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Usp7 contributes to the tail regeneration of planarians via Islet/Wnt1 axis

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Abstract

Background Regeneration plays a key role in energy recycling and homeostasis maintenance. Planarians, as ideal model animals for studying regeneration, stem cell proliferation, and apoptosis, have the strong regenerative abilities. Considerable evidence suggests that ubiquitin plays an important role in maintaining homeostasis and regulating regeneration, but the function of Ubiquitin specific proteases 7 (*Usp7*) on regeneration in planarians remains elusive.

Methods We identified an evolutionarily conserved gene, Usp7, and utilized RNA interference (RNAi), Quantitative real-time PCR (qRT-PCR), Whole-mount immunofluorescence, Tunnel, Whole-mount in situ hybridization (WISH), and western blotting to detect the function of *Usp7* during the planarian regeneration.

Results In this study, we found that the regenerative trunk fragments in the *Usp7* RNAi worms could not regenerate missing tails; meanwhile, the level of cell proliferation was decreased, while cell apoptosis was increased. Furthermore, the expression of *Islet* was inhibited in the *Usp7* RNAi worms during planarian regeneration. The hybridization signal of *wnt1/P-1* exhibited the dot-like pattern at the posterior of the regenerating planarians after *Usp7* RNAi at regenerative 1 day (R 1 d). However, the concentrated expression pattern *wnt1/P-1* dramatically declined at regenerative 3 days (R 3 d) and disappeared at regenerative 7 days (R 7 d). In addition, activating the Wnt pathway partially rescued regenerative defects induced by inhibition of *Usp7*.

Conclusions Collectively, *Usp7* is necessary for tissue regeneration and tail blastema formation partially by regulating the cell proliferation and apoptosis during planarian regeneration. It could also promote the posterior polarity reconstruction of the regenerative planarians via the Islet/Wnt1 axis.

Keywords USP7, Planarian, Regeneration, Islet, Wnt1

Background

Planarians are an outstanding model for studying organ formation, phylogeny, and regeneration [1]; they can regrow a complete and well-proportioned individual no matter what body part is cut off [2]. The basis for this powerful regenerative ability comes from planarian adult stem cells (Neoblasts), the only cells capable of mitosis in planarians; they can differentiate into various functional cells and tissue organs of the worm after splitting proliferation [3]. To maintain homeostasis, stem cells and their progeny must migrate and differentiate to the correct location, especially tissue damage caused by injury or pathogenic infection [4]. It is reported that the fantastic regeneration process of planarian is inseparable from cell proliferation, apoptosis, migration, and differentiation regulated by various signaling pathways [2, 5].

Deubiquitinating enzymes are involved in many important biological processes, such as the regulation of the cell cycle, proliferation, differentiation,

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and oncogenesis [6–8]. Ubiquitin-specific protease 7 (USP7) is a member of the ubiquitin specific processing enzymes (UBPs) family; it is located at the key node of many signaling pathways and participates in cell and other life activities by regulating many proteins, such as antioncogene, DNA repair proteins, etc. [9, 10]. Moreover, USP7, as a deubiquitinating enzyme, participates in many important signaling pathways, including Wnt and P53 signaling; its high expression is associated with poor prognosis of malignant tumors and is a potential therapeutic target for cancer [11, 12]. However, so far, there are few reports on the potential role of USP7 in the regeneration process, and the specific function of USP7 in planarians is even less clear.

In planarians, the neoblasts regenerated in the appropriate location is important for planarian regeneration, including the homeostasis maintenance and remodeling of the body polarity, which consists of the anterior–posterior axis (AP axis), mediolateral axis (ML axis), and dorsal–ventral axis (DV axis) [13–15]. Moreover, the position control gene (PCG) is vital in polarity reconstruction and maintenance during planarian regeneration [13]. During regeneration, PCG is regulated by external information, such as wound signals and reset location information [16]. Furthermore, the wnt signal has been proven to play an irreplaceable role in forming and maintaining position information of the AP axis polarity of the planarian [17]. Many genes encoding wnt family proteins are regionally transcribed along the AP axis of planarians: *wnt1/P-1*, *wnt11-1*, and *wnt11-2* are expressed at the posterior of planarian, which is primarily responsible for forming and maintaining the tail tissues [18–20]; and it was also reported that *islet* regulates Wnt signaling, which is required for posterior regeneration in planarians. The *Djislet* gene might be involved in the activation of the Wnt signaling pathway in the posterior blastema by perturbing the second phase *Djwnt1/P-1*-expressing cells at the posterior end in planarians [21].

In this study, we utilized a series of molecular biological techniques to detect the function of *Usp7* during planarians' regeneration. The results demonstrated that *Usp7* deficiency retarded the planarian tail regeneration, decreased the stem cell proliferation and differentiation and increased the cell apoptosis, even restrained the expression of *Islet,* and then reduced the second phase of *wnt1/P-1* expression. It is suggested that the failure of posterior blastema formation after silencing *Usp7* might be correlated with the Islet/Wnt1 axis. These findings provide evidence that *Usp7* may be an effective regulator of planarian regeneration. These results also provided experimental data for the analysis of the function of deubiquitination in the regenerating planarians. Also, they offered a reference for further investigation of the role of deubiquitination enzymes in freshwater animals.

Materials and methods

Animal preparation

Planarians (*Dugesia constrictive*) were collected from a stream in Yuquan country, Hebi City, Henan Province, China, and asexual clones were reproduced by cutting in the laboratory. The selected worm length is about 4–6 mm, and it is fed with beef liver once a week. Before the experiment, the worms were starved for at least 7 days.

Gene identification and sequence analysis

To identify a comprehensive set of planarian Usp7 homologs that may be involved in ubiquitylation, the potential Usp7 homologs were searched with the human USP7 proteins by tblastn in a *D. constrictiva* transcriptome database. Putative planarian Usp7 homologs were validated by executing a blastx search in the NCBI nr database. The amino acid sequences encoded by *Usp7*s were obtained from the NCBI ORFfnder website (https://www.ncbi.nlm.nih.gov/orfnder/). The Conserved Domain (CD) search tool of NCBI (https://www.ncbi.nlm.nih.gov/cdd) was used to identify the conserved domains of USP7 proteins, which were visualized by TBtools software (http://mdr.tbtools.org/).

RNA interference (RNAi) and Quantitative real-time PCR (qRT-PCR)

The feed-mediated RNAi technique is used to knock down target genes in planarians. The RNAi bacterial strain *Usp7* was constructed as previously described [22]. All the primers are listed in Table S1. RNAi was performed by feeding a mixture with beef liver homogenate, the ratio of bacterial pellets (1:1). L4440 vacant vector was used as the negative control. The worms were fed every 2 days, fed a total of 10 times over 19 days (Fig. 1F). Then qRT-PCR and Whole-mount in situ hybridization were utilized to test the effectiveness of RNAi, total RNA was extracted for starved a week after the last feeding. The total RNA extraction and qRT-PCR were performed as previously described [23]. We observed the intact and regenerative planarians (head, trunk, and tail fragments) every other day by stereomicroscope. The images were captured by a Leica camera (DFC300FX, Germany). First, the animals were cut posterior to the pharynx, and the second cut between the auricles and the pharynx. Furthermore, we determined the phenotype of the worms; only trunk fragments were used for the following experiments.

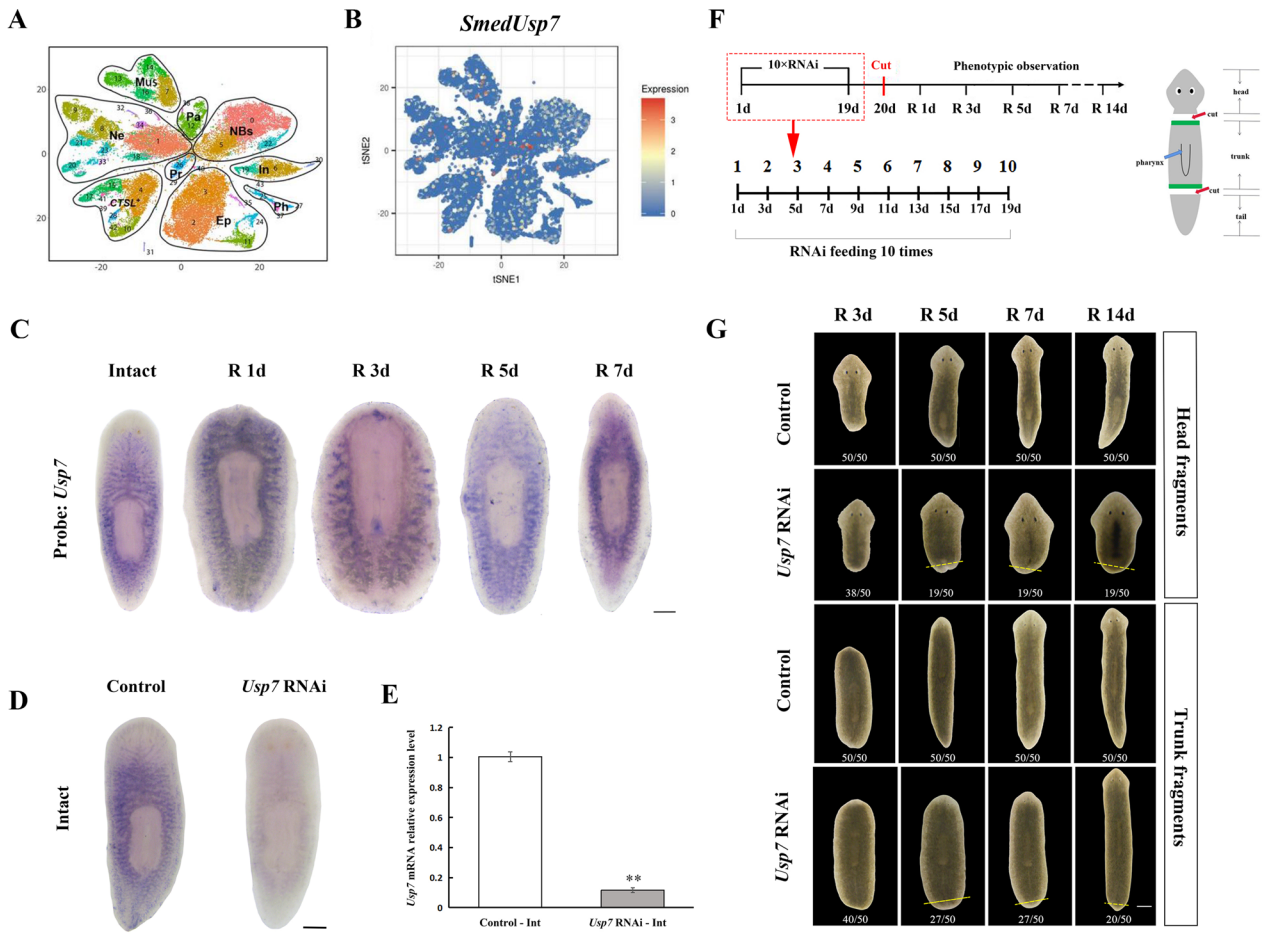

Fig. 1 *Usp7* Knockdown by RNAi in planarians disrupted the posterior regeneration. **A** 44 cell populations generated by single-cell sequencing that comprise nine planarian tissues. **B** *SmedUsp7* is expressed in various tissue cells, and the color represents the expression level, with red indicating high expression and blue indicating low expression level. **C** The spatiotemporal expression of *Usp7* in intact and regenerative trunk fragments of the planarian (n = 5). Scale bar: 500 μm. **D** The interference effect of *Usp7* in intact planarian by situ hybridization (n = 5). Scale bar: 500 μm. **E** The mRNA expression levels of *Usp7* in intact worms after *Usp7* RNAi. Vertical bars represent the mean ± SD (n = 3). **F** Schematic illustration of RNA interfering with feeding and amputation. Red dotted square: Worms were fed once every other day for 10 times, then cut after being starved for 7 days. **G** Morphological changes of regenerative planarians after *Usp7* RNAi. Yellow dotted line: The posterior blastema in *Usp7* RNAi worms could not regenerate a new tail like normal regenerating worms. Scale bar: 1 mm. Mus: muscle, Ne: Nerve, CTSL: cathepsin$^+$ cells, Ep: epidermis, Ph: pharynx, In: intestine, NBs: neoblasts, Pa: parenchyma, Pr: protonephridia, black outlines denote clusters assigned to that tissue class. R 1 d: Regenerative 1 day; R 3 d: Regenerative 3 days; R 5 d: Regenerative 5 days; R 7 d: Regenerative 7 days; R 14 d: Regenerative 14 days. Asterisks indicate statistical differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Whole-mount immunofluorescence and Tunel assay

In the study of planarians, Whole-mount immunofluorescence was used to label the proliferating stem cells of planarians, and TUNEL was a method to detect cell apoptosis in planarians. Whole-mount immunostaining was carried out as described previously [23]. Anti-phospho-Histone H3 (H3P) from Millipore (1: 200 dilution in PBST) was used for the neoblasts division assay. For TUNEL (Sigma-Aldrich, S7100), the experimental worms were fixed in the same way as Whole-mount immunostaining, then stained for Terminal-Deoxynucleotidyl Transferase Mediated Nick End Labelling (TUNEL). TdT reaction Mix (TdT enzyme and Reaction Buffer) was first added to the bleached worms for 4 h at 37 ℃; after rinsed with 1 × PBST 6 times, blocking solution and rhodamine-conjugated antibody were joined in the worms for 4 h, then rinsed with 1 × PBST 6 times. Fluorescent signals were observed and recorded through the stereo fluorescence microscope (Axio Zoom. V16, Germany). Graphics were taken with a digital camera, which was attached to a compound microscope. Positive signals for H3P and TUNEL were quantified with ImageJ software (http://rsb.info.nih.gov/ij/).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was utilized to detect the expression pattern of the genes in the intact and regeneration planarian. The in vitro labeling kit (Roche) was used to synthesize the antisense riboprobes, which were labeled with Digoxigenin (DIG) – 11-UTP or fluorescein (FAM) – 12-UTP. The method was in accordance with Dong et al. [24] with a few modifications as follows: treated with proteinase K (3 μg/ml), hybridized at 56 ℃, NBT/BCIP incubated at 35 ℃.

Western blot

In the study of planarians, Western blot was used to test the protein expression level changes of the genes in the planarians. Cell lysates were prepared from planarians with RIPA buffer in the presence of protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3. Proteins (20–40 μg) were loaded onto 8%–15% Tris-Acrylamide gels and blotted with primary antibodies that included: anti-β-actin (sc-47778, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-Cleaved Caspase-3, BCL-2, Cyclin B1 (Cat. # 9661, Cat. # 3498, Cat. # 12231, Cell Signal Technology Inc., Danvers, MA, USA), anti-phospho-histone H3 (Cat. # 06-570, Millipore, USA), anti-PCNA (Cat. # GB11010, Servicebio, China), anti-USP7, ISLET1, WNT1 (Cat. # ab108931, Cat. # ab178400, Cat. # ab15251, Abcam Inc., Cambridge, United Kingdom), and followed by incubation with horse-radish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Western blot results were detected by an ECL chemiluminescence kit (Cat. # P0018S, Beyotime, China). Images were acquired using the Amersham Imager 600RGB detection system (GE Healthcare). All western blot results were provided as representative images from three independent experiments.

Small molecule treatment

The small molecule was utilized to explore the gene and protein functions of the planarians. Wnt agonist 1 (Wnt signaling activator) (BML-284; 853,220–52-7) was purchased from Topscience. The reagent (purity > 99%) was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, 67–68-5) to a final concentration of 10 mM. In this experiment, worms were treated with 10 nM Wnt agonist 1 after diluting the stock to 10 nM with deionized water.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. Values were expressed as the mean ± SEM. Statistical significance was determined by the student's t-test. A value of $p < 0.05$ was considered statistically significant. * indicates significant difference with $p < 0.05$, ** indicates significant difference with $p < 0.01$, *** indicates significant difference with $p < 0.001$.

Results

Usp7 gene encodes a conserved protein

To screen potential candidate genes involved in planarian regeneration and ubiquitylation, we obtained the full-length cDNA of a USP7 homolog and named it *Usp7*. The online Prot Paramtool found that the gene encodes a protein consisting of 1209 amino acid residues. Moreover, the protein domain was predicted by online software PFAM (http://pfam.xfam.org/); conserved domain analysis revealed that this protein contains four domains: Meprin and TRAF homology domain (MATH domain); Ubiquitin carboxyl-terminal hydrolase (UCH); ICP0-binding domain of Ubiquitin-specific protease 7 (USP7 ICP0—bdg), Ubiquitin-specific protease C-terminal (USP7-C2), from its amino acids 107 to 208, 213–273, 291–595, 697–950, and from 961 to 1195, respectively (Fig.S1A). Further analysis showed that both its protein sequence and its domains were found to be conserved within invertebrates and vertebrates (Fig.S1B). Thus, we named this protein USP7.

Usp7 is required for the tail regeneration of the planarians

Planarian digiworm database (https://digiworm.wi.mit.edu) revealed that *Smedusp7* (*Usp7* homolog) was expressed in a variety of tissue cells, especially in neoblasts, parenchyma, epidermis, nerve, and intestine (Fig. 1A–B). To explore the function of *Usp7* in planarians, Whole-mount in situ hybridization (WISH) was utilized to display spatio-temporal expression patterns of *Usp7* in intact and regenerative planarians. The results showed that *Usp7* was expressed in parenchyma of the body sides and intestine, whether in the intact worms or the regenerating trunk fragments (Fig. 1C). Furthermore, we performed RNAi experiments on planarians (Fig. 1F), the results of WISH and qRT-PCR exposed that the expression levels of *Usp7* were obviously lower in RNAi group compared with the control after 10 times feeding (Fig. 1D–E). However, we detected that the phenotype of the *Usp7* RNAi group was as normal as the control group in the intact planarians (Fig.S2A). For the further test, the worms were amputated into head, trunk, and tail fragments for the following observation on regenerative 3 days (R 3 d), 5 days (R 5 d), 7 days (R 7 d), and 14 days (R 14 d), and the regenerating head and trunk fragments showed that the new blastema at posterior was very small and even hard to regenerate the new tail from R 3 d to R 14 d (Fig. 1G). However, the tail fragments could regenerate a new head normally (Fig.S2B). Based on the results, the regenerative trunk fragments of the planarians were chosen for the following experiments.

Usp7 deficiency inhibits the stem cell proliferation and differentiation

Because blastema formation and differentiation depend on pluripotent adult somatic stem cells (neoblasts) in planarians, we speculated that the defects observed in posterior blastema formation and growth might result from problems in neoblast maintenance or proliferation. So, the qRT-PCR was performed to examine the mRNA expression of stem cell marker genes in planarians on R 3 d (the critical period of stem cell differentiation in planarians) and R 7 d (completely regenerate all the missing structures in planarians), including neoblast markers *piwiA* and *pcna*, early epidermal-committed stem cell progeny maker *prog1*, late epidermal-committed stem cell progeny marker *agat1*, cell cycle marker *cyclinB*. The five markers decreased in the *Usp7* RNAi group compared to the control group (Fig. 2A).

Correspondingly, *piwiA*, *prog1*, and *agat1* changes were elevated via WISH in planarians on R 3d and R 7d. The results indicated that the trends of the three markers' hybridization signals were the same as those of qRT-PCR. Compared to the control group, the *PiwiA*$^+$ neoblast population visibly decreased in the blastema and parenchyma of the regenerating worms at R 3d. It also obviously decreased in the parenchyma of both body sides in the *Usp7* RNAi group at R 7d. In contrast, the quantity of both *Prog1* and *agat1* in *Usp7* RNAi worms was clearly reduced at R 3d and R 7d (Fig. 2B).

Simultaneously, Whole-mount immunofluorescence was utilized to detect the H3P nuclei positive numbers (labeling the stem cells) of the regenerative trunk fragments in planarians on R 6 h, R 48 h, R 3 d, and R 7 d after *Usp7* RNAi. The four time points' expression levels decreased compared to the control group, and the evident declines were observed at R 6 h, R 48 h, R 3d, and R 7 d in the *Usp7* RNAi group (Fig. 2C–D).

To further verify the results of qRT-PCR, WISH, and Whole-mount immunofluorescence, Western blot was utilized to detect the protein expression of H3P, PCNA (stem cell marker genes), and Cyclin B1 (cell cycle protein) in regenerating worms at R 3 d and R 7 d. Correspondingly, the protein expressions of the three marker genes at R 3 d and R 7 d in regenerative trunk fragments after *Usp7* RNAi were distinctly lower than those in control groups (Fig. 2E, Fig.S3A-B).

Based on the experiments' results, we confirmed that *Usp7* deficiency restricted the stem cell proliferation and differentiation in regenerative worms. This might explain

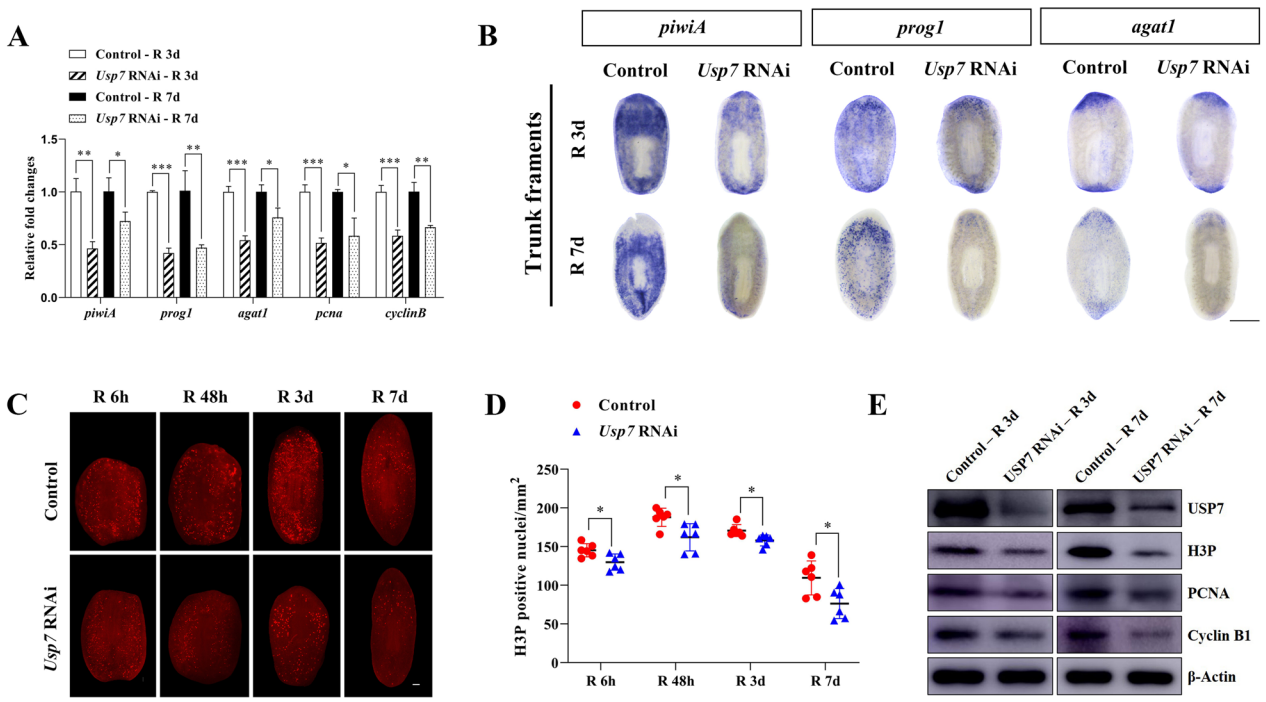

Fig. 2 The cell proliferation level of regenerative planarians after *Usp7* RNAi. **A** The mRNA expression of marker genes in regenerating trunk fragments after *Usp7* RNAi for R 3 d and R 7 d. Vertical bars represent the mean ± SD (n = 3). **B** WISH of *piwiA*, *prog1*, and *agat1* in regenerative trunk fragments after *Usp7* RNAi for R 3 d and R 7 d (n = 6). Scale bar: 200 μm. **C** Immunostaining with anti-H3P antibody in regenerating trunk fragments of the worms after *Usp7* RNAi for R 6 h, R 48 h, R 3 d, and R 7 d (n = 6). Scale bar: 200 μm. **D** Mitotic density in regenerative planarians. Vertical bars represent the mean ± SD (n = 6). **E** The protein expression level of USP7, H3P, PCNA, and Cyclin B1 in regenerating planarians after *Usp7* RNAi for R 3 d and R 7 d. R 6 h: Regenerative 6 h; R 48 h: Regenerative 48 h. Asterisks indicate statistical differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

why an abnormal phenotype occurred during planarian regeneration.

Usp7 knockdown promote the cell apoptosis during planarian regeneration

Beyond our proven hypothesis that *Usp7* RNAi inhibited cell proliferation and differentiation of the regenerative worms, we wondered how cell apoptosis would be affected by *Usp7* deficiency during regeneration. Whole-mount TUNEL was used to examine the apoptotic cells at R 3 h and R 3 d (the peak of cell apoptosis) after *Usp7* RNAi. Compared with the control group, the apoptotic signals of regenerative trunk fragments were visibly increased at R 3 h and R 3 d in the *Usp7* RNAi group (Fig. 3A–B). Moreover, we examined the mRNA expression of the cell apoptosis marker gene in regenerative planarians after *Usp7* RNAi. The results of qRT-PCR showed that the expression of *caspase3* was increased in the *Usp7* RNAi group compared to the control group at R 3d and R 7 d (Fig. 3C).

Western blot was used to quantify the apoptotic signals in regenerating planarians to further verify the results of Whole-mount TUNEL. Cleaved-Caspase3 (providing information on cell apoptosis) and B-cell lymphoma (BCL2) (antiapoptotic protein) were chosen to quantify the apoptotic signals in *Usp7* RNAi worms. Accordingly, Cleaved-Caspase3 protein expressions were dramatically higher than those of the control groups at R 3 d and R 7 d after *Usp7* RNAi. However, the protein expression of BCL2 were obviously decreased in *Usp7* RNAi worms at R 3 d and R 7 d, suggesting promoting of cell apoptosis after *Usp7* RNAi (Fig. 3D, Fig.S3C-D).

Usp7 RNAi inhibits the expression of *Islet* and *wnt1/P-1* during planarian regeneration

Based on the above results, *Usp7* RNAi suppressed tail regeneration in planarians. In addition to affecting stem cell proliferation and differentiation as a cause of this phenotype, we also speculated that it might be related to the Wnt pathway that controls the AP axis polarity. However, it was reported that *Djwnt1/P-1*(RNAi) appeared

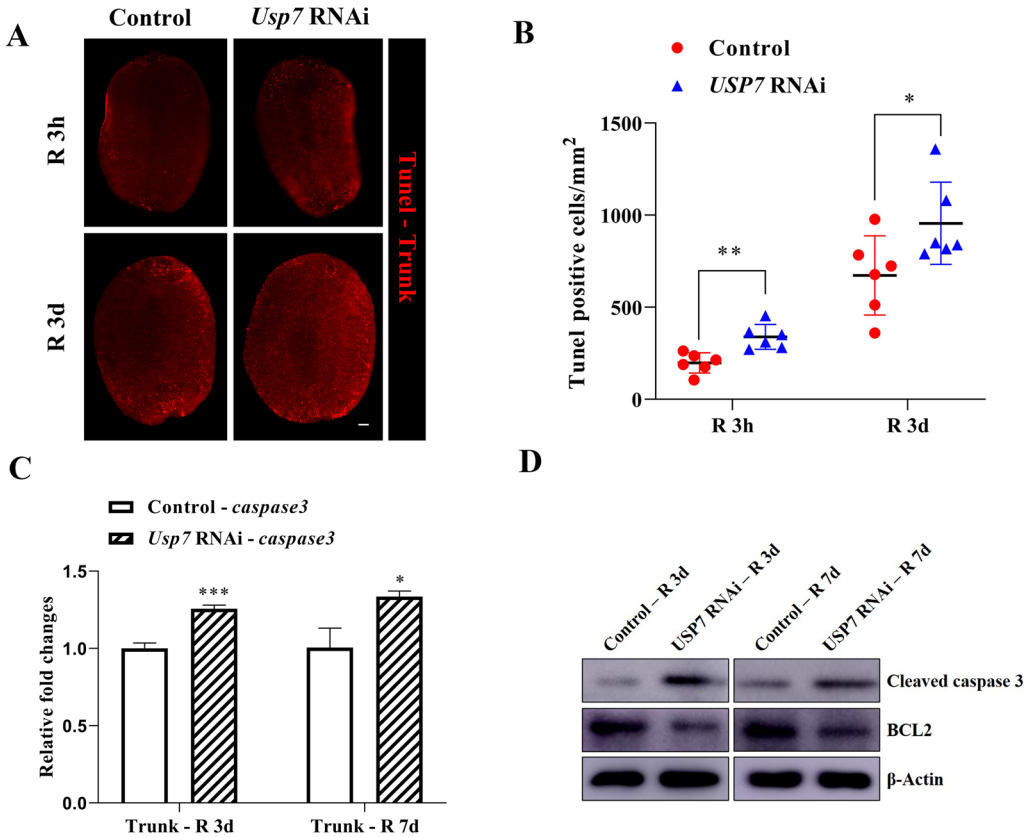

Fig. 3 Cell apoptosis in regenerating planarians after *Usp7* RNAi. **A** Immunostaining with whole-mount TUNEL of the regenerating trunk fragments. **B** Apoptosis density in regenerating trunk fragments. Vertical bars represent the mean ± SD (n = 6). Scale bar: 100 μm. **C** The mRNA expression level of *caspase3* in regenerating trunk fragments after *Usp7* RNAi for R 3 d and R 7 d. Vertical bars represent the mean ± SD (n = 3). **D** The expression level of Cleaved-Caspase3 and BCL2 in the trunk fragments after *Usp7* RNAi for R 3 d and R 7 d by Western blot. Asterisks indicate statistical differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Janus-heads in addition to some pharynx/tail-less (P/T-less) and *Djislet* (RNAi) exhibited tail-less (T-less) phenotype [14]. So, we utilized qRT-PCR to detect the mRNA expression of *Islet* and *wnt1/P-1* at R 1 d, R 3 d and R 7 d after *Usp7* RNAi. The results showed that the expression of *Islet* reduced at R 1 d, R 3 d, and R 7 d after *Usp7* RNAi compared to the control group. Interestingly, the expressions *wnt1/P-1* only decreased at R 3 d and R 7 d in *Usp7* RNAi worms, but there was no significant change at R 1 d. (Fig. 4A).

Furthermore, we detected the signals of *islet* and *wnt1/P-1* via WISH in planarian. The results indicated that the dot-like expression pattern of *islet* could be observed at the posterior region of the worms in the control group on R 1 d and R 2 d but not in the *Usp7* RNAi worms, and the signal of *islet* was expressed in a gradually concentrated pattern in the dorsal midline of the posterior blastema of the normal worms on R 3 d and R 7 d. However, there were no signals found in the *Usp7* RNAi worms (Fig. 4B). Moreover, in the control group, the hybridization signal of *wnt1/P-1* was expressed both at the anterior and posterior wound sites at R 1 d (the first phase) in the regenerative trunk fragments of the planarians, the signals began to gather at the two wound sites at R 2 d, then the anterior signals were disappeared. Enhanced hybridization signals were observed at R 3 d (the second phase), at the end of the central axis, an apparent point-like aggregation was existed at R 7 d. In the *Usp7* RNAi group, the expression of *wnt1/P-1* was exhibited dot-like expression pattern, the same as the control group on R 1 d, but the expression level of the hybridization signal was significantly lower than the control group at R 2 d and R 3 d. Moreover, the signal of *wnt1/P-1* was no longer detected at R 7 d (Fig. 4C).

In addition, the western blot was utilized to detect the protein expression of Islet and Wnt1 in the regenerative planarians. The data showed that the expression of Islet was significantly decreased in the *Usp7* RNAi group at

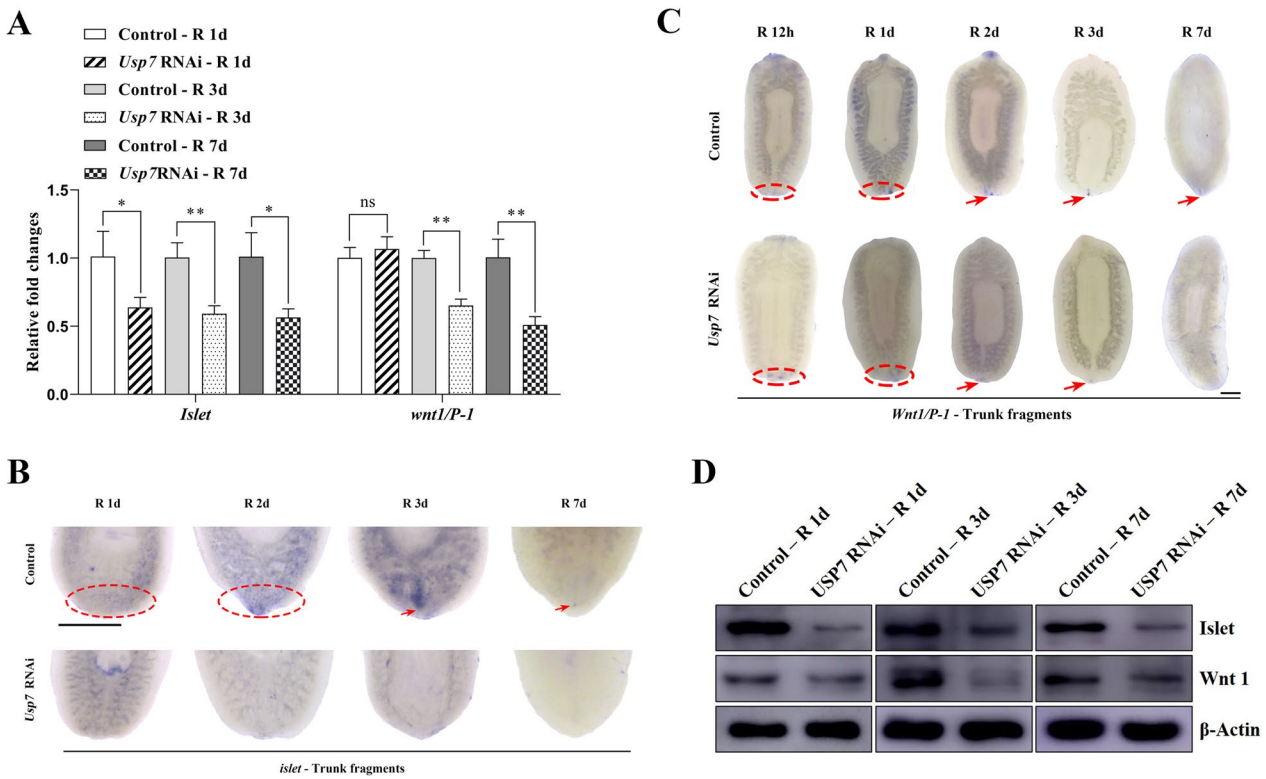

Fig. 4 Expression of genes related to Wnt pathway of planarian after *Usp7* RNAi. **A** The mRNA expression level of *islet* and *wnt1/P-1* in trunk fragments for R 1 d, R 3 d, and R 7 d after *Usp7* RNAi. Vertical bars represent the mean ± SD (n = 3). **B** The expression of *islet* in regenerative trunk fragments of the worms after *Usp7* RNAi by WISH at R 1 d, R 2 d, R 3 d, and R 7 d (n = 6). Red circle: The dot-like expression pattern of *islet* at the posterior of the worms in the control group. Red arrow: the concentrated pattern of *islet* was expressed in the dorsal midline of the posterior blastema of the normal worms. Scale bar: 500 μm. **C** The expression of *wnt1/P-1* in regenerative trunk fragments of the worms after *Usp7* RNAi by WISH at R 12 h, R 1 d, R 2 d, R 3 d, and R 7 d (n = 6). Red circle: The dot-like pattern of *wnt1/P-1* was expressed in the *Usp7* RNAi worms at the posterior. Red arrow: The aggregation pattern of *wnt1/P-1* was expressed in the *Usp7* RNAi worms at the posterior. Scale bar: 500 μm. **D** The protein expression level of Islet and Wnt1 in the trunk fragments after *Usp7* RNAi by Western blot for R 1 d, R 3 d, and R 7 d. R 2 d: Regenerative 2 days; R 12 h: Regenerative 12 h. Asterisks indicate statistical differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

R 1 d, R 3 d, and R 7 d compared to the control group. However, the expression of Wnt1 had no significant change at R 1d but was clearly reduced at R 3d and R7 d in *Usp7* RNAi worms (Fig. 4D, Fig.S3E-G).

In brief, we found the same trends in our experiment data. It only inhibited the point-like concentrated pattern expression of *wnt1/P-1* in regenerating worms after R 3 d; the dot-like pattern expression of *wnt1/P-1* in the posterior was not affected by *Usp7* RNAi during the early regeneration of the planarians. It demonstrated that the planarian tail could not regenerate because knockdown *Usp7* retarded the expression of *Islet*; *Islet* deficiency reduced the second phase of *wnt1/P-1* expression in the regenerating worms and prevented the polarity formation.

Usp7 promote the posterior formation in regenerating planarian by Wnt pathway

To further explore the function of Wnt signaling in the regeneration defect of the planarian induced by *Usp7* inhibition, 10 nM Wnt agonist 1 was employed to activate the Wnt pathway [25]. We observed that 10 nM Wnt agonist 1 could partly rescue the phenotype of the regenerative planarian after *Usp7* RNAi, the posterior of the regenerative trunk fragments could regenerate the new tail at R 7 d (Fig. 5A). Moreover, we utilized qRT-PCR, WISH, Whole-mount immunofluorescence, and Western blot detect the mRNA and protein expression of the related gene markers at R 3 d and R 7 d after Wnt agonist 1 activation.

The results showed that compared to the *Usp7* RNAi group, the data of qRT-PCR suggested that the mRNA expression levels of cell proliferation and cell cycle marker genes *piwiA*, *pcna*, *prog1*, *agat1*, and *cyclinB* were significantly increased at R 3 d in the Wnt activation group. However, the expression level of cell apoptosis marker gene *caspase3* was obviously decreased; moreover, the expression levels of *wnt1/P-1* were significantly increased at R 3 d after Wnt activation (Fig. 5B).

Meanwhile, we detected the signals of *piwiA*, *prog1*, *agat1*, and *wnt1/P-1* by WISH in planarian on R 3 d. The results indicated that the four markers' hybridization signal expression trends were the same as those of qRT-PCR. Compared to the control group, the $PiwiA^+$ neoblast population visibly decreased in the regenerating worms in the *Usp7* RNAi group and *Usp7* RNAi + DMSO group at R 3 d, the quantity of both *Prog1* and *agat1* was also reduced at this time, but increased in Wnt activation group. While the hybridization signal expression of *wnt1/P-1* was detected in *Usp7* RNAi worms reduced at R 3 d but was rescued in the Wnt activation group (Fig. 5C). Furthermore, the number of H3P-positive nuclei was increased at R 3 d and R 7 d after 10 nM Wnt agonist 1 rescued *Usp7* inhibition compared to the Usp7 RNAi group (Fig. 5D–E).

In addition, we utilized the Western blot to quantify the protein expression levels in regenerating planarians. Compared to the control group, the protein expression level of USP7 was decreased in the *Usp7* inhibition group and Wnt activation group. However, the protein expression levels of H3P, Cyclin B1, BCL2, and Wnt1 were decreased in the *Usp7* inhibition group at R 3 d and R 7 d. However, all the proteins were increased nearly to the normal group in the Wnt activation group. (Fig. 5F, Fig. S3H–I).

These results indicated that *Usp7* could regulate cell proliferation, differentiation, and apoptosis during planarian regeneration and could participate in the posterior formation of the regenerative planarian by the Wnt pathway (Fig. 6).

Discussion

Planarian regeneration is a complex process involving wound healing, blastema formation, and the success of differentiating blastema cells to form missing tissues and organs [8, 26]. The only known proliferative cells in planarians are adult pluripotent stem cells, which can proliferate and differentiate to form new tissues and organs if a planarian misses a part of its body and are the main contributors to planarians' extraordinary regenerative capacity [27, 28]. Studying planarian regeneration provides an opportunity further to investigate stem cell cellular mechanisms and functions in vivo. However, more research is needed to understand the molecular network that controls the development of the blastema and the differentiation of the cells.

In this context, we identified a gene *Usp7* from *D. constrictive*. We presented that *Usp7* was expressed in the parenchyma of the body sides and intestine, whether in the intact worms or the regenerating trunk fragments. This gene encodes ubiquitin specific protease 7 and regulates the deubiquitination of various proteins, including tumor suppressors (P53, PTEN), transcription factors, epigenetic regulators, DNA repair proteins, and immune response regulators [29]. Elevated levels of USP7 are associated with a poor prognosis for various cancers. For example, USP7 promotes cell proliferation by stabilizing the Ki-67 protein in lung cancer cells, spreading cancer cells [30].

Furthermore, USP7 is a potential drug target for cancer treatment [31], and the efficacy of USP7 small molecule inhibitors has been demonstrated in vivo. Chauhan et al. found that it induced apoptosis of multiple myeloma cells and overcame bortezomib resistance [32]. Moreover, inhibition of USP7 induces apoptosis and cell cycle arrest in the G2/M phase. It overcomes taxane resistance

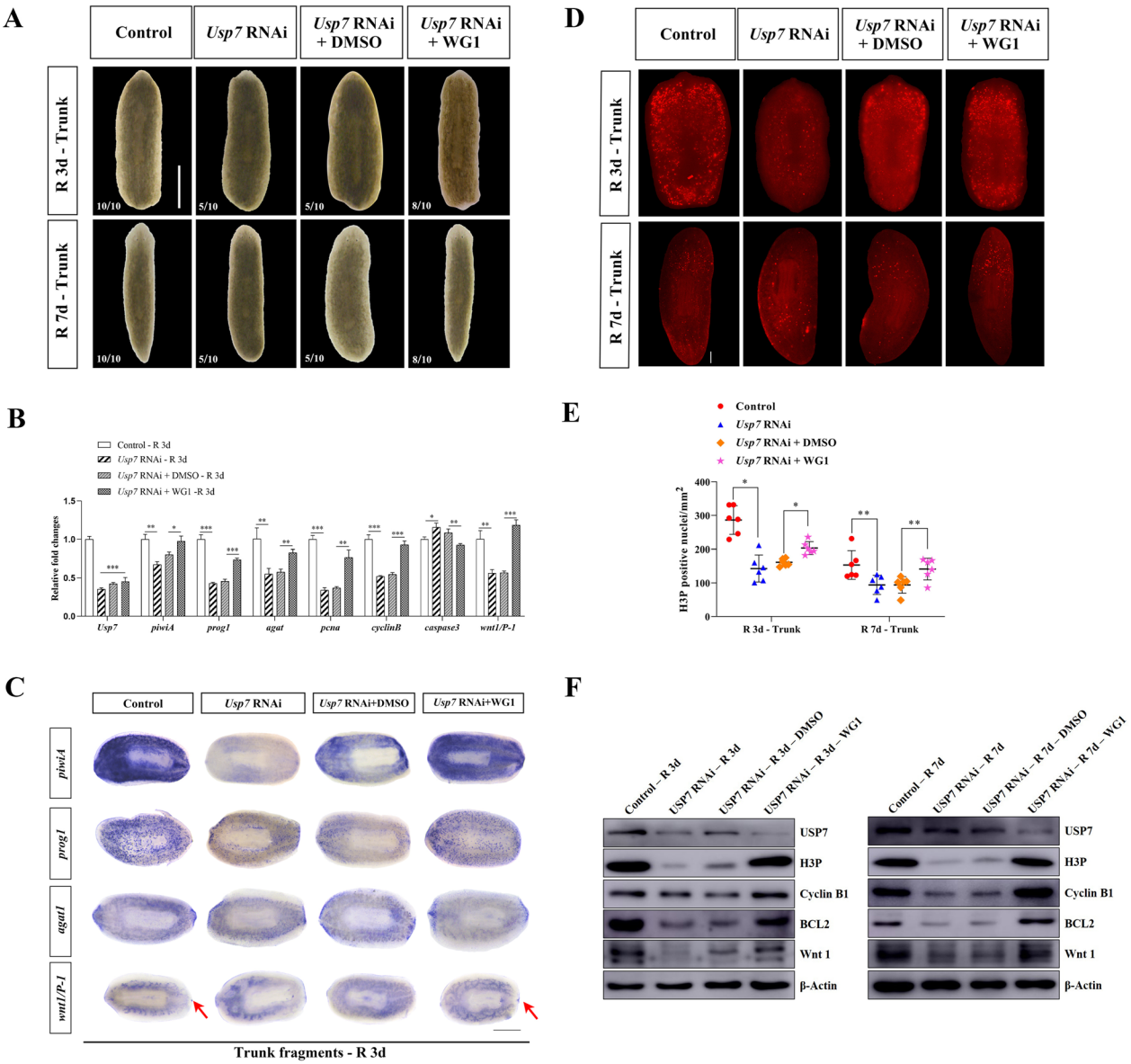

Fig. 5 Activation of Wnt signaling diminished the regeneration defect induced by *Djusp7* inhibition. **A** The phenotype of the regenerative trunk fragments in planarian after both *Usp7* RNAi and 10 nM Wnt agonist 1 treated (n = 10). Scale bar: 1 mm. **B** The mRNA expression level of marker genes of the regenerative trunk fragments in planarians at R 3 d. Vertical bars represent the mean ± SD (n = 3). **C** The expression of *piwiA*, *prog1*, *agat1*, and *wnt1/P-1* in regenerative trunk worms by WISH (n = 6). Scale bar: 200 μm. **D** Immunostaining with anti-H3P antibody of the regenerative trunk fragments of the worms at R 3 d and R 7 d (n = 6). Scale bar: 200 μm. **E** Mitotic density in regenerative planarians. Vertical bars represent the mean ± SD (n = 6). **F** The protein expression level of USP7, H3P, Cyclin B1, BCL2, and Wnt1 in the trunk fragments after Wnt activation by Western blot. Asterisks indicate statistical differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

by inducing the protein degradation of PLK1 (Polo-like Kinase 1), leading to chromosome dislocation in mitosis [33].

In planarians, maintaining homeostasis and the regeneration process is the balance between stem cell proliferation and cell death, the main events during regeneration [34]. Moreover, the cell cycle is a crucial cellular process that discreetly regulates proliferation during planarian regeneration [35]. In this study, the tail blastema of regenerative planarians grew slowly, the H3P immunofluorescence test showed a remarkable reduction in cell proliferation and a distinct increase in cell apoptosis during the regenerative period in *Usp7* RNAi worms. These results demonstrated that *Usp7* regulates cell proliferation and cell apoptosis and promotes blastema formation in regenerative planarians. Furthermore, the regeneration

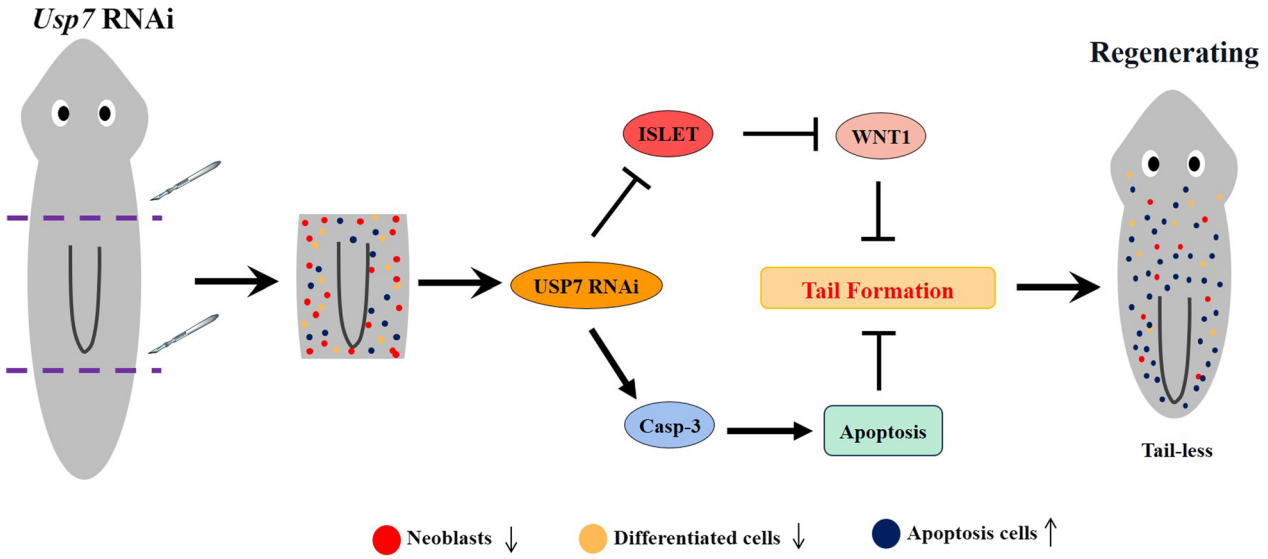

Fig. 6 Proposed model of Usp7 function in tail formation of planarians during the regeneration

of the planarian also involves the reconstruction of position information of the body, which leads to the establishment of new polarity. The reconstruction process of position information of different fragments is distinguishing, and crosscutting mainly relies on the reconstruction of the AP axis position information [36]. We observed that the ratios of the dorsal and ventral, medial and lateral were well-balanced; the anterior blastema of the regenerative worm could regenerate normally, while the posterior blastema regenerated slowly or could not regenerate the new tail after *Usp7* RNAi for 7 days during regeneration of the planarians, indicating that *Usp7* RNAi may seriously affect the reconstruction of the posterior position information of the regenerative planarians.

Actually, Wnt signals have been recognized to play an irreplaceable role in the formation and maintenance of position information of the AP axis polarity in planarians [37], and *Djwnt1/P-1* signal is upstream of the posterior Wnt genes, the primary function of it was the tail region formation and maintenance [36], moreover, the dot-like expression ablation of *Djwnt1/P-1* prompted Janus-heads and P/T-less phenotypes at R 1 d (the first phase of *wnt1/P-1* expression). However, the subsequent concentrated expression pattern ablation of *Djwnt1/P-1* at R 3 d (the second phase of *wnt1/P-1* expression) caused T-less phenotype in *Djislet* RNAi worms [14].

Our study showed that in *Usp7* RNAi worms, the trunk fragments were observed in the tail-less regeneration phenotype. Our result was just like the phenotype in *Djislet* RNAi worms, then the mRNA and protein expression of Islet was investigated in *Usp7* RNAi worms, and we found that they were obviously decreased at R 1 d and R 3 d. Moreover, *Djislet* is required for the second phase of *Djwnt1/P-1* expression [14], so the expression of *wnt1/P-1* was detected in *Usp7* RNAi worms. The data presented that *wnt1/P-1* was usually expressed in the posterior at the early stage (Islet-independent: the first phase of *wnt1/P-1* expression) of regeneration at R 1 d after *Usp7* RNAi, but *wnt1/P-1* signal was significantly weakened. It could not be gathered in the posterior of the regenerating worms at R 3 d (Islet-dependent: the second phase of *wnt1/P-1* expression) after *Usp7* RNAi. It demonstrated that in *Usp7* RNAi worms, the tail-less regeneration phenotype was regulated by the Islet/Wnt1 axis.

In addition, it was reported that USP7 is a tumor-specific Wnt activator that mediates the treatment of APC-mutated colorectal cancer [38], and the USP7 inhibitor P5091 attenuated wnt1 activity in the Wnt signaling pathway and inhibited the growth of colorectal cancer [39]. In our study, Wnt activation was used to rescue the phenotype of *Usp7* RNAi worms, and this rescue experiment was performed to confirm our hypothesis further. We found that the tail-less phenotype was partially rescued by 10 nM Wnt agonist 1 in the *Usp7* RNAi worms [25], and compared to the control worms, the stem cell proliferation was increased, the cell apoptosis was decreased in the Wnt activation rescue worms at R 3 d. Interestingly, the expression of *wnt1/P-1* was increased in the regenerating planarians of the rescue group at R 3 d, too. These data demonstrated that *Usp7* promotes the reconstruction of posterior position information during planarian regeneration by regulating the Islet/Wnt1 axis. Therefore, USP7 enzyme activity, which is essential for post-translational protein

regulation, is responsible for balancing the maintenance of pluripotency stem cell characteristics and the differentiation decisions of planarians.

Planarians' possible future research avenues are diverse and promising, given their unique regenerative abilities and biological characteristics. For example, we will intensively study the molecular mechanisms of regeneration. Planarians are an ideal model for studying regeneration due to their extraordinary regenerative capabilities. They possess a vast reservoir of adult stem cells, known as neoblasts, enabling them to regenerate almost any body part. By studying planarian regeneration, we can gain insights into the underlying biological processes of tissue and organ regeneration. Meanwhile, we will endeavor to develop biotechnological tools and methods to study planarian regeneration, such as advanced imaging techniques. These research directions have the potential to provide significant insights into the biology of regeneration and to advance the field of regenerative medicine.

Conclusions

In summary, we identified an evolutionarily conserved gene, *Usp7*, and detected the function of *Usp7* during the planarian regeneration. *Usp7* is partially necessary for tissue regeneration and tail blastema formation by regulating cell proliferation and apoptosis during planarian regeneration. Our findings provide a novel molecular mechanism for the second phase expression of Wnt1/P-1 enhancement via the mediation of Islet by USP7, which, intriguingly, is mediated by the freshwater planarians.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-025-06134-w.

Additional file 1

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Author contributions
A.L.: Methodology, Conceptualization, Investigation, Writing—original daft. J.L.: Methodology, Validation, Data curation. Z.Z.: Investigation, Software, Visualization. J.X.: Writing—reviewing and editing. D.L.: Methodology. Z.D.: Methodology, Investigation, Validation, Formal analysis, Funding acquisition. G.C.: Project administration, Resources, Conceptualization, Supervision, Writing—reviewing and editing.

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Availability of data and materials
Data will be made available on request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors consent to publication.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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