Original Article

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

Byulnim Hwang,¹ Yang An,¹ Kiyokazu Agata¹ and Yoshihiko Umesono²*

¹Department of Biophysics, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto, and
²Graduate 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).
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 Transcription 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: GACTGCTTTGTTGGGATTTTTG
reverse: GTCAAATACAAATGATCTCAAAGG
Djmats forward: GGTAGATCGGAAGGAATTAGCTCC
reverse: GACTGCTTGTTAGCTCTTGCT
DjaqpA forward: CTTTTGGACGGCTCTATTTG
reverse: ACAAGCTCCTAACCCAATGA
DjaqpB forward: CTTTTGGACGGCTCTATTTG
reverse: ACAAGCTCCTAACCCAATGA
DjaqpC forward: TATGTACGGCAGCAGACAAG
reverse: CAGAAATTCAGCAGACACAG
Djegfr5 forward: TGGGGACGAATCTGGAGTAT

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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 (H2O2) 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 4% paraformaldehyde for 90 min at 4°C) and Buffer II (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 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 (H2O2) 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) twice at RT, the 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 quantitave 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), Djegr5 (LC011530), DjCA (LC011531), and Djucubilin (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...
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
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.* 2000).
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 DjapA, 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 DjaqP B and C, DjaqP A 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 DjaqP A 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 DjaqP A depends on the activity level of Djyki during homeostasis. In contrast, the expression levels of DjaqP B and C were not changed in Djyki(RNAi) animals (Fig. S3A), showing that the Djyki activity is specifically required for the transcriptional regulation of DjaqP A during homeostasis. Furthermore, we also found that Djegfr5 RNAi did not affect the expression level of DjaqP A (Fig. S3B), suggesting that edema itself is not a cause of the increased expression of DjaqP A during homeostasis.

Next, we tested whether or not increased expression of DjaqP A has a role in Djyki RNAi-induced edema formation. Surprisingly, the edema phenotype induced by Djyki RNAi was rescued by simultaneous RNAi of DjaqP A (Fig. 5D), as well as by Djmats RNAi (Fig. 3A). These data suggest that increased expression of DjaqP A promotes edema formation in Djyki(RNAi) animals during homeostasis. Under this condition, interestingly, we found that simultaneous DjaqP A 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 DjaqP A enables us to conclude that Djyki plays at least two distinct roles in the regulation of stem cell dynamics and homeostasis in *D. japonica*.

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**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.

**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).
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 *Djyki*, which we showed here encodes an inhibitor of *DjaqpA* expressed in differentiated cells, as *Djyki* was (Fig. S4). These observations suggest that DjaqpA acts as a downstream effector 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.

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