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ERK signaling controls blastema cell differentiation during planarian regeneration

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SUMMARY

The robust regenerative ability of planarians depends on a population of somatic stem cells called neoblasts, which are the only mitotic cells in adults and are responsible for blastema formation after amputation. The molecular mechanism underlying neoblast differentiation associated with blastema formation remains unknown. Here, using the planarian *Dugesia japonica* we found that *DjmkpA*, a planarian mitogen-activated protein kinase (MAPK) phosphatase-related gene, was specifically expressed in blastema cells in response to increased extracellular signal-related kinase (ERK) activity. Pharmacological and genetic [RNA interference (RNAi)] approaches provided evidence that ERK activity was required for blastema cells to exit the proliferative state and undergo differentiation. By contrast, *DjmkpA* RNAi induced an increased level of ERK activity and rescued the differentiation defect of blastema cells caused by pharmacological reduction of ERK activity. These observations suggest that ERK signaling plays an instructive role in the cell fate decisions of blastema cells regarding whether to differentiate or not, by inducing *DjmkpA* as a negative regulator of ERK signaling during planarian regeneration.

KEY WORDS: Extracellular signal-related kinase (ERK), Blastema, Stem cells, Regeneration, Planarian

INTRODUCTION

Regeneration is the complex process by which animals properly reconstitute missing body parts after injury. The ability to regenerate varies greatly among animals, and is often higher in basal metazoans and lower in mammals. Key events of regeneration include successive phases of wound healing, the formation of a blastema (a mass of morphologically undifferentiated cells at the end of the stump) and differentiation to reconstitute lost tissues and organs. The origin and cellular state of blastema cells remains open to debate in many regeneration contexts (Stoick-Cooper et al., 2007) and a deeper understanding of the mechanisms underlying blastema formation and blastema cell differentiation is required to provide a basis for inducing regenerative responses in humans as novel clinical treatments for injury.

In the past decade, great advances in planarian studies have revealed many of the molecular mechanisms that regulate regeneration. The robust regenerative ability of planarians depends on a population of pluripotent stem cells called neoblasts (Baguñà, 1981; Baguñà et al., 1989; Agata and Watanabe, 1999; Newmark and Sánchez Alvarado, 2000). Neoblasts can self-renew and give rise to all missing cell types, including germ cells (Newmark et al., 2008). Neoblasts are defined morphologically as a unique population of cells (Coward, 1974; Hay and Coward, 1975; Hori, 1982; Hayashi et al., 2006; Higuchi et al., 2007) and express a specific set of 'stemness' genes (Shibata et al., 1999; Reddien et al., 2005; Guo et al., 2006; Oviedo and Levin, 2007; Rossi et al., 2007; Yoshida-Kashikawa et al., 2007; Eisenhoffer et al., 2008; Rouhana et al., 2010). X- or gamma-ray irradiation, which specifically eliminates neoblasts and not differentiated cells (Shibata et al., 1999), results in complete loss of regenerative ability in planarians (Wolff and Dubois, 1948). Another cellular characteristic of neoblasts is that they are the only mitotic somatic cells in adults and can therefore be visualized with universal mitotic cell markers and by BrdU incorporation (Newmark and Sánchez Alvarado, 2000; Salvetti et al., 2000; Orii et al., 2005; Hayashi et al., 2010). After amputation, neoblasts form a blastema at the end of the stump, which develops into distal structures such as the head or tail regions depending on the body axes (Agata and Watanabe, 1999; Agata et al., 2003; Agata and Umesono, 2008; Umesono and Agata, 2009). It has been reported that regeneration proceeds through two mitotic phases (Wenemoser and Reddien, 2010). The first phase occurs in response to wounding and the second occurs in response to loss of tissues after amputation. Active neoblast proliferation associated with blastema formation is found at the pre-existing tissue positioned most proximal to the blastema, termed the 'postblastema' region (Sálo and Baguñà, 1984), whereas no mitosis is found within the blastema itself despite the steadily increasing number of blastema cells during regeneration (Sálo and Baguñà, 1984; Eisenhoffer et al., 2008; Wenemoser and Reddien, 2010).

Much knowledge has been gained about the functional role of many genes involved in the regulation of neoblast maintenance (for a review, see Shibata et al., 2010) by using RNA interference (RNAi) (Sánchez Alvarado and Newmark, 1999). However, the molecular mechanism underlying the switch from neoblast proliferation to differentiation associated with blastema formation remains unknown. In order to gain insight into the molecular mechanisms that regulate differentiation of neoblasts during blastema formation, we performed

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high-coverage expression profiling (HiCEP), a cDNA-amplified fragment length polymorphism (AFLP)-based gene expression profiling method (Fukumura et al., 2003). From this, we identified a mitogen-activated protein kinase (MAPK) phosphatase-related gene (*DjmkpA*), as a reliable marker for blastema cells in the planarian *Dugesia japonica*. Furthermore, we demonstrated that MAPK extracellular signal-related kinase (ERK) and *DjmkpA* form a negative-feedback loop in blastema cells that is required for proper neoblast differentiation. Our findings here provide the first molecular evidence delineating cellular events required for proper differentiation of blastema cells in planarians.

MATERIALS AND METHODS

Animals

A clonal strain of the planarian *Dugesia japonica* derived from the Iruma River in Gifu prefecture, Japan, which was maintained in autoclaved tap water at 22-24°C, was used in this study. Planarians 6-8 mm in length were starved for at least 1 week before experiments.

HiCEP analysis

Total RNA from ~60 fragments in each sample (head-regenerating tissues at 12 or 24 hours after amputation, tail-regenerating tissues at 12 or 24 hours after amputation, and non-regenerating tissues as a reference) was isolated using Isogen (Nippon Gene). Using these five different sources of total RNAs as templates, HiCEP analysis was performed as described previously (Fukumura et al., 2003). Briefly, cDNA synthesized with oligo-d(T) primer was digested with two restriction enzymes, *Taq*I and *Mse*I, followed by the ligation of adapters. Selective PCR was then conducted using 256 primer sets, followed by capillary electrophoresis.

Cloning of the *DjmkpA* cDNA by 5′-RACE

To obtain the full-length *DjmkpA* sequence, 5′-RACE (rapid amplification of cDNA ends) was performed using a SMART TM RACE cDNA Amplification Kit (Clontech Laboratories). The PCR products were cloned into TA vector pGM-T Easy (Promega) and sequenced.

X-ray and gamma-ray irradiation

X-ray irradiation was performed as described by Yoshida-Kashikawa et al. (Yoshida-Kashikawa et al., 2007). Gamma-ray irradiation was performed with 15,000 cGy using a Gammacell 40 Exactor (Best Theratronics). Five days after irradiation, planarians were amputated and allowed to undergo regeneration.

Whole-mount in situ hybridization

Animals were treated with 2% hydrochloric acid (HCl) in 5/8 Holtfreter's solution for 5 minutes at 4°C and fixed in 5/8 Holtfreter's solution containing 4% paraformaldehyde and 5% methanol for a maximum of 2 hours at 4°C. Hybridization and color detection of digoxigenin (DIG)-labeled RNA probes were carried out as described previously by Umesono et al. (Umesono et al., 1997). TSA Labeling Kit No. 2 or No. 15 (Molecular Probes) or HNPP Fluorescent Detection Set (Roche) was used for detection of fluorescence (Yoshida-Kashikawa et al., 2007).

In situ hybridization of sections

Preparation of paraffin section samples was carried out as described by Takeda et al. (Takeda et al., 2009). Sections were deparaffinized with xylene and ethanol solutions of gradually decreasing concentration and acetylated with acetic acid anhydride. Then these samples were hybridized with a DIG-labeled *DjpiwiA* probe, as described by Umesono et al. (Umesono et al., 1997). To detect the signal, TSA Labeling Kit No. 15 (Molecular Probes) was used. Cell nuclei were labeled with Hoechst 33342 (Invitrogen).

Treatment with chemical inhibitors

The MAPK/ERK kinase (MEK) inhibitor U0126 (Cell Signaling Technology) was dissolved in DMSO and used at a final concentration of 25 μM (unless otherwise indicated) in 0.25% DMSO solution. Amputated planarians were allowed to regenerate in tap water supplemented with each inhibitor either immediately after amputation or from 12 hours after amputation until the indicated period of regeneration for each experiment.

Antibody generation and purification

Peptide GIL(pT)E(pY)VATR corresponding to the diphosphorylated form of the presumptive activation loop of the deduced protein encoded by the planarian *erk1/2*-related gene *DjerkA* was synthesized by Medical & Biological Laboratories (MBL). A rabbit polyclonal antibody against the diphosphorylated peptide was generated and affinity purified by MBL using a diphosphorylated DjERKA peptide column. Purified antibody fractions were further affinity purified by MBL using a non-phosphorylated DjERKA peptide column. Flow-through fractions from the peptide affinity column were pooled as anti-phosphorylated-specific antibody. The specificity of each antibody was confirmed by MBL by performing an enzyme-linked immunosorbent (ELISA) assay.

Whole-mount immunostaining

Planarians were treated with 2% HCl in 5/8 Holtfreter's solution for 5 minutes at room temperature and washed three times with 5/8 Holtfreter's solution at room temperature. They were then fixed in 5/8 Holtfreter's solution containing 4% paraformaldehyde, 5% methanol and PhosSTOP phosphatase inhibitor (Roche) for 3 hours at 4°C. To decrease background signals, fixed planarians were treated with hybridization buffer for whole-mount in situ hybridization overnight at 55°C. They were then blocked with 10% goat serum in PBST (phosphate buffered saline containing 0.1% Triton X-100) for 1 hour at 4°C, and incubated with mouse anti-DjPiwiA (1/1000) (Yoshida-Kashikawa et al., 2007), rabbit anti-phosphorylated histone H3 (1/200; Upstate Biotechnology) or diluted rabbit anti-phosphorylated ERK (1/1000) overnight at 4°C. The samples were washed four times with TPBS for 30 minutes per wash. Signals were detected with Alexa Fluor 488- or 594-conjugated goat anti-rabbit or mouse IgG (1/500; Invitrogen) in 10% goat serum in TPBS for 3 hours at room temperature in the dark. TSA Labeling Kit No. 2 (Molecular Probes) was used for signal amplification.

Western blotting

Sixty fragments from blastemas at 1 or 2 days of regeneration were dissolved in sample buffer (0.01 M Tris-HCl, 2% SDS, 6% 2-mercaptethanol, 10% glycerol) and were boiled for 5 minutes. The samples were then subjected to gel electrophoresis and the gel was subjected to western blotting. Blocking One-P (Nacalai Tesque) was used for membrane blocking. Western blotting was performed using rabbit anti-phosphorylated ERK (1/500), rabbit anti-DjPiwiA (1/1000) or mouse anti-α-tubulin monoclonal antibody DM 1A (1/5000; Sigma) as the primary antibody, and a 1/5000 dilution of each appropriate secondary antibody conjugated with horseradish peroxidase. Signal detection was performed using SuperSignal West Dura Extended Duration Substrate (Pierce).

BrdU incorporation and detection

Bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) labeling was performed by injection as described previously (Newmark and Sánchez Alvarado, 2000). Five days after injection, samples were fixed and sectioned as for in situ hybridization of sections. Deparaffinized samples were treated with 2N HCl for 30 minutes at room temperature. After washing with PBST, samples were treated overnight at 4°C with Anti-BrdU (1/25; Becton, Dickinson). BrdU signals were detected using a TSA Labeling Kit No. 2 (Molecular Probes).

RNA interference

Double-stranded RNA (dsRNA) was synthesized as previously described (Sánchez Alvarado and Newmark, 1999; Cebrià et al., 2002). Control animals were injected with DEPC-treated H_2O alone. One day after the second or third injection, planarians were amputated and allowed to undergo regeneration.

Reverse transcription and quantitative RT-PCR analysis

The reverse transcription reaction was carried out with total RNA from five intact planarians or 15 tail fragments using a QuantiTect Transcription Kit (Qiagen). RT-PCR was performed as reported previously (Yazawa et al., 2009).

The PCR primers used were as follows: *DjmkpA* forward, 5′-CACT-GATATCTACTTCACGAAAGCCAG-3′; *DjmkpA* reverse, 5′-AAG-GCATCCAGTTCATTTCCTAAAT-3′; *DjsFRP-A* forward, 5′-

TTGCTCTCTTTACGCTCCGGT-3′; *DjsFRP-A* reverse, 5′-CGCATAGTTCCCTGCATGGT-3′; *DjerkA* forward, 5′-GTGAAGGAGCTTATGGTCAAGTTGTAT-3′; *DjerkA* reverse, 5′-TGTCCGCTTTATCGCAACATT-3′; *DjpiwiA* forward, 5′-CGAATCCGGGAACTGTCGTAG-3′; *DjpiwiA* reverse, 5′-GGAGCCATAGGTGAAATCTCATTTG-3′; *Djpcna* forward, 5′-ACCTATCGTGTCACTGTCTTTGACCGAAAA-3′; *Djpcna* reverse, 5′-TTCATCATCTTCGATTTTCGGAGCCAGATA-3′; *DjMCM2* forward, 5′-CGCTGTTGGACAAGGTCAGAAGAATGAACA-3′; *DjMCM2* reverse, 5′-CCAGAAACACAAATCTACATCTTCCAAAGG-3′; *DjMCM3* forward, 5′-GAGTCAGTTCCAAATCATCGATTATATCCT-3′; *DjMCM3* reverse, 5′-TTCAAGGATGTCCTGAAGAAGACGAACAAG-3′.

RESULTS

Identification of *DjmkpA*, a planarian member of the mitogen-activated protein kinase phosphatase (mkp) gene family

We performed a detailed molecular analysis of early events in the process of blastema formation of the planarian *D. japonica* by using high-coverage expression profiling (HiCEP) (Fukumura et al., 2003). For HiCEP analysis, cDNA was prepared from five distinct sources, namely from head-regenerating tissues 12 and 24 hours after amputation, tail-regenerating tissues 12 and 24 hours after amputation, and non-regenerating tissues (time 0) as a reference (see Fig. S1 in the supplementary material). HiCEP analyses identified a gene fragment for which the expression level in each of these regenerating tissues was about ten times higher than that in the reference (data not shown). The sequence of the fragment perfectly matched that of a planarian expressed sequence tag (EST) clone encoding for a protein highly similar to mitogen-activated kinase phosphatases (MKP) (see Fig. S2 in the supplementary material). Phylogenetic analysis indicated that this fragment was not a clear ortholog of any specific vertebrate mkp gene (data not shown). Therefore, we named this gene *DjmkpA* (*Dugesia japonica mitogen-activated kinase phosphatase A;* accession number AB576208).

Spatiotemporal expression pattern of *DjmkpA* during regeneration

We examined the spatiotemporal expression pattern of *DjmkpA* during regeneration by whole-mount in situ hybridization. Planarians were transversely dissected into three pieces: head, trunk (including pharynx) and tail, and allowed to regenerate for 7 days. In intact animals, there was no obvious expression of *DjmkpA* (Fig. 1A). One day after amputation, strong expression of *DjmkpA* was observed at the anterior and posterior stumps (Fig. 1B,C). High levels of *DjmkpA* expression were detected in the presumptive head region and in the presumptive tail region of regenerating fragments 3 days after amputation; *DjmkpA* expression was also observed at lower levels in the prepharyngeal region (Fig. 1D). At this stage, *DjmkpA* was expressed in a subset of cells in the pharynx-forming region of head and tail fragments (Fig. 1D). The expression of *DjmkpA* gradually decreased as the regeneration became complete 7 days after amputation (Fig. 1E).

We next examined *DjmkpA* expression during the first hours of blastema formation (Fig. 2A-E). The expression of *D. japonica noggin-like gene A* (*DjnlgA*) (Ogawa et al., 2002) was used as an indicator of the onset of regeneration (Fig. 2F-J). The expression of *DjpiwiA* (Yoshida-Kashikawa et al., 2007; Hayashi et al., 2010) was used as a reliable marker of undifferentiated neoblasts (Fig. 2K-O). To assess whether *DjmkpA*-positive cells are derived from neoblasts or from already existing differentiated cells, the *DjmkpA* expression was also examined in X-ray-irradiated animals

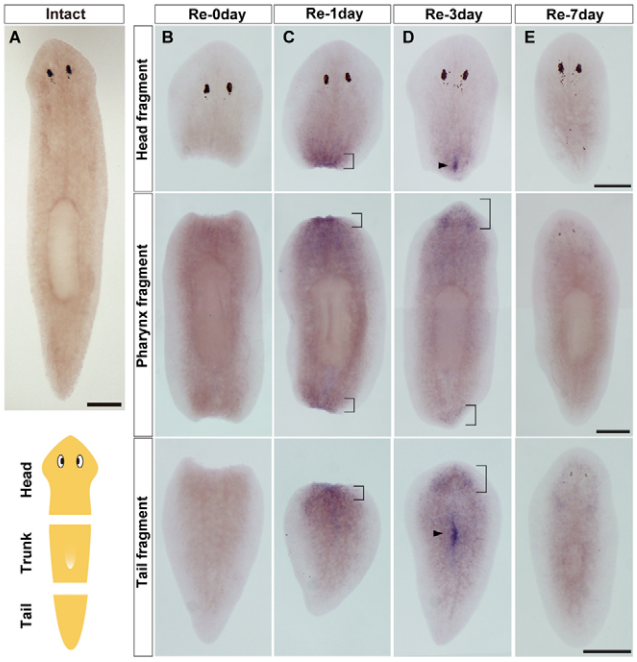

Fig. 1. *DjmkpA* expression during planarian regeneration. (**A**) *DjmkpA* expression in intact animals assessed by whole-mount in situ hybridization. There is no obvious expression of *DjmkpA*. (**B-E**) Expression pattern of *DjmkpA* in regenerants at 0 (B), 1 (C), 3 (D) and 7 (E) days after amputation. Strong expression of *DjmkpA* is transiently observed in regenerating regions. Brackets indicate regenerating head or tail regions. Arrowheads indicate the *DjmkpA* expression in the pharynx-forming region. Anterior is to the top. Scale bars: 500 μm.

selectively deprived of neoblasts (Fig. 2A′-E′,K′). During regeneration of trunk fragments, strong expression of *DjmkpA* was detected in the stump of the amputated regions within 3 hours of regeneration (Fig. 2B) and before the onset of *DjnlgA* expression at 6 hours of regeneration (Fig. 2H). *DjmkpA* expression at this time point was not affected by X-ray irradiation (Fig. 2B′), indicating that it originated from differentiated cells. We confirmed that the *DjmkpA* expression occurred in response to the wounding itself by performing a simple assay for wounding (see the detailed experimental scheme in Fig. 2P).

When the expression profiles of *DjmkpA* were compared between the normal and X-ray-irradiated animals, we found that expression of *DjmkpA* detected in differentiated cells 3 hours after amputation became apparent in X-ray-sensitive neoblast-derived blastema cells within 24 hours of amputation (Fig. 2A-E′). Thus, *DjmkpA* is a new reliable marker gene to monitor the processes of blastema formation during regeneration (Fig. 2Q).

DjmkpA functions as a negative-feedback regulator of ERK signaling in blastema cells

MAPK signaling pathways are evolutionarily conserved kinase cascades that regulate diverse cellular functions, including cell proliferation, differentiation, migration and response to stress (Nishida and Gotoh, 1993; Chang and Karin, 2001). In these networks, MKPs dephosphorylate active MAPK and play an important role in regulating MAPK activities (Theodosiou and Ashworth, 2002; Kondoh and Nishida, 2007). Based on the expression of *DjmkpA* in blastema cells, and its relationship to

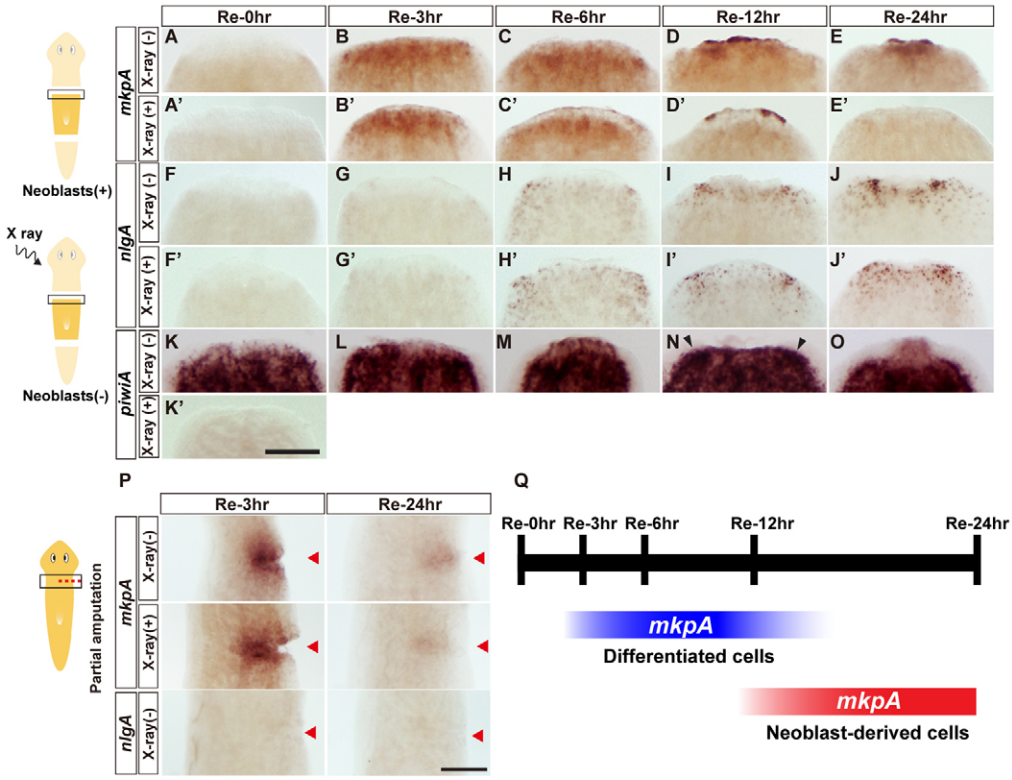

Fig. 2. Expression pattern of *DjmkpA* in the early phase of planarian regeneration from trunk fragments. (**A-E**) Temporal pattern of *DjmkpA* expression. (**A′-E′**) *DjmkpA* expression in X-ray-irradiated neoblast-deficient regenerants. (**F-J**) Temporal pattern of *DjnlgA* expression. (**F′-J′**) *DjnlgA* expression in X-ray-irradiated regenerants. (**K-O**) Temporal pattern of *DjpiwiA* expression. At around 12 hours of regeneration, *DjpiwiA*-positive cells are accumulated at each cut edge (arrowheads). (**K′**) *DjpiwiA* expression in X-ray-irradiated regenerant. *DjpiwiA* expression is not detected in the regenerant. Roughly the same patterns were commonly observed in both the anterior- and posterior-facing stumps (data not shown). (**P**) Simple assay for wounding. Strong expression of *DjmkpA* is induced in X-ray-insensitive differentiated cells at the wounded region at 3 hours post-partial amputation. By 24 hours, *DjmkpA* expression is dramatically reduced at the wounded region. The remaining faint signals at 24 hours after partial amputation are insensitive to X-ray irradiation, indicating that almost all of the *DjmkpA*(+) cells are differentiated cells. This means that partial amputation does not activate the *DjmkpA* expression in neoblasts. Arrowheads indicate the site of partial amputation. (**Q**) A summary of *DjmkpA* expression after amputation. *DjmkpA* expression gradually shifts from X-ray-insensitive differentiated cells to X-ray-sensitive blastema cells during regeneration. Anterior is to the top. Scale bars: 500 μm.

MAPK signaling, we examined the activation of the MAPKs ERK and c-Jun N-terminal kinase (JNK) early in the process of blastema formation by using an antibody against phosphorylated ERK (pERK, an indicator of ERK activity; Fig. 3A) and an antibody against phosphorylated JNK (pJNK, an indicator of JNK activity). We found that activated ERK was readily detected in the blastema, and confirmed the co-expression of pERK and *DjmkpA* in blastema cells at the single cell level (Fig. 3A,A′). By contrast, pJNK expression was observed not in the blastema, but in the ‘postblastema’ region (Tasaki et al., 2011).

To analyze the functional relationship between pERK and *DjmkpA* in the blastema cells, we examined the effects of various concentrations (maximum 25 μM) of a MEK inhibitor (U0126), which causes ERK inactivation (Fig. 3B), and of knockdown of the function of *DjmkpA* by RNAi of ERK signaling. We demonstrated by western blotting that pERK signal was already detected around the stump at time 0 (Fig. 3C). This signal was not sensitive to specific elimination of neoblasts by X-ray irradiation (Fig. 3C), indicating that ERK activation occurred in already existing differentiated cells. As regeneration proceeded, the majority of the pERK signal shifted from the differentiated cells to X-ray-sensitive neoblast-derived cells within 24 hours after amputation (Fig. 3C). Treatment with 10 μM U0126 markedly reduced the level of pERK signal derived from neoblasts (Fig. 3D), leaving the expression of phospho-histone H3 (pH3) roughly normal (Fig. 3D), demonstrating that U0126 did not affect phosphorylation levels of this different branch of the signaling pathway. We also found that treatment with U0126 reduced the *DjmkpA* expression in the blastema at 24 hours post-amputation in a dose-dependent manner (Fig. 3E,G). However, knockdown of the function of *DjmkpA* by RNAi increased the pERK level and caused robust resistance to pharmacological perturbation of the ERK phosphorylation level by 10 μM U0126 (Fig. 3F). The effect of *DjmkpA* RNAi on the ERK activation level in the blastema was confirmed by monitoring the expression level of *DjsFRP-A*, an ortholog of *Smed-sFRP-1*, which is specifically expressed in subsets of the anterior blastema-forming cells (Gurley et al., 2008; Petersen and Reddien, 2008). The expression dynamics of *DjsFRP-A* were highly correlated with alteration of the ERK activity levels by *DjmkpA* RNAi (Fig. 3G), demonstrating that *DjmkpA* tightly regulates the ERK downstream readout in the blastema through

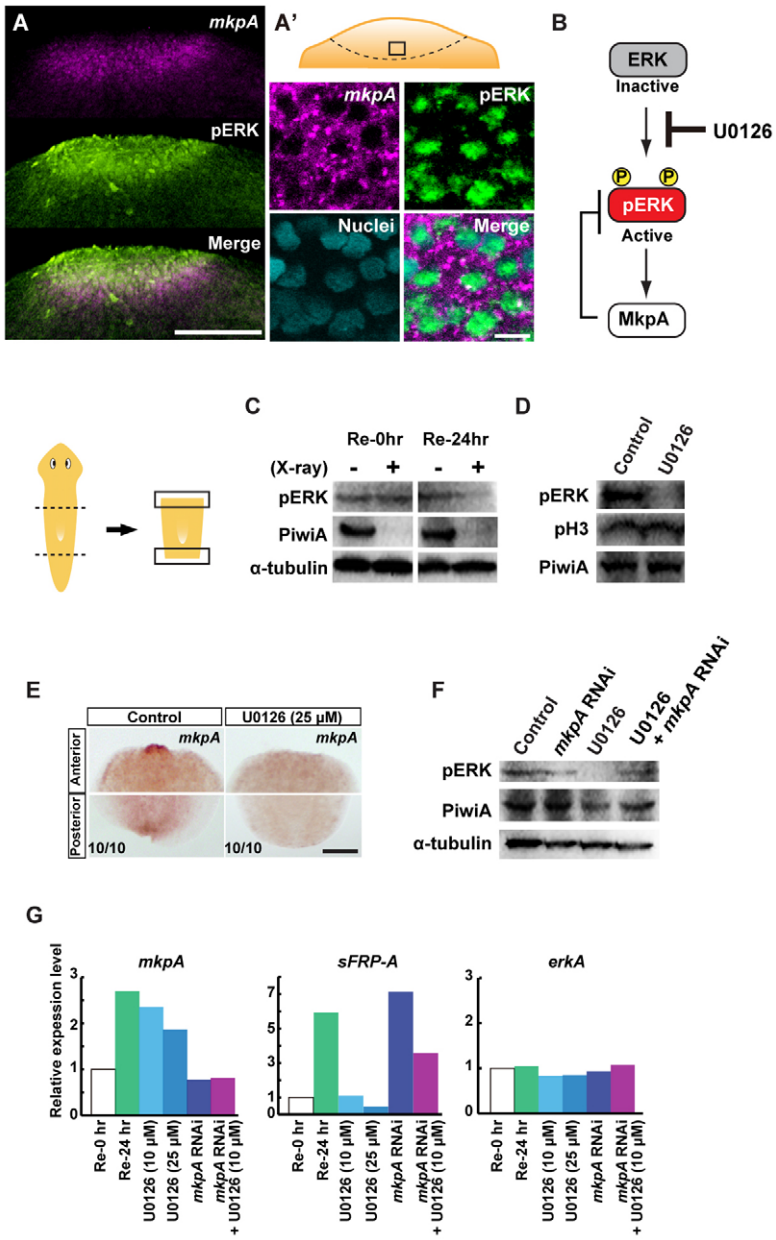

Fig. 3. *DjmkpA* functions as a negative-feedback regulator of ERK signaling in blastema cells. (**A**) Double staining with anti-pERK (green) and *DjmkpA* gene probe (magenta) at 24 hours of regeneration. (**A′**) Upper panel shows a schematic view of panel A. The dashed line indicates the border of the blastema region. The lower panels show higher magnification views of double staining with *DjmkpA* gene probe (magenta) and anti-pERK (green) in the region indicated by the black square in the schematic view. (**B**) Summary of biological activities of the specific MEK inhibitor U0126 and DjMkpA for the ERK signaling pathway. ERK is phosphorylated and activated by its upstream kinase MEK. (**C**) For western blotting analysis, protein extracts were made from X-ray-irradiated or non-X-ray-irradiated fragments including the anterior and posterior blastema that were collected at 2 days after amputation. The pERK signal was detected just after amputation (Re-0 hour). This activation was largely found in differentiated cells. However, the pERK signal at 24 hours after amputation was largely found in neoblast-derived cells. (**D**) Western blotting analysis also indicated that treatment with 10 μM U0126 markedly reduced pERK signals, but did not affect phospho-histone H3 signals. (**E**) *DjmkpA* expression in blastema cells was dramatically reduced at 24 hours of regeneration after 25 μM U0126 treatment. (**F**) *DjmkpA* RNAi increased the pERK level and caused robust resistance to pharmacological perturbation of the ERK phosphorylation level. (**G**) Relative expression levels of *DjmkpA*, *DjsFRP-A* and *DjerkA* genes determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR) at Re-24 hour. Anterior is to the top. Scale bars: 250 μm in A, 20 μm in A′.

the modulation of ERK signaling. Therefore, we concluded that *DjmkpA* functions as a negative-feedback regulator of ERK signaling in blastema cells (Fig. 3B).

ERK inhibition caused differentiation defects of neoblasts during regeneration

Our successful inactivation of ERK signaling by treatment of planarians with U0126 provides a method to examine the role of ERK signaling in blastema cells. Accordingly, we performed detailed histochemical analysis of the effect of ERK inhibition on temporally distinct phases of wound healing and blastema formation by assessing the expression profile of *DjmkpA* (Fig. 2 and Fig. 3A,A′). Planarians were allowed to regenerate in tap water supplemented with various concentrations of U0126 immediately after amputation, or 12 hours after amputation (that is, from the beginning of blastema formation after completion of wound healing) (Fig. 4A). We obtained the same results under these two conditions.

At 3 days post-amputation, we found that treatment with U0126 caused severe defects of both head and tail regeneration in a dose-dependent manner (Fig. 4B,C and Fig. 5B,D,F), although wound healing itself and the early expression of *DjmkpA* in differentiated cells were normal (see Fig. S3 in the supplementary material). We confirmed that this defect was also produced by U0126 treatment from 12 hours of regeneration (Fig. 4C). By contrast, as a negative control, treatment with 25 μM SB203580, a MAPK p38-specific inhibitor, resulted in normal regeneration (Fig. 4E). The regeneration defects caused by U0126 were similar to that caused by loss of neoblasts as a result of X-ray irradiation (Fig. 4C,D). However, we detected a large number of neoblasts in U0126-treated animals (Fig. 4C′,D′), as shown by anti-DjPiwiA antibody staining (Yoshida-Kashikawa et al., 2007). These observations suggest that ERK signaling regulates neoblast dynamics involved in blastema formation, and not neoblast survival itself.

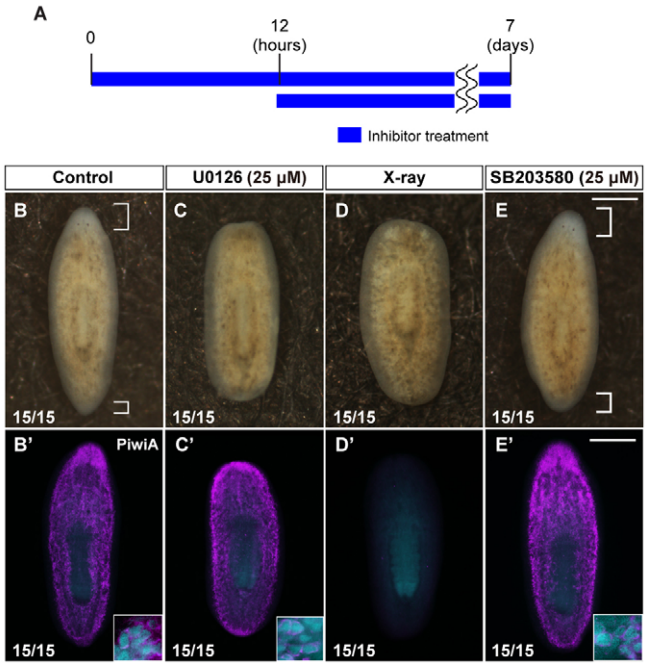

Fig. 4. Effect of U0126 on planarian regeneration. (**A**) Experimental time-schedule. Planarians were allowed to regenerate in tap water supplemented with U0126 either immediately after amputation or from 12 hours after amputation. (**B-E′**) Effect of the ERK inhibitor on regeneration starting from 12 hours after amputation. Upper panels show dorsal images of live regenerants at 3 days of regeneration (B-E). Brackets indicate regenerating head and tail. Lower panels show distribution pattern of anti-DjPiwiA-positive neoblasts (B′-E′). The inset shows a higher magnification view of the staining pattern with anti-DjPiwiA. Hoechst staining indicates nuclei (blue). Scale bar: 500 μm.

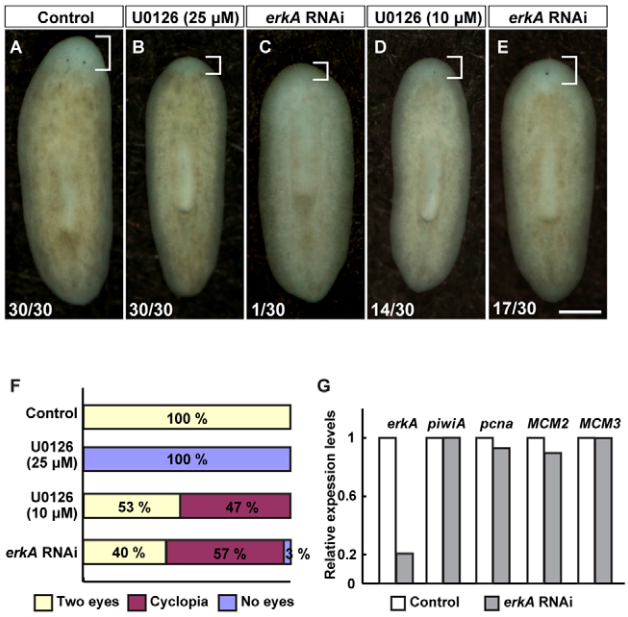

Fig. 5. *DjerkA* RNAi showed phenocopy of the U0126-treated animals. (**A**) At 3 days after amputation, control animals regenerated a head with two eyes (100% animals, *n*=30). (**B**) 25 μM U0126-treated animals showed a no-eye phenotype (100% animals, *n*=30). (**C**) *DjerkA(RNAi)* animals showed phenocopy of the severe defect of 25 μM U0126-treated animals (1/30). (**D**) 10 μM U0126-treated animals showed cyclopia (14/30). (**E**) *DjerkA(RNAi)* animals also showed cyclopia (17/30). (**F**) Quantification of the phenotype in A-E based on the eye number. (**G**) RT-PCR analysis. *DjerkA* RNAi led to severe reduction of *DjerkA* transcripts but not of the transcripts of neoblast-specific marker genes *DjpiwiA*, *Djpcna*, *DjMCM2* and *DjMCM3* in intact animals. Brackets in A-E indicate the regenerating head. Scale bar: 500 μm.

The planarian *erk1/2*-related gene *DjerkA* encodes an evolutionarily conserved amino acid sequence motif for phosphorylation by MEK kinase and was expressed in blastema cells at 24 hours post-amputation (see Fig. S4 in the supplementary material). We found that *DjerkA(RNAi)* animals phenocopied the U0126-treated animals (Fig. 5A-E). However, the majority of the *DjerkA(RNAi)* animals showed a head structure reduced in size and cyclopia similar to that induced by treatment with a moderate concentration (10 μM) of U0126 (Fig. 5D-F), presumably due to incomplete penetrance of *DjerkA* RNAi (Fig. 5G) as assayed by quantitative RT-PCR. This idea was supported by the fact that combined treatment with a relatively low concentration (6.25 μM) of U0126, which caused only a minor defect of head regeneration (cyclopia; less than 10%), and *DjerkA* RNAi had a synergistic effect on the regeneration defect (see Fig. S5 in the supplementary material), suggesting that *DjerkA* RNAi and U0126 treatment probably targeted the same signaling proteins.

ERK inhibition caused blastema cells to retain undifferentiated neoblast state

We analyzed further the regeneration defect early in the process of the blastema formation. At 24 hours post-amputation, pERK(+) cells can be recognized as a mass of non-proliferative blastema cells, in which DjPiwiA protein is strongly detected, but in which *DjpiwiA* mRNA expression has decayed, indicative of their undergoing a transition from the undifferentiated state to a differentiated state (Fig. 6A-C and see Fig. S6A-C in the supplementary material). Consistent with the normal survival of neoblasts, accumulation of DjPiwiA(+) cells at both the anterior and posterior stumps (presumptive blastema cells) was readily observed in U0126-treated regenerates at 24 hours post-amputation (Fig. 6D,E and see Fig. S7 in the supplementary material). However, these cells sustained high levels of *DjpiwiA* mRNA expression (Fig. 6D,E and see Fig. S7 in the supplementary material). This phenotype was also observed in *DjerkA(RNAi)* animals (Fig. 6F). Furthermore, U0126 treatment caused prolonged expression of *histone H2B* (accession number AB576209), a marker gene for proliferative neoblasts (Guo et al., 2006) in the blastema cells (see Fig. S8 in the supplementary material). Finally, we confirmed that 25 μM U0126 treatment induced ectopic mitotic cells in the blastema (Fig. 6G-I), as assayed with an antibody against pH3, a marker for M-phase of the cell cycle (Hendzel et al., 1997). These observations suggest that inhibition of ERK signaling caused blastema cells to retain their ability to proliferate at the expense of cell differentiation.

ERK is required for the differentiation of neoblasts into multiple cell lineages

The normal physiological cell turnover in non-regenerating intact planarians depends on neoblasts (Pellettieri and Sánchez Alvarado, 2007), and thus provides another opportunity to examine the effect of ERK inhibition on neoblast differentiation without injury to the animals. To trace neoblast progeny at the single cell level, neoblasts were specifically labeled by BrdU incorporation (Newmark and

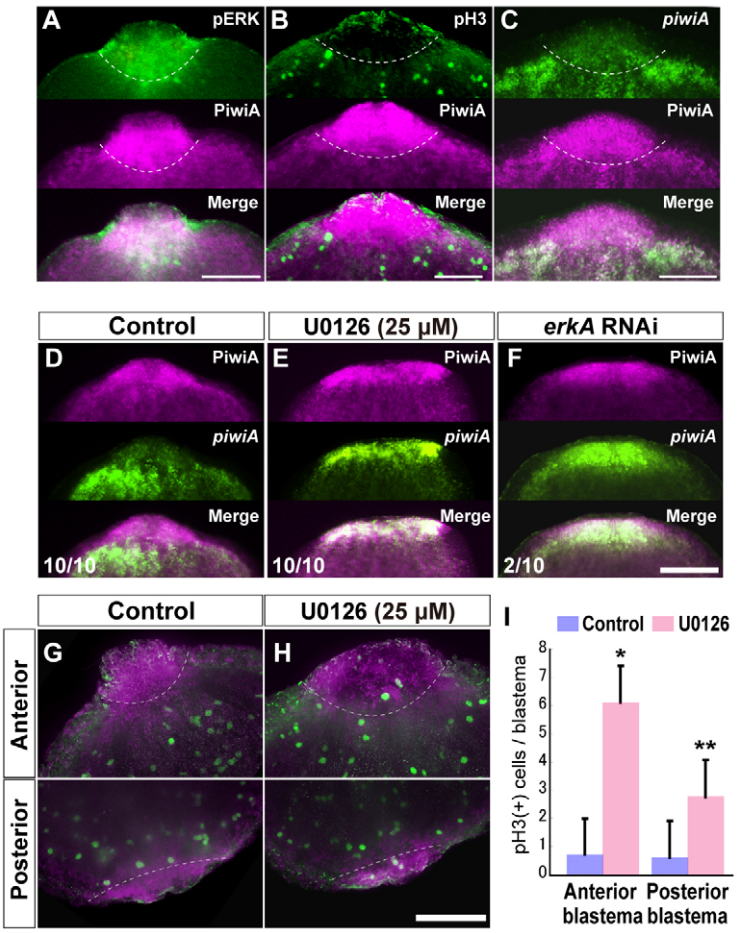

Fig. 6. Effect of U0126 on neoblast dynamics involved in the blastema formation. (**A**) Double staining with anti-pERK (green) and anti-DjPiwiA (magenta) at 24 hours of regeneration. pERK signals are detected in the blastema. (**B**) Double staining with anti-pH3 antibody (green) and anti-DjPiwiA (magenta) at 24 hours of regeneration. No anti-pH3(+) mitotic cells are detected in the blastema. (**C**) Double staining with *DjpiwiA* gene probe (green) and anti-DjPiwiA (magenta) at 24 hours of regeneration. Expression of *DjpiwiA* mRNA is dramatically decreased in the blastema. (**D-F**) Double staining with anti-DjPiwiA (magenta) and *DjpiwiA* gene probe (green) at 24 hours of regeneration. (**G**,**H**) Double staining with anti-DjPiwiA (magenta) and anti-pH3 (green) at 24 hours of regeneration. Dashed lines indicate the border of the blastema region in A-C,G,H. (**I**) Quantitative analysis of ERK inhibition-induced cell cycle progression in the blastema. The number of anti-pH3(+) cells in each blastema was counted (control animals, n=11; U0126-treated animals, n=9). U0126 treatment induces ectopic anti-pH3(+) cells in both the anterior blastema (*P<0.01) and posterior blastema (**P<0.05). Error bars represent s.d. Anterior is to the top in A-H. Scale bars: 500 μm in A-E, 250 μm in G,H.

Sánchez Alvarado, 2000), and allowed to undergo cell differentiation for 5 days in water (as control) or water supplemented with 25 μM U0126. In intact animals, brain, gut and pharynx structures were visualized by Hoechst staining (Fig. 7Ai-iii). The lack of *DjpiwiA* expression in these organs indicates that they are composed solely of differentiated cells (Fig. 7A). In control animals, the first detectable signal was observed only in *DjpiwiA* mRNA(+) undifferentiated neoblasts at 24 hours post-BrdU pulse (data not shown). Five days after the BrdU-pulse, BrdU incorporation was detected in *DjpiwiA* mRNA(–) neoblast progeny from a variety of tissues derived from the three germ layers, such as the brain, pharynx and intestinal tube (Fig. 7Ai-iii). At this time-point, we could not detect BrdU incorporation in epidermal cells. Consistent with our hypothesis, U0126-treated animals showed normal BrdU incorporation in *DjpiwiA* mRNA(+) undifferentiated neoblasts in the mesenchymal space 5 days after BrdU-pulse labeling (Fig. 7B,C), but a drastic decrease in the number of BrdU(+) cells in a variety of differentiated tissues, including the brain, pharynx and intestinal tube (Fig. 7Bi-iii,D). From these results, we conclude that ERK signaling is not required for survival and self-renewal of neoblasts, but is required for their differentiation into multiple cell lineages in planarians.

An ERK-*DjmkpA* feedback circuit makes the cell fate decision of blastema cells

To clarify whether *DjmkpA* indeed functions in the circuitry of the ERK-dependent cell fate decisions, we performed a functional assay to test the effect of *DjmkpA* RNAi on blastema cells sensitized by treatment with a concentration of U0126 that leads to inhibition of the ERK activity at a moderate level. Head regeneration from tail fragments was used as a model to assess the ability of *DjmkpA* RNAi to rescue these defects. U0126 (10 μM) caused clear differentiation defects in the head region, such as its reduction in size with cyclopia or no eyes, in all of the treated animals at 7 days post-amputation (Fig. 8C). This defect was robustly rescued by simultaneous *DjmkpA* RNAi (Fig. 8D), which accords with the restoration of the ERK phosphorylation level (Fig. 3F, lane 4). Quantitative analysis (counting the number of successfully regenerated eyes) confirmed that *DjmkpA* RNAi rescued the regenerative defects induced by U0126 treatment (Fig. 8E).

Based on all these findings, we conclude that negative-feedback regulation of ERK signaling by *DjmkpA* seems to establish a threshold level of ERK activation for binary cell fate decisions of blastema cells regarding whether to proliferate or to differentiate.

DISCUSSION

Role of ERK signaling in blastema cells

Here, we clarified the details of stem cell dynamics early in the process of blastema formation during regeneration of the planarian *Dugesia japonica*. On the basis of our findings, we propose that blastema cells originate from a population of proliferating neoblasts near the amputation stump (postblastema) (Sálo and Baguñà, 1984) and subsequently undergo activation of ERK signaling that allows the blastema cells to simultaneously exit from the proliferative state and enter the differentiating state. ERK-dependent switching of the

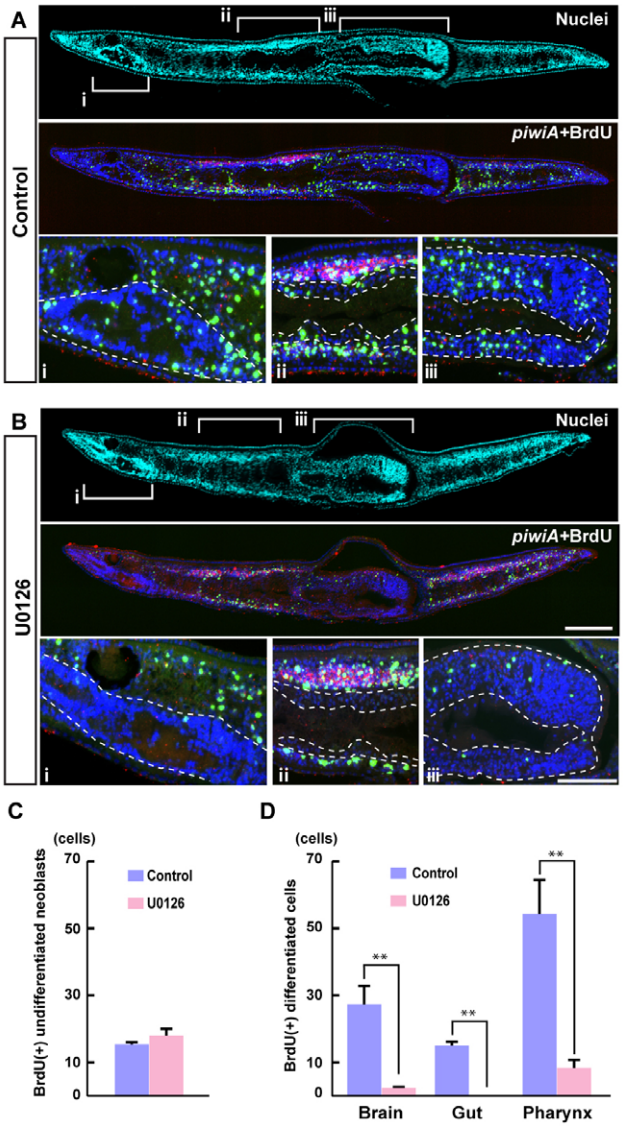

Fig. 7. Effect of U0126 on neoblast differentiation in intact animals. Sagittal view of double staining with anti-BrdU and *DjpiwiA* probe in non-regenerating intact animals. Hoechst staining visualizes the structures of brain (i), gut (ii) and pharynx (iii). The lack of *DjpiwiA* expression in these organs indicates that they are composed of differentiated cells. (**A**) All panels show a control animal at 5 days after BrdU (green) incorporation. BrdU is specifically incorporated into *DjpiwiA*-positive undifferentiated neoblasts (red) in the mesenchymal space and then transmitted into neoblast-derived differentiated cells (Newmark and Sánchez Alvarado, 2000). (**B**) All panels show a 25 μM U0126-treated animal at 5 days after BrdU (green) incorporation. Fewer BrdU signals are seen in a variety of differentiated cells, such as brain, gut and muscle cells (regions surrounded by dashed line). Hoechst staining indicates nuclei in cyan (upper panel) or blue (middle and lower panels). (**C**) Quantitative analysis of BrdU(+)/*DjpiwiA*(+) neoblasts in U0126-treated animals. In U0126-treated animals, the number of BrdU(+)/*DjpiwiA*(+) neoblasts residing in the mesenchymal space did not show a significant difference from that in control (control animals, *n*=3; U0126-treated animals, *n*=3). (**D**) Quantitative analysis of BrdU(+) differentiated cells in brain, gut and pharynx in U0126-treated animals. The number of BrdU(+) differentiated cells was significantly decreased in the brain (**P<0.05), gut (**P<0.05) and pharynx (**P<0.05) of inhibitor-treated animals (control animals, *n*=3; U0126-treated animals, *n*=3). Error bars represent s.d. Anterior is to the left. Scale bars: 500 μm in upper and middle panels, 250 μm in lower panels.

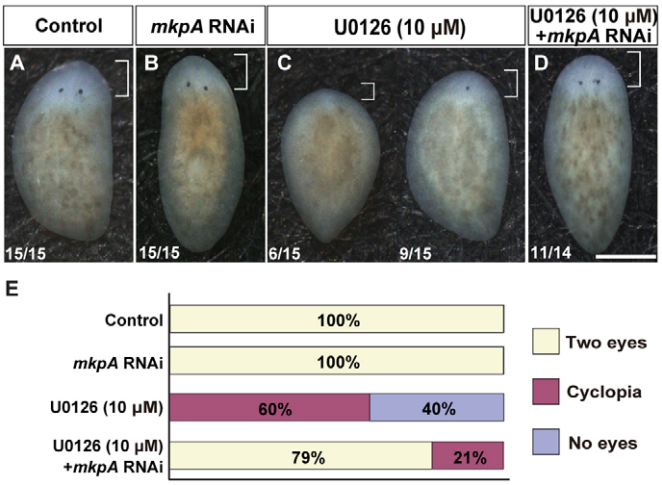

Fig. 8. U0126-induced failure of differentiation of neoblasts is rescued by *DjmkpA* RNAi. (**A-D**) Dorsal view of day 7 regenerants from tail fragments. Brackets indicate regenerated head region. (A) Control animals regenerate a head structure with two eyes (100% of animals, *n*=15). (B) *DjmkpA(RNAi)* animals seemed to undergo morphologically normal head regeneration (100% of animals, *n*=15). (C) Treatment with 10 μM U0126 causes severe defects of head regeneration, including a reduced head structure and no eyes (6/15) or cyclopia (9/15). (D) The 10 μM U0126-induced defect is rescued by *DjmkpA* RNAi. (**E**) Quantification of the phenotype shown in B-D based on eye number. Anterior is to the top. Scale bar: 500 μm.

cellular state of neoblasts results in a clear distinction between the blastema (non-proliferative) and postblastema (proliferative) regions (Fig. 9A,B).

ERK signaling is also known to regulate cytoskeletal machineries that drive cell migration in many developmental contexts (Huang et al., 2004; Pullikuth and Catling, 2007), but, in fact, treatment with U0126 did not affect the migration of blastema cells towards the amputation stump in *D. japonica*, because U0126-treated animals showed a blastema-like structure (Fig. 6E). However, reduced directional migration of blastema cells into their correct locations within the blastema could still account for the regeneration defect induced by U0126 (Fig. 9A,B). It has been reported that neoblast migration and differentiation are strongly coupled with each other in planarians (Newmark and Sánchez Alvarado, 2000). Therefore, our observations encourage us to speculate that ERK signaling might promote blastema cell differentiation and coordinate blastema cell migration, resulting in the robust formation of the blastema structure during regeneration.

Why did ERK inhibition result in a decreased rather than increased number of blastema cells? To ensure continuous recruitment of neoblast progeny to the blastema at a later stage, ERK signaling might play a role in directing active mitosis in a non-cell autonomous manner after the formation of the blastema. Agata et al. (Agata et al., 2003; Agata et al., 2007) previously proposed that a newly formed blastema acts as a signaling center that induces intercalation to restore patterning along the anterior-posterior axis during planarian regeneration. In this scenario, we suggest that ERK signaling ensures the differentiation of an initial cohort of blastema cells early in the process of regeneration (around 24 hours post-amputation). This is required for subsequent blastema function as a distalization signal that induces active

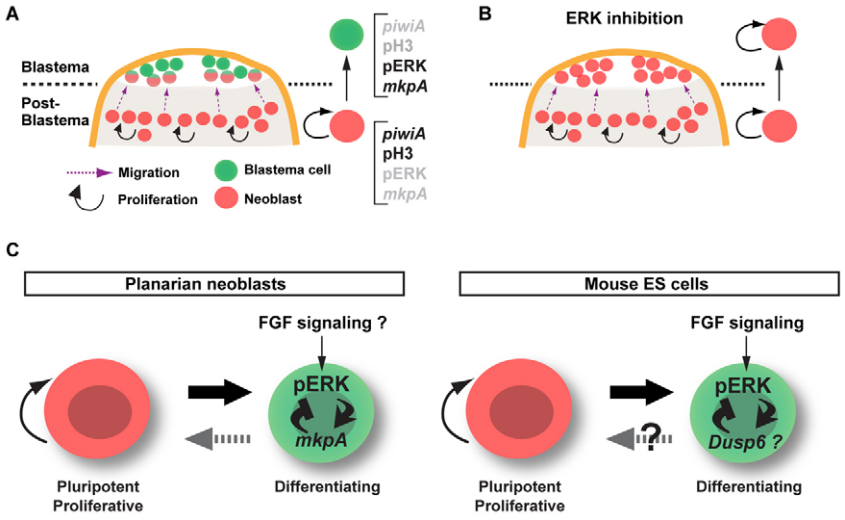

Fig. 9. A proposed model for the roles of pERK and *DjmkpA* in blastema formation. (**A**) The normal process of blastema formation in the planarian *Dugesia japonica*. Cell cycle progression leads to the generation of blastema cells at the end of the stump (recognized as a proliferative area, the postblastema), and then allows the blastema-forming cells to activate ERK signaling, which promotes exit from the proliferative state and simultaneous entry into the differentiating state, resulting in the fine distinction between blastema (non-proliferative) and postblastema (proliferative) regions. (**B**) ERK inhibition allows blastema cells to retain their ability to proliferate at the expense of differentiation. (**C**) Evolutionarily conserved role of ERK signaling in regulating the pluripotent state between neoblasts in planarians and embryonic stem (ES) cells in mice. Proliferation of neoblasts is highly sensitive to ERK signaling as a differentiation signal in a manner similar to the regulation of ES cells in mice (Ying et al., 2008). *Dusp6* (*Mkp3*) encodes a MAPK phosphatase gene in vertebrates and might share a common function with *DjmkpA* (dashed arrow).

mitosis of undifferentiated neoblasts outside the blastema to feed their progeny into a variety of regenerating tissues, including the blastema during intercalary regeneration. (We believe that mitosis in *D. japonica* is likely to correspond to the second mitotic phase in *Schmidtea mediterranea*). This idea at least would account for the most puzzling observation that ERK inhibition decreased number of blastema cells at 72 hours post-amputation (Fig. 4B′,C′). We are looking forward to assessing our proposed model in the future.

Recently accumulated knowledge about regenerative programs in many model organisms highlights crucial roles of MAPK signaling pathways early in the regenerative response (Tanaka and Galliot, 2009). Importantly, ERK activation has been found in developing blastemas in various animal species (Suzuki et al., 2007; Nakamura et al., 2008). These observations suggest that ERK activation might act as a common molecular indicator of the differentiating state of blastema cells among animal species.

DjmkpA functions as a negative-feedback regulator of the ERK signaling in blastema cells

MKPs inactivate JNK, ERK and p38 MAPKs by dephosphorylation with distinct specificities for individual MKPs (Theodosiou and Ashworth, 2002; Kondoh and Nishida, 2007). The first wave of *DjmkpA* expression occurred immediately in differentiated cells in response to wounding stimuli independently of ERK signaling. By contrast, we found that *DjmkpA* acts as a negative-feedback regulator of ERK signaling in blastema cells (Fig. 3 and Fig. 8C). This feedback circuit would ensure pivotal cell fate decisions of blastema cells, namely proliferation or differentiation, in the presence of fluctuations generated by upstream noise and variations of ERK signaling. We speculate that the expression of *DjmkpA* is involved in inactivation of ERK, but not JNK, based on the spatially distinct profiles of their activation during regeneration (Tasaki et al., 2011). Our recent study demonstrated that strong JNK activation is observed in the postblastema region around 24 hours post-amputation and is required for the entry of neoblasts into M-phase of the cell cycle and for the generation of blastema cells, in which ERK is highly activated (Tasaki et al., 2011) (J.T., unpublished data). If *DjmkpA* were required for inactivation of JNK in the blastema under the control of ERK activity, the U0126-induced differentiation defect would be enhanced by simultaneous *DjmkpA* RNAi, presumably owing to disturbance of the cell fate transitions of blastema cells by JNK-dependent active mitosis. In fact, *DjmkpA* (RNAi) did not seem to rescue the regeneration defect induced by the treatment with the JNK inhibitor (J.T., unpublished data).

Implications for stem cell biology

Historically, it was thought that during vertebrate regeneration blastema cells de-differentiate into a undifferentiated pluripotent state, as typified by embryonic stem (ES) cells, which are derived from the inner cell mass of mammalian blastocysts and have the ability to self-renew and differentiate into cells of ectodermal, endodermal and mesodermal germ layers (Evans and Kaufman, 1981; Martin, 1981). However, recent work on the salamander *Ambystoma mexicanum* showed that blastema cells have apparently restricted potential to differentiate during limb regeneration (Kragl et al., 2009). Although somatic cells can be reprogrammed to a pluripotent state by the expression of defined factors in mammalian cells (Takahashi and Yamanaka, 2006), during the normal course of regeneration, blastema cells in the salamander are in a cellular state apparently different from that of ES cells.

Our findings demonstrated that the cellular state of blastema cells in planarians fits the conventional concept of blastema cells when the ERK signaling is inhibited, for they maintain their pluripotent neoblast state. This leads to the interesting idea that neoblasts in planarians and ES cells in vertebrates might share a common molecular basis for their cell fate decisions (Fig. 9C). That

is, proliferation of neoblasts is highly sensitive to ERK signaling as a differentiation signal in a similar way to the regulation of ES cells in mice (Ying et al., 2008). Similarities between planarian neoblasts and mammalian ES cells have been postulated previously by their similar expression of homologous RNA-binding proteins (Salvetti et al., 2005; Shibata et al., 2010; Rouhana et al., 2010). It is known that fibroblast growth factor (FGF) signaling acts upstream of ERK signaling to promote cell fate decisions of ES cells in mice (Kunath et al., 2007). *Mkp3* (*Dusp6*) is a well-known target of the FGFR-mediated ERK signaling pathway in vertebrates, and encodes a key modulator that determines the magnitude and duration of ERK activity in a negative feedback manner (Eblaghie et al., 2003; Smith et al., 2006; Li et al., 2007). This suggests that *Mkp3* in vertebrates and *DjmkpA* in planarians share a similar function in the ERK-dependent differentiation network. Furthermore, identification of the *nou-darake* gene provided strong molecular evidence for the existence of a brain-inducing circuit based on FGF signaling in planarians (Cebrià et al., 2002). These observations lead us to speculate that FGF signaling and an ERK/*mkp* feedback circuit were integrated into the pluripotent stem cell system early in the course of evolution and together play a crucial role in balancing the self-renewal and differentiation of stem cells (Lanner and Rossant, 2010). Therefore, our study highlights the possible value of planarian studies for regenerative medicine via integration of regeneration biology and stem cell biology.

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Competing interests statement
The authors declare no competing financial interests.

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