Snail regulates Hippo signalling-mediated cell proliferation and tissue growth in Drosophila

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snail (sna) encodes an evolutionary conserved zinc finger transcription factor [13], which was first characterized in Drosophila as a critical regulator of embryonic mesoderm formation [14] and was later reported to play a key role in tumour invasion and metastasis, especially in epithelial-mesenchymal transition (EMT) [15,16]. Sna acts as a transcriptional repressor regulating a large number of genes involved in the EMT process [17,18]. For instance, the overexpression of SNAI1 in tumour cell lines promotes tumour metastasis [19,20]. Besides its well-known functions in embryo development and tumour metastasis, other studies suggest that Sna also plays important roles in regulating multiple biological processes including cell proliferation, cell differentiation and cell death [21–26]. However, the mechanism by which Sna regulates tissue homeostasis remains not fully understood. Due to the low redundancy, Drosophila is an excellent model system to investigate the physiological functions of Sna in tissue/organ development.

In this study, we identified Sna as a crucial modulator of Hippo signalling-mediated tissue growth in Drosophila development. Loss of sna inhibits, while overexpression of Sna promotes, Hippo signalling-mediated cell proliferation and tissue growth. In addition, Sna is physiologically required for tissue growth in normal development. The genetic epistasis analysis indicates that Sna acts downstream of Yki to promote target genes expression and cell proliferation. Mechanistically, Yki activates sna transcription, while elevated Sna binds to Sd and promotes Sd-dependent cell proliferation. In conclusion, our results identified Sna as an essential regulator of the Hippo pathway and revealed the underlying mechanism by which Sna modulates Hippo signalling-mediated cell proliferation, tissue growth and tumour progression.

2. Results

2.1. Loss of sna suppresses Hippo signalling-mediated tissue overgrowth

To investigate the genetic interaction between Sna and Hippo pathway, we first checked whether Sna is required for Hippo signalling-mediated overgrowth. Compared with the control (figure 1a), inactivated Hippo signalling by depleting hpo along the A/P compartment boundary in third-instar larval wing discs driven by ptc-Gal4 robustly increased the width of ptc-expressing stripe (figure 1b) [27]. This phenotype was significantly suppressed by expressing three independent sna-IR lines that target distinct regions of the sna transcript [23] (figure 1c–e), while sd-IR was included as a positive control (figure 1f). Hippo pathway inactivation promotes tissue overgrowth mainly through accelerated cell proliferation [2,28]. Consistently, the depletion of hpo promoted cell proliferation in the corresponding region detected by increased anti-PH3 staining, which was dramatically suppressed by knocking-down sna and sd (figure 1d–f,m,n; electronic supplementary material, figure S1). In addition, ptc > hpo-IR + LacZ animals displayed enlarged area between L3 and L4 in the adult fly wings, which was also suppressed by depleting sna and sd (figure 1g–l,n). A quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay was performed to assess the knockdown efficiencies of the three sna RNAi lines (electronic supplementary material, figure S2). By contrast, ptc > sna-IR did not cause any obvious change in cell proliferation or tissue growth (electronic supplementary material, figure S3). Furthermore, hpo-IR and hpo-IR + sna-IR did not affect cell death or cell size (electronic supplementary material, figure S4). Collectively, these results indicate that Sna is required for hpo depletion-triggered cell proliferation and tissue overgrowth.

To dissect the mechanism by which Sna modulates Hippo signalling, we performed genetic epistasis analysis between Sna and Hippo pathway core components. warts (wts) encodes a serine/threonine kinase acting downstream of Hpo, and ptc > wts-IR produced similar phenotypes as that of ptc > hpo-IR, including expanded ptc-stripe and increased PH3-positive cell density [29]. Both phenotypes were suppressed by expressing sna-IR or sd-IR (figure 2a–f), suggesting Sna modulates Hippo pathway downstream of Wts.

Impaired Hippo signalling leads to the nuclear translocation of Yki and promotes Yki-dependent cell proliferation [30]. Ectopic expression of Yki dramatically promoted tissue overgrowth and cell proliferation [31], which were partially suppressed by depleting sna or sd (figure 2g–l). In addition, expressing an activated form of Yki (YkiS168A) by en-Gal4 promoted tissue overgrowth and cell proliferation in the P-compartment of wing discs, both of which were significantly suppressed by knockdown of sna or sd (electronic supplementary material, figure S5).

While sd is reported to be specifically expressed in the wing pouch of third-instar larvae, we noticed that sd-IR suppressed Hippo-Yki signalling-induced tissue overgrowth and cell proliferation in the wing pouch as well as in the hinge region (figures 1 and 2e,k; electronic supplementary material, figure S5E). To explain this, we used the G-TRACE system and found sd-mediated GFP expression in the entire wing disc (electronic supplementary material, figure S6), suggesting sd is expressed in the entire wing disc at an early larval stage [32].

Together, these results indicate that Sna regulates Hippo signalling-mediated Yki-dependent tissue growth and cell proliferation, most likely downstream of Yki.

2.2. Loss of sna suppresses Hippo signalling-mediated target gene expression

To verify the role of Sna in regulating Hippo signalling, we checked the expression of Hippo pathway target genes, including diap1 and myc, which are required for cell survival and proliferation, respectively [33,34]. Compared with the controls, the overexpression of Yki driven by ptc-Gal4 resulted in upregulated expression of diap1-LacZ and Myc [35], which were suppressed by depleting sna, while sd-IR served as a positive control (figure 3). Moreover, upregulated diap1 expression in Yki overexpression clones was suppressed by depleting sna (electronic supplementary material, figure S7), confirming that Sna is required for Yki-triggered target gene expression.

2.3. Sna is necessary and sufficient for tissue growth

To test whether Sna is sufficient to promote tissue growth, we generated Flp-out clones that express UAS-transgenes by act-Gal4. Compared to the control, the expression of Sna resulted
in a mild increase of clonal size, while the expression of the activated YkiS168A, which was included as a positive control, caused dramatic overgrowth of the clones (figure 4a–d). In addition, Sna expression along the A/P boundary driven by ptc-Gal4 induced a mild overgrowth in the hinge region (electronic supplementary material, figure S8A,B), accompanied by upregulated expression of diap1 (electronic supplementary material, figure S8A,B). Furthermore, ectopic Sna expression in the P compartment of wing discs activated the transcription of diap1 (figure 4e,f) and ban (figure 4g,h), another Hippo pathway target gene [36]. Together, these data suggest that ectopic Sna is sufficient to

**Figure 1.** The loss of sna suppresses hpo depletion-induced cell proliferation and tissue overgrowth. (a–f) Fluorescence micrographs of third-instar larval wing discs stained with anti-pH3 (red) are shown. Compared with the ptc > GFP control (a,a’), hpo knockdown increases the width of ptc-stripe (b) and pH3-positive cell density within the stripe (b’), both of which are significantly suppressed by expressing three independent sna RNAi (c,c’, d,d’, e and e’), sd RNAi serves as a positive control (ff’). (g–l) Light micrographs of Drosophila adult wings are shown. Compared with the ptc-Gal4 control (g), the expression of hpo-IR + LacZ causes an expanded L3-L4 area (h), which is suppressed by depletion of sna (i–k) or sd (l). (m) Quantification of pH3-positive cell density ratio for GFP region/total region (left to right: n = 10, n = 9, n = 8, n = 9, n = 10, n = 7). (n) Quantification of size ratio for L3-L4 area/total area (left to right: n = 10, n = 15, n = 24, n = 24, n = 19, n = 20). One-way ANOVA was used to compute p-values, ****p < 0.0001. Scale bar: 100 µm in (a–f), 250 µm in g–l.
activate the expression of Hippo pathway target genes and promote tissue growth.

To further investigate the physiological function of Sna in development, we first checked the endogenous expression of sna in the wing discs. To this end, we used a Sna-GFP reporter, which carries a genomic fragment in which Sna has been fused in-frame at its C-terminus to GFP. We found that Sna is ubiquitously expressed in the third-instar wing discs (electronic supplementary material, figure S9A), and its expression in the p-compartment was significantly reduced upon hh-Ga4 driven sna-IR expression (electronic supplementary material, figure S9B,C). Next, we checked whether sna is required for normal wing growth. As ptc > sna-IR did not notably affect cell proliferation and tissue growth along the A/P compartment boundary in the developing wings (electronic supplementary material, figure S3), presumably due to the relative mild expression of the ptc-Ga4 driver, we raised ptc > sna-IR animals at 29°C to increase the Ga4 activity and observed reduced sizes of the L3-L4 area (electronic supplementary material, figure S10). Moreover, sna depletion in the P compartment of wing discs by hh-Ga4, a stronger Ga4 driver, resulted in diminished posterior areas in the adult wings (figure 4i–l), suggesting that sna is physiologically required for proper tissue growth in normal development.

2.4. Sna physically interacts with Scalloped to promote Scalloped-dependent cell proliferation

Since Sna is required for Yki-triggered tissue overgrowth, cell proliferation and target gene expression (figures 2g–j and 3; electronic supplementary material, figure S5), we reasoned that Sna might act downstream of Yki. In agreement with this hypothesis, Sna-induced cell proliferation remained unchanged upon yki depletion (figure 5a–c,e), while sna transcriptor was upregulated by activated Yki (figure 5f). Together, these results indicate that Yki activates the expression of Sna, which acts as a downstream mediator of Yki activity.

The Hippo-Yki signalling modulates tissue growth through the transcription factor Sd [5,37], which has been
Snail (Sna) belongs to the Snail superfamily of C2H2-type zinc finger proteins [42], which functions as a transcription factor by binding to the consensus sequence CAGGTG [13]. Sna was first identified in *Drosophila* as a transcription regulator involved in embryonic patterning [14] and was later characterized as a key regulator of EMT and tumour metastasis by repressing E-cadherin expression [43]. However, the role of Sna in normal tissue growth has remained unknown [22]. In this study, we employed *Drosophila* as a model organism to investigate the physiological functions of Sna in tissue growth and revealed that Sna is not only required for impaired Hippo signalling-induced accelerated cell proliferation and tissue overgrowth, but also contributes to proper...
tissue growth in normal development. Our genetic epistasis analysis showed that the loss of sna suppressed hpo or wts depletion, or Yki overexpression-induced cell proliferation and tissue overgrowth, whereas ectopic Sna-induced cell proliferation was not suppressed by yki depletion, suggesting Sna acts downstream of Yki to regulate Hippo signalling-mediated tissue growth and cell proliferation. Consistently, sna expression is upregulated by Yki, which provides a molecular explanation for the above genetic data. Moreover, Sna forms a transcriptional complex with Sd by direct physical interaction and promotes Sd-dependent cell proliferation.

In support of our findings, ectopic Sna has previously been shown to activate the expression of diAP1 and Myc, both of which are targets of Yki, yet the role of Sna in Hippo-Yki signalling was not further investigated in the research [44]. Thus, our study represents the first report that Sna is involved in

Figure 4. Sna promotes Yki target gene expression and tissue growth. Fluorescence micrographs of third-instar larval wing discs with clones (marked by GFP) (a–c), stained with anti-β-Gal antibody (e–h) are shown. GFP-labelled Sna (b) or YkiS168A (c) overexpression clones are larger than wild-type controls (a). (d) Quantification of clone size/total size shown in (a–c) (n = 8, n = 10, n = 11). One-way ANOVA was used to compute p-values, ****p < 0.0001, *p < 0.05. Expression of Sna activates Hippo reporter diap1-LacZ (f) and ban-LacZ (b), compared with the controls (e,g). (i–k) Light micrographs of Drosophila adult wings are shown. Compared with the hh-Gal4 control (i), the depletion of sna reduces the size of posterior compartment (j,k). (l) Statistical analysis of the adult wing size (P/A) is shown (n = 14, n = 11, n = 12). One-way ANOVA was used to compute p-values, ****p < 0.0001. Scale bar: 100 µm in (a–c), 50 µm in (e–h), 250 µm in (i–k).
Hippo signalling-mediated tissue overgrowth and that Sna is also required for normal tissue growth in development. Although we provide evidence here that Sna promotes cell proliferation and tissue growth, previous studies have shown that Sna overexpression also triggers cell death and affects cell size [44,45]. Therefore, as a result of the comprehensive effect of these cellular processes, Sna overexpression promotes a mild growth phenotype, much less prominent than that induced by Yki overexpression (figure 4a–d). Besides regulating tissue/organ growth and tumour formation, the Hippo pathway is also involved in other functions, including stem cell self-renewal and differentiation [46]. Intriguingly, murine Snail/Slug were reported to form complexes with YAP/TAZ in regulating skeletal stem cell development and functions [21,44], suggesting Sna family members may regulate the Hippo-Yki signalling by distinct mechanisms in a context-dependent manner. Both Sna and Hippo signalling play pivotal roles in tumour progression [47,48]; therefore, this study also shed light on the interaction and underlying mechanism between Sna and Hippo signalling in cancer development.

4. Methods

4.1. Fly strains

All flies were raised on a standard cornmeal and agar medium at 25°C unless otherwise indicated. Fly strains used in this
article have been described previously: ptc-Gal4 [49], en-Gal4, hh-Gal4, Sd-Gal4, UAS-LacZ, UAS-GFP, UAS-hpo-IR and UAS-sir-IR [50], UAS-Yki, daupl-LacZ, UAS-Sna, UAS-Flip UAS-RFP act > y + > EGFP, tub-Gal80°. cx-LacZ, ban-LacZ, UAS-sd-IR and UAS-HA-Sd were gifts from professor Lei Zhang. UAS-sna-IR (28679) and UAS-YkiS168A (28816) were obtained from the Bloomington stock centre, UAS-sna-IR (28679) and Sna-GFP (318402) were obtained from the Vienna Drosophila RNAi Center, UAS-sna-IR (3956R-5) was obtained from Japanese National Institute of Genetics (NIG). 

Transgenic flies expressing UAS-Myc-Snail was generated by standard P element-mediated transformation.

Figure 6. Sna physically interacts with Sd. Co-IP experiment showing that Sna physically interacts with Sd (a), but not Yki (c) in S2R+ cells. Co-IP experiment showing that Sna interacts with Sd in vivo (b). Co-IP experiment analysis showing that Sna interacts with Sd_N, but not Sd_C (d). Diagram of Sd protein and truncated fragments (e). GST pull-down assay showing that Sna directly binds to Sd (f). (g–i) Fluorescence micrographs of subcellular localization of HA-Sd and Myc-Sna are shown. HA-Sd and Myc-Sna expression in the wing pouch are driven by Sd-Gal4. Scale bar: 50 µm in (g–i).
To induce Flp-out clones, animals were reared at 25°C for 3 days, heat-shocked at 37°C for 15 min and recovered at 29°C for 2 days prior to dissection. To obtain the hh > snai-IR wing phenotype, animals were raised at 29°C to enhance the Gal4 activity. For ptc > Sna experiments, animals were raised at 20°C to avoid ectopic Sna-induced larval lethality. When tub-Gal80<sup>op</sup> was used to regulate Sna expression, animals were raised at 25°C for 3 days, then shifted to 29°C for 2 days before dissection.

### 4.2. Immunostaining

Antibody staining was performed by standard procedures for third-instar larval imaginal discs. Primary antibodies included rabbit anti-Phospho-Histone H3 (1:400, Cell Signaling Technology, CST, cat. no. 9701), mouse anti-β Gal (1:500, Developmental Studies Hybridoma Bank, DSHB, cat. no. 40-1a), rabbit anti-Myc (1:500, Santa Cruz Biotechnology, d1-717), rabbit anti-Cleaved Caspased-3 (1:400, CST, cat. no. 9661), mouse anti-Myc-Tag (1:100, CST, cat. no. 2276), rabbit anti-HA-Tag (1:100, CST, cat. no. 3724) and mouse anti-GFP (1:200, Roche, cat. no. 1181446001). Secondary antibodies were goat anti-rabbit Cy3 (1:1000, Life technologies, cat. no. A10520), goat anti-mouse Cy3 (1:1000, Life Technologies, cat. no. A10521) and goat anti-rabbit Alexa Flour 488 (1:1000, Life Technologies, cat. no. A32731).

### 4.3. Image and quantification of fly wings

Wings were dissected and placed on slide with alcohol/glycerol (1:1) medium. Light images of wing were taken by Olympus BX51 microscope. Adobe Photoshop 2020 was used to retouch the images.

### 4.4. Reverse transcription polymerase chain reaction

For heat shock experiment, animals were raised at 25°C, heat-shocked at 37°C for 30 min and recovered at 29°C for 2 h before experiments. Total RNAs were isolated from whole third-instar larvae.

For hh > snai-IR experiments, animals were raised at 25°C. Total RNAs were isolated from the wing disc of third-instar larvae, and RT-qPCR was performed as previously described. RP49 served as the internal control.

Primers used are provided:

- rp49-FP: TACAGGCCCAAGATCGTGAA
- rp49-RP: TCTCITGCGCTTCTTGGA
- snai-FP: ATGGCGGCCAATCACAAAAAG
- snai-RP: GCAAACGTTGAGCTCCTGGTC

### 4.5. Co-Immunoprecipitation

*Drosophila* S2R+ cells were cultured in Corning Insectagro DS2 with 10% FBS (HyClone). Effectene Transfection Reagent (Qiagen) was used for co-transfection of Plasmids pUAST-Flag-Yki, pUAST-Myc-Sna, pUAST-HA-Sd, pUAST-HA-Sd<sub>N</sub>, pUAST-HA-Sd<sub>C</sub> and Actin-GAL4 as indicated. Cells were lysed in RIPA buffer with PMSF 48 h after transfection and proceeded with the standard co-immunoprecipitation protocols.

For *in vivo* co-immunoprecipitation experiment, third-instar larval wing discs over-expressing UAS-Myc-Sna and UAS-HA-Sd driven by Sd-GAL4 were used. Tissues were lysed in RIPA buffer with PMSF and proceeded with the standard co-immunoprecipitation protocols.

Antibodies used in this study were as follows: rabbit anti-HA (CST, cat. no. 3724), mouse anti-HA (CMCTAG, cat. no. AT0024), normal rabbit IgG (CST, cat. no. 2729), mouse anti-Myc (CST, cat. no. 2276), rabbit anti-flag (CST, cat. no. 14793), mouse anti-flag (Sigma, cat. no. F3165), goat anti-rabbit IgG (Abways, cat. no. AB0101) and goat anti-mouse IgG (Abways, cat. no. AB0102).

### 4.6. GST pull-down assays

Plasmids for Sd-His, GST-Sna and GST-His were transfected into *E. coli*. Bacterial cell lysates were prepared as described [51]. Pierce GST Protein Interaction Pull-Down Kit (Thermo 21516) was used for pull down and analysed by western blot. Antibodies used in this study were as follows: rabbit anti-His (CST, cat. no. 2365), rabbit anti-GST (Rackland, cat. no. 24833) and goat anti-rabbit IgG (Abways, cat. no. AB0101).

#### Data accessibility

The main data supporting this work are available within the article and additional data are provided in the electronic supplementary material.

#### Author contributions

X.D.: data curation, investigation, visualization, writing—original draft and writing—review and editing; Z.L.: conceptualization, data curation, investigation, validation, visualization, writing—original draft and writing—review and editing; K.P.: investigation, validation and visualization; R.Z.: investigation, validation and visualization; C.W.: resources and supervision; G.L.: resources and supervision; W.L.: investigation, visualization, validation, writing—original draft and writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

#### Competing interest

We declare we have no competing interests.

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