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Functional characterization of *salvador* and *warts* in planarians

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Abstract

In the last years, the Hippo signaling has emerged as an essential pathway to control tissue and organ homeostasis, and its deregulation leads to tumoral transformation in all animals studied. However, the underlying cellular and molecular mechanism of this transformation are still not clear. The core of the Hippo pathway includes a kinase cassette composed by the kinases Hippo, Salvador, Mats, and Warts that in turn phosphorylates the nuclear effector of the pathway, Yorkie, targeting it to proteasomal degradation. Recently, the functional study of *hippo*, in planarians, flatworms that continuously change their size and renew their tissues according to nutrients, allowed demonstrating that the underlying mechanism of cellular transformation was not an increase in cell proliferation but inducing cell cycle arrest and promoting cell dedifferentiation. However, the functional study of *yorkie* did not allow to relate it with the Hippo function. Here, we show that inhibition of *salvador* and *warts* phenocopies the defects observed in *hippo* RNAi planarians. Since Warts is responsible for phosphorylating *yorkie,* this data suggests that not only the kinase cassette of the Hippo pathway is functionally conserved but also the nuclear effector.

Introduction

The Hippo signaling plays a central role in the control of essential cellular mechanisms required for proper cell turnover in all organisms, as stem-cell maintenance, cell differentiation, cell fate decisions, and cell survival [1] [2] [3] [4] [5] [6]. Although the down-regulation of Hippo signaling is clearly related with the appearance of overgrowths and tumorigenesis, the underlying mechanism is still poorly understood [7] [8] [9] [10]. The core of the Hippo pathway is a kinase cassette, composed by the kinases Hippo, Salvador, Mats, and Warts [11]. Warts, in turn, phosphorylates the nuclear effector of the pathway, Yorkie, targeting it to proteasomal degradation. Thus, only when the Hippo kinases are not active Yorkie is not phosphorylated and can enter the nucleus to regulate transcription [11]. There are multiple upstream signals that regulate the pathway, most of them related to cell-cell and cell-matrix contact [12]. Recently, the role of *hippo* during cell renewal has been functionally studied in planarians, flatworms well known for their abilities to regenerate any missing organ, even the head, in a few days [13]. Planarians do not only regenerate but continuously renew their tissues since they grow and shrink in size according to nutrients throughout their lives. These features are due to the presence of a unique population of adult stem cells, together with the continuous activation of signaling cues that coordinate cell proliferation, cell death, and cell fate decisions, allowing planarians to maintain proportioned and functional organs during homeostasis and regeneration [14] [15] [16]. This continuous active regulation of the stem cell and postmitotic cell compartments makes the planarian an ideal in vivo model to understand the mechanisms underlying homeostatic cell renewal [17] [18]. It was recently shown that inhibition of *hippo* in planarians promoted the appearance of undifferentiated regions and overgrowths [19]. Cellular studies demonstrated that the underlying mechanism of overgrowth formation was not an increase in cell proliferation but in arresting the cell cycle, inhibiting apoptosis and promoting cell dedifferentiation [19]. Due to the complex phenotype obtained, the functional study of *yorkie* did not allow to relate it with the Hippo function [19] [20]. Here, we show that inhibition of the core kinase elements *salvador* and *warts* promotes the appearance of undifferentiated regions and overgrowths, phenocopying the *hippo* RNAi phenotype. Since Warts is responsible to phosphorylate *yorkie,* this data suggests that not only the kinase cassette

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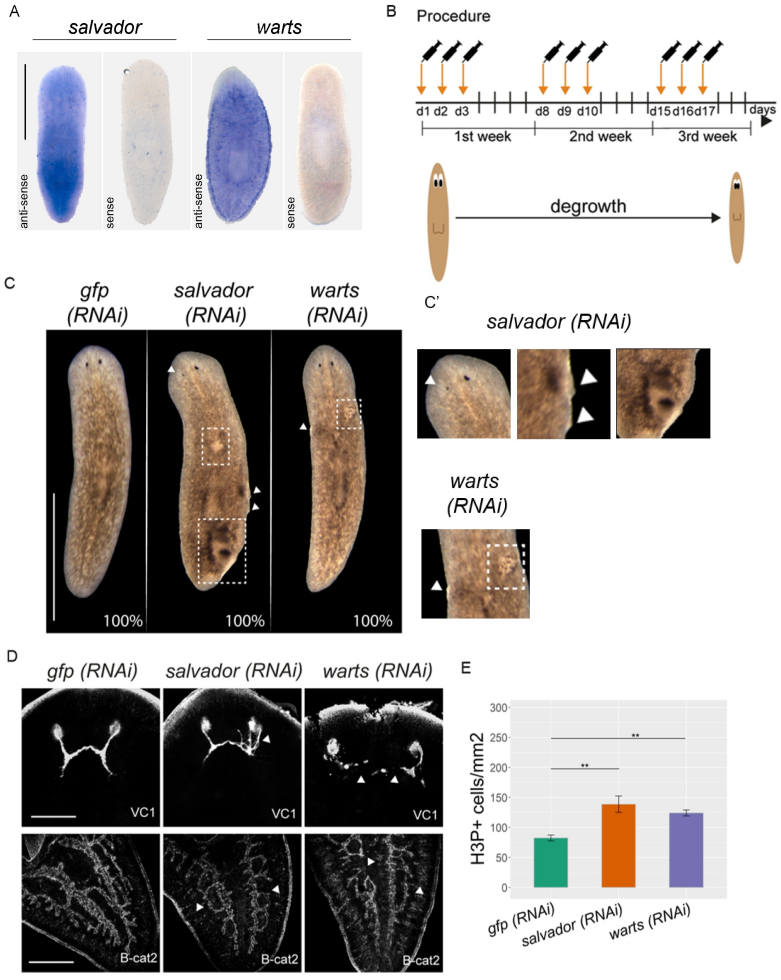
of the Hippo pathway is functionally conserved but also the nuclear effector.

Objective

The specific objective is to check whether inhibition of the core kinase elements of Hippo pathway *salvador* and *warts* promotes the appearance of overgrowths in planarians, thus phenocopying the *hippo* RNAi phenotype. The general objective is to demonstrate that, despite the functional study of *yorkie* did not allow to demonstrate its role as an effector of the pathway in planarians, the main elements, including Warts, which directly regulates Yorkie nuclearization, are indeed conserved.



a

Figure Legend

Figure 1. Functional characterization of *salvador* and *warts* in planarians.

(A) In situ hybridization for *salvador* and *warts* with sense and anti-sense probe. Anti-sense probe reveals a ubiquitous expression pattern.

(B) Cartoon illustrating the experimental design used for *salvador* and *warts* RNAi during planarian homeostasis. Animals were starved for 1 week before the experiment

and were then injected on 3 consecutive days each week for 3 weeks. Starvation was maintained throughout.

(C) Stereomicroscopic image of live *salvador (RNAi)* and *warts (RNAi)* planarians showing the overgrowths and unpigmented regions in marginal regions of the body (n = 10). White arrows indicate overgrowths and white discontinuous boxes indicate unpigmented regions. **(C')** Magnifications of unpigmented areas and overgrowths.

(D) Analysis of unpigmented regions. Top panel: the visual system stained with anti-arrestin (VC-1). Bottom panel: digestive system labeled with the anti-Bcat2 antibody. White arrows indicate defects in the visual and digestive system.

(E) Quantification of mitotic cells (H3P+) in *salvador* and *warts (RNAi)* animals (n ≥ 10). Error bars represent standard deviation. Data were analyzed by Student t-test. $^{**}p < 0.01$. Scale bars: (**A**) and (**D**) 1 mm; (**E**) top panel 100 µm; (**E**) bottom panel 250 µm.

Results & Discussion

The two genes of the Hippo pathway core kinase *Smed-salvador* and *Smed-warts* (for simplicity, s*alvador* and *warts*) were identified in the *Schmidtea mediterranea* transcriptomes database (PlanMine) [21] (Suppl. Info. 1). An alignment of the planarian sequences with the homologs of different model species demonstrates the sequence conservation (Suppl. Info. 2). *In situ* hybridization (ISH) showed that both genes are ubiquitously expressed since they do not show any tissue-specific pattern (Fig. 1A). An *in silico* search in a single cell database of planarians SCS [22] also corroborates that they are present in several planarian cell types, as also found for *hippo* and *yorkie* genes (Suppl. Fig. 1).

To inhibit the function of *salvador* and *warts* during homeostatic cell renewal in planarians, we injected dsRNA of both genes into planarians during 3 weeks in the ventral pre-pharyngeal region (see Methods and Fig. 1B). Planarians were starved during the experiments; thus they were shrinking in size. After the inhibition of *salvador* or *warts,* animals presented unpigmented regions, mainly around the body margin, which became bigger or evolved into unpigmented overgrowths (Fig. 1C and C'). Taking into account that, the region where were performed injections is not the same where we observe phenotype, and that control animals were also injected and did not present any phenotype, we can conclude that the injections are not related with the appearance of the overgrowths/unpigmented regions. The analysis of the expression pattern of genes associated with cell differentiation (*arrestin* and *b-catenin2*) revealed that *salvador* and *warts (RNAi)* animals are not able to properly maintain differentiated structures as the visual and the digestive system (labeled with *arrestin* (VC1) and *b-catenin2,* respectively (Fig. 1D). The eyes of these animals are smaller, and the optic chiasm is thinner and interrupted in the case of *warts* (RNAi). The digestive system is also thinner and interrupted in *warts* (RNAi) animals (Fig. 1D). The loss of differentiated structures in these animals could be due to the inability of cells to maintain the differentiated state, as previously shown for *hippo* RNAi animals [19].

It has been shown that, despite not showing an increase in the number of cells, neither an increase in cell proliferation, *hippo* RNAi animals show an increase of cells in M phase. To test if this was also the case for *salvador* and *warts (RNAi)* animals, we analyzed the expression of pH3 with a specific antibody. The results show that the number of pH3+ cells was increased after three weeks of *salvador* and *warts* inhibition (Fig. 1E).

Conclusions

The observation that *salvador* and *warts (RNAi)* animals show the same phenotype as that of *hippo* RNAi animals suggests that the kinase cassette of the Hippo pathway appears conserved in planarians. In *salvador* and *warts (RNAi)* animals the mitotic arrest and the inability of cells to maintain a stable differentiated fate could underlay the appearance of the overgrowths, as observed in *hippo* RNAi animals [19]. Since Warts is

the kinase dedicated to Yorkie phosphorylation [11], the results also suggest that Yorkie could be the effector of the pathway in planarians and that its activation leads to the observed phenotype.

Limitations

To demonstrate that the Hippo kinase core elements regulate Yorkie phosphorylation, specific experiments would be required, for instances to test the levels of phosphorylated Yorkie in *warts* and *Salvador* RNAi planarians with respect to controls.

Alternative Explanations

It would be possible that the kinase cassette of the Hippo pathway is conserved, and even that Yorkie also acts downstream of the pathway in planarians. However, it could also be that some of the defects observed in planarians depend on the activity of the kinases but not of Yorkie, since the Hippo kinase cassette has been demonstrated to exert several Yorkie independent function [23].

Conjectures

It would be necessary to characterize more in deep the cellular origin of the overgrowths, and if their appearance depends on the activation of Yorkie.

Additional Information

Methods

Planarian culture

Asexual planarians from a clonal strain of *S. mediterranea* BCN-10 were maintained at 20℃ in PAM water (planarian artificial medium) as described [24]. Animals were fed with veal liver and starved for at least 1 week before starting the experiments.

RNA interference analysis

dsRNA was synthesized by in vitro transcription (Roche) and microinjection performed as previously described [25], following the standard protocol of 3×32 nl injection of dsRNA for 3 consecutive days. To achieve the inhibition of salvador and warts, we performed this protocol during 3 consecutive weeks in starved planarians.

Whole-mount in situ hybridization

Sense and anti-sense RNA probes were synthesized in vitro using Sp6 or T7 polymerase (Roche) and DIG-modified (Perkin Elmer) nucleotides. RNA probes were purified and precipitated with ethanol and 7.5 M ammonium acetate. For in situ hybridization, animals were fixed and processed as previously described [26]. After probe development, animals were mounted with 70% glycerol in PBS.

Whole-mount immunostaining

Immunostaining was performed as described [27]. The following antibodies were used: rabbit anti-phospho-histone-H3-Ser10 (anti-H3P) (1:500; Cell Signaling Technology) and rabbit anti-β-catenin2 (anti-Bcat2) ([28]; 1:2000). To avoid technical variance and obtain a reliable quantification of H3P+cells, at least 2 independent experiments were performed.

Imaging

Immunostained samples were imaged using a MZ16F stereomicroscope (Leica) equipped with a ProgRes C3 camera (Jenoptik). Images were processed using Fiji software. Quantifications were performed by hand using the "multi-point selection" tool of Fiji. Data were analyzed by Student t-test.

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Ethics Statement

Not Applicable.

Citations

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