcbc-1* in newly regenerated planarians under this condition will more easily result in the abnormal phenotype. We hypothesize that depletion of DjCBC-1 alters the proliferating potency and property of neoblasts and impairs the ability of planarian to overcome or recover from stress, thus leads to death (Fig 13). However, the direct

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mechanism of DjCBC-1-induced death in newly regenerated planarians is still unknown and expected to be revealed in the future.

4.3 Planarian behavior and the subtype neurons of CNS

Many genes have been found in planarian neural networks and control the behavior of planarian. For example, DjTRPMa is expressed in the thermosensory neurons of planarian (Inoue et al., 2014). Moreover, the GABAergic neurons (e.g. the DjGAD-positive neurons) for light stimulation and serotonergic neurons (e.g. the DjTPH-positive neurons) for temperature stimulation compose the primary center of sensory in planarian brain (Inoue et al., 2015). The motor neurons include dopaminergic neuron (e.g. DjTH-positive) and cholinergic neurons (e.g. DjChAT-positive) (Inoue et al., 2015). In addition, previous study has shown that regeneration-dependent conditional gene knockdown (readyknock) of a conserved synaptic protein, *Djsnap-25* (synaptosomal- associated protein 25 kDa), reduced the negative phototaxis of planarian while the head structure remains normally (Takano et al., 2007).

Depletion of *Djcbc-1* mainly affects the expression patterns of *Djchat, Djsyt, and* Synapsin (Fig 15 and Fig 17). The following will detail describe the function and mechanism of each protein.

First, *Djchat* encoded the choline acetyltransferase (ChAT or CAT), which is required for the synthesis of neurotransmitter acetylcholine (Ray et al., 1999; Yasuyama et al., 1995). ChAT is expressed in cell body and then transported to synapse. Therefore, it can be used as a marker for cholinergic neurons. In *C. elegans*, the decrease of ChAT activity results in several phenotypes, including the slow growth, small size, and uncoordinated behavior (Rand and Russell, 1984). In *D. melanogaster*, ChAT is expressed in the visual system of larval for acetylcholine synthesis, and is involved in the light

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avoidance of larval (Keene et al., 2011; Yasuyama et al., 1995; Yasuyama and Salvaterra, 1999). Collectively, disturbing the expression of ChAT may result in loss of normal behavior, and affects the function of neurons by reducing the level of acetylcholine for release.

Second, *Djsyt* encode a conserved synaptotagmin protein, and is a membrane Cs^{2+} sensor protein. Djsyt is expressed at pre-synaptic axon terminal, and is involved in the neurotransmitter release and hormone secretion in all neurons (Lee et al., 2010; Perin et al., 1991; Tazaki et al., 1999). It also showed that SYT interacts with SNAP-25 (Gerona et al., 2000; Zhang et al., 2002). Moreover, the planarian studies have reported that readyknock of *Djsyt* caused the loss of negative phototaxis, negative thermotaxis, chemotactic, and negative thigmotactic while the mobile ability was unaffected (Inoue et al., 2015; Inoue et al., 2014; Takano et al., 2007). These data provided supports to our results that depletion of *Djcbc-1* may reduce the negative phototaxis of planarian by affecting the expression pattern of *Djsyt*.

In our IF data, we used the antibody of SYNORF1 (Synapsin) to label the Synapsin protein family. Synapsin regulates the neurotransmission and synaptogenesis by controlling the exocytosis of synaptic vesicles, and are widely used as a marker for presynaptic terminals (Evergren et al., 2007; Ferreira and Rapoport, 2002; Gitler et al., 2004; Kao et al., 1999). Interestedly, a study in mice showed that the expression of some synaptic proteins was reducing when the photoreceptors signaling was lost (Kihara et al., 2008). It provides the possibility that the expression of Synapsin may be also decreased when the photoreceptors signaling was decreased in *Djcbc-1*-depleted planarian. However, whether the decreased level of Synapsin will also affect the photoreceptors signaling is still unknow and reqire to be examined in the future.

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4.4 The function of DjCBC-1 in neurons

Orthologs of DjCBC-1 in other animals have been reported to be expressed in neurons and is usually localized in RNA granules for the function of translation repression or mRNA degradation (Yoshida-Kashikawa et al., 2007). For example, the ortholog of DjCBC-1 in *D. melanogaster*, Me31B, was reported to localize at dendritic RNA granules of olfactory neurons (Barbee et al., 2006).

Local protein synthesis in dendritic cells is critical for neurons to change the synaptic activity in response to stimuli and maintain the potency of synapse (Bramham and Wells, 2007). Certain amounts of mRNA are transcribed at cell body then transported to the dendrites by specific dendritic RNA granules, known as transport ribonucleoprotein particles (RNPs). It has been reported that Me31B may be involved in the translation repression of mRNA when it is transported from neuron cell body to dendrites (Pimentel and Boccaccio, 2014; Savas et al., 2010; Zeitelhofer et al., 2008). These research indicate that DjCBC-1 may play similar role in regulating neurons mRNA at post-transcriptional levels.

4.5 Perspective

Because no antibody is available for detecting the DjCBC-1 protein levels so far, we mainly used WISH and quantitative PCR to detect the expression of DjCBC-1 at RNA level in this study. If the DjCBC-1 antibody is available, we will utilize it for IF and Western blot to examine the expression level and pattern of DjCBC-1 in planarian. In addition, we can also use it to pull-down the DjCBC-1-associated complex. We will be able to discover the DjCBC-1-interacting protein, and the associated RNAs as well.

To investigate more down-stream targets of *Djcbc-1* globally, we will perform the

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NGS for transcriptome in *Djcbc-1*-depleted planarian. By comparing with the control planarian, we can profile the direct or indirect down-stream targets of DjCBC-1 and provide insight into the mechanism for its function in regeneration.

One of the possible effector pathways of DjCBC-1 is through the mRNA decapping mechanism that has been confirmed conservatively in other organism. Similar to its human orthologs, DDX6, multiple functions of DjCBC-1 are occurred by interacting with mRNA decapping complex. We will examine the phenotype of planarian with double depletion of *Djcbc-1* and other members of decapping complex, such as *Djdcp1a*, *Djdcp2*, *Djedc3*, *Djedc4*, and *Djpatl1* (Ayache et al., 2015; Nishimura et al., 2015). Results from this approach will help us to dissect whether decapping complex play potential roles in planarian homeostasis and regeneration at post-transcriptional levels, and link function of DjCBC-1 in controlling RNA stability.

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5. Figures

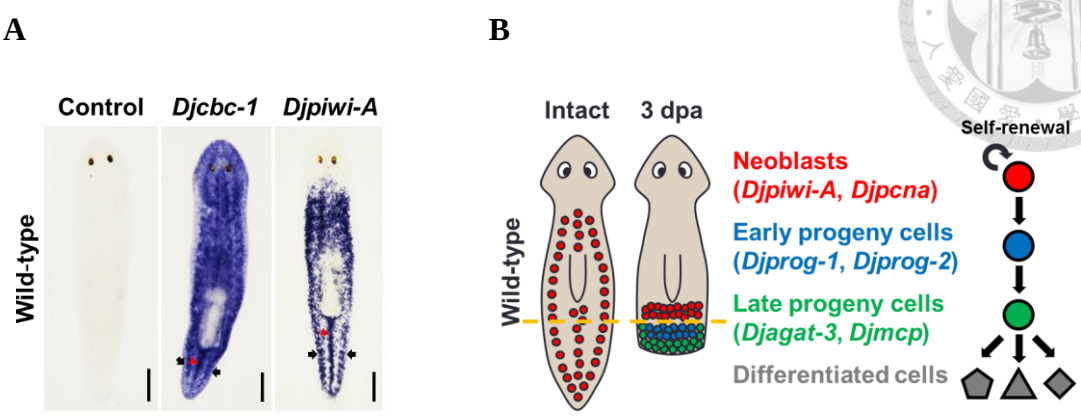

Figure 1. *Djcbc-1* was highly expressed in brain and neoblasts of planarian

(A) *Djcbc-1* was detected in brain, dorsal midline (red arrow head), and lateral lines (black arrow) of wild-type planarian by WISH. Sense strand probe of *Djcbc-1* was used as a control. *Djpiwi-A* which is highly expressed in the dorsal midline and lateral lines was used as the marker for neoblasts. (B) The schematic diagram of the lineages of cells. The localization and marker genes for each cell type are shown in different colors. (Scale bars: 250 μm.)

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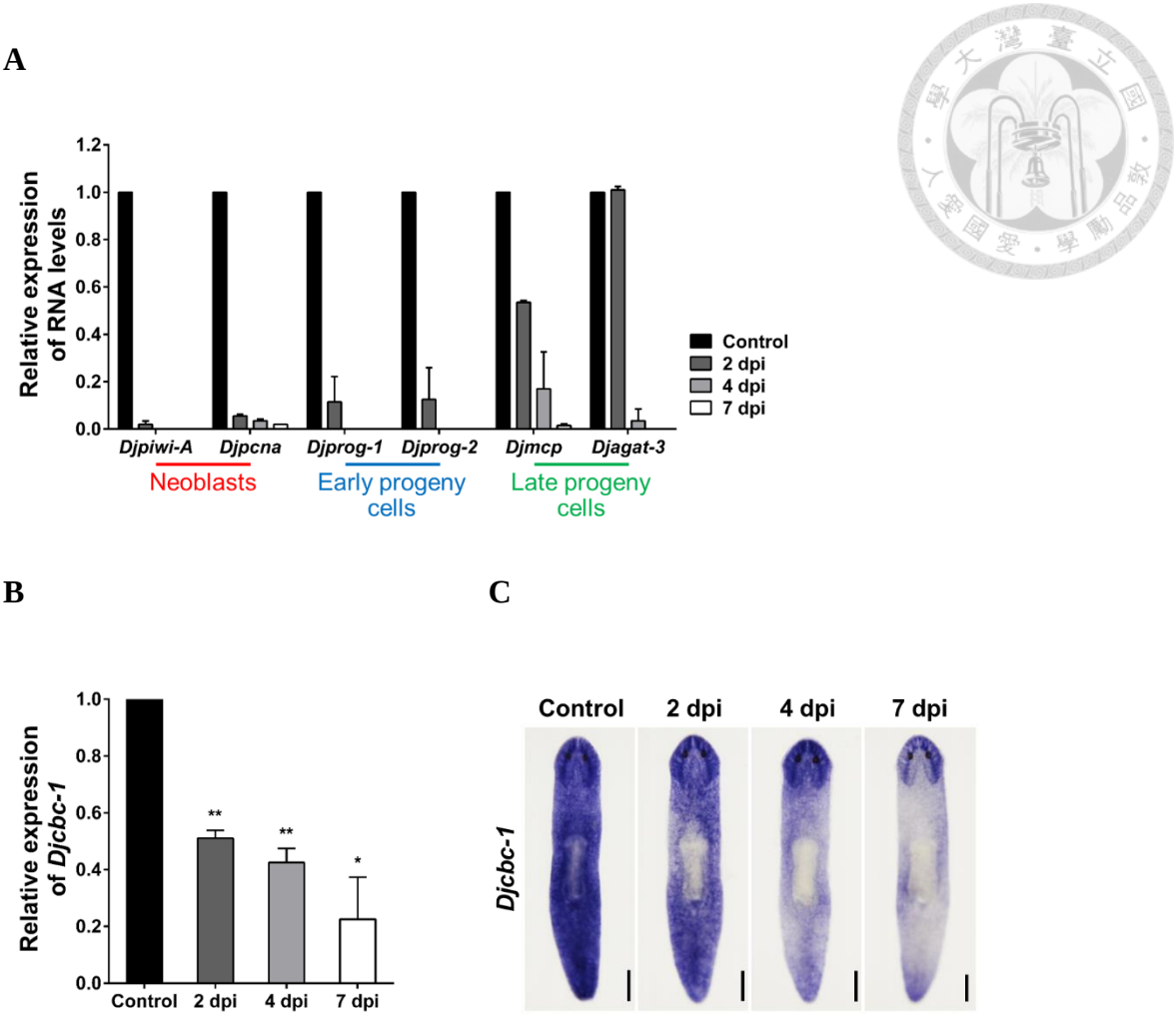

Figure 2. The expression level of *Djcbc-1* was decreased after γ-irradiation

(A) The RT-qPCR for lineage marker genes in wild-type planarian on 2, 4, or 7 days post γ-irradiation (90 Gy). The expression levels of marker genes for neoblasts (*Djpiwi-A* and *Djpcna*, in red), early progeny cells (*Djprog-1* and *Djprog-2*, in blue), and late progeny cells (*Djmcp* and *Djagat-3*, in green) were decreased after γ-irradiation. The un-irradiated wild-type planarian was used as a control. (B and C) Expression of *Djcbc-1* after γ-irradiation. The RT-qPCR (B) and WISH (C) showed that expression level of *Djcbc-1*, which was sensitive to γ-irradiation (90 Gy), was similar to that of other marker genes for neoblasts and progeny cells (*P*-value was determined by comparing with control, $^{**}P<0.01$, $^{*}P<0.05$, Student's t-test). (Scale bars: 250 μm.)

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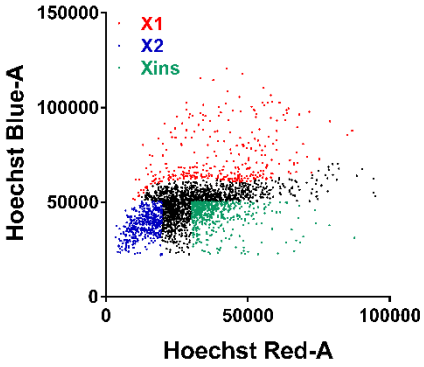
A



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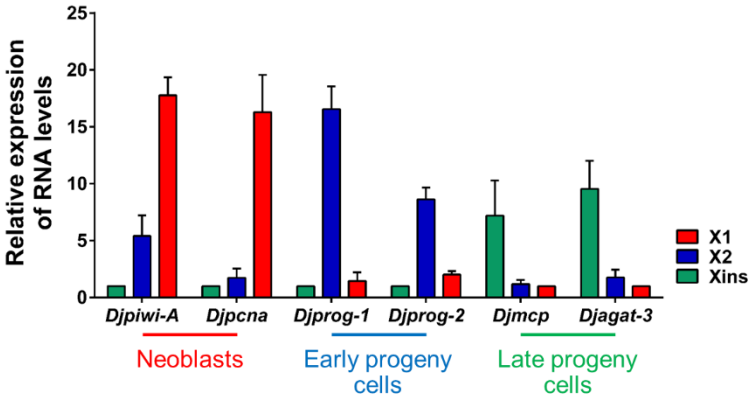

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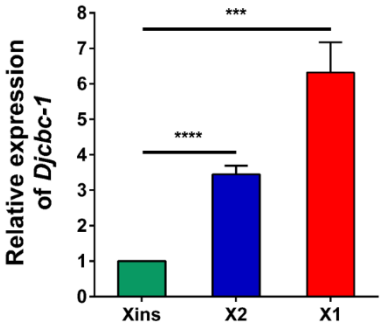

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Figure 3. *Djcbc-1* was highly expressed in FACS isolated X1 and X2 populations from the body of planarian

(A) Mitotic neoblasts (X1), non-mitotic neoblasts and early progeny cells (X2), late progeny cells and differentiated cells (Xins) were separated by fluorescence-activated cell sorting (FACS) according to the published methods. To specific monitor the expression of *Djcbc-1* in neoblasts and progeny cells, instead of the CNS, we removed the head fragment, where the *Djcbc-1* is highly expressed, before cell sorting. (B) The composition of isolated X1, X2, and Xins cells were confirmed with various markers. The RT-qPCR results showed that *Djpiwi-A* and *Djpcna* were highly expressed in X1 population, and *Djprog-1* and *Djprog-2* were highly expressed in X2 population (normalized to Xins population). *Djmcp* and *Djagat-3* were highly expressed in Xins population (normalized to X1 population). (C) RT-qPCR result showed that *Djcbc-1* was highly expressed in isolated X1 and X2 populations from trunk fragments. The Xins population was used as a control for normalization and statistics (****P<0.0001, ***P<0.001, Student's t-test).

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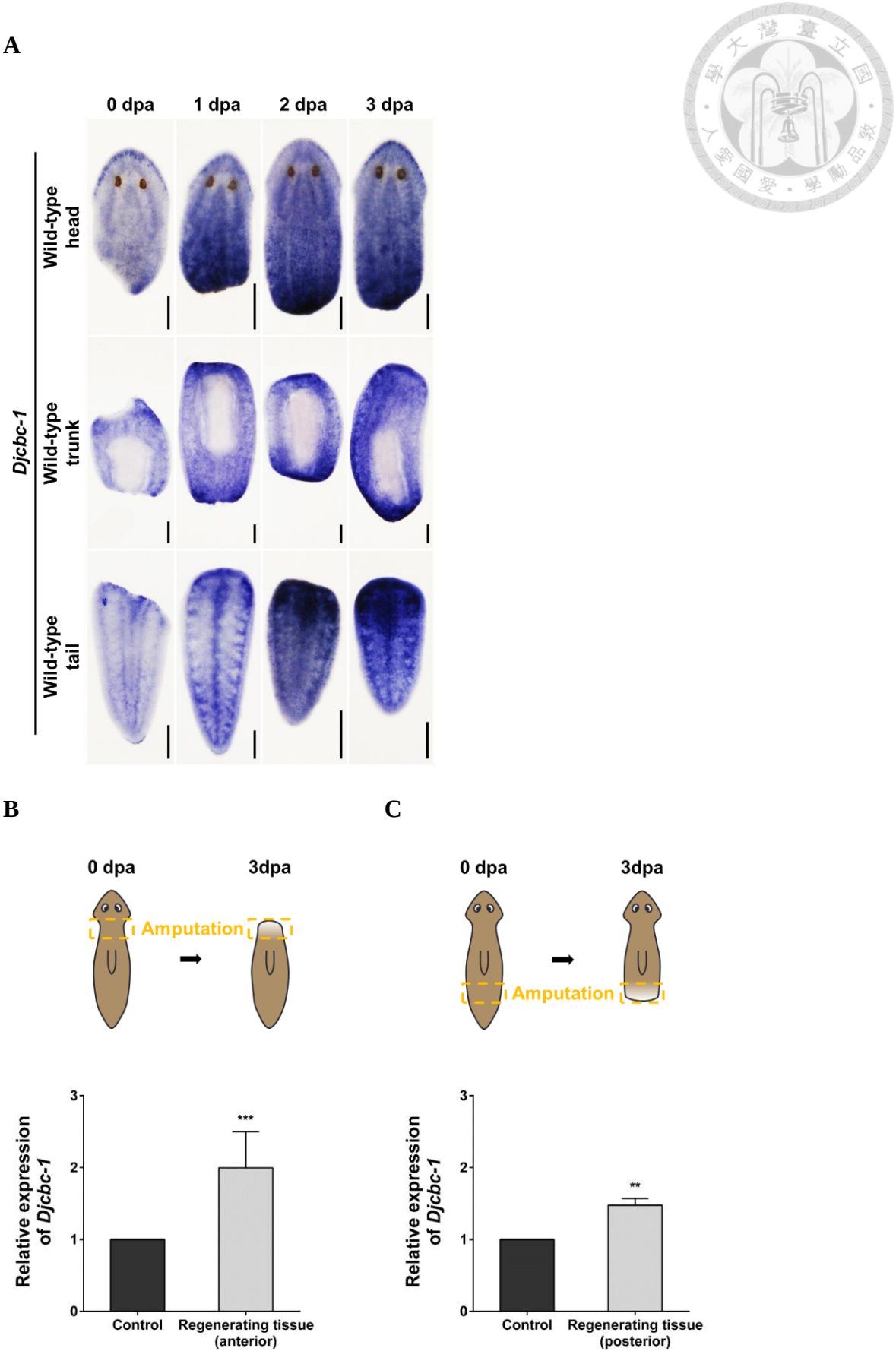

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Figure 4. *Djcbc-1* was highly expressed in the regenerating tissue of planarian

(A) The wild-type planarian amputated at pre- and post-pharyngeal region were fixed at 1, 2, or 3 days post-amputated (dpa). The planarian that was fixed immediately after amputation (0 dpa) was used as a control. Results from WISH showed that *Djcbc-1* was highly expressed in the regenerating tissue at 1 dpa to 3dpa. (B and C) RT-qPCR analysis showed the increased level of *Djcbc-1* in regenerating tissue of head regeneration (B) and tail regeneration (C) at 3 dpa (***P<0.001, **P<0.01, Student's t-test). (Scale bars: 250 μm.)

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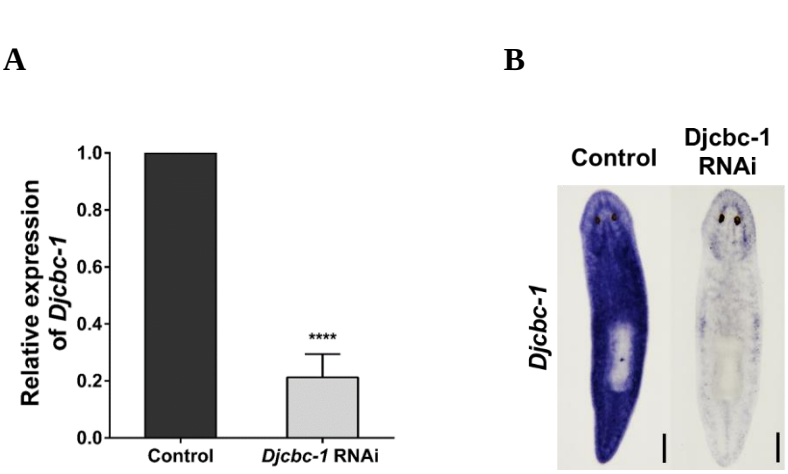

Figure 5. *Djcbc-1* RNAi was sufficient to deplete 80% of *Djcbc-1* in planarian

(A) The RNAi efficiency was determined by RT-qPCR. The endogenous level of *Djcbc-1* was reduced to 20% after *Djcbc-1* RNAi. YFP RNAi was used as the control. (****P<0.0001, Student's t-test). (B) The WISH results showed a high efficiency of *Djcbc-1* RNAi.

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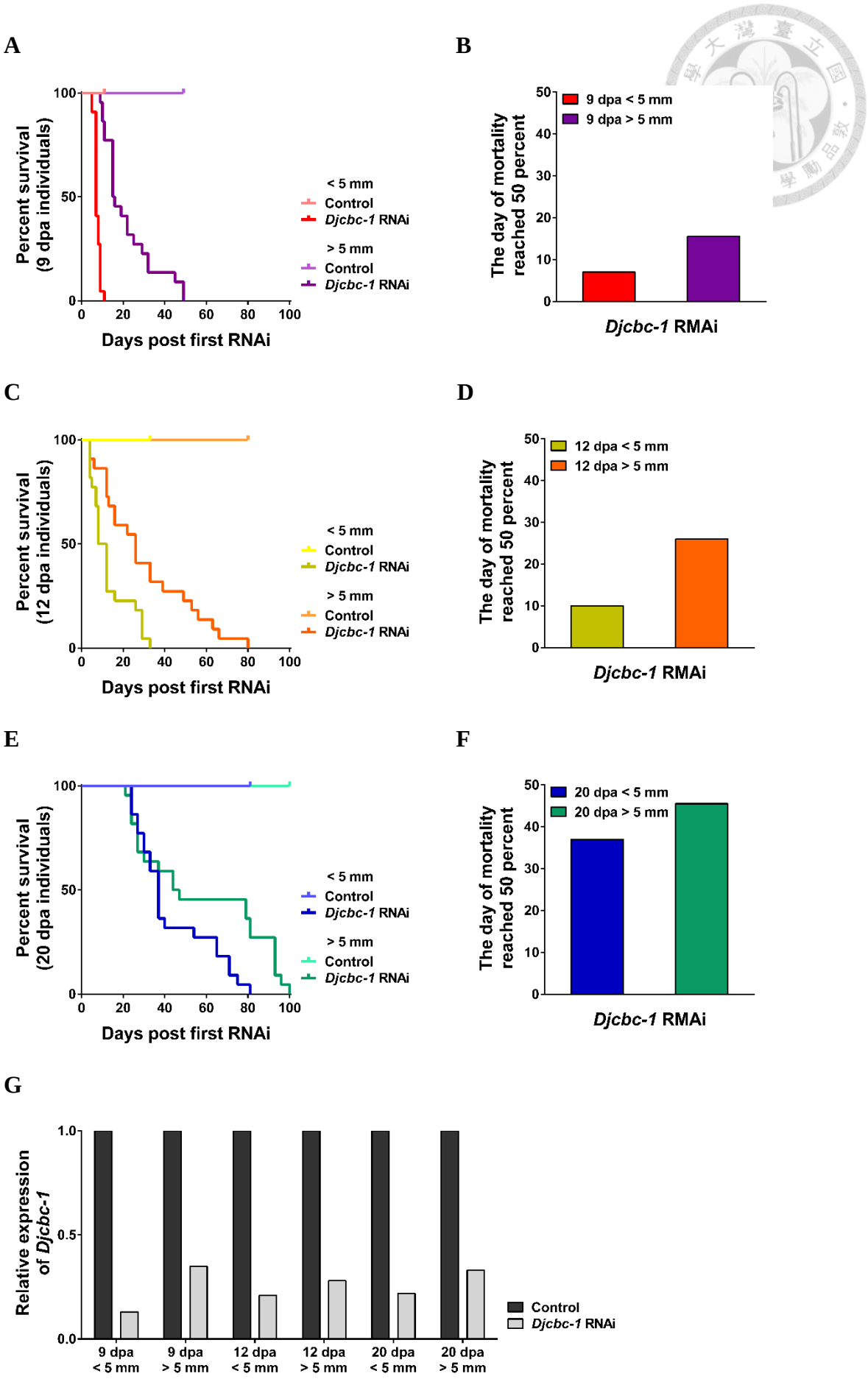

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Figure 6. Small planarians were more sensitive to *Djcbc-1* RNAi

(A, C and E) Depletion of *Djcbc-1* in newly regenerated planarians resulted in death. Percent survival of small (<5mm) or large (>5mm) planarians with *Djcbc-1* RNAi started at 9 dpa (A), 12 dpa (C), and 20 dpa (E) (n=22). (B, D and F) The median survival for planarians with *Djcbc-1* RNAi at 9 dpa (B), 12 dpa (D), and 20 dpa (F) in size < 5mm or > 5mm. Days reached 50% mortality were calculated from panel A, C and E. (G) The knockdown efficiencies after *Djcbc-1* RNAi were confirmed by RT-qPCR for each group.

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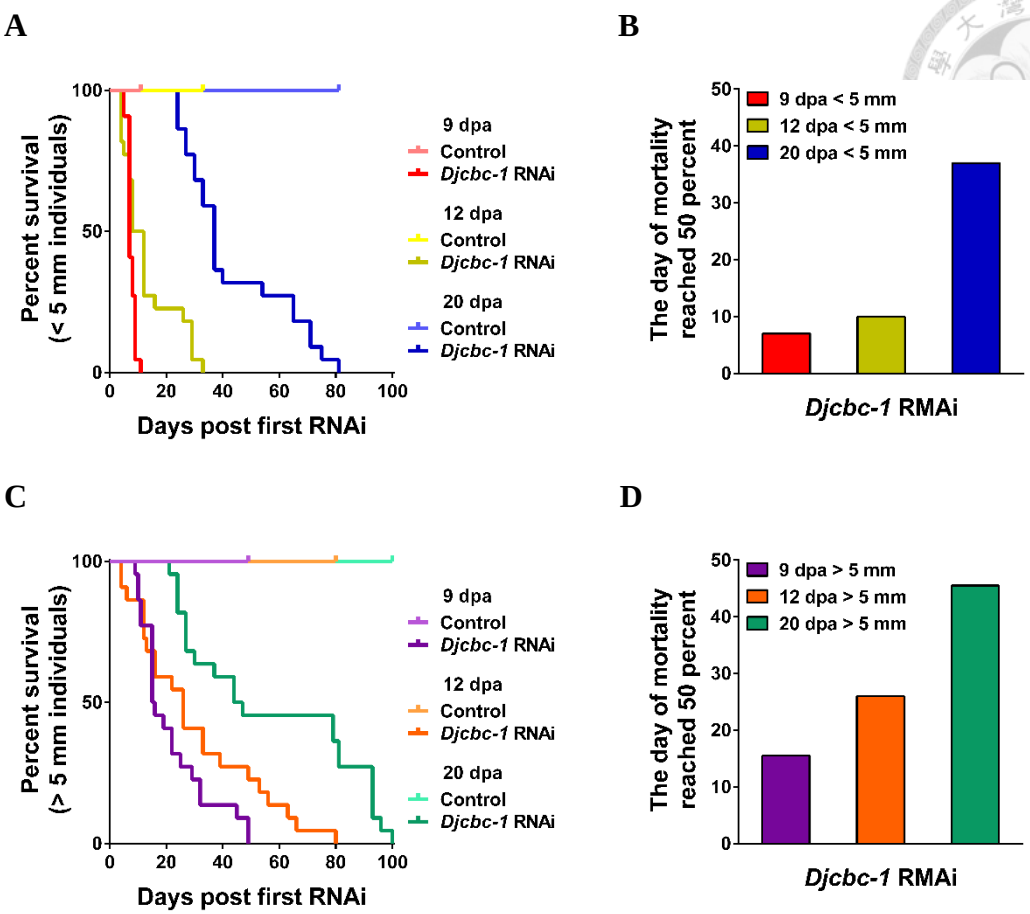

Figure 7. Newly regenerated planarians were more sensitive to *Djcbc-1* RNAi

(A and C) Percent survival of newly regenerated planarian with size < 5mm (A) or > 5mm (C). *Djcbc-1* RNAi was started at 9 dpa, 12 dpa, or 20 dpa (n=22 for each group). Planarians fed with YFP dsRNA were used as a control and were 100% survived. (B and D) The median survival for size < 5mm (B) or > 5mm (D) planarians with *Djcbc-1* RNAi started at various time points. Days reached 50% mortality were calculated from panel A and C.

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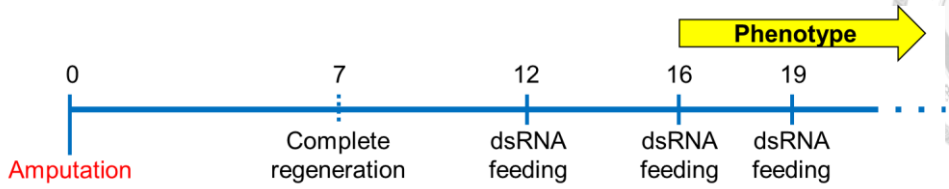

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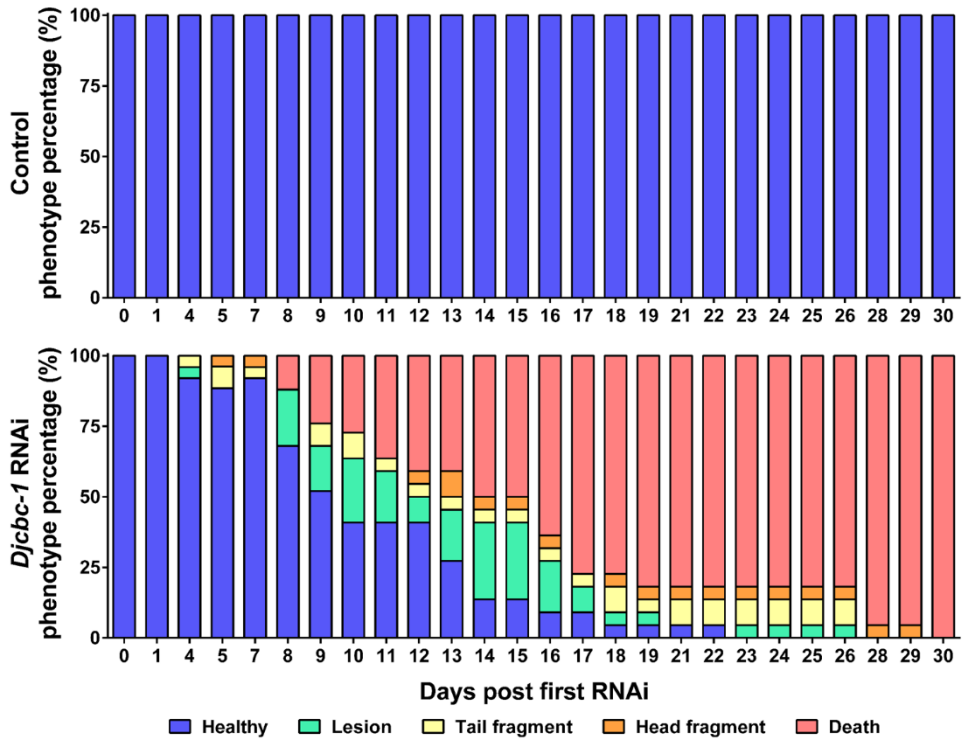

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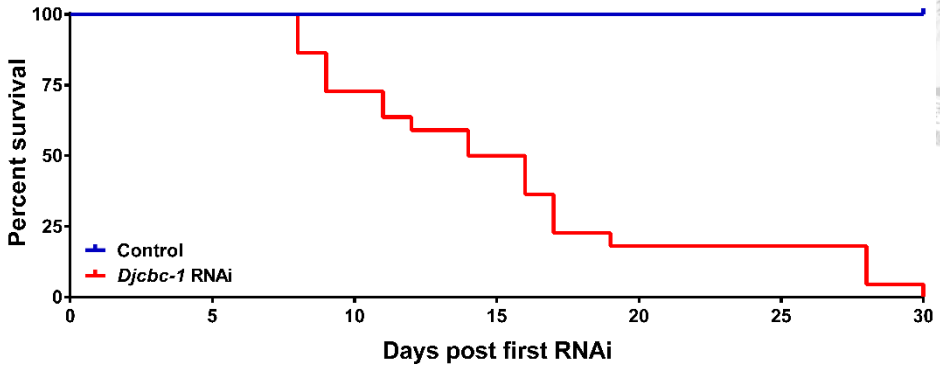

Figure 8. *Djcbc-1* was essential for the survival of newly regenerated planarian

(A) The schematic diagram of experimental schedule. Planarians with *Djcbc-1* RNAi started at 12 dpa were used in this experiments. (B) Depletion of *Djcbc-1* in newly regenerated planarians (12 dpa) resulted in a lesion or fragmentation phenotype and led to death (n=22). (C and D) The phenotype progress (C) and survival rate record (D) of planarian treated with *Djcbc-1* RNAi at 12dpa (n=22). YFP RNAi was used as a control. It showed a significant difference between the survival rate of control and *Djcbc-1* RNAi planarians. This result showed that *Djcbc-1* is essential for planarian survival (****$P<$ 0.0001, Log-rank (Mantel-Cox) test).

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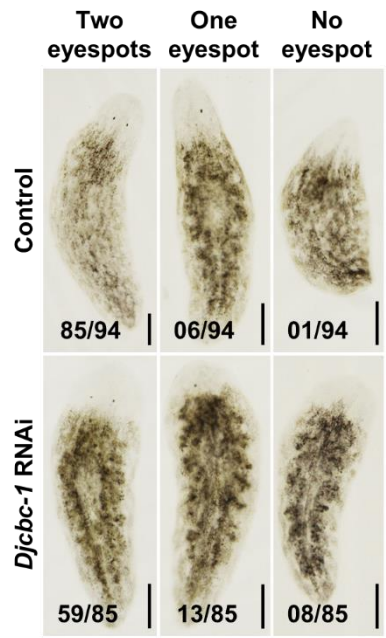

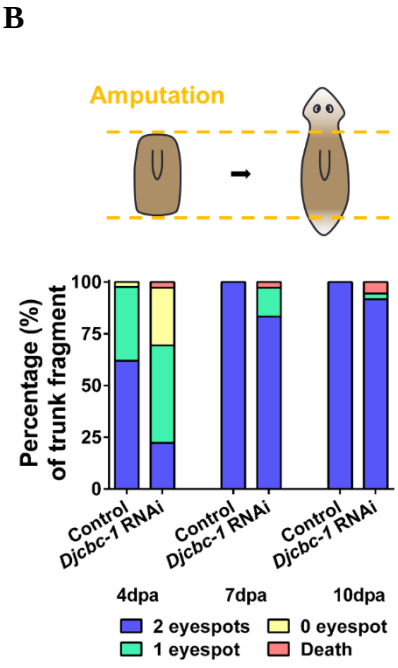

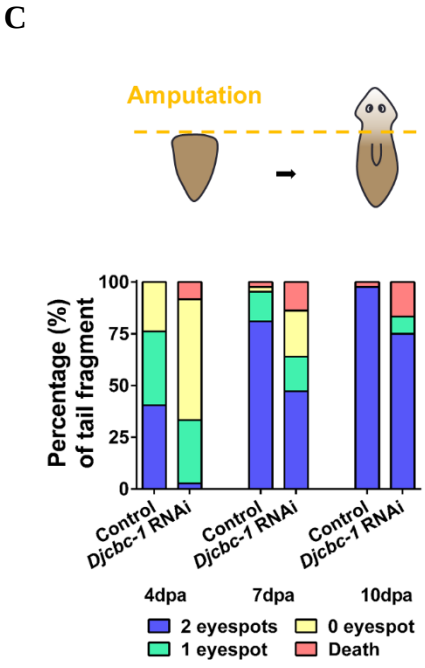

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Figure 9. Depletion of *Djcbc-1* delayed the eyespots formation during planarian head regeneration

(A) The planarians were amputated at pre- and post-pharyngeal region. Images represent the three phenotypes and their ratio in tail fragments at 7dpa. The planarian with YFP RNAi was used as a control. (B and C) Depletion of *Djcbc-1* prolonged the head regeneration process and delayed the eyespots formation in both trunk (B) and tail (C) fragments after amputation. Various phenotypes, defined as two eyespots (normal), one eyespot (cyclopia), no eyespot, or death, were recorded at 4 dpa, 7 dpa, and 10 dpa. Treatment: *Djcbc-1* RNAi (n=85). Control: YFP RNAi (n=94).

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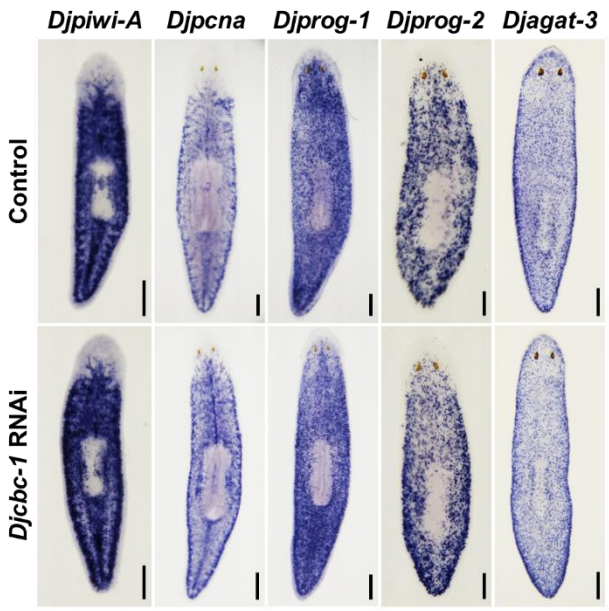

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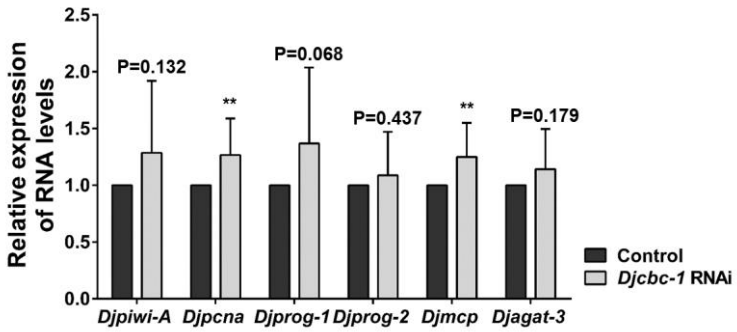

Figure 10. The expression patterns and levels of neoblasts-related marker genes after *Djcbc-1* RNAi

(A) The WISH results showed that depletion of *Djcbc-1* did not alter the expression pattern of neoblasts and progeny cells markers. (B) The RT-qPCR results showed that depletion of *Djcbc-1* up-regulated the expression levels of *Djpcna* and *Djmcp* (Error bar represent SD, ***P*<0.01, Student's t-test).

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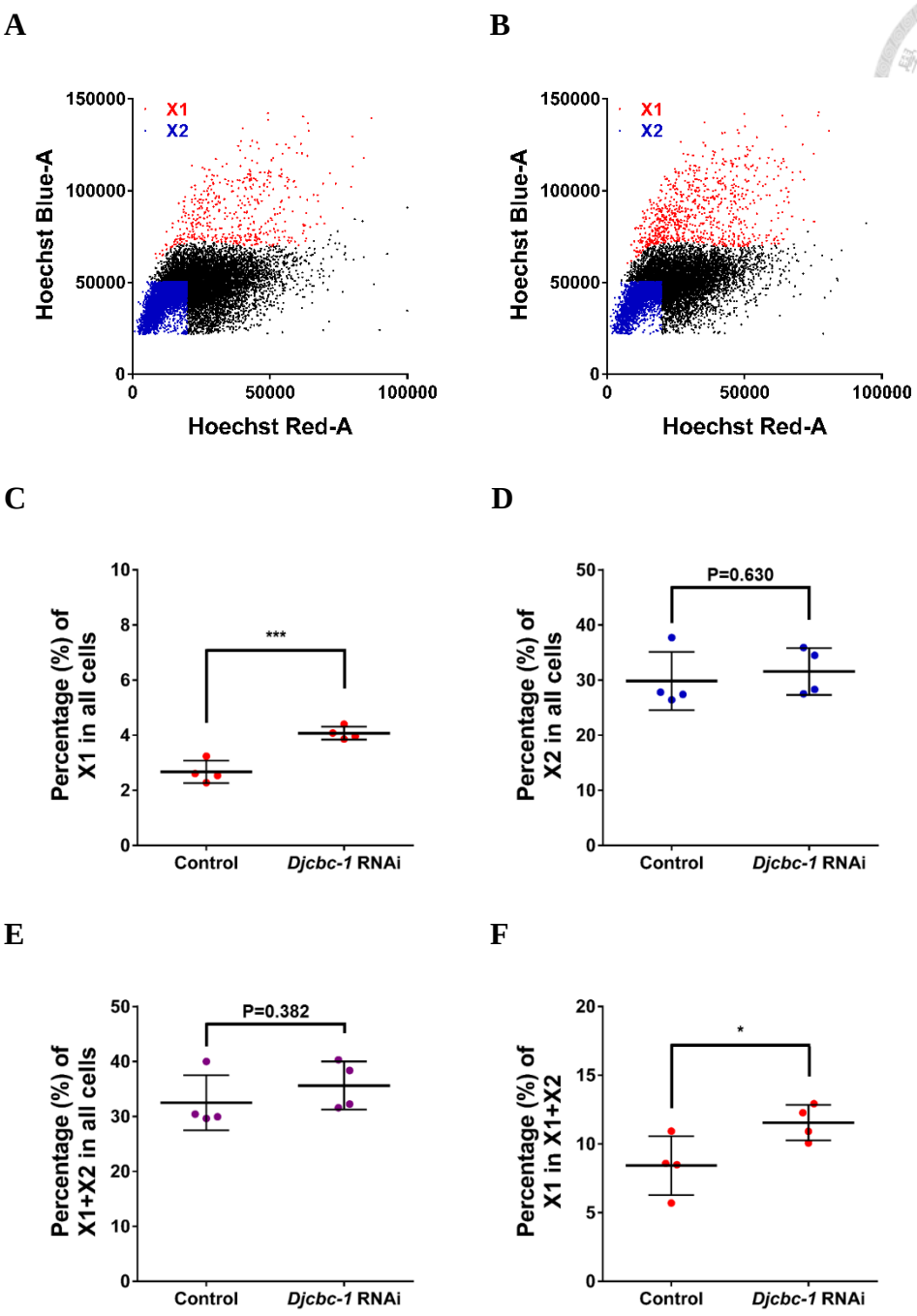

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Figure 11. Depletion of *Djcbc-1* increased the relative amounts of isolated X1 but not the X2 cells.

(A and B) Gating and isolating the X1 and X2 cell populations by FACS. Single cell suspensions from planarian with YFP RNAi (A) or *Djcbc-1* RNAi (B) were analyzed to quantify the relative amounts of X1 and X2 cells (n=4). (C, D, and E) The FACS results showed that depletion of *Djcbc-1* increased the ratio of X1 population (C), but did not affect the ratio of X2 population (D) or the ratio of X1+X2 (E) in all cells (n=4) (***P<0.001 with a paried on-tailed Student's t-test). (F) These results showed that depletion of *Djcbc-1* increasing the ratio of X1 population in X1 and X2 population, which refer to mitotic neoblasts and non-mitotic neoblasts, respectively (*P<0.05, Student's t-test).

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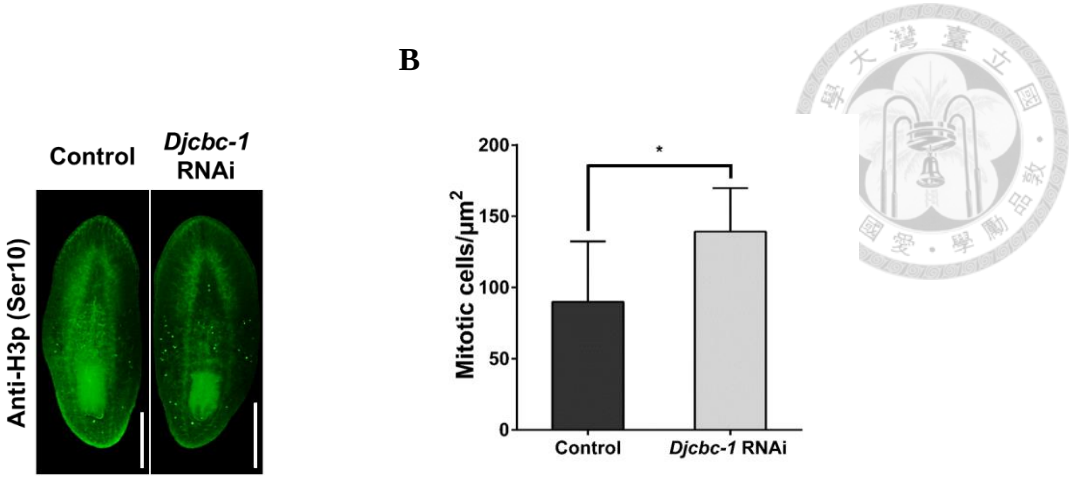

Figure 12. Depletion of *Djcbc-1* increased the mitotic cells of planarians

(A) Planarians were amputated after treated with *Djcbc-1* RNAi for 13 times and the head fragments (n=6) were fixed at 13dpa. The YFP RNAi was used as a control. The mitotic cells were detected by immunofluorescence staining with anti-phospho-Histone 3 (H3p) antibody. (B) Quantitative analysis of mitotic cells ($H3p^+$ cells) was normalized to the quantified area (n=6) (*P<0.05, Student's t-test). (Scale bars: 250 μm.)

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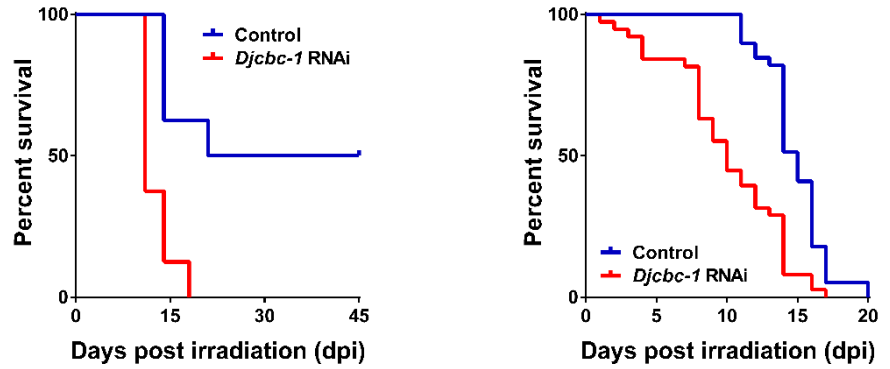

Figure 13. *Djcbc-1*-depleted planarians were more sensitive to γ-irradiation with the dosage of 5 Gy or 90 Gy

(A) Percent survival of planarian with 5 Gy γ-irradiation showed a significant difference between the control and *Djcbc-1* RNAi planarians (n=8) (***P*< 0.01, Log-rank (Mantel-Cox) test). (B) Percent survival results of planarian with 90 Gy γ-irradiation. A significant difference was shown between the control and *Djcbc-1* RNAi planarians (n=38). (*****P*< 0.0001, Log-rank (Mantel-Cox) test).

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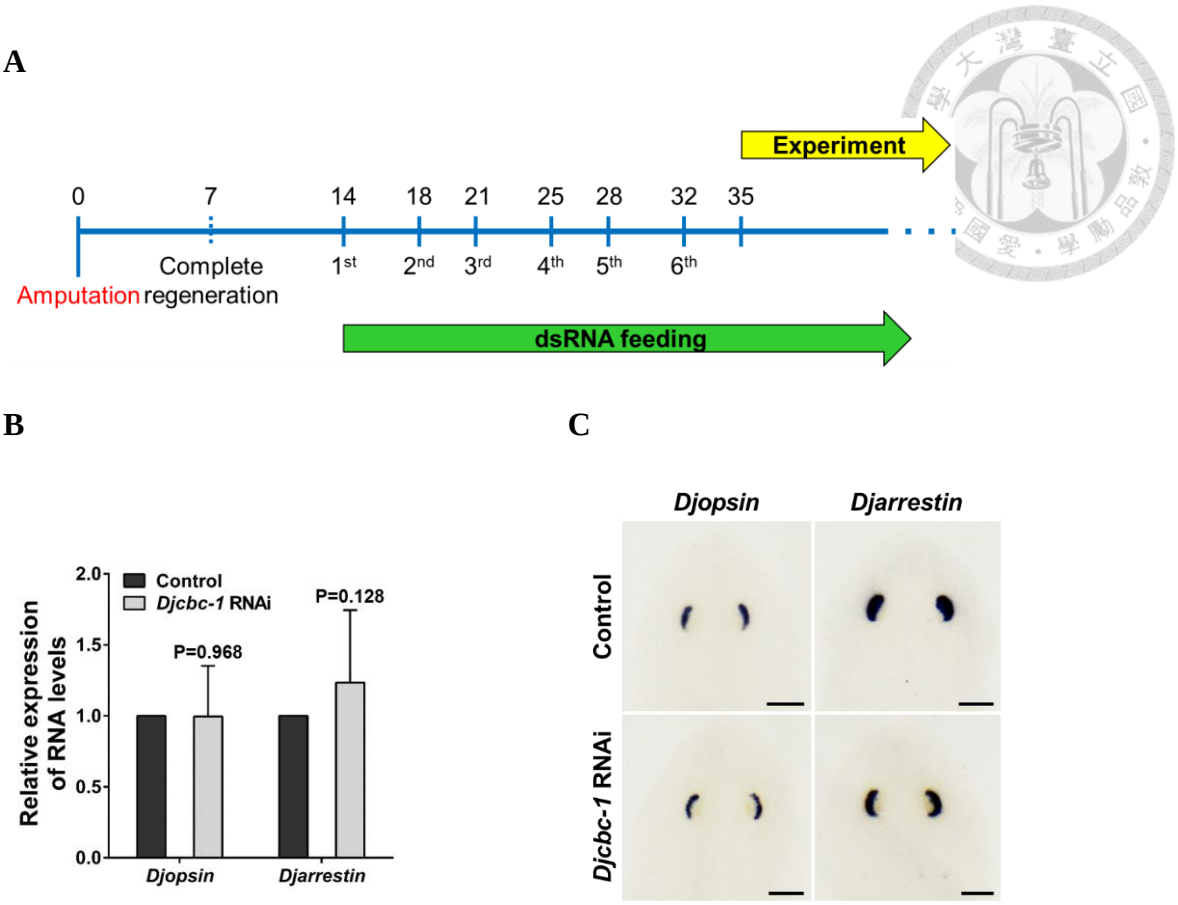

Figure 14. Depletion of *Djcbc-1* had no effect on the planarian eyespots during homeostasis

(A) The schematic diagram of experimental design for long-term RNAi. Planarians treated with long-term *Djcbc-1* RNAi more than 6 rounds were examined for the following experiments. (B) The RT-qPCR results showed that depletion of *Djcbc-1* did not significantly change the expression levels of *Djopsin* or *Djarrestin*, the two essential genes for eyespots formation and function (*P*=0.968 or 0.128, respectively, Student's t-test). (C) The WISH results showed that depletion of *Djcbc-1* did not change the expression patterns of *Djopsin* or *Djarrestin* in long-term *Djcbc-1*-depleted planarian. (Scale bars: 100 μm.)

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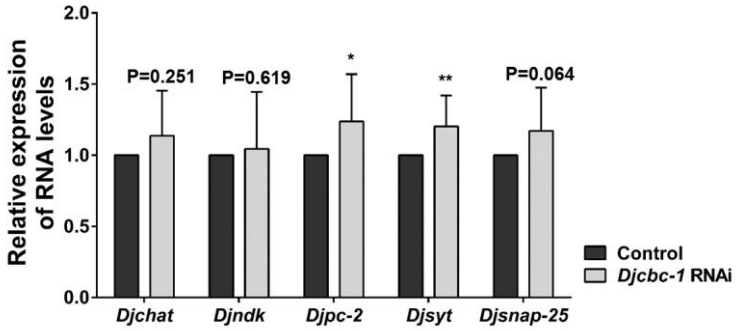

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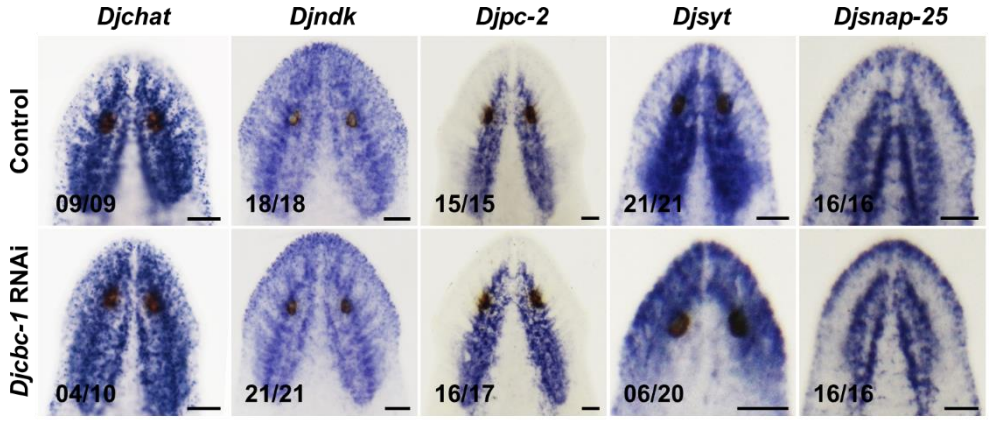

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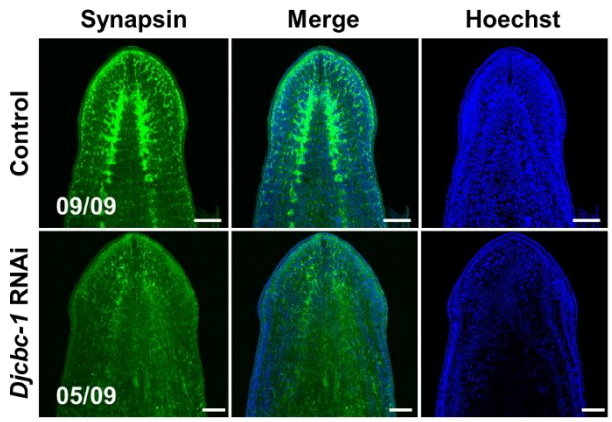

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Figure 15. Depletion of *Djcbc-1* perturbed the planarian brain during homeostasis

(A) Depletion of *Djcbc-1* did not significantly change the expression levels of *Djchat*, *Djndk* and *Djsnap-25* while slightly increased the levels of *Djpc-2* and *Djsyt* (*P<0.05, **P<0.01, Student's t-test). (B and C) The WISH and immunofluorescence staining results showed that depletion of *Djcbc-1* perturbed the expression patterns of *Djchat*, *Djsyt*, and Synapsin in planarians with long-term *Djcbc-1* RNAi. Population sizes for the observed morphology were showed at lower left corner of each panel. (Scale bars: 100 μm.)

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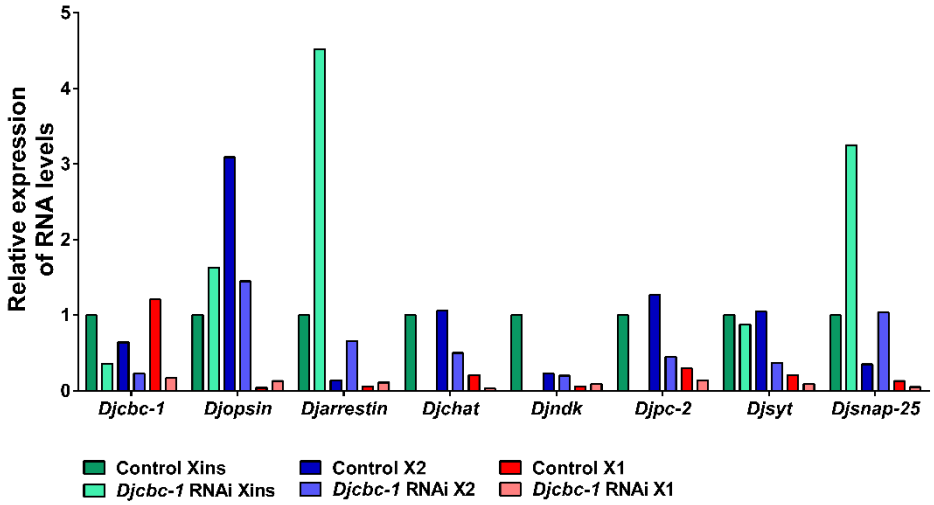

Figure 16. Quantitative analysis of CNS specific genes in isolated X1, X2, and Xins populations

Three populations of different cell types from *Djcbc-1*-depleted planarian were isolated by FACS. The RT-qPCR was used to determine the expression of various CNS marker genes in each cell population. The data were normalized by the expression levels of each gene in the Xins population of control. Control: YFP RNAi.

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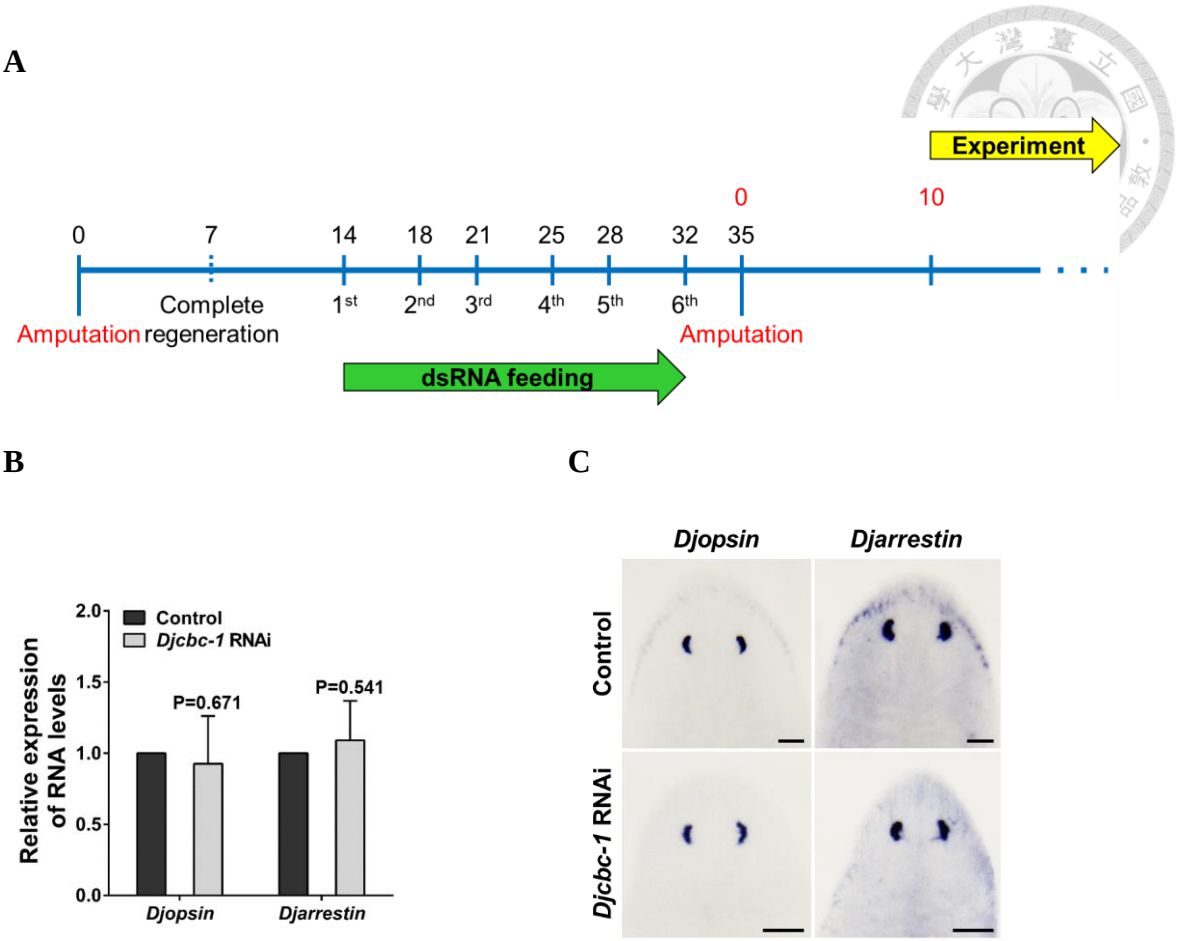

Figure 17. Depletion of *Djcbc-1* did not impair the eyespots regeneration of planarian

(A) The schematic diagram of experimental conditions. Planarians amputated after RNAi and regenerated for 10 to 13 days were used in the following experiments. (B) The RT-qPCR results showed that depletion of *Djcbc-1* did not significantly change the expression levels of *Djopsin* and *Djarrestin* at 11-13 dpa during regeneration (*P*=0.671 or 0.541, respectively, Student's t-test). (C) The WISH results showed that depletion of *Djcbc-1* did not affect expression patterns of *Djopsin* and *Djarrestin* at 11-13 dpa during regeneration. (Scale bars: 100 μm.)

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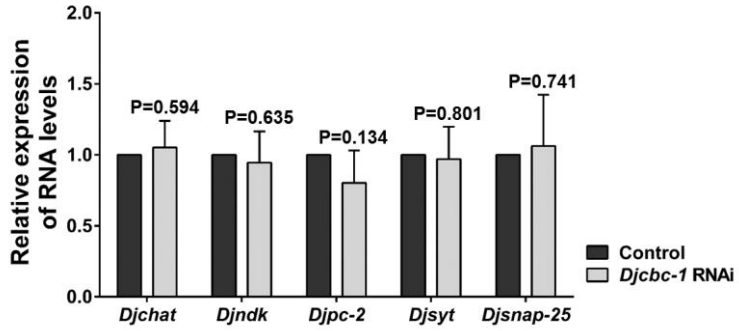

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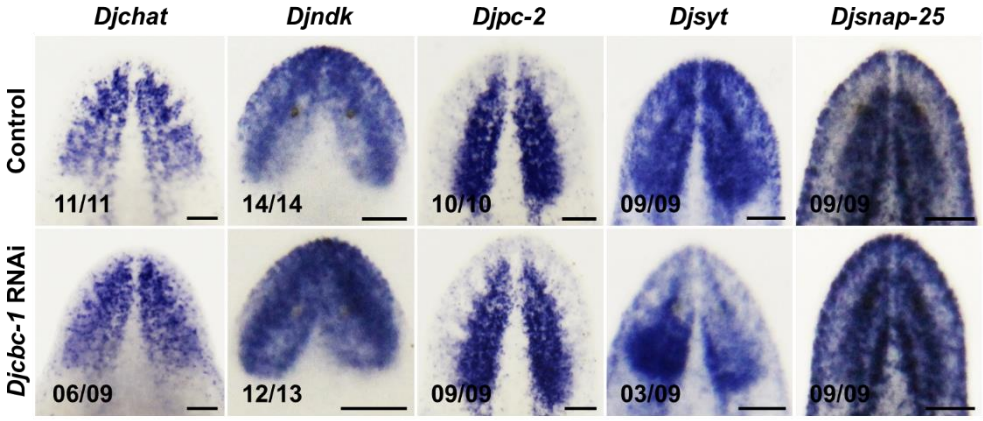

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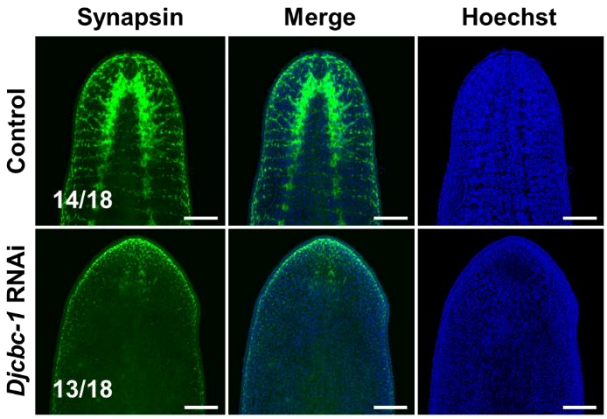

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Figure 18. Depletion of *Djcbc-1* impaired the brain regeneration of planarian

(A) The RT-qPCR results showed that the expression levels of brain marker genes did not significantly change in *Djcbc-1*-depleted planarians at 13 dpa during regeneration (P-values were determined by Student's t-test). (B and C) Depletion of *Djcbc-1* perturbed the expression patterns of *Djchat*, *Djsyt*, and Synapsin in planarians at 13 dpa during regeneration. Ratios for the represented morphology were showed at lower left corner of each panel. (Scale bars: 100 μm.)

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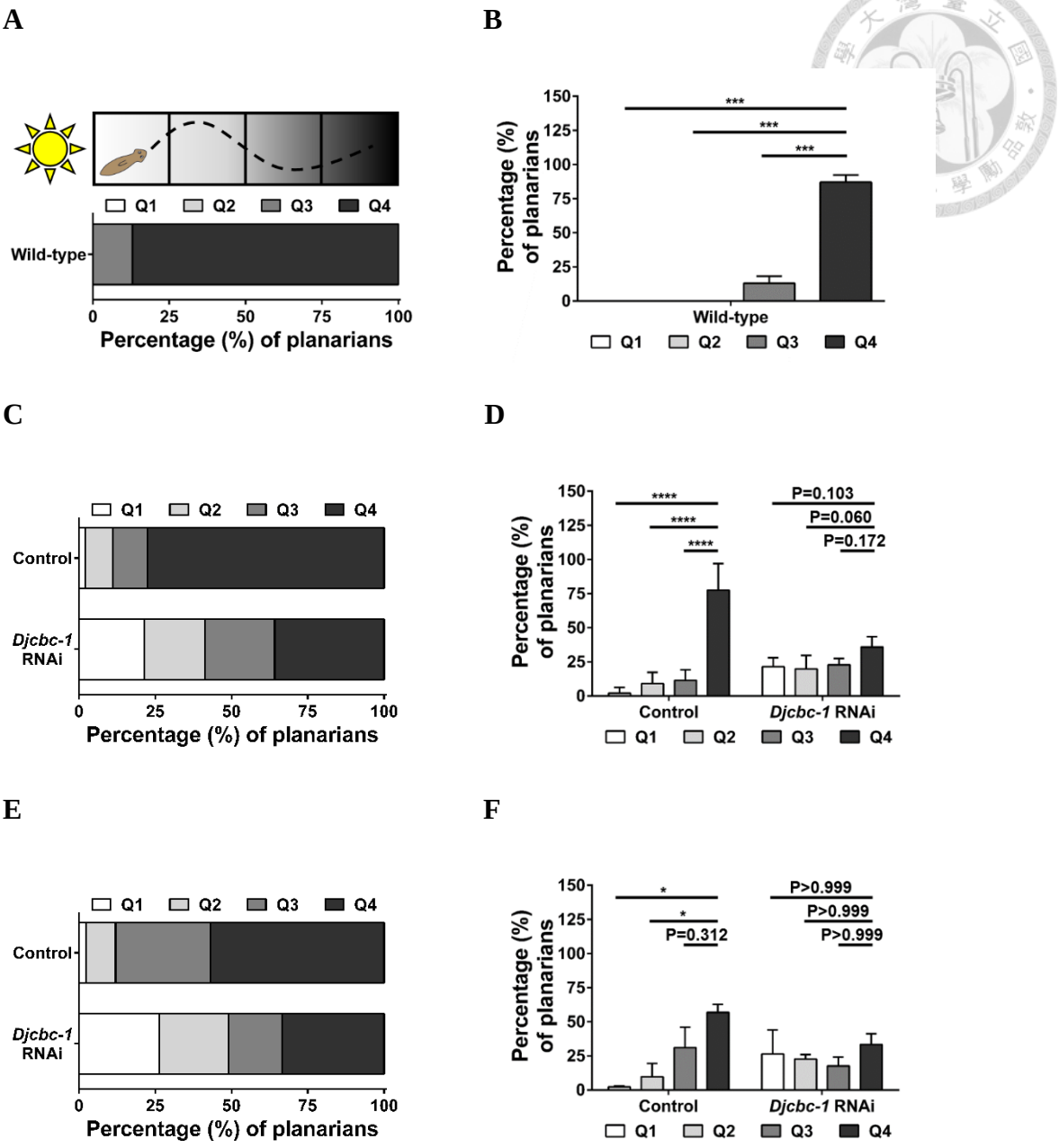

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Figure 19. Depletion of *Djcbc-1* abolished the negative phototaxis of planarian

(A and B) The negative phototaxis behavior of planarian was quantified by photophobia assay. Planarian was initially placed at Q1 in dark, and the numbers of planarian in each zone (Q1-Q4) were recorded at 2 mins after light exposure from the Q1 side. Wild-type planarian tends to move away from Q1 rapidly and stays in Q4 (n=30) (***P< 0.001, ANOVA, Bonferroni's multiple comparisons test). (C and D) The photophobia assay of long-term *Djcbc-1*-depleted planarian. Planarians with long-term *Djcbc-1* RNAi (more than 6 rounds of RNAi) were used for photophobia assay (control: n=48, *Djcbc-1* RNAi: n=51) Percentage of planarian in each zone in the assay was quantified and analyzed in (D) (****P< 0.0001, ANOVA, Bonferroni's multiple comparisons test). (E and F) The photophobia assay of *Djcbc-1*-depleted planarian with regeneration at 11 dpa (n=30). Both results of *Djcbc-1*-depleted planarians in homeostasis and regeneration suggested that depletion of *Djcbc-1* reduced the negative phototaxis (*P< 0.05, ANOVA, Bonferroni's multiple comparisons test).

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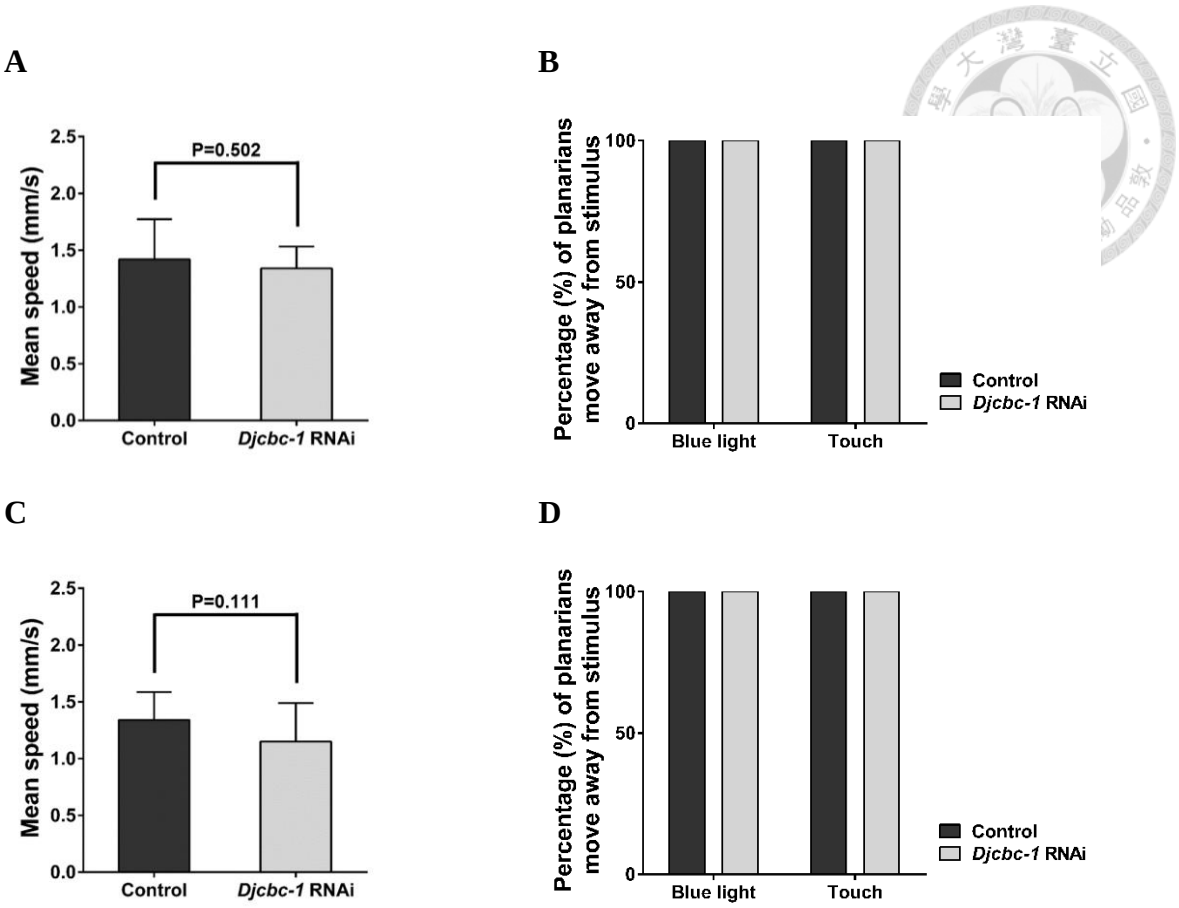

Figure 20. Depletion of *Djcbc-1* did not affect the planarian motility and the stimulating-escape response

(A and C) The moving ability of planarian was examined by a motility assay. Planarian was initially placed at the center of a 9-cm petri dish and the behavior was recorded for more than two minutes. We then used software to analysis the distance that planarian moved in two minutes. The results showed no significant difference between control and *Djcbc-1*-depleted planarian in the mean speed (mm/s) (*P*=0.502, Student's t-test). (B and D) The percentage (%) of planarians moved away from blue light or physical touching stimuli showed that depletion of *Djcbc-1* did not affect the ability of escape from other stimuli of planarian. (A and B) Assays were performed in long-term *Djcbc-1*-depleted planarian (n=12). (C and D) Assays for *Djcbc-1*-depleted planarian after regeneration at 11 dpa (n=12).

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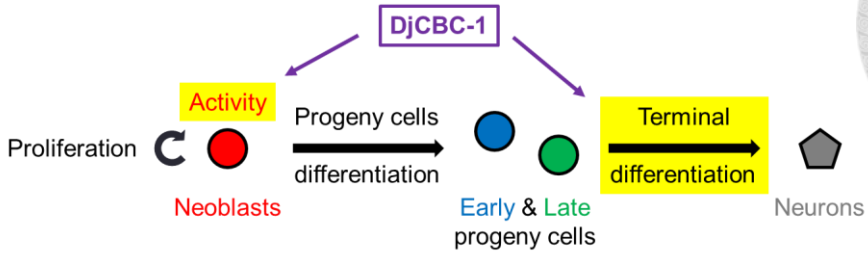

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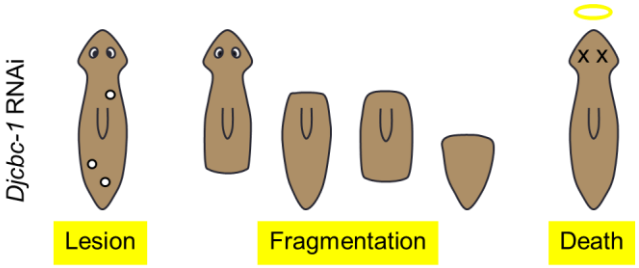

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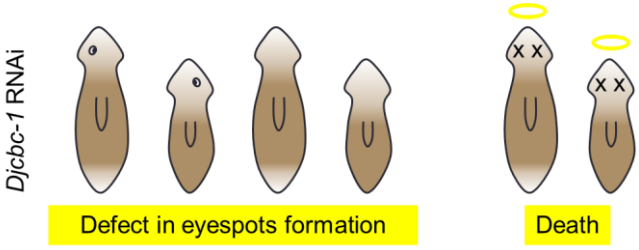

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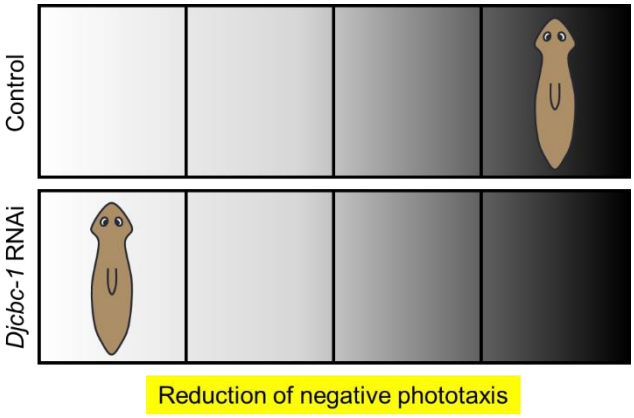

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Figure 21. Summary of the function of DjCBC-1 in planarian

(A) DjCBC-1 may be involved in the maintenance of functional active neoblasts and differentiation of neurons. (B) DjCBC-1 is critical for the survival of newly regenerated planarian in maintaining the homeostasis. (C) DjCBC-1 plays an important role in eyespots and brain formation during regeneration. (D) DjCBC-1 is required for the negative phototaxis behavior of planarian.

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