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

(*Dugesia japonica* プラナリアで *yorkie/ yap* 遺伝子の 2 つの機能)

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ABSTRACT

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 accord 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: planarian, stem cells, osmoregulation, Yorkie/Yap, homeostasis.

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1. Introduction

Planarians possess somatic pluripotent stem cells, neoblasts, in adults, which provide us a good opportunity to investigate the molecular mechanisms underlying stem cell dynamics *in vivo* (Agata & Watanabe, 1999;; Agata & Umesono, 2008; Umesono & Agata, 2009). In addition, planarians have regenerative ability from tiny fragment after amputation, based on their somatic pluripotent stem cells system, suggesting that planarians are powerful experimental animals to understand molecular mechanisms underlying regeneration.

The neoblasts were defined by their morphological features, which were observed by electron microscopy (EM) (Pedersen 1959; Morita 1969; Hori 1992). They are small in size and have scanty cytoplasm with a few mitochondria, many free ribosomes, no endoplasmic reticulum, and chromatoid bodies, which is a unique cytoplasmic structure. When planarians are irradiated with X-ray, neoblasts are specifically eliminated and planarians lose the ability of regeneration (Wolff & Dubois 1948). It has been reported that the transplantation of a single neoblst into irradiated planarian restores regeneration activity (Wagner et al., 2011), demonstrating the pluripotency of neoblasts in planarian. Initially, *DjvlgA* (vasa-like gene A) was identified as a neoblast-marker gene (Shibata et al. 1999). After that, several molecular markers to distinguish neoblasts have been identified. PCNA, S-phase-specific gene, was revealed as a powerful tool for detection of proliferating stem cells (Orii et al., 2005). Furthermore, staining with BrdU and anti-phosphorylated histone H3 antibody (pH3), markers of mitotic cells (S- and M-phase, respectively), visualized neoblasts (Newmark & Sánchez Alvarado, 2000), since only the neoblasts maintain proliferative activity in planarian somatic cells.

Based on the feature, planarian excretory system defines as protonephridial, which is closed up by a terminal cell, in contrast to metanephridial system, which is opened into the coelomic cavity such as vertebrate nephron. The excretory system consists of a series of tubules and flame cells. By beating of the flame cell cilia bundle, body fluid is filtered through the tubules and excess water and liquid waste

are secreted from the body. This excretory system is required for osmotic regulation, critically, to the freshwater planarian (Wilson & Webster, 1974). However, the structure and function of planarian protonephridia is still not well understood.

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) (Fig. 1). Hippo (Hpo) interacts with the scaffolding protein Salvador (Sav) and then activates Warts (Wts) and Mats by phosphorylation (Udan et al., 2003; Wei et al., 2007; Wu et al., 2003). Yki is inactivated through phosphorylation by the Warts/Mats complex and is tethered in the cytoplasm by binding to 14-3-3 (Oh & Irvine, 2008). Dephosphorylation of Yki enables it to translocate into the nucleus and interact with Scalloped (Sd), a DNA-binding transcription factor, to promote target gene expression (Wu et al., 2008; Zhang et al., 2008).

The kinase cascade of hippo signaling pathway is well conserved in vertebrate also (Fig. 1). STE20 family protein kinase, MST1/2 (Hpo orthologs), make a complex with Salvador1 to enhance kinase activity and then directly phosphorylate Lats1/2 (Wts orthologs) and Mob1 (Mats ortholog) (Wu et al., 2003; Chan et al., 2005; Praskova et al., 2008). This phosphorylation leads to Lats1/2 activation. Activated Lats1/2 interact with and phosphorylate YAP (Dong et al., 2007; Huang et al., 2005). When Yap is phosphorylated, Yap is sequestered in the cytoplasm by promoting its interaction with 14-3-3 and degraded in an ubiquitin-proteasome-dependent manner (Zhao et al., 2010). Dephosphorylated Yap translocates into the nucleus and activates target gene expression through the interaction with TEAD (Sd ortholog) (Wanget al., 2009).

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 (Dong et al., 2007;
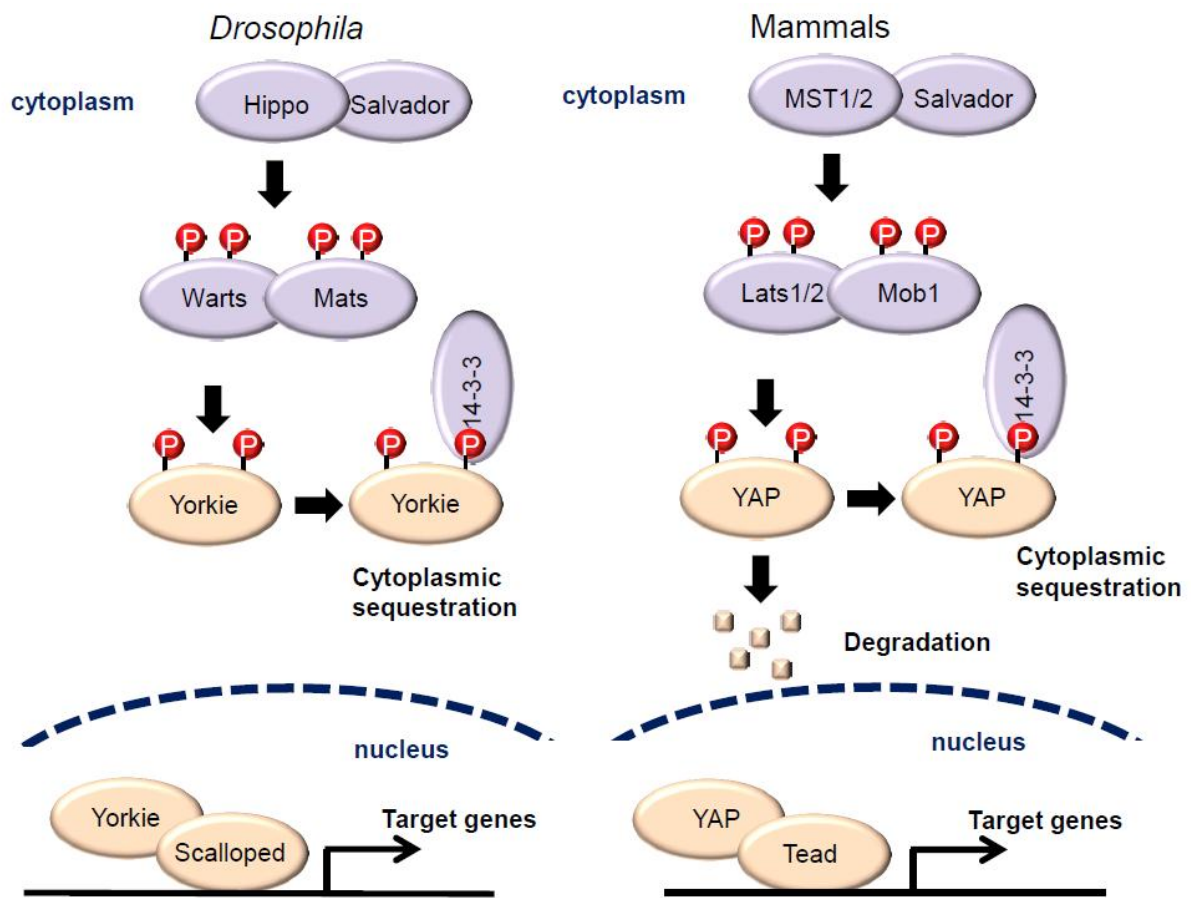
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Fig. 1. Schematic models of the Hippo pathway in *Drosophila* and mammals.

Huang et al., 2005). 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).

In addition, it has been reported that the Hippo signaling pathway is involved in tissue regeneration in *Drosophila* and mammal. In the *Drosophila* midgut, expression of Yki is mostly restricted to the cytoplasm of intestinal stem cells (ISC), suggesting that Yki might be inactivated. In response to injury, however, Yki is translocated into nucleus and facilitates ISC proliferation (Karpowicz et al., 2010; Shaw et al., 2010). Furthermore, expression of Yap is increased by intestinal damage and deficiency of Yap leads to malfunction of regeneration, showing Yap has an important role in tissue regeneration in mammal (Cai et al., 2010).

Recently, two groups have reported the function of Hippo signaling pathway, specially focusing on *yki/yap*-related genes using two different free-living flatworm species as models (Table. 1). 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). In contrast, deficiency of *Mac-Hpo, Mac-Sav, Mac-Wts*, and *Mac-Mats* caused increase proliferative activity and formed overgrowth demonstrating that *yki/yap* is functionally conserved between flatworms and mammals. In the case of the freshwater planarian *Schmidtea mediterranea,* however, *Smed-yki* RNAi led to hyperproliferation of neoblasts during homeostasis, resulting in the opposite phenotype to that in *M. lignano* (Lin & Pearson, 2014). Specially, *Smed-yki* is also required for maintenance of excretory system, but *Mac-Yap* is not. Furthermore, they showed the possibility that *Smed-sd* might function as co-activator of *Smed-yki*. Even though *Mac-Yap* and *Smed-yki* showed opposite function in stem cell dynamics, both *Mac-Yap* RNAi and *Smed-yki* RNAi failed to regenerate after amputation.

The aquaporins (AQPs) are water channels to facilitate transcellular water flow across cell

Table 1. Comparisons between *Smed-yki* and *Mac-Yap*.

	Smed-yki (Lin & Pearson, 2014)	*Mac-Yap* (Demircan & Berezikov, 2013)
Proliferative activity	repression	activation
Regeneration	required	required
Excretory system	required	-
Inhibitor	-	*Mac-Hpo, Sav, Wts, Mats*
Co-activator	*Smed-sd*	-

membranes in response to osmotic gradients (Carbrey & Agre, 2009; Verkman, 2012). Commonly, water molecules are transported by AQP-dependent passive mechanism, however, AQP Knockout studies demonstrate that AQPs is controlled by lots of regulation and have diverse distribution depending on cell and tissue type (Papadopoulos & Verkman, 2013). In human, thirteen AQPs have been identified and these AQPs express in a wide range of tissue; brain, kidney, Heart, and lung (Day et al., 2014). AQP4, the most well studied AQP, is known to have a critical role for maintenance of CNS functions. It has been reported that water permeability was reduced a sevenfold in primary cultured astrocytes derived from AQP4-deficient mice (Solenov et al., 2004). Moreover, AQP4 deletion showed a tenfold reduction in blood-brain barrier (BBB) water permeability in mouse brain (Papadospoulos et al., 2005). Even though hundreds of AQP homologues have been already discovered from the vertebrate to lower organisms (Tanghe et al., 2006; Soveral et al., 2010), it is unknown whether AQPs exist and regulate water flow in planarian. Edema, swelling, is caused by abnormal accumulation of fluid in the space that surrounds the body's tissues and organs. Normally, edema is well studied in human and mouse. AQP4 is one of the key regulators for edema formation (Badaut et al., 2011) and is the most abundant AQP in brain (Badaut et al., 2002).

Since Hippo signaling pathway is already known to have functions to regulate proliferative activity and regeneration in *Drosophila* and mammal, we expected that Yki/Yap might be a key clue to elucidate stem cells dynamics and regeneration process of planarian in the molecular level. To assess the role of *yki/yap,* 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.* As expected, *Djyki* RNAi showed regeneration defect after amputation. 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*.

2. Materials and methods

2.1 Animals

A clonal strain of the planarian *D. 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 which were 6 to 8 mm in length and that had been starved for at least 1 week were used in all experiments.

2.2 X-ray irradiation

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

2.3 Feeding RNA interference

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, 3 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 RT-PCR, using a set of primers specific to the gene that was targeted.

2.4 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 minutes, 95°C for 15 minutes, 50 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 1 minute. 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: ACCTATCGTGTCACTGTCTTTGACCGAAAA

    reverse: TTCATCATCTTCGATTTTCGGAGCCAGATA

2.5 Whole-mount in situ hybridization

The plasmid pBluescript SK containing the gene from the EST clone was used as the template for RNA probe synthesis. The plasmid was linearized with NotI or Kpn I at the 5' or 3' region of the target genes. The linearized DNA was used as a template for antisense or sence RNA probe by T7 or T3 RNA polymerase (Fermentas). Antisense and sense RNA probe was labeled with digoxigenin (Dig) according to the manufacturer's instructions (Roche Diagnostics). 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 washed by methanol. After washing by methanol, the samples were rinsed with 100%, 75%, 50% and 25% ethanol in Holtfreter's solution consecutively for 30 min each at 4°C. After rinsing with ethanol, the samples were washed one more time by PBST (phosphate buffered saline containing 0.1% Triton X-100) for 30 min at 4°C and then treated with 5 mg/ml proteinase K in PBST for 10 min at 37°C. After washing with cold PBST, the samples were 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 hour at 55°C. Digoxigenin (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 6 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 6 times for 30 minutes 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). After the color detection, the samples were rinsed with TE buffer to stop the reaction and then stored in TE buffer at 4°C.

2.6 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 for overnight at 50°C. After washing with Buffer I (maleic acid buffer containing 0.1% Triton X-100) 6 times for 30 min each at RT, Buffer II (Buffer I containing 1% blocking reagent (Roche Diagnostics)) was treated 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 6 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 6 times for 30 min each at RT, and mounted with Fluorescent Mounting Medium (Dako).

2.7 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 $\pm$ standard error of the mean (SEM) of three independent biological replicates.

2.8 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

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similarity in a previously constructed library of expressed sequence tags (ESTs) (Mineta et al., 2003)

using tblastn program.

3. Results

3.1 Identification of homologs

We identified a single *yki*-related gene (termed *Djyki*) from our cDNA database of *D. japonica* (Nishimura et al., 2012) using BLAST search based on protein sequence similarity to figure out the role of Hippo signaling pathway in *D. japonica*. *Djyki* encodes a protein with a tead-binding domain, which is necessary for the interaction with Sd to activate transcriptional activity, 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. 2A). We also identified homologs of the hippo pathway components, a single *mats*-related gene (termed *Djmats*), a single *hpo*-related gene (termed *Djhpo*), a single *wts*-related gene (termed *Djwts*), which provided us a good opportunity to assess whether *Djmats, Djhpo,* and *Djwts* protein functions as an evolutionarily conserved negative regulators of *Djyki* in this planarian species. Furthermore, to check conserved enhanced activity, we identified a single *sd*-related gene (termed *Djsd*).

Firstly, we analyzed the expression patterns of these five genes in non-regenerating intact animals. Whole-mount in situ hybridization (WISH) demonstrated that *Djyki, Djmats, Djwts,* and *Djsd* were ubiquitously expressed throughout the body (Fig. 2B). Only *Djhpo* localized cephalic ganglia (CG), ventral nerve cord (VNC), and pharynx (Fig. 2B).
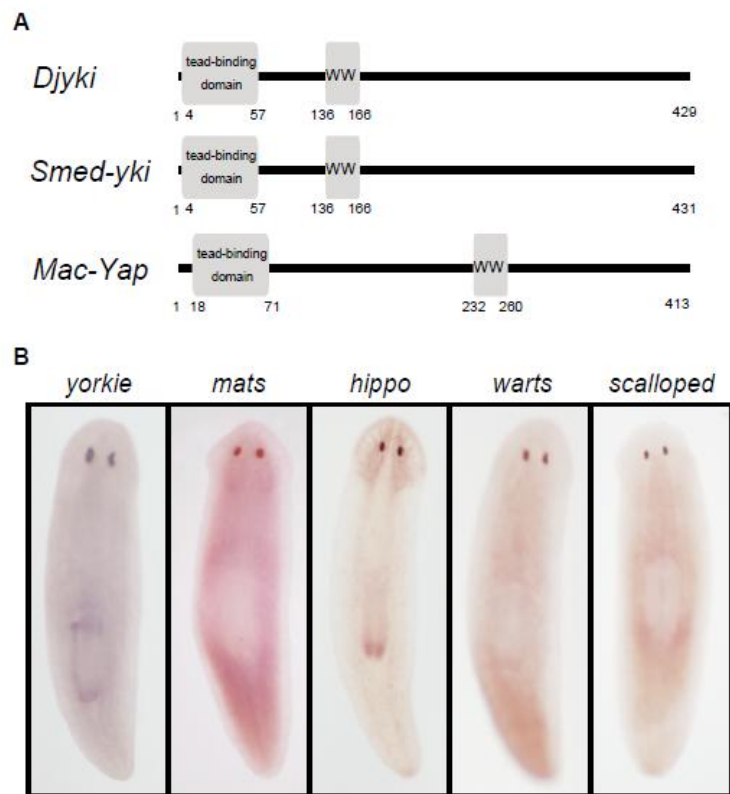
Fig. 2. Identification of Hippo pathway components and their expression. (A) Schematic representation of the protein structures of DjYki, Smed-Yki, and Mac-Yap. (B) Expression patterns of homologs by performing Whole-mount in situ hybridization (WISH).

3.2 *Djyki* RNAi caused edema formation during homeostasis

Next, we performed RNAi experiments of *Djyki, Djmats, Djhpo, Djwts,* or *Djsd*. We fed double-strand RNA (dsRNA) three times in a week with two days interval. In single *Djmats(RNAi), Djhpo(RNAi), Djwts(RNAi),* and *Djsd(RNAi)* animals, we could not detect any obvious defect during homeostasis or regeneration (data not shown). In contrast, we found that *Djyki(RNAi)* non-regenerating intact animals showed the edema phenotype, swelling by an excessive accumulation of water into the body, during homeostasis (Fig. 3A). All of these animals died within 17 days after the first feeding of *Djyki* dsRNA (Fig. 3B). We performed double RNAi to analyze whether the edema phenotype induced by *Djyki* RNAi was rescued by simultaneous *Djmats, Djhpo,* or *Djwts* RNAi. Since *mats, hpo*, and *wts* showed conserved inhibitory functions against *yki* from drosophila to vertebrate, we expected clear rescue phenotypes after double RNAi experiments. Surprisingly, only *Djmats* RNAi rescued *Djyki* RNAi induced edema (Fig. 3A), but *Djhpo* and *Djwts* did not (data not shown). 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). Single *Djmats(RNAi)* did not affect survival of planarians. There is no obvious difference between control (*EGFP(RNAi)*) and *Djmats(RNAi)*. The survival rate of *Djyki(RNAi)* or/and *Djmats(RNAi)* was confirmed by performing an independent experiment (data not shown). Since *Djmats* showed conserved negative activity for the function of *Djyki*, we tested whether *Djsd* also have conserved function as positive regulator of *Djyki*. However, double RNAi of *Djyki* and *Djsd* did not show any significant difference compared to *Djyki* single RNAi animals (data not shown). Even though we identified several key genes of Hippo pathway, only *Djmats* functions against *Djyki* as a conserved inhibitory regulator.
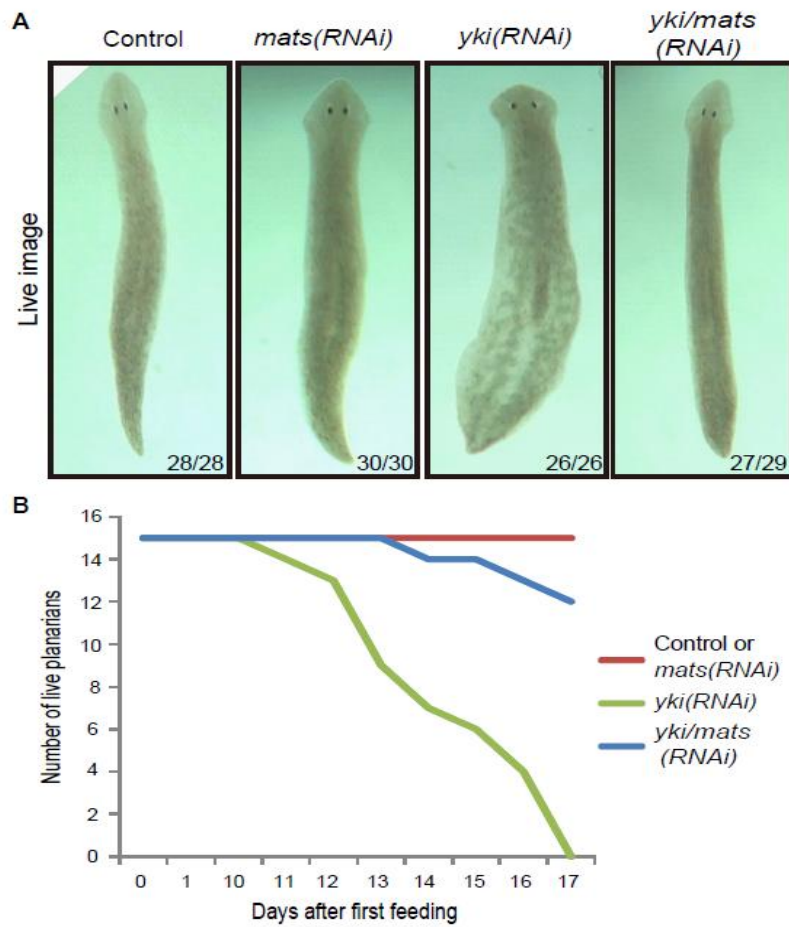
Fig. 3. *Djyki* **RNAi leads to edema, which is rescued by** *Djmats* **RNAi.** (A) Live image of intact animals at 10 days after last RNAi feeding. (B) Survival curves for RNAi-treated planarians. n=15. The experiment was performed twice independently to confirm that the results were reproducible.

3.3 *Djyki* and *Djmats* expressed in differentiated cells, but not in stem cells

Based on results of RNAi assay, we were focusing on demonstration of the relationship between *Djyki* and *Djmats*. 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. 4A). We used antisense and sense mRNA probes, respectively, to demonstrate specificity of antisense mRNA probe. 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. 4B). These data suggested that *Djyki* and *Djmats* located in differentiated cells, but not in stem cells.
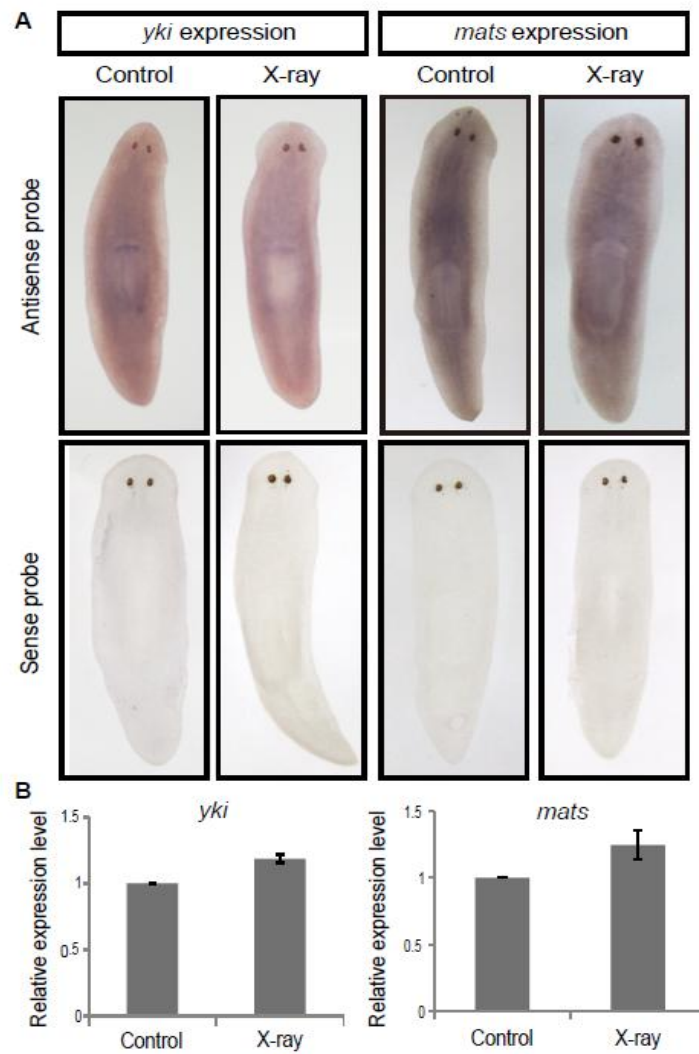
Fig. 4. *Djyki* and *Djmats* are expressed in differentiated cells. (A) Expression patterns of *Djyki* and *Djmats*, analyzed by WISH after X-ray irradiation. (B) Expression levels of *Djyki* and *Djmats*, analyzed by qRT-PCR after X-ray irradiation. No significant variation was observed between control and X-ray irradiated animals.

3.4 *Djyki* RNAi caused a decrease of neoblast proliferation

Since *yki/yap* is known as a co-transcription factor to facilitate proliferation, we tested proliferative activity in *Djyki* RNAi. 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 the expression levels of both *DjpiwiA* and *Djpcna* when compared to the control during homeostasis (Fig. 5A). 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. 5B). In addition, we also detected head-regeneration defects in *Djyki(RNAi)* animals (Fig. 5C). *Djyki(RNAi)* animals failed to regenerate their eyes after amputation. As shown in Fig. 5D, over 60% of *Djyki(RNAi)* have one or no eye at 3 day post amputation (dpa), suggesting that they have regeneration defect. In control animals, however, 15% of planarians showed cyclopia or no eye at 3dpa also. To confirm the regeneration defect in *Djyki* RNAi, we tested later time point, 6dpa. At 6dpa, all of control planarians successfully regenerated two eyes. In contrast, 35% of *Djyki(RNAi)* still failed to regenerate normal eyes at 6dpa. Even though success rate of head regeneration was slightly increased from 3dpa to 6dpa, *Djyki* RNAi planarians showed distinct regeneration defect. As we expected, all of the defects related to neoblast activity and regeneration defect in *Djyki(RNAi)* animals were rescued by simultaneous RNAi of *Djmats* (Fig. 5A, B, D), while leaving *Djyki* RNAi was effective. These observations suggest that *Djyki* is required for active proliferation of neoblasts and regeneration, in which processes *Djmats* negatively regulates *Djyki*.

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
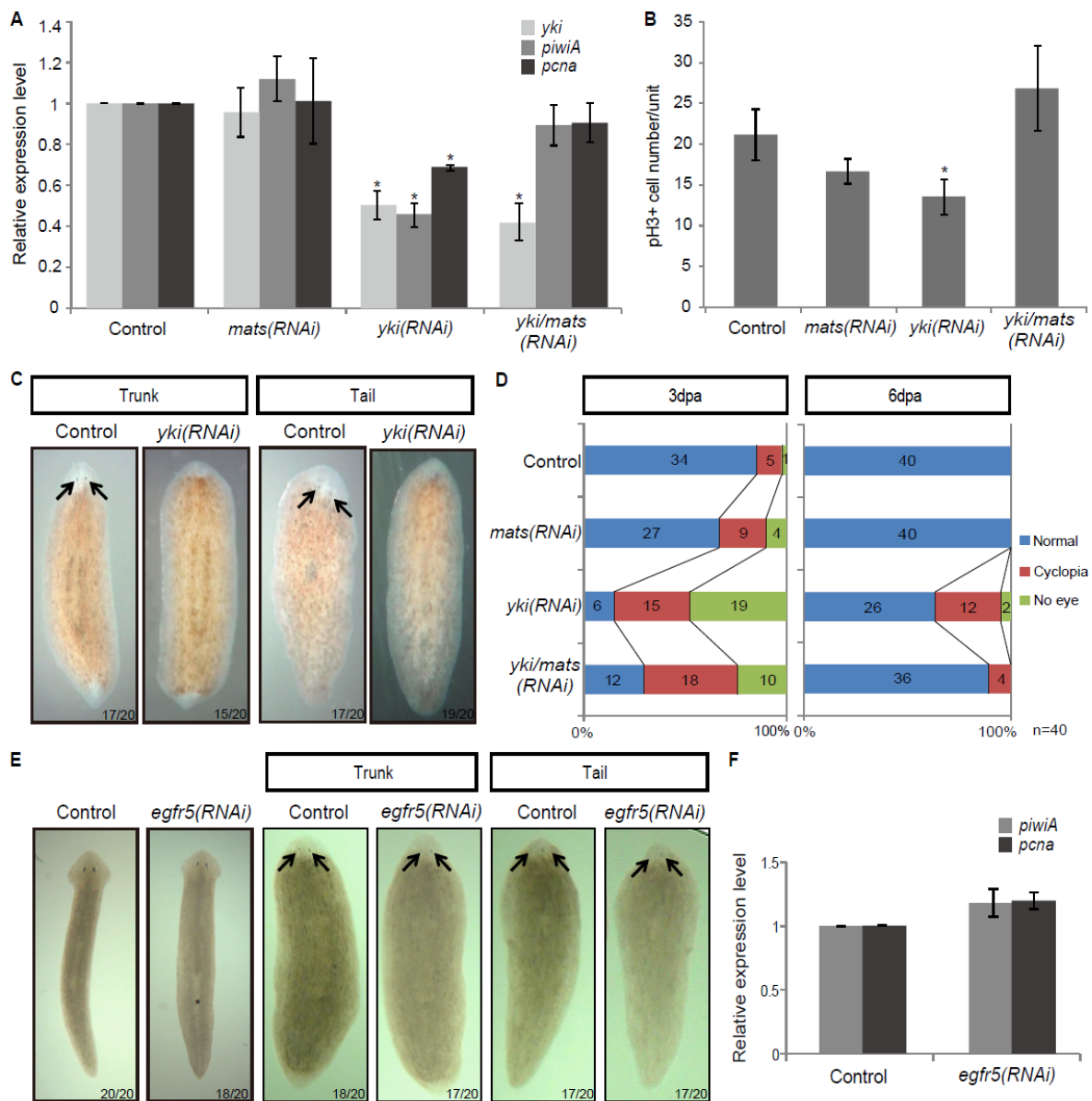
Fig. 5. Reduced proliferation and defective regeneration caused by *Djyki* RNAi. (A) Significant decrease of *DjpiwiA* and *Djpcna* in *Djyki(RNAi)* at 3 days after the last feeding. * P<0.05. (B) The number of pH3-positive cells (n=5). * P<0.05. Unit volume: 1.1 x 10^{-2}mm^3. (C) Head regeneration 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. (E) *Djegfr5* RNAi caused the edema phenotype and normal head regeneration. Live image. Asterisk indicates bloated region. Arrows indicate eyes. (F) Graphs display levels of *DjpiwiA* and *Djpcna* expression in *Djegfr5(RNAi)* animals.

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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. 5E). In addition, qRT-PCR analysis demonstrated that *Djegfr5* RNAi did not affect the expression levels of *DjpiwiA* or *Djpcna* during homeostasis (Fig. 5F).

3.5 *Djyki* is not required for maintenance of protonephridial system

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 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. 6A). In contrast, we did not detect any significant difference of the number of these two clusters between control and *Djyki(RNAi)* animals (Fig. 6B). Furthermore, qRT-PCR analysis demonstrated that *Djyki* RNAi did not affect the expression level of *Djegfr5* (Fig. 6C). Consistent with this observation, double *Djyki* and *Djegfr5* RNAi resulted in a dramatic increase of the number of dead planarians when compared to single *Djyki* or *Djegfr5* RNAi (Fig. 6D).

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.
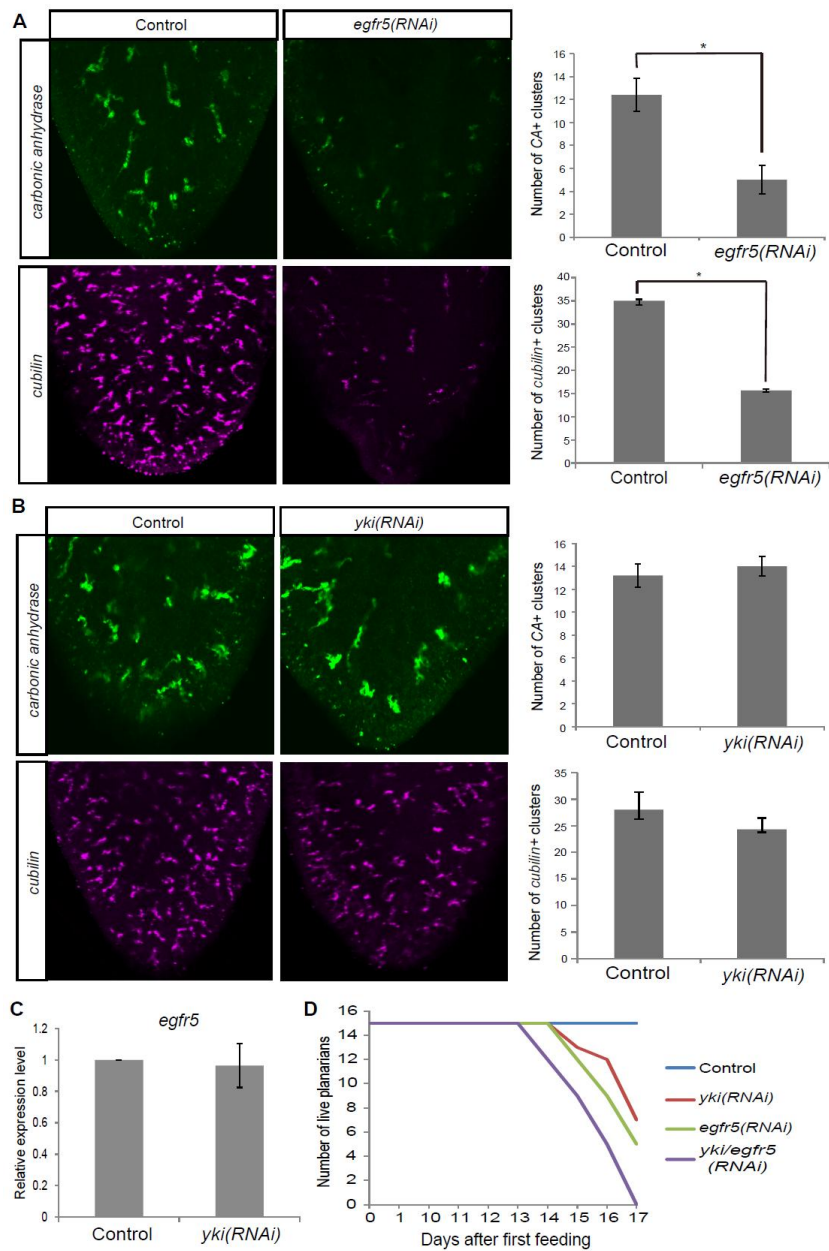
Fig. 6. ***Djyki*** **is not required for excretory system.** (A), (B) Fluorescence in situ hybridization (FISH) for staining RNAs transcribed from *DjCA* (green), *Djcubilin* (magenta), and protonephridial marker genes, after *Djegfr5* and *Djyki* RNAi. Graphs display number of *DjCA*[+] expression and *Djcubilin*[+] expression clusters (right). n=3. * P<0.05. (C) *Djegfr5* level in *Djyki(RNAi)* analyzed by qRT-PCR. (D) Survival curves after RNAi feeding.

3.6 *DjaqpA* was upregulated in *Djyki* RNAi

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 (Papadopoulos & Verkman, 2005, 2007; Sun et al., 2003; Zador et al., 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. 7A). In contrast to *DjaqpB* and *C*, *DjaqpA* was also expressed strongly in the brain-branch region (Fig. 7A). 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. 7B, C). This increase was suppressed by simultaneous *Djmats* RNAi (Fig. 7C), suggesting that the expression level of *DjaqpA* depends on the activity level of *Djyki* during homeostasis. By contrast, the expression levels of *DjaqpB* and *C* were not changed in *Djyki(RNAi)* animals (Fig. 7D), 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. 7E), suggesting that edema itself is not a cause of the increased expression of *DjaqpA* during homeostasis.
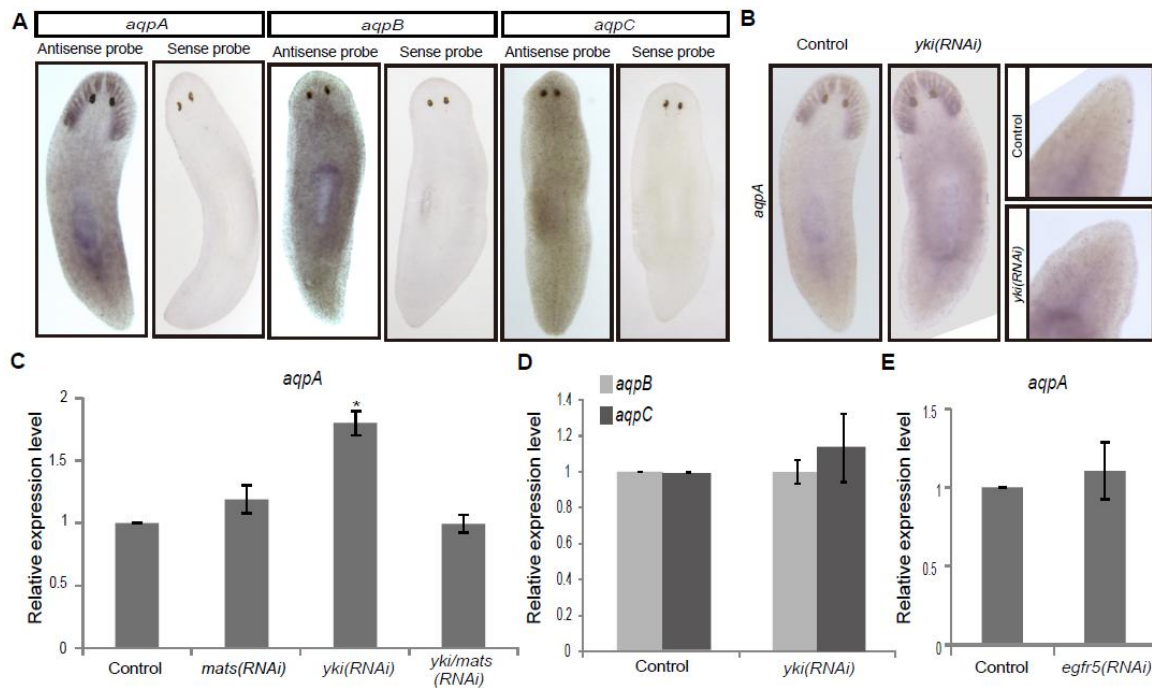
Fig. 7. *Djyki* **RNAi Upregulates expression of *DjaqpA*.** (A) Expression patterns of *DjaqpA*, *B*, and *C* in intact animals. (B), (C) Level of *DjaqpA* in *Djyki(RNAi)* planarian as determined by WISH and qRT-PCR. * P<0.05. (D)There was no significant change of *DjaqpB* or *C* expression level after *Djyki* RNAi. (E) *DjaqpA* expression level in *Djegfr5(RNAi)* planarians analyzed by qRT-PCR.

3.7 *Djyki* negatively regulates the expression of *DjaqpA* to block edema formation 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. 8A), as well as by *Djmats* RNAi (Fig. 4A). 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. 8B, C).

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*.
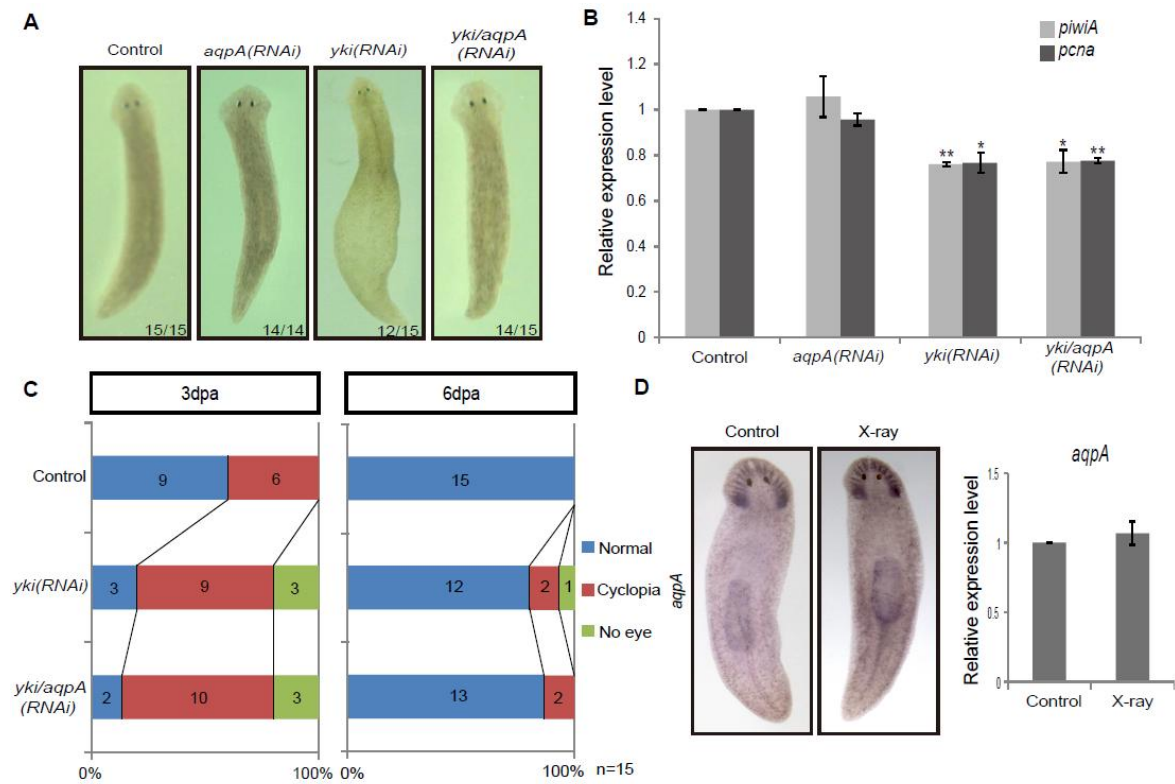
Fig. 8. Upregulation of *DjaqpA* is necessary for *Djyki* RNAi-induced edema. (A) Phenotypes of *Djyki* or/and *DjaqpA* RNAi at 10 days after last RNAi feeding. (B) Levels of *DjpiwiA* and *Djpcna* expression in *Djyki* or/and *DaqpA* RNAi planarians. ** P<0.01, * P<0.05. (C) Classification by number of eyes at 3 days (left) and 6 days (right) post amputation. n=15. (D) WISH and qRT-PCR data showed that *DjaqpA* is expressed in X-ray-insensitive cells.

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4. Discussion

We showed here that in *D. japonica*, *Djyki* is required for stem cell proliferation and regeneration, and also for osmoregulation (Fig. 2, 4). 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, 4). Furthermore, the most interesting discovery here was that *Djyki* negatively regulates the expression of *DjaqpA* and thereby blocks edema formation during homeostasis (Fig. 6, 7).

From mammals to flatworms, *yki/yap* has a conserved role to activate stem cell proliferation (Demircan & Berezikov, 2013; Schlegelmilch et al., 2011; Zhi et al., 2012). 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 (Fig. 9). However, it is possible to speculate that regulation of body size and osmotic regulation should be coupled to maintain homeostasis of body conditions. 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.

We identified five homologs, *yki, mats, hpo, wts*, and *sd* in *D. japonica* (Table. 2), and confirmed functional activity of only two genes, *yki* and *mats,* by performing RNAi assay. In *M. lignano*, *Mac-Hpo, Mac-Wts*, and *Mac-Mats* showed opposite phenotype against *Mac-Yap* after RNAi assay

Table 2. Comparisons between *Djyki*, *Smed-yki* and *Mac-Yap*.

Gray parts indicates new discoverywhich we have demonstrated and identified in *D. japonica.*

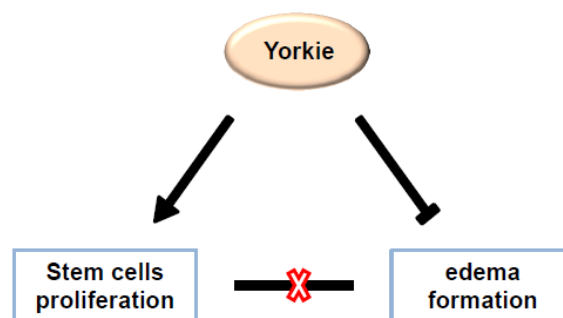|	*Djyki*(Hwang, 2015)	*Smed-yki*(Lin & Pearson, 2014)	*Mac-Yap* Demircan & Berezikov, 2013)
Proliferative activity	activation	repression	activation
Regeneration	required	required	required
Excretory system	not required	required	-
aqp expression	required	-	-
Co-activator	-	*Smed-sd*	-
Inhibitor	*Djmats*	-	*Mac-Hpo, Sav, Wts, Mats*

Fig. 9.   Yorkie activates stem cells proliferation and inhibits edema formation in independent

manner in *D. japonica*

(Demircan & Berezikov, 2013). Two homologs of *sd* were identified in *S. mediterranea* and showed similar function as *Smed-yki* did (Lin & Pearson, 2014). In *D. japonica*, we found that *yki* is negatively regulated by *mats* and required for stem cell proliferation and osmoregulation (Fig. 10). Still, we do not understand how yki regulates those things and why other homologs, *hpo*, *wts*, and *sd*, did not show any phenotype when they were deficient. In Drosophila and vertebrate, Mats inhibits Yki activity by binding to Wts. In other ward, Mats is not able to phosphorylate and regulate Yki directly. Therefore, further studies are necessary to elucidate how *Djmats* regulates *Djyki* and what gene has a role as the bridge to connect between *Djmats* and *Djyki*.

Even though *D. japonica* and *S. mediterranea* have similar biotic features as a sister species (Nishimura et al, 2012), *yki/yap*-related genes have different functions in stem cells dynamics and homeostasis in both species. Surprisingly, *Djyki* and *Smed-yki* showed opposite functions to regulate stem cells proliferation (Table. 2). To inhibit edema formation, *Djyki* and *Smed-yki* chose different mechanism, regulation of *Djaqp* expression and protonephridial system, respectively (Table. 2). The hippo pathway is well known to regulate diverse cellular processes such as proliferation, development, regeneration, and metastasis (Harvey et al, 2013). Specially, Yki, a key factor of the hippo pathway, has functions to be involved in various processes and is controlled by multiple mechanisms. Therefore, it is difficult to pick only a couple of function of *yki* by performing RNAi analysis. Complex and tangled functions of *yki* might show distinct phenotype after knockdown of *yki* in *D.japonica* and *S. meditteranea*.

Since planarians live in water, it is important to maintain the internal water balance of the in 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). By contrast, we found that increased expression of *DjaqpA* induced by *Djyki* RNAi could also
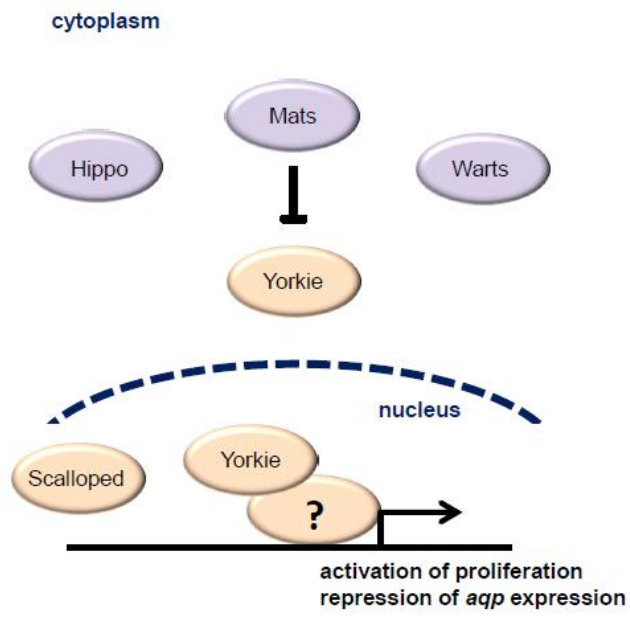
Fig. 10. The functions of key components of Hippo pathway in *D. japonica*.

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. 7D). 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 kinds 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.

Acknowledgements

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