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Cdc42 regulate the apoptotic cell death required for planarian epidermal regeneration and homeostasis

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ABSTRACT

Rho GTPases have been shown previously to play important roles in several cellular processes by regulating the organization of the actin and microtubule cytoskeletons. However, the mechanisms of Rho GTPases that integrate the cellular responses during regeneration have not been thoroughly elucidated. The planarian flatworm, which contains a large number of adult somatic stem cells (neoblasts), is a unique model to study stem cell lineage development *in vivo*. Here, we focus on *cdc42*, which is an extensively characterized member among Rho GTPases. We found that *cdc42* is required for the maintenance of epidermal lineage. *Cdc42* RNAi induced a sustained increased of cell death and led to a loss of the mature epidermal cells but without affected cell division. Our results indicate that *cdc42* function as an inhibitor to block the excessive apoptotic cell death in planarian epidermal regeneration and homeostasis.

1. Introduction

The family of Rho GTPases is a member of the Ras superfamily of GTPases(Van Aelst and D'Souza-Schorey, 1997; Boettner and Van Aelst, 2002). Rho GTPases function as molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state (Schmitz et al., 2000; Boettner and Van Aelst, 2002). They play important role in several cellular processes by regulating the organization of the actin and microtubule cytoskeletons, such as cell migration, morphogenesis and axon guidance (Luo et al., 1997; Nobes and Hall, 1999; Settleman, 1999; Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002).

Cdc42 (cell division cycle 42) as the most extensively characterized member among Rho GTPases plays a crucial role in actin cytoskeletal organization and assembly (Xiao et al., 2018). It induced the formation of focal adhesion complexes and filopodia and involved in morphogenesis (Nobes and Hall, 1995; Settleman, 1999; Erickson and Cerione, 2001). Knockdown of *cdc42* by RNA interference in *Drosophila*, the epithelial wounds cannot be repaired (Verboon and Parkhurst, 2015). In *X. laevis*, interference with *cdc42* function causes an arrest of gastrulation and an inhibition of convergent extension movements (Choi and Han, 2002). In *C. elegans*, the lack of *cdc42* causes embryos arrest during elongation with epidermal ruptures (Zilberman et al., 2017). The abnormal renal vesicle originating from epithelial cell polarity defects is also observed for *cdc42* mutation in mouse (Elias et al., 2015). The previous studies indicated *cdc42* regulated morphogenesis through controlling various biological activities, such as cell cycle progression, cell proliferation and cell polarity. However, mechanisms of *cdc42* in controlling stem cell lineage development have not been thoroughly elucidated.

Regeneration is the process that body replaces the damaged tissue or organs through proliferation, differentiation (Barrero and Izpisua Belmonte, 2011). It is currently believed that body regeneration and damage repair of animal are mainly based on the presence and differentiation of adult stem cells (ASCs) (Pearson, 2001; Salvetti et al., 2005). Planarians are considered as an important model system because of a population of pluripotent ASCs *in vivo* (Agata and Watanabe, 1999; Newmark and Sanchez Alvarado, 2000; Tasaki et al., 2011). Adult stem cells, called neoblasts, account for approximately 10% of all planarian cells (Reddien et al., 2005). It makes that planarians have remarkable ability to regenerate complete worms from any part of their body fragments. When injured, neoblasts can migrate to the wound position and differentiate into the missing organs or tissues(Aboobaker, 2011; Wagner et al., 2011; Rink, 2013; Henderson et al., 2015). Therefore, the planarian, which contains a large number of adult somatic stem cells

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(neoblasts), is a unique model to study stem cell lineage development *in vivo*.

In this study, we identified *cdc42* homolog in planarians and determined its expression patterns by whole mount in situ hybridization (WISH), which showed that it is highly expressed in wound region. When *cdc42* is targeted by RNAi, the defects of abnormal epidermis could be observed in both regenerating and intact worms. We found that *cdc42* RNAi induced an excessive apoptosis. Meanwhile, the mature epidermal cell was decreased along with a significant increased epidermal progeny cells. These results suggest that *cdc42* might function as an inhibitor to block the excessive apoptotic cell death in planarian epidermal regeneration and homeostasis.

2. Materials and methods

2.1. Species and culture conditions

A clonal strain of the planarian *Dugesia japonica* used in all experiments were maintained autoclaved stream water at 20 C. Planarians were starved for at least 1 week and select 4-6 mm in length when used for experiments.

2.2. Gene cloning and RNA interference

Djcdc42 were found in the planarian transcriptome. The pairs of specific primers (5′-ATGGTACAAACTATTAAATGTGTTGTCG-3′and 5′-TATCAATGAACATTTTCGTTTACTATGG-3′) were designed to amplify fragment of *cdc42* from cDNA. Double-stranded RNAs (dsRNAs) were synthesized by in vitro transcription as previously described (Rouhana et al., 2013). DsRNA was injected to animals using a Drummond microinjector. Control animals were injected with water treated by DEPC. At 24 h after the last injecting, worms were amputated into three fragments anterior and posterior of the pharynx and allowed to undergo regeneration.

2.3. Whole-mount in situ hybridization

Whole-mount ISH (WISH) was performed as previously described (Pearson et al., 2009). After bleaching and rehydration, the animals were treated for 10 min at 37 °C using Proteinase K (20 mg ml^{-1} in PBS containing 0.3% Triton X-100) for 10 min at 37 °C and fixation in 4% paraformaldehyde. Then these samples were hybridized with a DIG-labeled probe at 56 °C for 16-17 h. Digoxigenin-labeled RNA probes were synthesized using an RNA in vitro transcription kit (Roche). After proper washing and antibody incubation (anti–digoxigenin-AP,1:4000; Roche), colorimetric (NBT/BCIP) were used to detect probe hybridization. For the regeneration experiments, the animals were cut transversely into 3 pieces as follows: the head; trunk (including the pharynx) and tail, and the fragments were fixed at 1, 3 and 7 d after amputation.

2.4. Whole-mount immunostaining

Animals were killed with 5% NAC in PBS (phosphate buffered saline) for 5 minutes at room temperature and washed three times with PBST (phosphate buffered saline containing 0.1% Triton X-100) at RT. Then they were fixed in PBST containing 4% paraformaldehyde for 2 -4 h at 4 °C and incubated in 100% methanol for 1 h at −20 °C. They were then blocked with 10% goat serum in PBST for 2 to 4 hours at 4 °C or RT, and incubated with primary anti-synapsin (1:100; Developmental Studies Hybridoma Bank) or anti-H3P (1:250; Millipore, 05-817R) antibodies overnight at 4 °C. After the samples were washed six times with PBST for 30 minutes per wash, Signals were detected with goat anti-mouse Alexa Fluor 488 (1:500; Invitrogen, 673,781) and goat anti-rabbit Alexa Fluor 568 (1:500; Invitrogen, 11,036). Stained planarians were visualized using NIS element software (Nikon).

2.5. Quantitative real-time PCR

Total RNA was extracted from three intact planarians using Trizol (TaKaRa), and reversed transcripted with oligo-dT primers and reverse transcriptase (TaKaRa). Real-time quantitative PCR was performed using SYBR green qPCR Master Mix (Vazyme). The primers are listed below. Three samples were run in parallel for each condition. Elongation factor 2 (EF-2) was used as the alternative internal reference.

2.6. Whole-mount TUNEL

Whole-mount TUNEL assay using the planarians were performed as described previously (Pellettieri et al., 2010). Briefly, the worms were sacrifced in 10% n-acetyl cysteine (diluted in PBS) at room temperature (RT), and fixed in 4% formaldehyde for 20 min. Then samples were permeabilized in 1% SDS (diluted in PBS) for 20 min. Fixed animals were next bleached overnight in 6% H2O2 (diluted in PBST)at RT. Following the washes in PBST and PBS, the worms were incubated for 4 h at 37 °C in terminal transferase enzyme (Chemicon,Cat. No. 90,418) diluted in reaction buffer (Chemicon, Cat. No.90,417). Enzyme-treated worms were stopped by washing in stop/wash buffer (90,419; EMD Millipore) and rinsed in PBSTB (PBST with 0.25% BSA), and these animals were incubated in anti–digoxigenin-rhodamine (90,429; EMD Millipore) diluted in blocking solution (90,425; EMD Millipore) for 4 h at RT. Stained animals were rinsed on a platform shaker at room temperature in PBSTB for 4 × 10 min and mounted under cover slips on glass slides. The signals were detected using transmission electron microscopy.

2.7. Statistical analyses

Data were presented as means ± SD, and statistical analyses were performed by Students. The datas of two groups or one-way analysis of variance for three or more groups were analysed. $P < 0.05$ was considered a significant difference.

3. Results

3.1. Spatiotemporal expression pattern of cdc42 during regeneration

We identified a homologue of *cdc42* in the *D.japonica* transcriptome (see alignment in Fig. S1). To determine the expression patterns of *cdc42* in planarians, whole-mount RNA in situ hybridization (WISH) was performed in intact and regenerative worms. Worms were amputated into three pieces before and after the pharynx for regeneration experiments. The fragments were fixed at 1, 3, and 7 d post amputation. In intact animals, we observed a ubiquitous expression throughout the body with no specific localization to tissue or organs (Fig. 1A). One day after amputation, the expression level of *cdc42* was higher in wound region (Fig. 1B). The high expression levels of *cdc42* in the presumptive head and tail region were continuously during the course of regeneration (Fig. 1B). When the regeneration became complete 7 d after amputation, the expression of *cdc42* mainly distribute in the newly regenerated head and tail and the pharynx-forming region (Fig. 1B). Together these results indicate that *cdc42* might function as a wound-induced gene during the regeneration process.

3.2. Cdc42 is required for proper regeneration and tissue homeostasis

To investigate the function of *cdc42* in planarians, we performed RNAi to knock down individual *cdc42* genes in intact and regenerating animals. Animals were microinjected containing double-stranded RNA (dsRNA) twice a day, and then amputated transversely into three fragments on day 2 after injecting continuously for 6 d. At 7 d after amputation, we observed that control (RNAi) planarians have

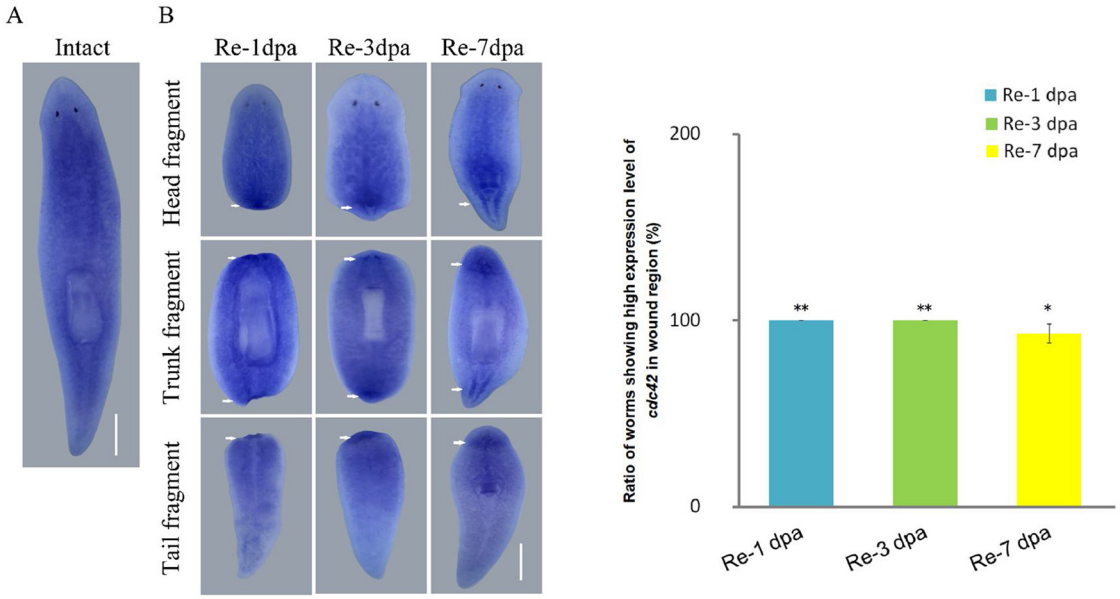

Fig. 1. *Cdc42* expression patterns in planarians. (A) *Cdc42* expression in intact worms (n = 20) detected by whole-mount in situ hybridization. **(B)** Expression patterns of *cdc42* in regenerating worms (n = 20) at 1, 3 and 7 d after amputation. The arrowheads point to the higher expression of *cdc42* in wound region. For each time point, three experimental replicates were performed. Error bars represent SEM. *P < 0.05, **P < 0.001; The scale bars: 300 μm.

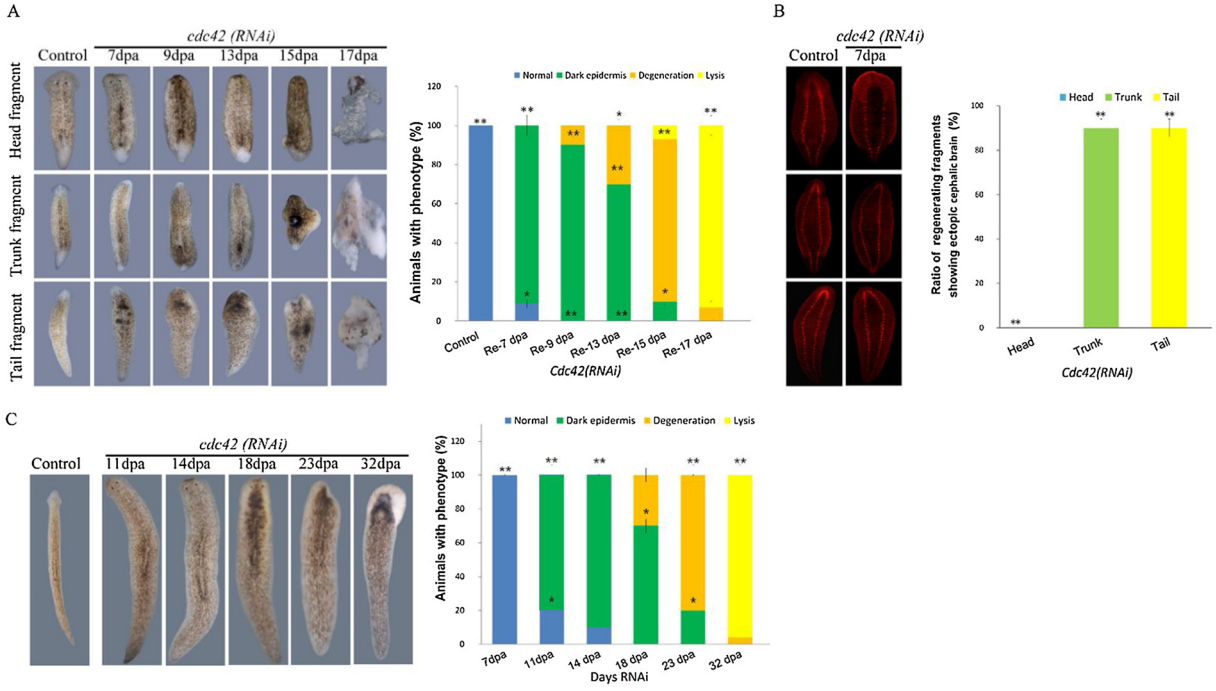

Fig. 2. RNAi knockdown of *cdc42* causes regeneration and tissue homeostasis defects. (A) Regeneration defects with injecting *cdc42* dsRNA into planarians. Worms are amputated 2 d after RNAi injecting 6 d. **(B)** Immunostaining with anti-synapsin (Syn). The worms were fixed at 7 d post amputation. **(C)** Phenotypes of intact *cdc42(RNAi)* planarians. First panel shows a view of a control(RNAi) worm. Uninjured *cdc42(RNAi)* worms exhibited head degeneration phenotype around day 11. The defects characterized by darkening of the epidermis in the ante-pharyngeal region and their head were complete loss by day 23. The arrowheads point to the darker region of epidermis. *n* = 30 total animals used in three biological replicates. Error bars represent SEM. *P < 0.05, **P < 0.001; Scale bars: 300 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

regenerated complete heads and tails (20/20) (Fig.2A). In contrast, the regeneration of *cdc42(RNAi)* animals was impaired. The body fragments failed to regenerate a normal head or tail. A darker region of epidermis could be observed by 7 d of regeneration (27/30) (Fig.2A). All body fragments followed by the more severe degeneration and completely loss of their head or tail by day 15. After 17 d of regeneration, the *cdc42(RNAi)* animals began to lyse (28/30) (Fig. 2A). Additionally, the development of cephalic brain was inhibited even though nerve cord regenerated completely in the trunk and tail fragments of *cdc42(RNAi)* animals (Fig. 2B). These data indicate that *cdc42* is required for proper regeneration in planarians.

We next tested whether *cdc42* was also required for tissue homeostasis. RNAi experiments were performed on intact adult animals. Indeed, we found that worms subjected to *cdc42*(*RNAi*) exhibited macroscopic signs of tissue degrowth (30/30) (Fig. 2C). The epithelia are also abnormal in the ante-pharyngeal region. Eventually, their

bodies are completely lysed by day 32 (28/30) (Fig. 2C). These data suggest that *cdc42* is required for both regeneration and tissue homeostasis in planarians. According to the defect of epidermis, we propose that knockdown of *cdc42* cause a profound defect in epidermis regeneration. It may disrupt the maintenance of epidermal cell lineages during tissue regeneration and homeostasis. If the defect is in the epidermal neoblasts, progeny cells, or a combination of both, it is possible that a series of defective phenotypes appear. Therefore, we next analysis the effect of *cdc42* on cell division and the maintenance of epidermal lineage during the course of the *cdc42(RNAi)* phenotype.

3.3. The effect cdc42(RNAi) on cell division and differentiation of epidermal lineage

To assess the effect of *cdc42* RNAi on cell division, we performed wholemount immunofluorescence for phosphorylated histone H3 (H3ser10p), a marker of dividing cells beginning at the G2 M^{-1} cell-phase (Lin and Pearson, 2017), in *cdc42(RNAi)* regenerating heads, trunks, and tails 7 d post amputation. However, the number of Phospho-$H3^{+}$ cells in *cdc42(RNAi)* animals was comparable to that of control (RNAi) animals (Fig. S2). Consistently, the *cdc42* RNAi did not affect the number of Phospho-$H3^{+}$ cells in intact animals either (Fig. 3A). These results indicate that knockdown of *cdc42* has no effect on cell division. We next assessed that whether the abnormal phenotypes during regeneration and tissue homeostasis were resulted from the perturbation of the maintenance of epidermal lineage.

To determine which cell types were affected, we examined the expression patterns of epidermal cell lineage makers in the *cdc42(RNAi)* phenotype. The *piwi-1*, *prog-1 and agat-1* were assigned to epidermal-lineage cell types and mark undifferentiated neoblasts, early progeny and late progeny, respectively (Eisenhoffer et al., 2008; Hayashi et al., 2010; van Wolfswinkel et al., 2014). The expression patterns of the neoblasts marker *piwi-1* showed unaltered compared with controls at day 3 and 11 in *cdc42(RNAi)* animals (Fig. 3B). Correspondingly, the qRT-pCR analysis also showed that the expression levels of *piwi-1* were unaltered after *cdc42* RNAi (Fig. 3B). These results further indicate that *cdc42* does not affect the proliferation of epidermal cells. Conversely, the expression levels of early progeny *prog-1* was significantly increased in *cdc42(RNAi)* at day 3 and 11 after RNAi (Fig. 3C). The *agat-1* cell population was increased only at day 11 (Fig. 3D). As *agat-1* is expressed in stem cell progeny at 3-4 d after their birth, it is normal that the expression of *agat-1* has no change at day 3 (Fig. 3D). These results were supported by parallel experiments using qRT-PCR (Fig. 3C–D). Together, these data show that *cdc42* RNAi promote the differentiation from neoblasts to the epidermal lineages.

We have identified that *cdc42* have effect on early and late stage epidermal progenitors. However, the *prog-1* (early progeny) and *agat-1* (late progeny) are not expressed in the mature epidermis cell. To further detect the effect on epidermal differentiation, we performed whole-mount in situ hybridizations (WISH) with epidermal boundary marker *laminB*, which had been identified to be expressed only in the mature epidermal cells (Tazaki et al., 2002; Wurtzel et al., 2017). We found that the *cdc42(RNAi)* animals lost the *laminB*$^{+}$ cells at the wound sites in regenerating fragments (Fig. 3E). The decreased expression levels of *laminB* could also be observed in intact animals especially at the head region (Fig. 3F). These results suggest that *cdc42* RNAi lead to the disruption of the epidermal lineage maintenance at the mature stage in both regeneration and homeostasis.

3.4. Cdc42 involved in the regulation of apoptotic cell death

The tissue maintenance and repair require the balance between stem cell proliferation, differentiation, and cell death. We next asked whether *cdc42* is required for cell death. Using TdT-mediated dUTP nick-end labeling (TUNEL) assay (Gold et al., 1993), we first observed an excessive number of apoptotic cells in the intact *cdc42(RNAi)* animals at day 11 when the degrowth phenotype appeared (Fig. 4A). To further analysis the effect of *cdc42* RNAi on apoptosis, we next performed TUNEL assay at day 1, 3, 5, and 7 after *cdc42* RNAi. The excessive apoptosis appears at the first day and followed by a consistently dramatic increase during the course of *cdc42* RNAi phenotypes (Fig. 4B). These results suggest that *cdc42* RNAi induced an excessive apoptotic cell death, and this effect become more and more significant as the *cdc42* RNAi proceeds. We propose that the over apoptotic response caused by *cdc42* RNAi is probably responsible for the phenotype of worms lysis and the decreased mature epidermal cells in intact worms.

Apoptosis controls the precise number of cells for different types of organs or tissues. It is crucial for in tissue remodeling during planarian regeneration (Pellettieri et al., 2010; Beane et al., 2013). The defects of lysis during regenerating in *cdc42(RNAi)* planarians are also possibly associated with excessive apoptosis. Amputation in planarians triggers two peaks of apoptosis. The initial wave localized in near the wound region and can be detected 4 hours after amputation. The second peak, which appears 3 d after injury, spreads throughout the organism (Pellettieri et al., 2010; Almuedo-Castillo et al., 2014). We performed an analysis of apoptotic cell death at 4 hours and 3 d after anterior amputation by using the TUNEL assay. A significant increase was observed in wound region at 4 hours after amputation compared with controls. The apoptosis cells in pre-existing regions of *cdc42(RNAi)* animals was apparently increased and occurred 3 d after amputation (Fig. 4C). These findings indicate that *cdc42* is essential to controlling wound-induced apoptotic cell death only in homeostasis but also in tissue regeneration process.

4. Discussion

The planarian is a unique model system to investigate stem cell lineage development in the context of homeostasis and regeneration. The mechanisms underlying post-mitotic differentiation of neoblasts to generate mature tissue cell types is still not well understood. In this work a major new role for *cdc42* in planarian regeneration is presented. The removal *cdc42 by* employing RNAi led to an increased of epidermal progenitors and a loss of the mature epidermal cells. In addition, we show that *cdc42* is required for planarian regeneration by regulating proper apoptotic cell death without affecting cell division.

It has been reported that *cdc42* can control many cellular functions from yeast to mammals (Zhang et al., 2019). Previous study suggested that cdc42 influence cell proliferation and the deletion of cdc42 inhibit cardiomyocyte proliferation during the heart development of mice(Li et al., 2017). Additional findings suggest cdc42 palys critical role in the cell differentiation, such as chondrocyte, Th2 cell, β cell and leukemia cell(Kesavan et al., 2014; Suzuki et al., 2015; Yang et al., 2019). In this report, we demonstrate that *cdc42* is required for epidermal cell differentiation in planarian regeneration. We observed the epithelia of all *cdc42* RNAi phenotype were abnormal and darker (Fig.2A and C). The *cdc42* RNAi epidermal defects was further underscored by a significant decreased in mature epidermal cell during regeneration and homeostasis (Fig.3E–F). The expression of epidermal progeny markers *prog-1* and *agat-1* was significant increased in different time point after RNAi (Fig.3C–D). Combined with the decreased mature epidermal cell, these results indicate that *cdc42* is required for the differentiation of epidermal cell lineages. This requirement provides an explanation for the higher expression level of *cdc42* in injury region during regeneration in planarians (Fig.1A). For the population difference between epidermal progenitors and mature epidermal cell, we propose that *cdc42* RNAi lead to a reduced mature epidermal cells, the increased epidermal progenitors might attempt to differentiate into mature epidermal cells to restore the maintenance of epidermal cell lineages.

Although *cdc42* is widely expressed and plays critical functions in embryogenesis and tumorigenesis, but its exact function in adult homeostasis and regeneration is unknown. It has shown that the

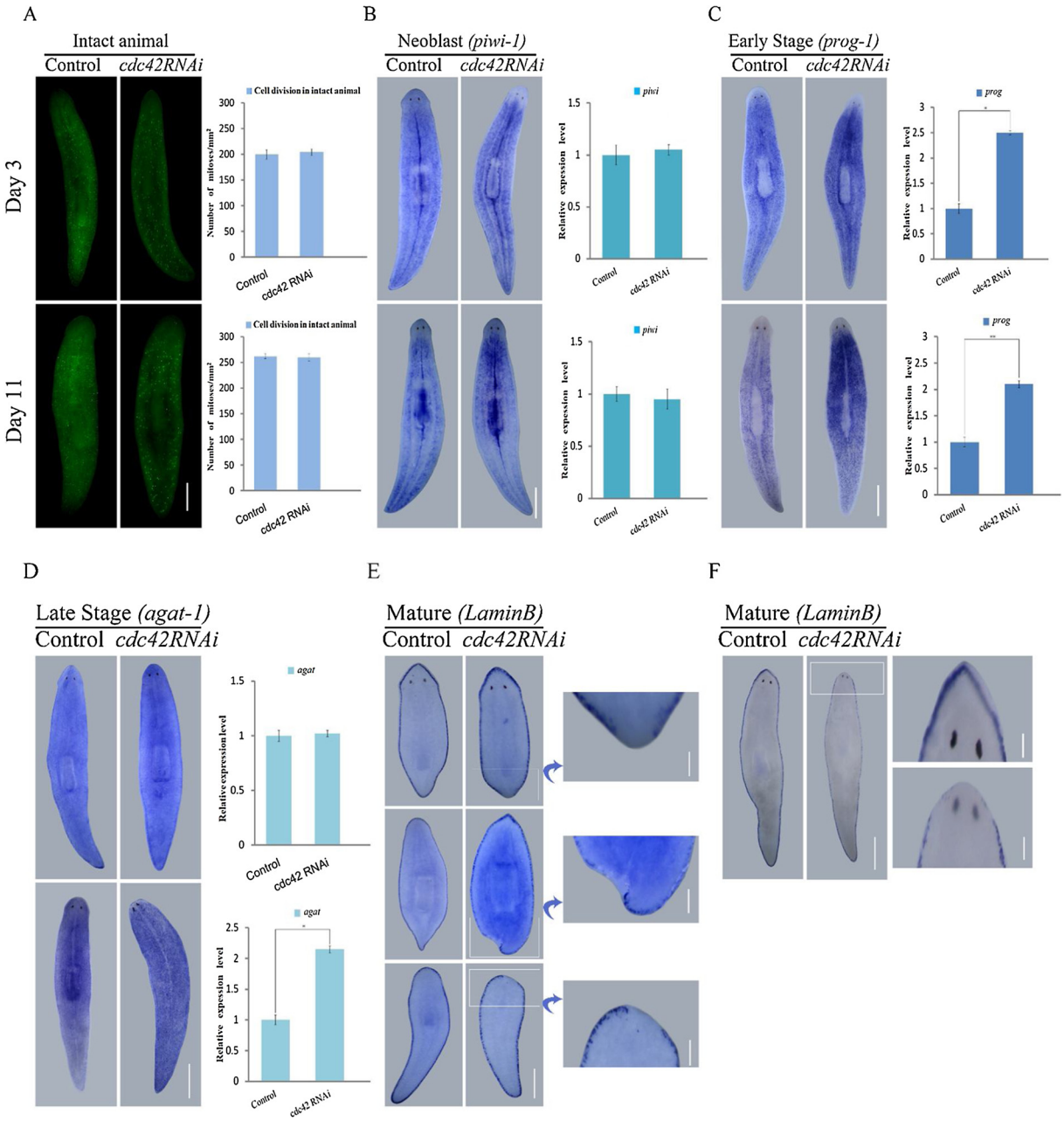

Fig. 3. Analysis of cell division and stem cell lineage in the *cdc42(RNAi)* phenotype. (A) Phospho-H3 + staining in intact *cdc42(RNAi)* animals. **(B-D)** WISH of uninjured intact animals stained for neoblasts markers *piwi-1***(B)**, early progeny marker *prog-1***(C)** and late progeny marker *agat-1***(D)** during the initial phase (day 3) and the late phase (day 11) of the *cdc42(RNAi)* phenotype. **(E)** WISH for the expression of *laminB*, which is expressed only in the mature epidermal cells. **(F)** The reduced expression level of *laminB* in intact animals after 11 d of RNAi. For each time point, $n = 30$ animals with three experimental replicates. Error bars represent SEM. *P < 0.05, **P < 0.001; Scale bars: 300 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

function of *cdc42* is controlling cell death in neuroblastoma, T lymphocytes and colorectal cancer(Chuang et al., 1997; Stengel and Zheng, 2011; Xu et al., 2017). Similarly, *cdc42* is crucial in the regulating of apoptotic cell death during adult homeostasis and regeneration. A global increase in TUNEL + cells was found in tissue regeneration and homeostasis after *cdc42* RNAi-treating (Fig.4A–C). These results suggest that the effect of *cdc42* RNAi on apoptosis is independent of the regeneration process. The excessive apoptosis also provide an explanation for the reduced mature epidermal cells. These data indicate cdc42 is required for cell differentiation and controlling cell death during epidermal morphogenesis. Taken together, we reason that the down-regulation of *cdc42* causes epidermal cells apoptotic uncontrollable, epidermal progeny cells hyper-proliferated to maintain epidermal mature cell proper population but unaffected the differentiation from neoblasts to epidermal progenitors.

The epithelium surrounding the planarian body is a simple, monostratified tissue (Rompolas et al., 2010). When injured, the epidermis would stretch out over the amputation-induced wound site through cell spreading (Morita and Best, 1974; Tu et al., 2015). This process is required for worms survival. The epidermal progenitors derived from mitotic neoblasts and undergo differentiation in a spatially and temporally and ultimately integrate into the mature epidermis, a single-layered epithelial sheet (Tu et al., 2015; Wurtzel et al., 2017). However, the mechanisms about how the worm's adult stem cells develop into the

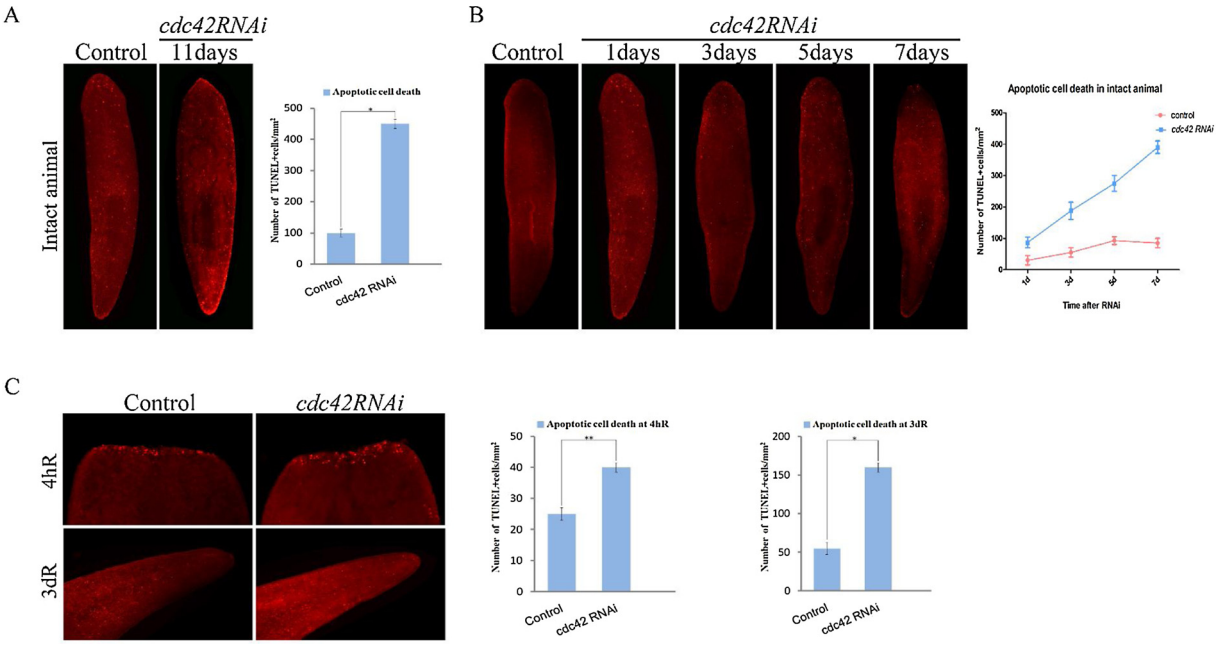

Fig. 4. ***Cdc42*** **RNAi induces an excessive cell apoptosis in planarian regeneration and tissue homeostasis. (A)** Whole-mount TUNEL staining showing apoptotic cells at day 11 when the degrowth phenotype appeared. **(B)** TUNEL assays were performed at different time point in uninjured worms. Graph depicting the quantification of TUNEL + cells in *cdc42*(RNAi) and control*(RNAi)* animals. **(C)** Apoptosis in regenerating planarians. Whole-mount TUNEL staining showing apoptotic cells in the wound region (4 hR) and in pre-existing regions (3 dR) after anterior amputation. $n = 30$ animals used in experiment at different time point. Data obtained from triplicates per experiment of three biological replicates. *P < 0.05; Scale bars: 300 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

epidermal cells to form planarian skin remains unclear. Our results reveal that this process requires precise coordination between stem cell proliferation, differentiation, and cell death. For example, cdc42 RNAi influence the differentiation of epidermal cell lineages by just disrupting the proper apoptotic cell death without affecting cell division.

Taken together, we propose that *cdc42* is required for the epidermal regeneration in planarians. The knockdown of *cdc42* induces the excessive apoptosis, and leads to the loss of mature epidermal cells. The epidermal progenitor cells attempt to repair these defects through hyper-proliferation. However, the sustained increased cell death results in a collapse of the epidermis cell lineage and eventually leads to the lysis of the *cdc42* RNAi worms. Our study helps to elucidate the mechanism of epidermal cell differentiation and apoptosis during epidermal morphogenesis.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.05.008.

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