ISJ 14: 1-8, 2017 ISSN 1824-307X

RESEARCH REPORT

RNAi of CNS-expressed gene *DjSlk* induces morphogenetic malformation and death in planarian *Dugesia japonica*

X Chen[1,2], H Zhen[1], S Wu[1], Q Lu[1], Q Pang[1], B Zhao[1]

[1]Laboratory of Developmental and Evolutionary Biology, School of Life Sciences, Shandong University of Technology, Zibo 255049, P.R. China
[2]Translational Medicine Center, The Sixth People's Hospital of Zhengzhou, Zhengzhou 450000, P. R. China

Accepted December 13, 2016

Abstract

Ste20-like kinases are critically multifuctional proteins which play important roles in varieties of celluar processes and physiological events. Here, We characterized a Ste20-like kinase gene (*DjSlk*) in planarian *Dugesia japonica*. Whole-mount *in situ* hybridizations revealed that *DjSlk* was expressed in the central nervous system (CNS) including cephalic ganglia and ventral nerve cords (VNCs) in intact and regenerating animals. After RNA interference (RNAi) of *DjSlk*, adult planarians became immobilized and wrinkled, then swelled and lysed eventually. *DjSlk* RNAi treated regenerating planarians could form the entire animals, and then displayed the similar phenotype transformation. These results suggest that loss of function of *DjSlk* leads to morphogenetic malformation of planarian *D. japonica* probably via regulating cell volume instead of disrupting the balance between cell proliferation and apoptosis.

Key Words: CNS; *DjSlk*; expression; morphogenetic malformation; cell volume

Introduction

Endowed with abundant pluripotent stem cells named neoblasts which proliferate and differentiate to all cell types in response to amputation and/or injury，planarians can regenerate the complete worms with all organs from almost any tiny fragments (Newmark and Sánchez Alvarado, 2002; Reddien and Sánchez Alvarado, 2004). Neoblast is the only source to provide new cells during turnover and regeneration (Wagner DE *et al.*, 2011). Elimination of neoblasts by irradiation, planarians demonstrate the typical phenotype defects such as head regression, dorsal curling and lesions during homeostasis and fail to shape the whole animals for amputated pieces (Newmark and Sánchez Alvarado, 2002; Reddien and Sánchez Alvarado, 2004; Saló *et al.*, 2009; Scimone *et al.*, 2010). Planarians can acquire similar phenotypes by silencing some genes related to neoblast maintenance, proliferation, differentiation, and apoptosis (Reddien *et al.*, 2005; Guo *et al.*, 2006; Pearson and Sánchez Alvarado, 2010; Li *et al.*, 2011).

Corresponding author:
Bosheng Zhao
School of Life Sciences
Shandong University of Technology
266 Xincun Western Road, Zibo 255049, P.R. China
E-mail: zhaobosheng@sdut.edu.cn

Sterile 20 (ste20) is originally found as mitogen-activated protein kinase kinase kinase kinase (MAP4K) involved in the mating response of haploid yeast *Saccharomyces cerevisiae* (Wu *et al.*, 1995). Its homologs in other organisms form the ste20-like kinase (SLK) superfamily and are mainly regarded as upstream regulators of the MAPK pathways (Dan *et al.*, 2001; Ling *et al.*, 2008). Ste20-like kinases play crucial roles in various cellular processes including cell growth, cell migration, apoptosis, cell-cycle control, cell shape change and stress responses (Dan *et al.*, 2001; Strange *et al.*, 2005; Ling *et al.*, 2008). In this paper, we identified a *Slk* gene in planarian *Dugesia japonica* and studied its tempospacial expression pattern and loss-of-function phenotype in intact and regenerating animals.

Materials and Methods

Animals

The planarians *Dugesia japonica* collected in Boshan, Shandong, China, are maintained in autoclaved tap water at 20 ℃ and 6 - 10 mm long animals were starved for at least one week before use in experiments.

Cloning and Sequence Analysis of cDNA

The *DjSlk* cDNA was derived from random

sequencing of a planarian cDNA library. Comparison against the GenBank protein database was performed using the BLAST network server at the National Center for Biotechnology Information (NCBI). Multiple protein sequences were aligned using the MegAlign program by the CLUSTAL method in DNASTAR software package (Burland, 2000).

Whole-mount in situ hybridization

Whole-mount *in situ* hybridizations were performed as described previously (Pearson *et al.*, 2009). The digoxigenin (DIG)-labelled antisense RNA probes were synthesized *in vitro*. Hybridizations were carried out at 56 ℃ for 17 h, the BCIP/NBT mixture solution (Roche) was used for color development. For regeneration experiments, animals were amputated pre- and post-pharyngeally and left to regenerate, the head-, trunk-, and tail-pieces were collected at the times indicated.

Quantitative real-time PCR

Quantitative real-time PCR was used to monitor the quantitative expression of the *DjSlk* as described previously (Yu *et al.,* 2015) in intact planarians, regenerating trunk fragments， and regenerating trunk fragments of RNAi-treated planarians at different times after amputation. The cDNA was synthesized using a First-Strand System kit from Invitrogen after total RNAs were extracted using RNAiso Reagent (TaKaRa). qPCR reactions were performed using Fast Start Universal SYBR Green Master (Rox) (Roche, Switzerland) according to the manufacturer's protocol. Three samples for each condition were run in parallel by a 7,500 Real Time PCR System (Applied Biosystems). Data were normalized to the expression of the internal control *DjEF2*. The following sets of specific primers were used: *DjSlk* mRNA, 5′-CGAAGGACAAAGGCACAT-3′, 5′-GAGCGAACACCAGGAACT-3′. *DjEF2* mRNA, 5′-TTAATGATGGGAAGATATGTTG-3′; 5′-GTACCATAGGATCTGATTTTGC-3′.

The data were analyzed using SPSS 16.0 software. The significance of differences was analyzed by one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc analysis to identify differences between the experimental and intact planarians. Data presented are means ± SD. Values of $p < 0.05$ were considered to be significant.

RNA interference

DjSlk was cloned into the L1440 plasmid with two T7 primers, and then dsRNA was synthesized according to the manufactrue's instructions (MEGAscript® RNAi Kit). Animals were injected at day 0, day 2, 4, and 6. For regeneration studies, animals were cut at day 10. Control animals were injected with deionized sterile water.

Immunostaining

Animals were killed in 2 % HCl for 5 min at RT and fixed in 4 % paraformaldehyde solution for 3 h at 4 ℃, then dehydrated in 100 % methanol solution for 1 h at -20 ℃. The following procedures were processed as described elsewhere (Robb and Sánchez Alvarado, 2002; Inoue *et al.*, 2007; Cebria, 2007, 2008). The primary antibody anti-SYNORF1, a mouse monoclonal antibody specific for synapsin (Developmental Studies Hybridoma Bank) was used at a dilution of 1:25. The secondary antibody Dylight 594 AffiniPure Goat anti-mouse IgG(H+L) (EarthOX) was used at a dilution of 1:200.

Results

Sequence analysis of DjSlk

The cDNA clone obtained from planarian *D. japonica* cDNA library is about 1,100 bp with the longest open reading frame of 879 bp. It encodes for a deduced protein of 292 amino acids with predicted molecular mass of approximately 32.4 kDa (Fig. 1)

```
  3 CCC TGG TTT ATA TAG GGG CGC TAG CTC GCC GCA GCC GAA CGA CCG AGC GCA GCG AGT CAG TGA GCG AGG AAG CGG CCG CAT AAC TTC GTA    92
  1                                                                                                                        30

 93 TAG CAT ACA TTA TAC GAA GTT ATC AGT CGA CGG TAC CGG ACA TAT GCC CGG GAA TTC GGC CAT TAC GGC CGG GGG ATG CGA GTA TTA TTT   182
 31                                                                                                   M  R  V  L  F      60

183 CTT ATT CCT AAA AAT CCT TCT CCT CAG TTA GAT GGG TCT TTC TCA AAA TTA TTC CGG GAT TTT GTC GAC TGT TGC TTG AAT AAG GAA CCA   272
 61 L  I  P  K  N  P  S  P  Q  L  D  G  S  F  S  K  L  F  R  D  F  V  D  C  C  L  N  K  E  P      90

273 GAA AAT AGA CCA TCT GCC AAG GAA TTA CTC CAT CAT AAC TTC ATT AAG AAA GCA AAG AGA ACC GCA TTT CTG CAG GAA TTG ATT GAT CGA   362
 91 E  N  R  P  S  A  K  E  L  L  H  H  N  F  I  K  K  A  K  R  T  A  F  L  Q  E  L  I  D  R    120

363 TAT CAA AAA TGG AAA ACT GAA GCC GAG AAC CAA GGT GAC AGT GAT TCC GAT GAT GAT GGC TTG GTT CCA GAT GAC GAT CAC AAA GCG CAC   452
121 Y  Q  K  W  K  T  E  A  E  N  Q  G  D  S  D  S  D  D  D  G  L  V  P  D  D  D  H  K  A  H    150

453 GGA TCG AAG GAC AAA GGC ACA TTC AAA TGG AAT TTC GAT ACT GTG AAA GCA AGC GGA GCG GCA GTG ATG GCA ACC AAT CCT GAG ATT ATC   542
151 G  S  K  D  K  G  T  F  K  W  N  F  D  T  V  K  A  S  G  A  A  V  M  A  T  N  P  E  I  I    180

543 ATG CGA GAA CCG ACT GTC CAG ATT CCT CGC TCC AGT CTT ATC TCG AGT CCT TCT TCT CCT GGA CGT ATT TCC ACT CCG CAA GGA TTT GTC   632
181 M  R  E  P  T  V  Q  I  P  R  S  S  L  I  S  S  P  S  S  P  G  R  I  S  T  P  Q  G  F  V    210

633 GGC TCT CCT ACA GAT GTG AGA CGC AGC ATG CCT CCT GCT GGC AGT GTC CAA GTG CTG CCC TTA GTT CCT GGT GTT CGC TCA AGT TGT ACC   722
211 G  S  P  T  D  V  R  R  S  M  P  P  A  G  S  V  Q  V  L  P  L  V  P  G  V  R  S  S  C  T    240

723 TCC ACA AGA TCC CCC ACG TGT CGG ACA AGC TTC TTC TAC TCT GAG ACA ACA AAT ACT TCC GGT TCT CAA AGA TTT GGA ATC AGT TTA TTC   812
241 S  T  R  S  P  T  C  R  T  S  F  F  Y  S  E  T  T  N  T  S  G  S  Q  R  F  G  I  S  L  F    270

813 TCA CGT GTC GGG AGG ACG ACA TCA GCC AAT AGC AGA ACT CAC TGC TGC ATT CGA AAT GGT CGA TTC CAT TTC TCC AAC TTA CAC TTC TCA   902
271 S  R  V  G  R  T  T  S  A  N  S  R  T  H  C  C  I  R  N  G  R  F  H  F  S  N  L  H  F  S    300

903 GTT TCT TTC CGA GCT GCT TTT CCG AGT CTT CGA CAA TTG TCC GAG ATT GTC CGA GGC GCA GAA CGA CGA GCA CTC AGT CAA ATC AGA GGT   992
301 V  S  F  R  A  A  F  P  S  L  R  Q  L  S  E  I  V  R  G  A  E  R  R  A  L  S  Q  I  R  G    330

993 TGA CAT TAT CTA TAT ATC TGT GTC TGT CTG ACT GTC TGT CTG TCT GTC TTT GAT TGC AGC AAC CAC ATG ATC GTT CGG              1070
331 *                                                                                                                    360
```

Fig. 1 The nucleotide and deduced amino acid sequence of *DjSlk*.

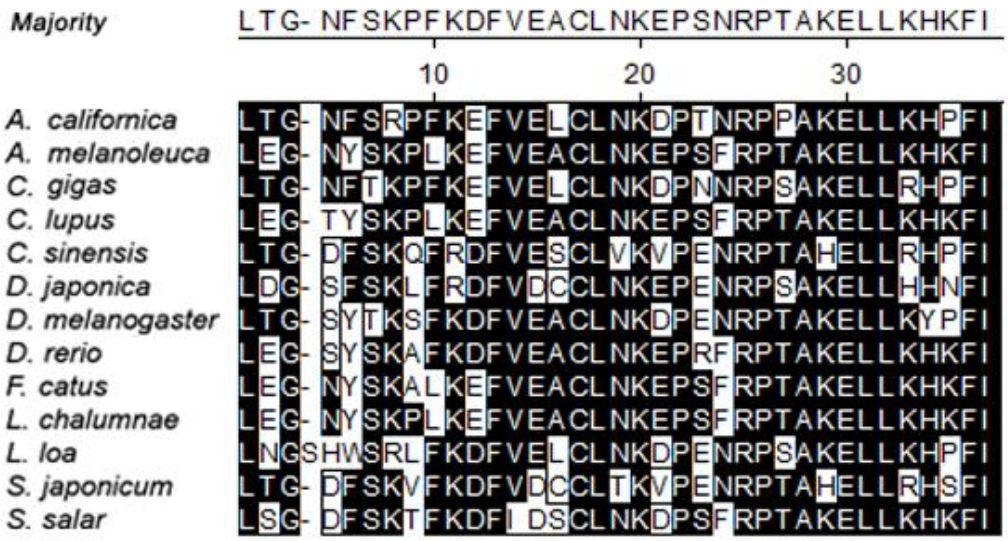

Fig. 2 Alignment of subdomain XI of Ste20-like kinases, including DjSLK using the MegAlign program (DNASTAR) by the CLUSTAL W method. Shaded (with solid black) residues are the amino acids that match the consensus. GenBank accession numbers: *Aplysia californica* (XP_005111485.1), *Ailuropoda melanoleuca* (XP_002914907.1), *Crassostrea gigas* (EKC21462.1), *Canis lupus* (XP_003433112.1), *Clonorchis sinensis* (GAA52112.1), *Drosophila melanogaster* (NM_142339.2), *Danio rerio* (XP_005165982.1), *Felis catus* (XP_003980538.1), *Latimeria chalumnae* (XP_005992350.1), *Loa loa* (EJD76726.1), *Schistosoma japonicum* (CAX753595.1), *Salmo salar* (ACI33699.1).

Initial BLASTP search at NCBI revealed that this gene belongs to Ste20-like kinase. However, its 5′ end is missed and only subdomain XI is entire (Hanks and Hunter, 1995). The deduced amino acids aligned with other subdomain XI of Ste20-like kinases showed that DjSLK shares 39.2 % - 72.2 % similarity with its homlogs in other organisms (Fig. 2). Ste20 kinases consist of the P21-activated kinase (PAK) and germinal center kinase (GCK) families according to the relative location of kinase domain, these two families can be further subdivided into PAK I and PAK II and GCK I to VIII subfamilies, respectively (Dan *et al.*, 2001; Strange *et al.*, 2005; Ling *et al.*, 2008). Due to loss of most subdomains, the closest homlogs can not be ascertained and the gene is termed ste20 like kinase (*DjSlk*) in this study.

DjSlk expression pattern in adult and regenerating planarians

In order to analyse the expression pattern of the planarian *DjSlk* gene we performed whole mount *in situ* hybridization on intact and regenerating animals. In intact planarians, *DjSlk* was expressed in central nervous system which possesses an inverted U-shaped pair of cephalic ganglia and two longitudinal ventral nerve cords that project posteriorly along the worm (Cebrià *et al.*, 2002; Cebrià, 2007; Agata and Umesono, 2008) (Fig. 3B). And *DjSlk* localized in both the central spongy region and the lateral branches in the cephalic ganglia (Fig. 3B). In regenerating animals, *DjSlk* transcripts could always be detected in CNS in the head-, trunk-, and tail-pieces. During the initial regeneration stages after amputation, *DjSlk* expression was not detected

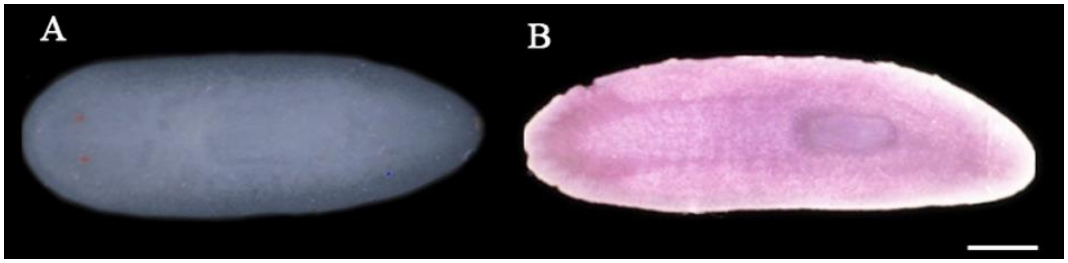

Fig. 3 Expression of *DjSlk* in intact planarian (A) An intact planarian processed and hybridized using the *DjSlk* sense probe. No signal was seen in the control. (B) Ventral view of intact planarian, expression of *DjSlk* is mainly present in the CNS. Anterior is to the left. Scale bar: 500 μm.

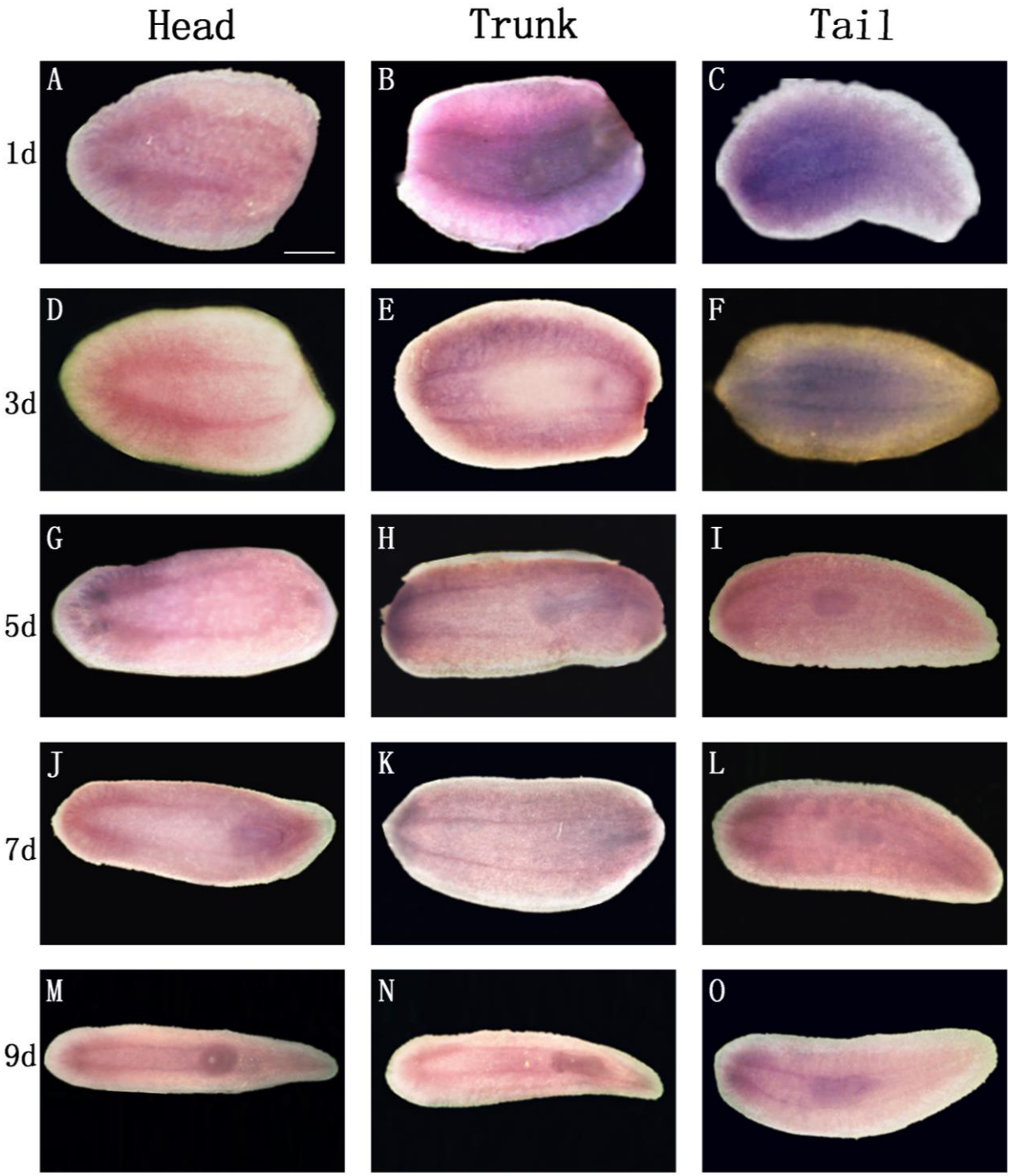

Fig. 4 Expression of *DjSlk* during regeneration. Expression of *DjSlk* in regeneration of day 1 (A - C), day 3 (D - F), day 5 (G - I), day 7 (J - L), and day 9 (M - O) after amputation. *DjSlk* is detected in the pre-exiting and newly regenerated CNS. Anterior is to the left. Scale bar: 300 μm.

within the head and tail blastema, but it was detected in the preexisting CNS (Figs 4A - C). At 3 and 5 days of regeneration, new neural cells in front of the commissure differentiated, and CNS recovered most of its function (Cebria, 2007). *DjSlk* expression was detected in the preexisting and newly regenerated CNS (Figs 4D - I). With the development of regeneration, the original expression was gradually reestablished (Figs 4J - O). The Relative quantitative real-time PCR analysis was performed to investigate the change of expression of *DjSlk* mRNA during planarian regeneration. We examined RNA samples from normal intact planarians and trunk fragments regenerated for 1 day,

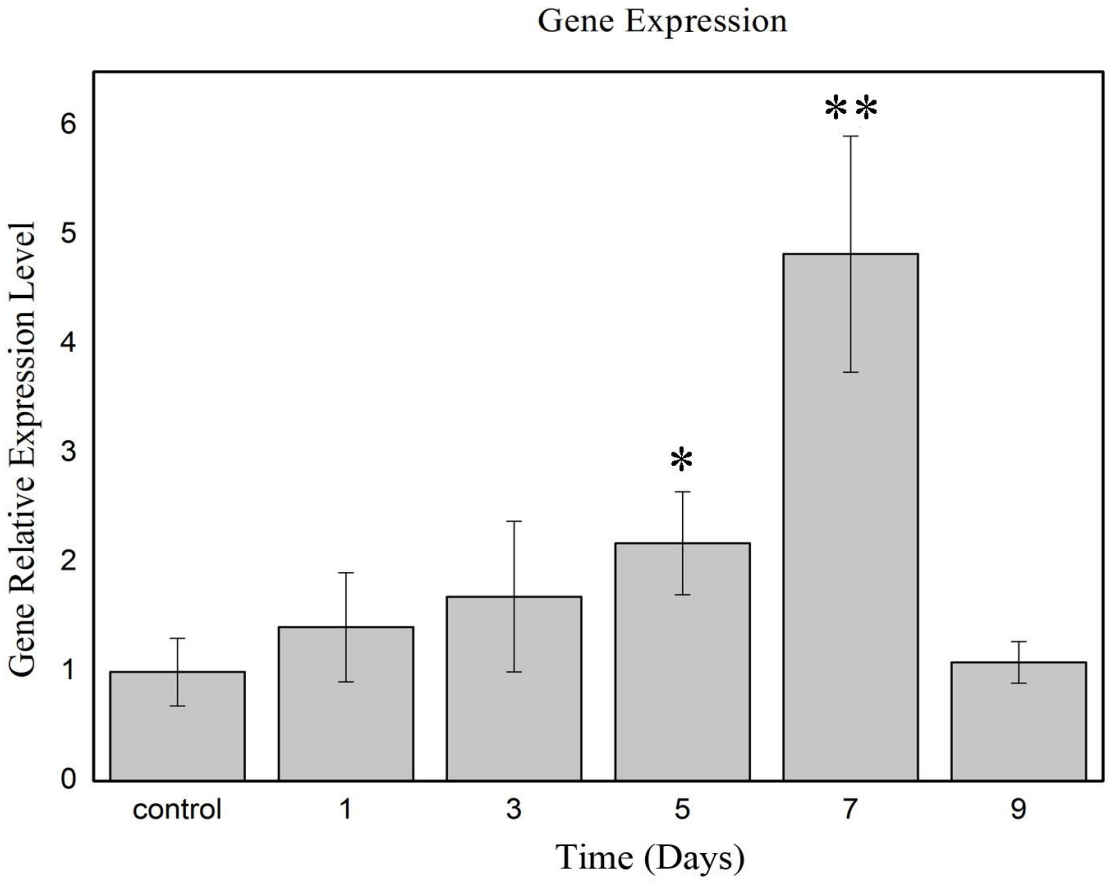

Fig. 5 qRT-PCR analysis of *DjSlk* expression in intact and regenerating truck fragments at 1, 3, 5, 7, 9 days after amputation. Data was expressed as the ratio of *DjSlk* to *DjEF2α* mRNA. Error bars represent the ± SD for three independent PCR amplifications and quantifications. *$p < 0.05$ or **$p < 0.01$ compared to control intact planarians.

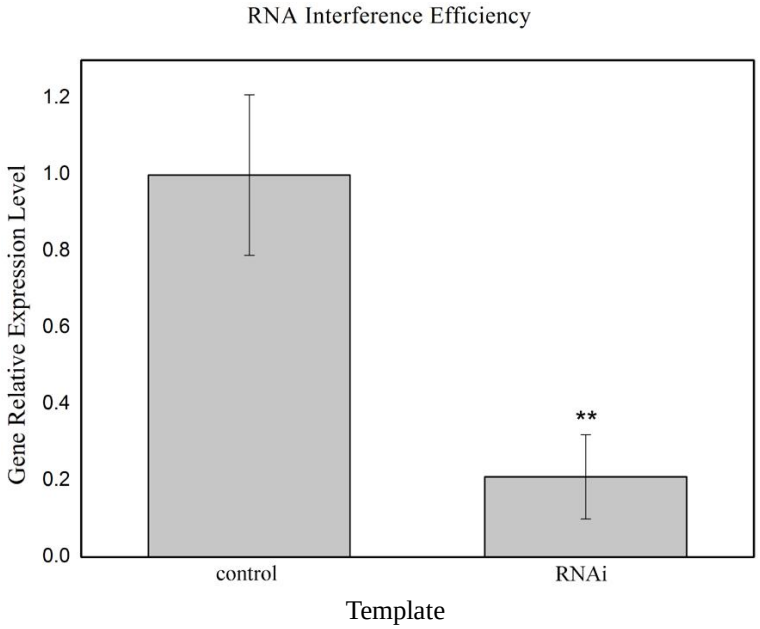

Fig. 6 qRT-PCR analysis of *DjSlk* RNAi efficiency in adult intact planarians. Error bars represent the ± SD for three independent PCR amplifications and quantifications. **$p < 0.01$ is the comparison between control intact animals and RNAi intact animals.

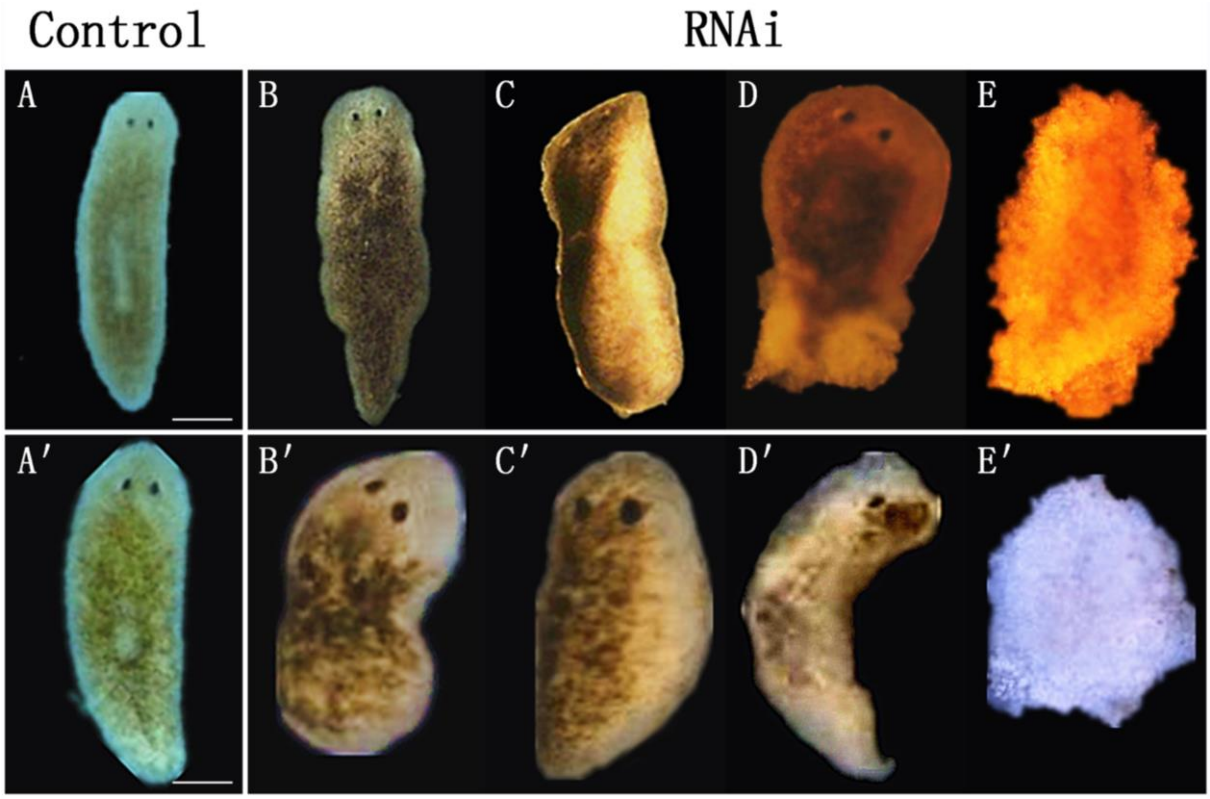

Fig. 7 Abnormal appearance change in intact (B-E) and regenerating planarians (B′-E′) after *DjSlk* RNAi. (A) Control animal, microinjection of water in adult animal after 2 months. (B-E) After RNAi, planarians became immobilized and wrinkled (B), swelled (C), lysed (D), and died (E). (A′) Contral animal, day 30 of regeneration after amputation. (B′-E′) The appearance transformation in regenerating trunk fragments after RNAi-treated planarians. Anterior is to the top. Scale bar: A - E = 800 μm, A′- E′ = 200 μm.

3 days, 5 days, 7 days, and 9 days, respectively. The resluts indicated that *DjSlk* mRNA was gradually increased during regeneration compared to normal intact planarians and achieved to the maximal leval at 7 days ($p < 0.01$) after amputation. Then it declined to almost normal level at regeneration day 9 (Fig. 5).

DjSlk RNAi induces morphogenetic defects and death of planarians

To study the role of *DjSlk* gene in the homeostasis and regeneration of the planarian, we knocked down the endogenous expression of *DjSlk* using RNAi. Real-time PCR analysis of *DjSlk* mRNA showed that the RNAi-treatment efficiently down-regulated the expression of *DjSlk* in intact planarians ($p< 0.01$) (Fig. 6). After 5 days of injecting *DjSlk* dsRNA, the intact animals became immobilized and wrinkled (Fig. 7B, n = 6/13). Then the planarians swelled and started to lyse at day 9 and day 15, respectively (Figs 7C and D, n = 6/13). And the animals completely lysed at about 25 days after the treatment (Fig. 7E, n = 6/13). In contrast, control animals lived without any abnormal change even for 2 months (Fig. 7A, n = 10/10). *DjSlk* RNAi treated trunk fragments could regenerate the whole bodies, and then showed the same appearance change starting at 14 days after amputation (Figs 7B' - E'). Immunostaining with an anti-SYNORF1 antibody against synapsin revealed that *DjSlk* RNAi didn't interfere with CNS intactness and regeneration (Fig. 8).

Discussion

Ste20 kinases function in morphological events in different organisms (Strange *et al.*, 2005). In yeast mating pathway, cell shrinkage activates ste20 kinase and suppresses mating defects (Strange *et al.*, 2005). One ste20 kinase named proline-alanine-rich ste20-related kinase (PASK) is strongly expressed in neurons and transporting epithelia in rats (Ushiro *et al.*, 1998). PASK can interact with and phosphorylate Na-K-2CL (NKCC) and K-CL (KCC) cotransporters to regulate cell volume (Piechotta *et al.*, 2002; Dowd and Forbush, 2003; Strange *et al.*, 2005). During cell swelling, loss of kinase activity of PASK results in dephosphorylation of both cotransporters which lead to inhibit NKCC and activate KCC (Strange *et al.*, 2005). In this study, *DjSlk* was expressed in CNS and RNAi enventully induced swelling and lysing of planarians. And the defects probably resulted from cell swelling and osmotic lysis after loss of kinase

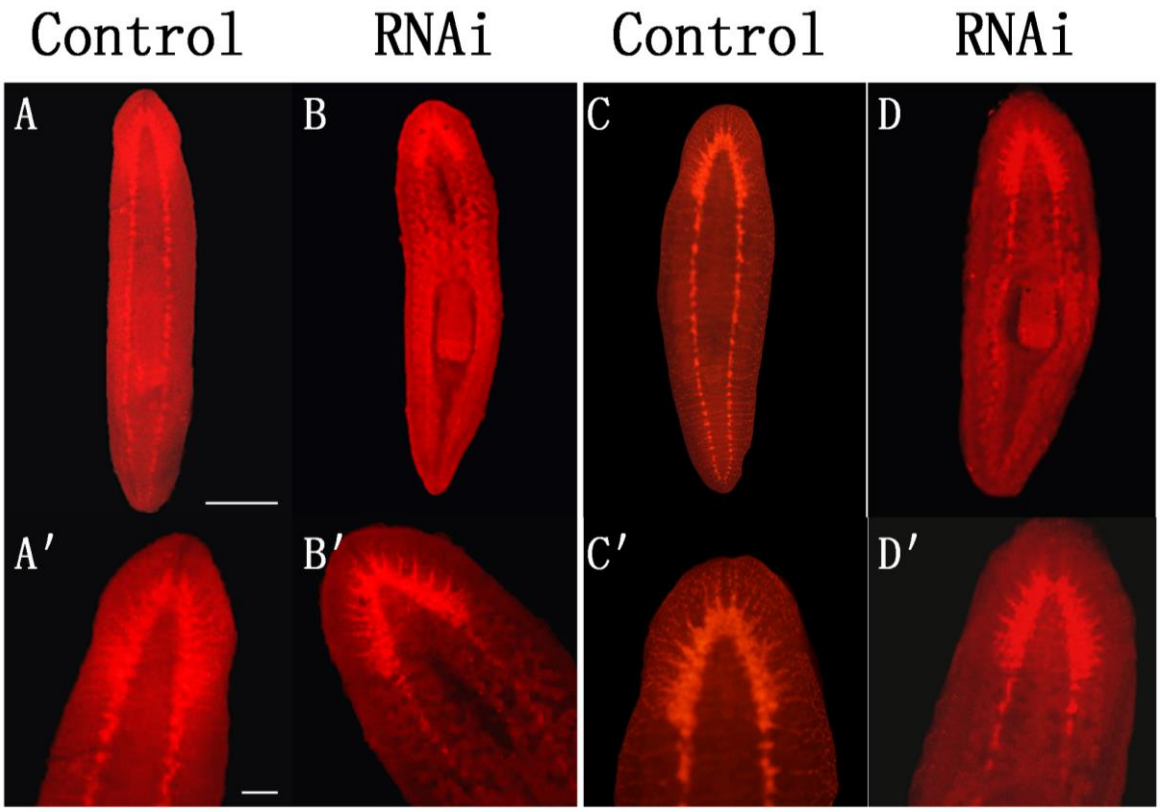

Fig. 8 Immunostaining with anti-SYNORF1 in *DjSlk*-RNAi-treated planarians during homeostasis (A - B and A' - B') and regeneration (C - D and C' - D'), the defects of CNS including brain and VNC were not detected. (A) Intact planarian was injected with water as control. (B) *DjSlk*-RNAi-treated intact planarian at 9 days. (C) Immunostaining of normally regeneration of the cutting head sample was detected at 15 days after amputation. (D) *DjSlk*-RNAi-treated intact planarian was cut head to regenerate at 15 days. A′- D′ the magnification of head in A - D, respectively. Anterior is to the up. Scale bars: A - D = 500 μm; A′- D′= 100 μm.

activity of *DjSlk*. Meanwhile, the irradiation-treated typical phenotype change did not occur in intact planarians, and amputated fragments could regenerate the whole bodies after silencing *DjSlk*. All these results suggest that, just like PASK, *DjSlk* regulates cell volume instead of involving in neoblast maintenance, proliferation, differentiation, and apoptosis like other neoblast-related genes.

Acknowledgments

We thank Dr. Yuqi Huo from Translational Medicine Center, The Sixth People's Hospital of Zhengzhou, for reading and discussing the manuscript. This work was supported by the National Natural Science Foundation of China (grant numbers 31572263, 31172074) and Shandong Province Natural Science Foundation of China (grant number ZR2013CM011).

References

Agata K, Umesono Y. Brain regeneration from pluripotent stem cells in planarian. Phil Trans R Soc B. 363: 2071-2078, 2008.

Burland TG. DNASTAR's Lasergene sequence analysis software. Methods Mol. Biol. 132: 71-91, 2000.

Cebria F. Regenerating the central nervous system: how easy for planarians! Dev. Genes Evol. 217: 733-748, 2007.

Cebria F. Organization of the nervous system in the model planarian *Schmidtea mediterranea*: An immunocytochemical study. Neurosci. Res. 61: 375-384, 2008.

Cebria F, Nakazawa M, Mineta K, Ikeo K, Gojobori T, Agata K. Dissecting planarian central nervous system regeneration by the expression of neural-specific genes. Dev. Growth Differ. 44: 135-146, 2002.

Dan I, Watanabe NM, Kusumi A. The Ste20 group kinases as regulators of MAP kinase cascades. Trends Cell Biol. 11: 220-230, 2001.

Dowd BFX, Forbush B. PASK (proline-alanine-rich ste20-related kinase), a regulatory kinase of the Na-K-CL cotransporter (NKCC1). J. Biol. Chem. 278: 27347-27353, 2003.

Guo T, Peters HFM, Newmark PA. A *bruno-like* gene is required for stem cell maintenance in planarians. Dev. Cell 11: 159-169, 2006.

Hanks SK, Hunter T. Protein kinase 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J. 9:576-596, 1995.

Li Y, Zeng A, Han X, Wang C, Li G, Zhang Z *et al*.Argonaute-2 regulates the proliferation of adult stem cells in planarian. Cell Res.151: 1-5, 2011.

Ling P, Lu T, Yuan C, Lai M. Biosignaling of mammanlian Ste20-related kinases. Cell Signal. 20: 1237-1247, 2008.

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

Pearson BJ, Sánchez Alvarado A. A planarian p53 homolog regulates proliferation and self-renewal in adult stem cell lineages. Development 137: 213-221, 2010.

Pang Q, Liu X, Zhao B, Jiang Y, Su F, Zhang X, *et al*. Detection and characterization of phenoloxidase in the freshwater planarian *Dugesia japonica*. Comp. Biochem. Physiol. 157B: 54-58, 2010.

Pearson BJ, Eisenhoffer GT, Gurley KA, Rink JC, Miller DE, Sánchez Alvarado A. Formaldehyde-based whole-mount *in situ* hybridization method for planarians. Dev. Dyn. 238: 443-450, 2009.

Piechotta K, Lu J, Delpire E. Cation chloride cotransporters interact with the stress-related kinases ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1). J. Biol. Chem. 277: 50812-50819, 2002.

Reddien PW, Sánchez Alvarado A. Fundamentals of planarian regeneration. Annu. Rev. Cell. Dev. Biol. 20: 725-757, 2004.

Reddien PW, Oviedo NJ, Jennings JR, Jenkin JC, Sánchez Alvarado A. Smedwi-2 is a PIWI-like protein that regulates planarian stem cells. Science 310: 1327-1330, 2005.

Robb SMC, Sánchez Alvarado A. Identification of immunological reagents for use in the study of freshwater planarians by means of Whole-mount immunofluorescence and confocal microscopy. Genesis 32: 293-298, 2002.

Strange K, Denton J, Nehrke K. Ste20-type kinases: evolutionarily conserved regulators of ion transport and cell volume. Physiology 21: 61-68, 2005.

Saló E, Abril JF, Adell T, Cebrià F, Eckelt K, Fernàndez-Taboada E, *et al.* Planarian regeneration: achievements and future directions after 20 years of research. Int. J. Dev. Biol. 53: 1317-1327, 2009.

Scimone ML, Meisel J, Reddien PW. The *Mi-2-like Smed-CHD4* gene is required for stem cell differentiation in planarian *Schmidtea mediterranea*. Development 137: 1231-1241, 2010.

Takano T, Pulvers JN, Inoue T, Tarui H, Sakamoto H, Agata K, *et al.* Regeneration-dependent conditional gene knockdown (Readyknock) in planarian: demonstration of requirement for Djsnap-25 expression in the brain for negative phototactic behavior. Dev. Growth Differ. 49: 383-394, 2007.

Ushiro H, Tsutsumi T, Suzuki K, Kayahara T, Nakano K. Molecular cloning and characterization of a novel ste20-related protein kinase enriched in neurons and transporting epithelia. Arch. Biochem. Biophys. 355: 233-240, 1998.

Ultanir SK, Yadav S, Hertz NT, Oses-Prieto JA, Claxton S, Burlingame AL, *et al.* MST3 kinase phosphorylates TAO1/2 to enable myosin Va function in promoting spine synapse development. Neuron 84: 968-982, 2014.

Wagner DE, Wang IE, Reddien PW. Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. Science 332: 811-816, 2011.

Wu C, Whiteway M,Thomas DY, Lerberer E. Molecular characterization of ste20p, a potential mitogen-activated protein or extracellular signal-regulated kinase kinase (MEK) kinase kinase from *Saccharomyces cerevisiae*. J. Biol. Chem. 270: 15984-15992, 1995.

Yu S, Chen X, Yuan Z, Zhou L, Pang Q, Mao B, *et al.* Planarian myosin essential light chain is involved in the formation of brain lateral branches during regeneration. Mol. Genet. Genomics 290: 1277-1285, 2015.