Development, Growth & Differentiation
The Japanese Society of Developmental Biologists

Develop. Growth Differ. (2015) **57**, 209–217 doi: 10.1111/dgd.12195

Original Article

Two distinct roles of the yorkie/yap gene during homeostasis in the planarian *Dugesia japonica*

Byulnim Hwang,[1] Yang An,[1] Kiyokazu Agata[1] and Yoshihiko Umesono[2]*

[1]*Department of Biophysics, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto, and*
[2]*Graduate School of Life Science, University of Hyogo, Ako-gun, Hyogo, Japan*

Adult planarians possess somatic pluripotent stem cells called neoblasts that give rise to all missing cell types during regeneration and homeostasis. Recent studies revealed that the Yorkie (Yki)/Yes-associated protein (YAP) transcriptional coactivator family plays an important role in the regulation of tissue growth during development and regeneration, and therefore we investigated the role of a planarian *yki*-related gene (termed *Djyki*) during regeneration and homeostasis of the freshwater planarian *Dugesia japonica*. We found that knockdown of the function of *Djyki* by RNA interference (RNAi) downregulated neoblast proliferation and caused regeneration defects after amputation. In addition, *Djyki* RNAi caused edema during homeostasis. These seemingly distinct defects induced by *Djyki* RNAi were rescued by simultaneous RNAi of a planarian *mats*-related gene (termed *Djmats*), suggesting an important role of *Djmats* in the negative regulation of *Djyki*, in accordance with the conservation of the functional relationship of these two genes during the course of evolution. Interestingly, *Djyki* RNAi did not prevent normal protonephridial structure, suggesting that *Djyki* RNAi induced the edema phenotype without affecting the excretory system. Further analyses revealed that increased expression of the *D. japonica* gene *DjaquaporinA* (*DjaqpA*), which belongs to a large gene family that encodes a water channel protein for the regulation of transcellular water flow, promoted the induction of edema, but not defects in neoblast dynamics, in *Djyki(RNAi)* animals. Thus, we conclude that *Djyki* plays two distinct roles in the regulation of active proliferation of stem cells and in osmotic water transport across the body surface in *D. japonica*.

Key words: homeostasis, osmoregulation, planarian, stem cells, Yorkie/Yap.

Introduction

In *Drosophila*, the Hippo signaling pathway involves a kinase cascade that controls the activity of a transcriptional co-activator protein, Yorkie (Yki) (Huang *et al.* 2005; Pan 2010). Hippo phosphorylates and activates Warts and Mats (Udan *et al.* 2003; Wu *et al.* 2003; Wei *et al.* 2007). Yki is inactivated through phosphorylation by the Warts/Mats complex and is tethered in the cytoplasm by promoting its interaction with 14-3-3 (Oh & Irvine 2008). Dephosphorylation of Yki enables it to translocate into the nucleus and interact with Scalloped, a DNA-binding transcription factor, to promote target gene expression (Wu *et al.* 2008; Zhang *et al.* 2008).

The Hippo signaling pathway controls organ size by regulating cell proliferation and apoptosis in animals (Harvey *et al.* 2003; Jia *et al.* 2003; Pantalacci *et al.* 2003; Udan *et al.* 2003; Wu *et al.* 2003). Deficiency of *yap*, a homolog gene of *yki* in vertebrates, caused decreased proliferation in breast and epidermal cells (Schlegelmilch *et al.* 2011; Zhi *et al.* 2012). By contrast, overexpression of *yki/yap* caused ectopic cell proliferation, resulting in overgrowth of organs (Huang *et al.* 2005; Dong *et al.* 2007). These observations suggest that the expression level of Yki/Yap directly influences cell proliferation to control organ size during development in flies and vertebrates. Furthermore, a recent study highlighted the crucial role of Yap1 in active cell proliferation during limb regeneration in *Xenopus* (Hayashi *et al.* 2014).

*Author to whom all correspondence should be addressed.
Email: yumesono@sci.u-hyogo.ac.jp
Received 18 November 2014; revised 18 December 2014; accepted 21 December 2014.

Flatworms possess somatic pluripotent stem cells in adults, which provide us a good opportunity to investigate the molecular mechanisms underlying stem cell dynamics *in vivo* (Agata & Watanabe 1999; Newmark & Sánchez Alvarado 2002; Agata & Umesono 2008; Umesono & Agata 2009). Recently, two groups have reported the function of *yki/yap*-related genes using two different free-living flatworm species as models. In the basal flatworm *Macrostomum lignano,* knockdown of the function of the gene *Mac-Yap* by RNA interference (RNAi) resulted in reduced proliferation of pluripotent stem cells (called neoblasts) during homeostasis (Demircan & Berezikov 2013), demonstrating that *yki/yap* is functionally conserved between flatworms and mammals. In the case of the freshwater planarian *Schmidtea mediterranea, Smed-yki* RNAi led to hyperproliferation of neoblasts during homeostasis, resulting in the opposite phenotype to that in *M. lignano* (Lin & Pearson 2014).

To further assess the role of *yki/yap* in the regulation of stem cell dynamics in flatworms, we used *Dugesia japonica,* another species of free-living freshwater planarian, and performed RNAi experiments of its *yki/yap*-related gene (termed *Djyki*). We found that *Djyki* RNAi resulted in decreased rather than increased proliferation of neoblasts during homeostasis, a situation similar to that in *M. lignano*, but not to that in *S. mediterranea.* In addition, *Djyki* RNAi also caused edema formation during homeostasis, as *Smed-yki* RNAi did in *S. mediterranea*. It has been reported that *Smed-yki* RNAi caused an aberrant protonephridial (excretory) system, resulting in the edema formation (Lin & Pearson 2014). However, we revealed that *Djyki* RNAi caused edema formation by increased expression of the gene *D. japonica aquaporinA (DjaqpA)*, which belongs to a large gene family that encodes a water channel protein involved in the regulation of transcellular water flow, without affecting protonephridial structures. Thus, our findings represent qualitatively different aspects of the function of *Djyki* from that of *Smed-yki* in the two respective freshwater planarians, *D. japonica* and *S. mediterranea*.

Materials and methods

Animals

A clonal strain of the planarian *Dugesia japonica* was used. Planarians were cultured at 24°C in artificial diluted sea water consisting of sea water powder (Instant Ocean, Aquarium systems) in dissolved water. They were fed chicken liver one or two times per 2 weeks. Planarians that were 6–8 mm in length and that had been starved for at least 1 week were used in all experiments.

X-ray irradiation

One week starved planarians were irradiated at 18 kV, 5 mA, by using an X-ray generator (SOFTEX B-5; SOFTEX, Tokyo, Japan). Five days after irradiation, animals were used for experiments.

Feeding RNA interference feeding

Double-stranded RNA (dsRNA) was synthesized as previously described (Rouhana *et al.* 2013). Fifteen planarians were fed a mixture of 25 μL of chicken liver solution, 5 μL of 2% agarose, and 10 μL of 4 μg/μL dsRNA, three times at an interval of 2 days (Sakurai *et al.* 2012). For regeneration studies, planarians were amputated into three body fragments (head, trunk containing a pharynx, and tail) 1 day after the last dsRNA feeding. Control animals were fed *egfp* dsRNA. The effect of RNAi was confirmed by quantitative reverse transcription-polymerase chain reaction (RT-PCR), using a set of primers specific to the gene that was targeted.

Quantitative RT-PCR

Total RNA was extracted by using ISOGEN-LS (Wako) and cDNA was synthesized from 1 μg of total RNA using a QuantiTect Reverse Transciription Kit (Qiagen). The synthesized cDNAs were diluted 10-fold and used for gene expression analysis performed using an ABI PRISM 7900 HT (Applied Biosystems). The following series of incubation conditions was used for each PCR reaction: 50°C for 2 min, 95°C for 15 min, 50 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 1 min. Quantitative analysis of the amount of each gene product was carried out as previously described (Ogawa *et al.* 2002). All quantitative RT-PCR data were normalized against expression level of *DjGAPDH*, a housekeeping gene. A fold-change of the expression level of genes between control and RNAi-treated animals was reported using the mean of three biological replicates of quantitative RT-PCR assays.

The primer sets for each target gene were as follows:

Djyki forward: GACTGCTTGTTGGGATTTTTG
reverse: GTCAAATACAAAATGATCTCAAAGG
Djmats forward: GGTAGATCGGAAGGAATTAGCTCC
reverse: TGAGCTGTTTGATCTGTTTGGCT
DjaqpA forward: CTTTTGGACGGCTCTATTTG
reverse: ACAAGCTCCTAACCCAATGA
DjaqpB forward: CAGCTGCTAGTTTGGGAAAA
reverse: CCACCTAAAAGCGGTCCTAT
DjaqpC forward: TATGTACAGGCAGCACAGGA
reverse: CAGAATTCCAGCCAAAAATC
Djegfr5 forward: TGGGGACGAATTCTGGAGTA

reverse: TGCCGATTTAGTTGACTCTCTG
DjpiwiA forward: CGAATCCGGGAACTGTCGTAG
reverse: GGAGCCATAGGTGAAATCTCATTTG
Djpcna forward: ACCTATCGTGTCACTGTCTTTGA CCGAAAA
reverse: TTCATCATCTTCGATTTTCGGAGCCAGATA

Whole-mount in situ *hybridization*

Planarians were treated with 2% hydrochloric acid (HCl) in 5/8 Holtfreter's solution for 5 min at room temperature (RT) and fixed in 5/8 Holtfreter's solution containing 4% paraformaldehyde for 90 min at 4°C. The samples were bleached with 5% hydrogen peroxide (H_2O_2) in methanol overnight at RT under fluorescent light. Then, bleached samples were washed with a xylene and ethanol mixture (1:1) for 1 h at 4°C and rinsed with 100%, 75%, 50% and 25% ethanol in Holtfreter's solution consecutively for 30 min each at 4°C. After washing with PBST (phosphate buffered saline containing 0.1% Triton X-100) for 30 min at 4°C, samples were treated with 5 mg/mL proteinase K in PBST for 10 min at 37°C. The samples were then re-fixed with 4% paraformaldehyde in 5/8 Holtfreter's solution for 30 min at 4°C and rinsed with PBST twice each for 5 min 4°C. The samples were incubated in hybridization buffer for 1 h at 55°C. Digoxygenin (Dig)-labeled RNA probes were denatured for 10 min at 65°C and then mixed with the samples in hybridization. After 38 h of incubation at 55°C, the samples were washed in washing solution six times for 30 min each at 55°C and rinsed in Buffer I (maleic acid buffer containing 0.1% Triton X-100) twice at RT. The rinsed samples were treated with Buffer II (Buffer I containing 1% blocking reagent [Roche Diagnostics]) for blocking for 30 min at RT and treated with 1/2000 alkaline phosphatase-conjugated anti-Dig antibody (Roche Diagnostics) in Buffer II overnight at 4°C. Samples were rinsed in Buffer I six times for 30 min each at RT and washed in TMN solution two times at RT. A mixture of 3.5 mg/mL 5-bromo-4-chloro-3-indolyl phosphatase (Roche Diagnostics) and 2.7 mg/mL 4-nitro blue tetrazolium chloride (Roche Diagnostics) in TMN solution was used for detection of colored signals (Umesono *et al.* 1997).

Whole-mount immunohistochemistry

The processing of samples was the same as for whole-mount *in situ* hybridization before hybridization. In the case of immunohistochemistry, the samples were incubated overnight at 50°C. After washing with Buffer I six times for 30 min each at RT, Buffer II was added for blocking for 30 min at RT. After blocking, the samples were incubated in Buffer II containing 1/1000 diluted primary antibody overnight at 4°C. The samples were washed in Buffer I six times for 30 min each at RT, and incubated in Buffer II containing 1/1000 fluorescent-labeled secondary antibody (Alexa Fluor 594 or Alexa Fluor 488 [Molecular Probes]) and 1/1000 Hoechst33342 (Calbiochem) for 3 h at 4°C. The samples were rinsed in Buffer I six times for 30 min each at RT, and mounted with Fluorescent Mounting Medium (Dako).

Statistical analysis

The quantitative data were analyzed by one-way analysis of variance (ANOVA) and the statistical significance of differences was determined by Student's *t*-test. *P* values more than 0.05 were taken as not significant and error bars represent ± standard error of the mean (SEM) of three independent biological replicates.

cDNA clones

cDNA clones encoding the respective proteins *Djyki* (accession number LC011458), *Djmats* (LC011527), *DjaqpA* (LC012043), *DjaqpB* (LC011528), *DjaqpC* (LC011529), *Djegfr5* (LC011530), *DjCA* (LC011531), and *Djcubilin* (LC011532) were identified based on deduced protein sequence similarity in a previously constructed library of expressed sequence tags (ESTs) (Mineta *et al.* 2003) using tblastn program.

Results

Djyki RNAi caused a decrease of neoblast proliferation

We identified a single *yki*-related gene (termed *Djyki*) from our cDNA database of *Dugesia japonica* (Nishimura *et al.* 2012) using BLAST search based on protein sequence similarity. *Djyki* encodes a protein with a TEAD-binding domain with 38% and 92% identity to *Mac-Yap* and *Smed-Yki*, respectively, and also a WW domain, which is known to be required for the interaction with other proteins (Zhao *et al.* 2009), with 52% and 87% identity to *Mac-Yap* and *Smed-Yki*, respectively (Fig. 1A). We also identified a single *mats*-related gene (termed *Djmats*), which provided us a good opportunity to assess whether *Djmats* protein functions as an evolutionarily conserved negative regulator of *Djyki* in this planarian species.

Firstly, we analyzed the expression patterns of these two genes in non-regenerating intact animals. Whole-mount *in situ* hybridization (WISH) demonstrated that *Djyki* and *Djmats* were ubiquitously expressed

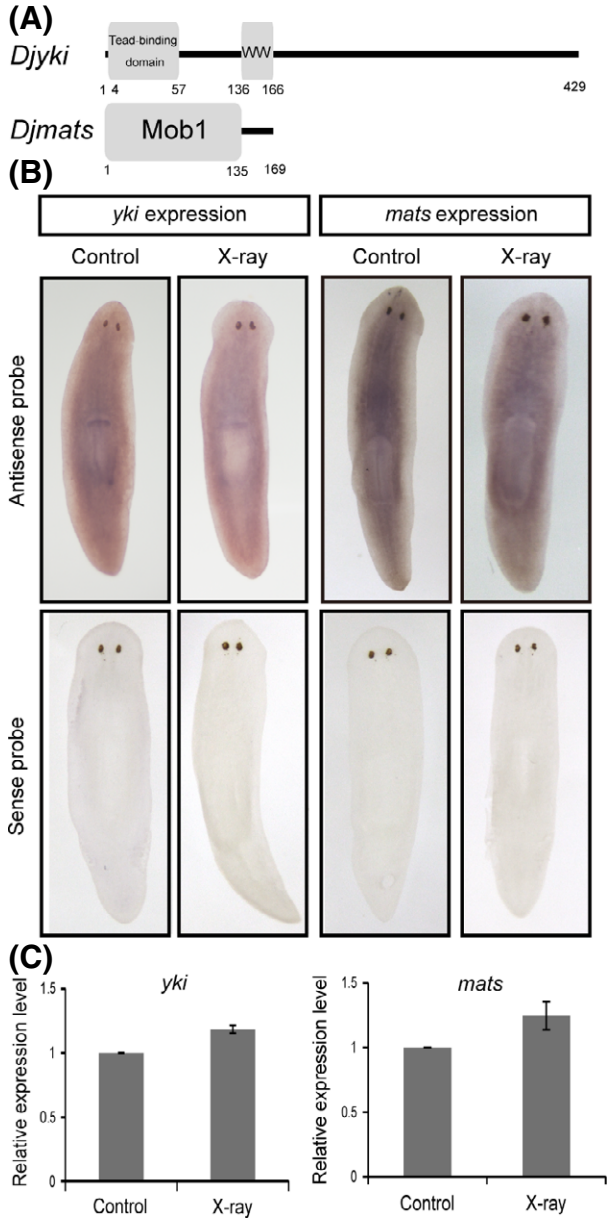

Fig. 1. *Djyki* and *Djmats* are expressed in differentiated cells. (A) Schematic representation of the protein structures of DjYki and DjMats. (B) Expression patterns of *Djyki* and *Djmats*, analyzed by whole-mount *in situ* hybridization (WISH) after X-ray irradiation. (C) Expression levels of *Djyki* and *Djmats*, analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) after X-ray irradiation. No significant variation was observed between control and X-ray irradiated animals.

throughout the body (Fig. 1B). Since X-ray irradiation specifically eliminates somatic pluripotent stem cells (neoblasts) in planarians, the lack of change of the expression patterns of *Djyki* and *Djmats* after X-ray irradiation suggests that *Djyki* and *Djmats* are expressed in X-ray-insensitive differentiated cells, not in neoblasts (Fig. 1B). To further assess the effect of X-ray irradiation, we also performed quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and confirmed that there was no reduction of the expression level of these two genes by X-ray irradiation (Fig. 1C).

Next, we performed RNAi experiments of *Djyki* and *Djmats*. In *Djmats(RNAi)* animals, we could not detect any obvious defect during homeostasis or regeneration (Fig. 2A, B, D). By contrast, quantification of the expression levels of *DjpiwiA*, a neoblast-specific marker gene, and *Djpcna*, a proliferative cell marker gene, by qRT-PCR revealed that *Djyki* RNAi caused a decrease rather than an increase in the expression levels of both *DjpiwiA* and *Djpcna* when compared to the control during homeostasis (Fig. 2A). Consistent with this observation, *Djyki* RNAi resulted in a decrease of the number of mitotic cells, as assayed by staining with anti-phospho-histone H3 antibody (Fig. 2B). In addition, we also detected head-regeneration defects in *Djyki (RNAi)* animals (Fig. 2C). Interestingly, all of the defects related to neoblast activity in *Djyki(RNAi)* animals were rescued by simultaneous RNAi of *Djmats* (Fig. 2A, B, D), while leaving *Djyki* RNAi was effective.

These observations suggest that *Djyki* is required for active proliferation of neoblasts and regeneration, the process in which *Djmats* negatively regulates *Djyki*.

Djyki RNAi caused edema formation during homeostasis

We also found that *Djyki(RNAi)* non-regenerating intact animals showed the edema phenotype during homeostasis (Fig. 3A). All of these animals died within 17 days after the first feeding of *Djyki* dsRNA (Fig. 3B). As we expected, the edema phenotype induced by *Djyki* RNAi was rescued by simultaneous *Djmats* RNAi (Fig. 3A). As a consequence, the survival rate of double *Djyki* and *Djmats(RNAi)* animals was increased prominently when compared to that of single *Djyki (RNAi)* animals (Fig. 3B). The survival rate of *Djyki (RNAi)* or/and *Djmats(RNAi)* was confirmed by performing an independent experiment (data not shown).

These observations raised the possibility that edema itself may decrease the number of neoblasts and also cause regeneration defects, and therefore we carefully examined this possibility.

To verify the relationship between edema and regeneration defects, we first examined the role of the gene *D. japonica epidermal growth factor receptor 5* (*Djegfr5*), a *D. japonica* ortholog of the *Smed-egfr5* gene in *S. mediterranea*, since it has been reported that *Smed-egfr5* RNAi induced edema by causing an aberrant protonephridial (excretory) system during homeostasis (Rink *et al.* 2011). We confirmed that

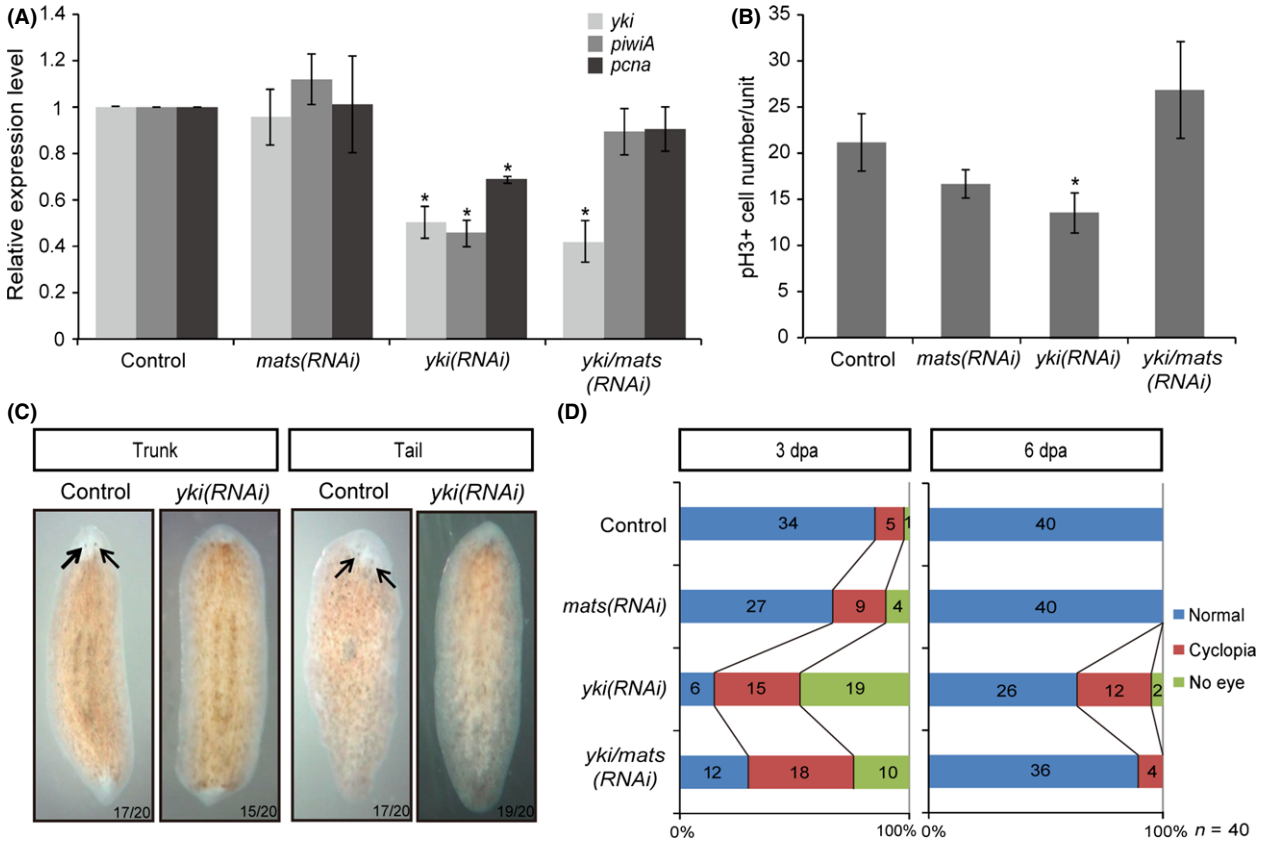

Fig. 2. Reduced proliferation and defective regeneration caused by *Djyki* RNAi. (A) Significant decrease of *DjpiwiA* and *Djpcna* in *Djyki (RNAi)* at 3 days after the last feeding. $^*P < 0.05$. (B) The number of pH3-positive cells ($n = 5$). $^*P < 0.05$. Unit volume: 1.1×10^{-2} mm^3. (C) Head regeneration at 3 dpa in trunk and tail fragments. Live image. Arrows indicate eyes. (D) Quantification of number of eyes at 3 days (left) and 6 days (right) post amputation ($n = 40$).

Djegfr5 RNAi also caused edema formation in *D. japonica*; however, *Djegfr5(RNAi)* animals seemed to undergo normal head regeneration after amputation, in contrast to *Djyki(RNAi)* regenerating animals (Fig. S1A). In addition, qRT-PCR analysis demonstrated that *Djegfr5* RNAi did not affect the expression levels of *DjpiwiA* or *Djpcna* during homeostasis (Fig. S1B).

We next examined the protonephridial system in *Djyki(RNAi)* animals since *Smed-yki* RNAi caused edema due to dysfunction of the protonephridial system in *S. mediterranea* (Lin & Pearson 2014). We used two protonephridial marker genes, *D. japonica carbonic anhydrase* (*DjCA*) and *Djcubilin,* and counted the number of clusters of *DjCA*-positive cells or *Djcubilin*-positive cells in *Djyki(RNAi)* animals, and compared them to those in control animals. Fluorescent *in situ* hybridization (FISH) assay demonstrated that the number of clusters of these two cell types were indeed significantly decreased in *Djegfr5(RNAi)* animals (Fig. 4B). In contrast, we did not detect any significant difference in the number of these two clusters between control and *Djyki(RNAi)* animals (Fig. 4C). Furthermore, qRT-PCR analysis demonstrated that *Djyki* RNAi did not affect the expression level of *Djegfr5* (Fig. S2A). Consistent with this observation, double *Djyki* and *Djegfr5* RNAi resulted in a dramatic increase in the number of dead planarians when compared to single *Djyki* or *Djegfr5* RNAi (Fig. S2B).

These observations suggest that: (i) edema itself may not affect the proliferative activity of neoblasts or regeneration; and that (ii) *Djyki* and *Djegfr5* may have different mechanisms of blocking edema formation during homeostasis.

Djyki negatively regulates the expression of DjaqpA to block edema formation during homeostasis

It has been demonstrated that aquaporin has an important role in the regulation of osmotic water transport across cell plasma membranes (Carbrey & Agre 2009; Verkman 2012). Specifically, dysregulation of aquaporin-4 correlates with the formation of brain edema in rodents and humans (Sun *et al.* 2003; Papadopoulos & Verkman 2005, 2007; Zador *et al.*

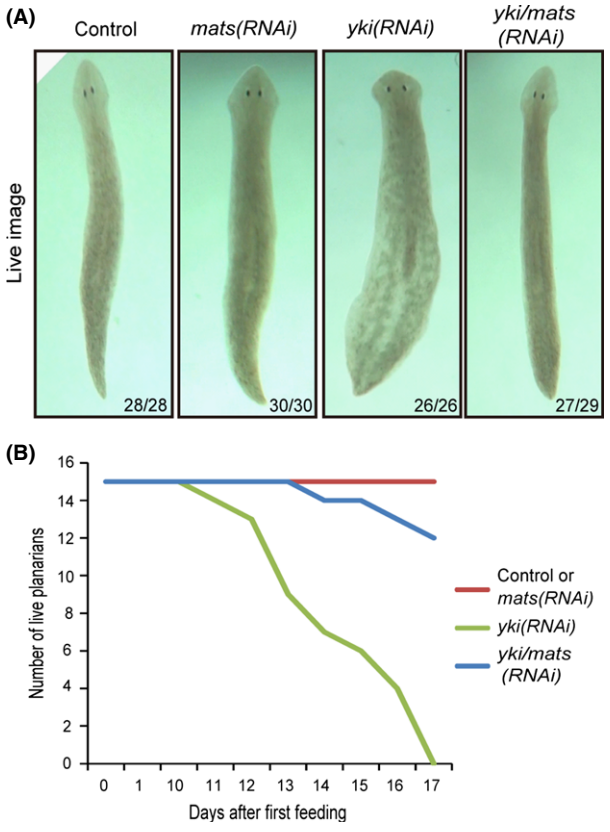

Fig. 3. *Djyki* RNAi leads to edema, which is rescued by *Djmats* RNAi. (A) Live image of intact animals at 10 days after last RNAi feeding. (B) Survival curves for RNAi-treated planarians (n = 15). The experiment was performed twice independently to confirm that the results were reproducible.

2009). These observations encouraged us to propose the idea that dysregulation of *aquaporin-4*-related genes in *Djyki(RNAi)* animals might cause the edema phenotype during homeostasis.

Firstly, we succeeded in identifying three distinct *aquaporin-4*-related genes, which we termed *DjaqpA, B,* and *C*, respectively, in the genome sequences of *D. japonica* and examined the expression patterns of these three *aquaporin* genes by WISH. All three genes were expressed ubiquitously throughout the body (Fig. 5A). In contrast to *DjaqpB* and *C*, *DjaqpA* was also expressed strongly in the brain-branch region (Fig. 5A). Interestingly, WISH and qRT-PCR analyses showed that *Djyki* RNAi caused a significant increase of the level of expression of *DjaqpA* during homeostasis when compared to that in control (Fig. 5B, C). This increase was suppressed by simultaneous *Djmats* RNAi (Fig. 5C), suggesting that the expression level of *DjaqpA* depends on the activity level of *Djyki* during homeostasis. In contrast, the expression levels of *DjaqpB* and *C* were not changed in *Djyki(RNAi)* animals (Fig. S3A), showing that the *Djyki* activity is specifically required for the transcriptional regulation of *DjaqpA* during homeostasis. Furthermore, we also found that *Djegfr5* RNAi did not affect the expression level of *DjaqpA* (Fig. S3B), suggesting that edema itself is not a cause of the increased expression of *DjaqpA* during homeostasis.

Next, we tested whether or not increased expression of *DjaqpA* has a role in *Djyki* RNAi-induced edema formation. Surprisingly, the edema phenotype induced by *Djyki* RNAi was rescued by simultaneous RNAi of *DjaqpA* (Fig. 5D), as well as by *Djmats* RNAi (Fig. 3A). These data suggest that increased expression of *DjaqpA* promotes edema formation in *Djyki(RNAi)* animals during homeostasis. Under this condition, interestingly, we found that simultaneous *DjaqpA* RNAi did not rescue either the reduced proliferative activity of neoblasts or the regeneration defects induced by *Djyki* RNAi (Fig. 5E, F).

Therefore, the identification and characterization of *DjaqpA* enables us to conclude that *Djyki* plays at least two distinct roles in the regulation of stem cell dynamics and homeostasis in *D. japonica*.

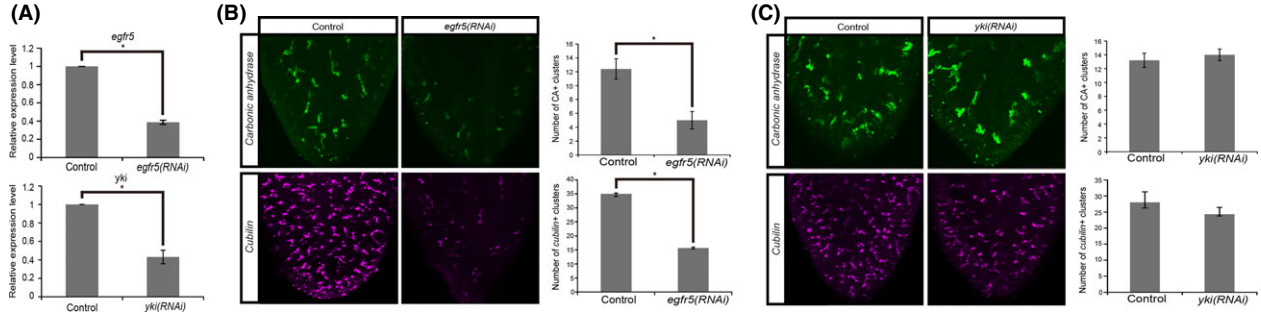

Fig. 4. *Djyki* is not required for excretory system. (A) Significant decrease of *Djegfr5* and *Djyki* by RNAi at 3 days after the last feeding. *P < 0.05. (B), (C) Fluorescence *in situ* hybridization (FISH) for staining RNAs transcribed from *DjCA* (green), *Djcubilin* (magenta), and protonephridial marker genes, after *Djegfr5* and *Djyki* RNAi. Graphs display number of *DjCA*+ expression and *Djcubilin*+ expression clusters (right) (n = 3, *P < 0.05).

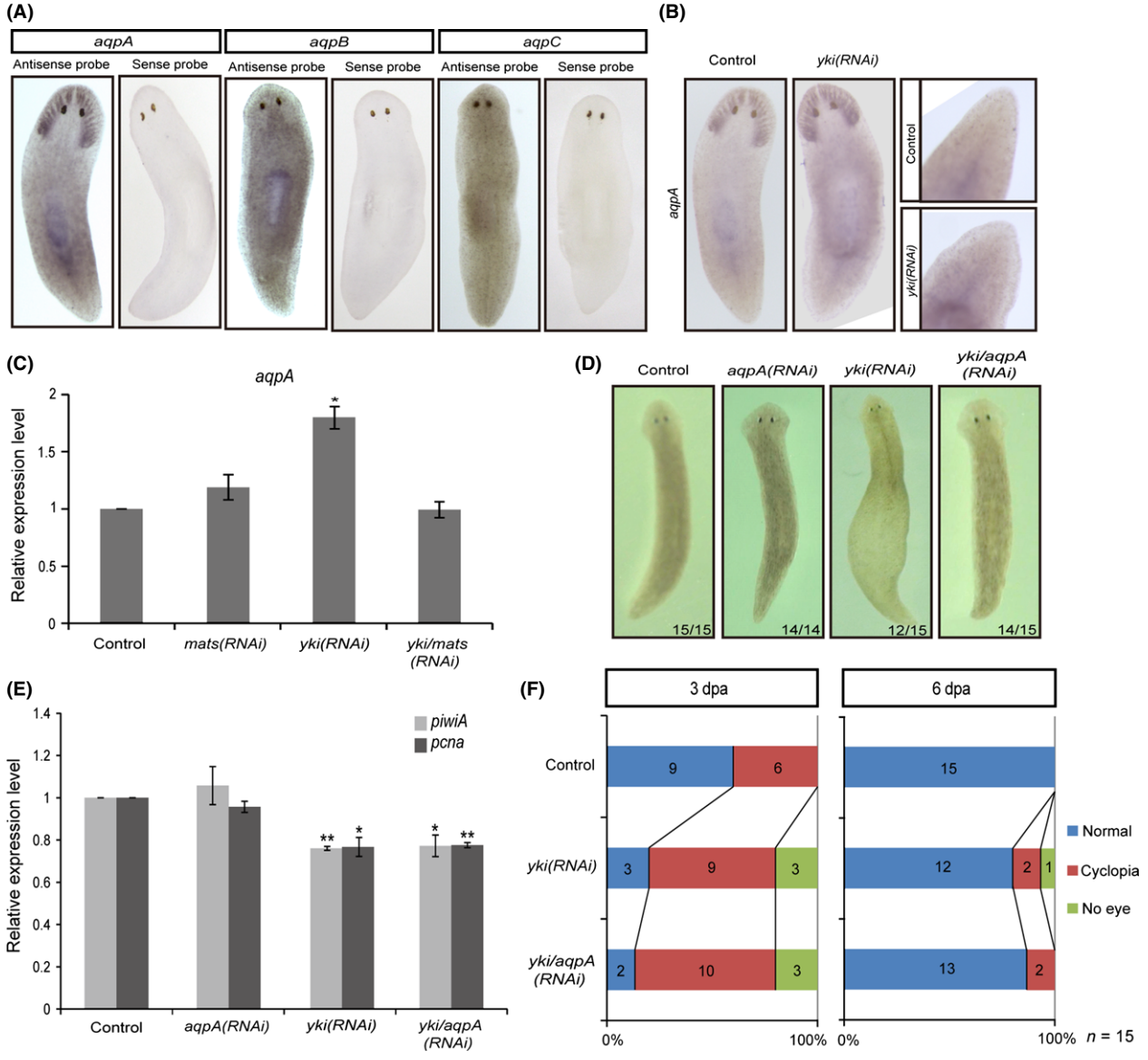

Fig. 5. Upregulation of *DjaqpA* is necessary for *Djyki* RNAi-induced edema. (A) Expression patterns of *Djaqp A*, *B*, and *C* in intact animals. (B), (C) Level of *DjaqpA* in *Djyki(RNAi)* planarian as determined by whole-mount *in situ* hybridization (WISH) and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). $*P < 0.05$. (D) Phenotypes of *Djyki* or/and *DjaqpA* RNAi at 10 days after last RNAi feeding. (E) Levels of *DjpiwiA* and *Djpcna* expression in *Djyki* or/and *DaqpA* RNAi planarians. $**P < 0.01$, $*P < 0.05$. (F) Classification by number of eyes at 3 days (left) and 6 days (right) post amputation ($n = 15$).

Discussion

We showed here that in *D. japonica*, *Djyki* is required for stem cell proliferation and regeneration, and also for osmoregulation (Fig. 2, 3). In addition, we found that *Djmats* has an evolutionarily conserved inhibitory function against *Djyki* and is involved in all of the contexts in which *Djyki* is required (Fig. 2, 3). Furthermore, the most interesting discovery here was that *Djyki* negatively regulates the expression of *DjaqpA* and thereby blocks edema formation during homeostasis (Fig. 5).

From mammals to flatworms, *yki/yap* has a conserved role to activate stem cell proliferation (Schlegelmilch *et al.* 2011; Zhi *et al.* 2012; Demircan & Berezikov 2013). Our study demonstrated that *Djyki* RNAi reduced the proliferative activity of neoblasts in *D. japonica*. We speculate that this defective proliferative activity may lead to the regeneration defect seen in *Djyki(RNAi)* animals. Indeed, our data in *D. japonica* fit with the general conception about the function of the *yki/yap* gene family among animal species. As far as we were able to determine, however, *Djyki* is not

highly expressed in neoblasts. For this reason, we attempted to further assess the relationship between edema and reduced proliferation of neoblasts in *Djyki (RNAi)* animals, and we concluded that they are mutually independent phenotypes induced by *Djyki* RNAi. These observations suggest that *Djyki* regulates neoblast proliferation in a non-cell-autonomous manner. Further investigations will be required to understand the non-cell-autonomous function of *Djyki* in the regulation of neoblast proliferation in *D. japonica*. For this, it will be very important to identify which types of *Djyki*-expressing differentiated cells promote neoblast proliferation in a non-cell-autonomous manner.

Since planarians live in water, it is important to maintain the internal water balance of the body by modulating osmotic water transport across the body surface depending on the environmental conditions under which they are living during homeostasis. The edema phenotype is an obvious sign that signifies the dysfunction of osmoregulation. Previous reports demonstrated that destruction of the excretory (protonephridial) system leads to edema in *S. mediterranea* (Rink *et al.* 2011; Scimone *et al.* 2011). In contrast, we found that increased expression of *DjaqpA* induced by *Djyki* RNAi could also induce edema in *D. japonica*, while leaving the protonephridial system normal. This increase of expression was restored to the normal level by simultaneous RNAi of *Djmats*, which we showed here encodes an inhibitor of *Djyki*, resulting in a lack of edema formation. X-ray irradiation of planarians demonstrated that *DjaqpA* was expressed in differentiated cells, as *Djyki* was (Fig. S4). These observations suggest that *DjaqpA* acts as a downstream effecter in the transcriptional circuit of *Djyki* for the regulation of osmotic water transport across the body surface. Interestingly, *DjaqpB* and *C* are not involved in this circuit.

It is still largely unknown what kind of signaling pathways regulate the expression of aquaporin genes in animals. Our data for the first time suggest the possibility that Hippo signaling might be involved in the regulation of *aquaporin* expression during homeostasis. It will be interesting to further assess this possibility in other animals, especially in mammals, including human.

Acknowledgments

We thank Dr Elizabeth Nakajima for critical reading of the manuscript, and all other laboratory members for their help and encouragement. This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas to Y. U. (22124004), a Grant-in-Aid for Scientific Research on Innovative Areas to K. A. (22124001), and a scholarship from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to B. H.

References

Agata, K. & Umesono, Y. 2008. Brain regeneration from pluripotent stem cells in planarian. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **363**, 2071–2078.

Agata, K. & Watanabe, K. 1999. Molecular and cellular aspects of planarian regeneration. *Semin. Cell Dev. Biol.* **10**, 377–383.

Carbrey, J. M. & Agre, P. 2009. Discovery of the aquaporins and development of the field. *Handb. Exp. Pharmacol.* **190**, 3–28.

Demircan, T. & Berezikov, E. 2013. The Hippo pathway regulates stem cells during homeostasis and regeneration of the flatworm *Macrostomum lignano*. *Stem Cells Dev.* **22**, 2174–2185.

Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S. A., Gayyed, M. F., Anders, R. A., Maitra, A. & Pan, D. 2007. Elucidation of a universal size-control mechanism in Drosophila and mammals. *Cell* **130**, 1120–1133.

Harvey, K. F., Pfleger, C. M. & Hariharan, I. K. 2003. The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* **114**, 457–467.

Hayashi, S., Tamura, K. & Yokoyama, H. 2014. Yap1, transcription regulator in the Hippo signaling pathway, is required for Xenopus limb bud regeneration. *Dev. Biol.* **388**, 57–67.

Huang, J., Wu, S., Barrera, J., Matthews, K. & Pan, D. 2005. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell* **122**, 421–434.

Jia, J., Zhang, W., Wang, B., Trinko, R. & Jiang, J. 2003. The Drosophila Ste20 family kinase dMST functions as a tumor suppressor by restricting cell proliferation and promoting apoptosis. *Genes Dev.* **17**, 2514–2519.

Lin, A. Y. T. & Pearson, B. J. 2014. Planarian yorkie/YAP functions to integrate adult stem cell proliferation, organ homeostasis and maintenance of axial patterning. *Development* **141**, 1197–1208.

Mineta, K., Nakazawa, M., Cebria, F., Ikeo, K., Agata, K. & Gojobori, T. 2003. Origin and evolutionary process of the CNS elucidated by comparative genomics analysis of planarian ESTs. *Proc. Natl Acad. Sci. USA* **100**, 7666–7671.

Newmark, P. A. & Sánchez Alvarado, A. 2002. Not your father's planarian: a classic model enters the era of functional genomics. *Nat. Rev. Genet.* **3**, 210–219.

Nishimura, O., Hirao, Y., Tarui, H. & Agata, K. 2012. Comparative transcriptome analysis between planarian *Dugesia japonica* and other platyhelminth species. *BMC Genom.* **13**, 289.

Ogawa, K., Ishihara, S., Saito, Y., Mineta, K., Nakazawa, M., Ikeo, K., Gojobori, T., Watanabe, K. & Agata, K. 2002. Induction of a *noggin-like* gene by ectopic DV interaction during planarian regeneration. *Dev. Biol.* **250**, 59–70.

Oh, H. & Irvine, K. D. 2008. In vivo regulation of Yorkie phosphorylation and localization. *Development* **135**, 1081–1088.

Pan, D. 2010. The hippo signaling pathway in development and cancer. *Dev. Cell* **19**, 491–505.

Pantalacci, S., Tapon, N. & Léopold, P. 2003. The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. *Nat. Cell Biol.* **5**, 921–927.

Papadopoulos, M. C. & Verkman, A. S. 2005. Aquaporin-4 gene disruption in mice reduces brain swelling and mortality in pneumococcal meningitis. *J. Biol. Chem.* **280**, 13906–13912.

Papadopoulos, M. C. & Verkman, A. S. 2007. Aquaporin-4 and brain edema. *Pediatr. Nephrol.* **22**, 778–784.

Rink, J. C., Vu, H. T.-K. & Sánchez Alvarado, A. 2011. The maintenance and regeneration of the planarian excretory system are regulated by EGFR signaling. *Development* **138**, 3769–3780.

Rouhana, L., Weiss, J. A., Forsthoefel, D. J., Lee, H., King, R. S., Inoue, T., Shibata, N., Agata, K. & Newmark, P. A. 2013. RNA interference by feeding in vitro-synthesized double-stranded RNA to planarians: methodology and dynamics. *Dev. Dyn.* **242**, 718–730.

Sakurai, T., Lee, H., Kashima, M., Saito, Y., Hayashi, T., Kudome-Takamatsu, T., Nishimura, O., Agata, K. & Shibata, N. 2012. The planarian P2X homolog in the regulation of asexual reproduction. *Int. J. Dev. Biol.* **56**, 173–182.

Schlegelmilch, K., Mohseni, M., Kirak, O., Pruszak, J., Rodriguez, J. R., Zhou, D., Kreger, B. T., Vasioukhin, V., Avruch, J., Brummelkamp, T. R. & Camargo, F. D. 2011. Yap1 acts downstream of α-catenin to control epidermal proliferation. *Cell* **144**, 782–795.

Scimone, M. L., Srivastava, M., Bell, G. W. & Reddien, P. W. 2011. A regulatory program for excretory system regeneration in planarians. *Development* **138**, 4387–4398.

Sun, M.-C., Honey, C. R., Berk, C., Wong, N. L. M. & Tsui, J. K. C. 2003. Regulation of aquaporin-4 in a traumatic brain injury model in rats. *J. Neurosurg.* **98**, 565–569.

Udan, R. S., Kango-Singh, M., Nolo, R., Tao, C. & Halder, G. 2003. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat. Cell Biol.* **5**, 914–920.

Umesono, Y. & Agata, K. 2009. Evolution and regeneration of the planarian central nervous system. *Dev. Growth Differ.* **51**, 185–195.

Umesono, Y., Watanabe, K. & Agata, K. 1997. A planarian *orthopedia* homolog is specifically expressed in the branch region of both the mature and regenerating brain. *Dev. Growth Differ.* **39**, 723–727.

Verkman, A. S. 2012. Aquaporins in clinical medicine. *Annu. Rev. Med.* **63**, 303–316.

Wei, X., Shimizu, T. & Lai, Z.-C. 2007. Mob as tumor suppressor is activated by Hippo kinase for growth inhibition in Drosophila. *EMBO J.* **26**, 1772–1781.

Wu, S., Huang, J., Dong, J. & Pan, D. 2003. Hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell* **114**, 445–456.

Wu, S., Liu, Y., Zheng, Y., Dong, J. & Pan, D. 2008. The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. *Dev. Cell* **14**, 388–398.

Zador, Z., Stiver, S., Wang, V. & Manley, G. T. 2009. Role of aquaporin-4 in cerebral edema and stroke. *Handb. Exp. Pharmacol.* **190**, 159–170.

Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B. & Jiang, J. 2008. The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. *Dev. Cell* **14**, 377–387.

Zhao, B., Kim, J., Ye, X., Lai, Z.-C. & Guan, K.-L. 2009. Both TEAD-binding and WW domains are required for the growth stimulation and oncogenic transformation activity of yes-associated protein. *Cancer Res.* **69**, 1089–1098.

Zhi, X., Zhao, D., Zhou, Z., Liu, R. & Chen, C. 2012. YAP promotes breast cell proliferation and survival partially through stabilizing the KLF5 transcription factor. *Am. J. Pathol.* **180**, 2452–2461.