Article

Fsh–Pc–Sce complex mediates active transcription of Cubitus interruptus (Ci)

Xiangdong Lv1,†, Hao Chen1,†, Shuo Zhang1, Zhao Zhang1, Chenyu Pan1, Yuanxin Xia1, Jialin Fan1, Wenqing Wu1, Yi Lu1, Lei Zhang1,2, Hailong Wu1,*, and Yun Zhao1,2,∗

1 State Key Laboratory of Cell Biology, CAS Center for Excellence in Molecular Cell Science, Innovation Center for Cell Signaling Network, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai 200031, China
2 School of Life Science and Technology, ShanghaiTech University, Shanghai 201210, China
† These authors contributed equally to this work.
* Correspondence to: Yun Zhao, E-mail: yunzhao@sibcb.ac.cn; Hailong Wu, E-mail: wu.hailong@sibcb.ac.cn

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The Hedgehog (Hh) signaling pathway plays important roles in both embryonic development and adult tissue homeostasis. Such biological functions are mediated by the transcription factor Cubitus interruptus (Ci). Yet the transcriptional regulation of the effector Ci itself is poorly investigated. Through an RNAi-based genetic screen, we identified that female sterile (1) homeotic (Fsh), a transcription co-activator, directly activates Ci transcription. Biochemistry assays demonstrated physical interactions among Fsh, Sex combs extra (Sce), and Polycomb (Pc). Functional assays further showed that both Pc and Sce are required for Ci expression, which is not likely mediated by the derepression of Engrailed (En), a repressor of Ci, in Pc or Sce mutant cells. Finally, we provide evidence showing that Pc/Sce facilitates the binding of Fsh at Ci locus and that the physical interaction between Fsh and Pc is essential for Fsh-mediated Ci transcription. Taken together, we not only uncover that Ci is transcriptionally regulated by Fsh–Pc–Sce complex but also provide evidence for the coordination between Fsh and PcG proteins in transcriptional regulation.

Keywords: Ci, Fsh, Hedgehog, polycomb complex, Sce, transcriptional activation

Introduction

The Hedgehog (Hh) signaling pathway plays important roles in pattern formation and tissue homeostasis from Drosophila to mammals. Dysfunction of Hh pathway causes numerous disorders, such as birth defects and cancer (Jiang and Hui, 2008). In Drosophila, without of Hh ligand binding, Patched (Ptc) inhibits Smoothened (Smo) (Beachy et al., 2004), and the downstream transcription factor Ci is sequentially phosphorylated and subsequently targeted to ubiquitin/proteasome-mediated proteolysis to generate a truncated form, which represses expression of its target genes (Jia and Jiang, 2006). The inhibitory effect of Ptc on Smo is relieved upon Hh binding to Ptc, leading to cell surface accumulation and activation of Smo (Hooper and Scott, 2005). Thus, full-length Ci is protected from phosphorylation and processing, and activates its target genes.

Ci functions as the pivot in the Hh pathway, thus it must be tightly controlled to precisely regulate the Hh signaling activity. The research focusing on its protein regulation, such as stability, localization, and transcriptional activity, has been extensively carried out (Hui and Angers, 2011; Infante et al., 2015). Yet, mechanism controlling the transcription of Ci itself is still largely unknown.

Female sterile (1) homeotic (Fsh) belongs to the Bromo and extra C-terminal (BET) family of proteins (Digan et al., 1986), which are characterized by two bromodomains capable of binding with acetylated lysine and an extra-terminus domain (ET domain). The fs(1)h gene encodes two isoforms, long (Fsh-L) and short (Fsh-S), both of them contain the tandem bromodomains and the ET domain. The long isoform is a C-terminal extension of its short relative. It has been shown that Fsh-S can bind to enhancers and promoters and activate gene transcription (Chang et al., 2007; Kellner et al., 2013; Kockmann et al., 2013), whereas Fsh-L may be involved in chromatin architecture (Kellner et al., 2013).

Polycomb group (PcG) proteins were initially identified in Drosophila to maintain heritable repression of homeotic genes, including Ultrabithorax (Ubx) (Struhl and Brower, 1982; Sawarkar and Paro, 2010). Further investigations uncovered that those highly conserved PcG proteins form multi-protein complexes,
such as polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2), to control the transcription of many key factors which are essential for development and cell fate determination in various species (Laugesen and Helin, 2014). Recently, we and others uncovered a positive role for PcG proteins in transcriptional regulation, which is critical for animal development (Schaf et al., 2013; Gao et al., 2014; Kono et al., 2014; Morey et al., 2015; Xu et al., 2015; Lv et al., 2016a). Since the Hh signaling is a key regulator for both development and cell fate determination, it is, therefore, curious to investigate whether PcG proteins can positively or negatively regulate Ci transcription and Hh signaling activity.

Here, we identify Fsh as a positive transcriptional regulator of Ci. We further provide genetic and biochemistry evidence supporting that Pc and Sce are required for Fsh-mediated transcriptional activation of Ci. Such Fsh-mediated Ci transcription relies on the physical interaction between Fsh and Pc/Sce, which facilitates Fsh binding to Ci locus. These findings expand our knowledge of Ci transcriptional regulation, and imply the existence of coordination between genetic and epigenetic regulation on Ci transcription.

Results

Fsh positively regulates Ci transcription

Ci is a key transcription factor in Hh signaling pathway and its expression and transactivity are tightly regulated to ensure proper Hh signaling activity. Despite the critical role of Ci in Hh signaling pathway, the mechanism regarding transcriptional regulation of Ci itself is still largely unknown. To explore potential transcriptional regulator(s) of Ci, we performed an RNAi-based genetic screen by using Drosophila wing disc development as a model. Wing discs expressing individual dsRNAs were dissected and stained to monitor the levels of Ci. fsl(1)h was emerged from the screen as a positive regulator for Ci. As shown in Figure 1A–B’, knockdown of Fsh led to a significant decrease in Ci levels. Such phenotype was further confirmed in several other fsl(1)h RNAi lines (Supplementary Figure S1A–D’), indicating that fsl(1)h RNAi-mediated decrease in Ci expression is not due to the off-target effects of RNAi assays. To determine whether Fsh transcriptionally regulates Ci expression, we introduced the Ci-lacZ fly, an enhancer trap line that recapitulates the transcription of Ci (Eaton and Kornberg, 1990). Compared with WT control clones (GFP-positive region, Figure 1C and C’), knocking down fsl(1)h decreased the Ci-lacZ level (GFP-positive region, Figure 1D and D’). Consistently, qPCR assays showed that the mRNA levels of Ci were compromised upon fsl(1)h suppression (Figure 1E), indicating that Fsh positively regulates Ci transcription. In addition, ChIP-qPCR assays with an anti-Fsh antibody (Chang et al., 2007) revealed binding of Fsh to Ci locus, mainly around the transcriptional start site (TSS) in developing wing discs (Figure 1F), suggesting a direct role of Fsh on Ci transcription. Since Fsh has two protein isoforms (Fsh-L and Fsh-S) with different regulatory functions, we further determined which Fsh isoform regulates Ci transcription. We examined Ci-lacZ changes when over-expressing Fsh-L or Fsh-S. Fsh-S but not Fsh-L showed regulation capacity to Ci transcription (Figure 1G–H’). Taken together, these results suggest that Fsh is a positive transcriptional regulator of Ci.

Fsh physically interacts with Pc–Sce

To further investigate the mechanism by which Fsh positively regulates Ci, we performed mass spectrometry (MS) assays using Fsh as a bait. Our MS analysis identified Polycomb (Pc), a PcG protein, as an Fsh potential interaction partner. Since Pc and Sex combs extra (Sce), another PcG protein, commonly form a complex to regulate gene expression, we then examined whether Fsh can form a complex with Pc/Sce. As shown in Figure 2A, two-step co-immunoprecipitation (co-IP) assays showed co-IP of Fsh, Pc and Sce in either first-round of Fsh IP or 2nd-round of Pc IP, suggesting that Fsh could form a complex with Pc and Sce. GST pull-down assays further confirmed this notion (Figure 2B). We then characterized that the E fragment of Fsh is critical for such interaction (Figure 2C and D). Interestingly, this E fragment contains a ET domain which has been suggested to be important for protein–protein interaction (Florence and Faller, 2001). Therefore, these findings indicate that Fsh can form a complex with Pc and Sce.

Pc and Sce are required for Ci transcription

Since Fsh could directly regulate Ci transcription and form a complex with Pc/Sce by physical interaction, we further determined whether Pc and Sce are involved in Ci transcriptional regulation. We first examined the Ci levels in Pc null mutant (PcT109) or Sce knockout (SceKO) clones. Consistent with previous studies indicating that Pc and Sce are PcGs responsible for repression of homeotic genes such as Ubx, we detected great induction of Ubx in clones with the loss of either Pc or Sce (Supplementary Figure S2A–A’ and B–B’), suggesting that our system works well. In contrast to the changes of Ubx, Ci levels were markedly decreased in either Pc null mutant (PcT109) or SceKO clones (Figure 3A–B’, GFP-negative region), indicating that Pc and Sce are required for Ci expression. To further support this notion, we also measured Ubx and Ci expression in several other Sce mutant lines. Similar to the results we got from SceKO clones, we also observed decreased Ci but increased Ubx levels in Sce mutant clones (SceT109 and Sce2) (Supplementary Figure S2C–E’). To investigate whether Pc- and Sce-mediated Ci expression occurs at the transcriptional level, we performed qPCR assays to detect Ci mRNA levels in either Pc or Sce RNAi wing discs. In line with the immunofluorescent staining results (Figure 3A–B’ and Supplementary Figure S2), either Pcsr Sce RNAi resulted in significant induction of Ubx and reduction of Ci at mRNA levels (Figure 3C), suggesting that Pc and Sce may regulate Ci expression at transcriptional level. To confirm this conclusion, we further detected Ci-lacZ levels in the context of Pc or Sce knockdown. Knockdown of Pc or Sce obviously inhibited the transcription of Ci as revealed by decreased Ci-lacZ levels (GFP-positive region, Figure 3D–F’). In response to the decreased Ci transcription, ChIP assays showed less RNA polymerase II (Pol II) binding at Ci locus upon Pc or Sce knockdown.
Figure 1 Fsh positively regulates Ci transcription. (A–B') Immunofluorescence analysis of Ci (red) in representative wing discs carrying indicated clones (GFP-positive regions). Ci level was dramatically decreased in Fsh RNAi clones (arrowheads, B–B') compared to WT clones (arrowheads, A–A'). (C–D') Immunofluorescence analysis of Ci-lacZ (blue) in representative wing discs carrying indicated clones (GFP-positive regions). Ci-lacZ level was dramatically decreased in Fsh RNAi clones (arrowheads, D–D') compared to WT clones (arrowheads, C–C'). (E) Transcription levels of Fsh and Ci in wing discs from WT or Fsh RNAi flies. (F) ChIP analyses on Ci locus. Upper panel, diagram shows the PCR amplicons at Ci locus used for following ChIP–qPCR assay. Lower panel, ChIP assays were performed with IgG control and anti-Fsh antibody in wing discs. Relative enrichment to IgG control is shown. (G–H') Immunofluorescence analysis of Ci-lacZ (red) in representative wing discs carrying indicated clones (CD2-negative regions). Ci-lacZ level was increased in Fsh-S clones (arrowheads, H–H'), but not Fsh-L clones (arrowheads, G–G'). Data are represented as mean ± SEM, ***P < 0.001, unpaired, two-tailed Student's t-test.
Figure 2 Fsh physically interacts with Pc–Sce. (A) Two-step co-IP to detect the interaction among Fsh, Sce, and Pc. Cell lysates were first incubated with anti-Flag M2 beads for 2 h. After three times washing, beads were eluted with Flag peptide and subjected to 2nd-round IP with anti-V5 antibody. Cell lysates or IP-ed samples were then analyzed by WB using the indicated antibodies. (B) GST pull-down assay to detect the interaction between purified GST-Sce and Fsh in S2 cell lysate. (C and D) Schematic illustration and domain mapping assay for different fragments of Fsh. Bromo, bromodomain; ET, extraterminal domain.
Figure 3 Pc and Sce are required for Ci transcription. (A–A’) Immunofluorescence analysis of Ci (red) in a representative wing disc carrying PcAT109 clones (GFP-negative regions). Ci level was dramatically reduced in PcAT109 clones (arrowheads). (B–B’) Immunofluorescence analysis of Ci (red) in a representative wing disc carrying SceKO clones (GFP-negative regions). Ci level was dramatically reduced in SceKO clone (arrowheads). (C) RT-qPCR shows transcription levels of Sce, Pc, Ci, and Ubx in wing discs from WT, Sce RNAi, or Pc RNAi flies, respectively. (D–F’) Immunofluorescence analysis of Ci-lacZ (red) in representative wing discs with indicated genotypes (WT, Sce RNAi, or Pc RNAi). Sce or Pc was knocked down specifically in GFP-positive regions below the dashed lines. (G) Diagram shows the PCR amplicons at Ci locus used for following ChIP–qPCR assay. (H) ChIP assays were performed with anti-Pol II antibody in wing discs with indicated genotypes. Relative enrichment to input is shown. Data are represented as mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, unpaired, two-tailed Student’s t-test.
The decrease of Ci transcription in Pc mutants is not due to derepression of Engrailed

It is unexpected that Ci transcription was compromised by loss-of-Pc considering the well-known repressive role of Pc. In addition, Engrailed (En) has been reported as a transcriptional repressor of Ci (Eaton and Komberg, 1990). To test whether the decrease of Ci transcription in Pc mutants is caused by the derepression of En, we first monitored En expression in Pc mutant clones. Interestingly, no obvious staining of En was observed whereas Ci levels were dramatically decreased in the same mutant clones of Pc in the wing pouch (Figure 4A–B”). Therefore, the decrease of Ci level in Pc clones, at least in those clones located in the wing pouch, is not likely caused by En. Of note, we also observed a derepression of En in the dorsal wing hinge (Figure 4A and C–C”), which is consistent with previous reports (Oktaba et al., 2008; Gutierrez et al., 2012).

To further exclude the possibility that the decrease of Ci levels caused by loss-of-Pc is mediated by derepression of En in A compartment, we tried epistasis assays. It is very hard to make double knockout clones for En and Pc since both genes locates in the third chromosome, we then tried to make the fly stock for Pc RNAi; En RNAi and tested whether knockdown of En could rescue the reduction in Ci expression in Pc RNAi region.

Compared with control, knockdown of En did decrease the level of En and increase the level of Ci around the A/P boundary (Figure 4D–G”, pointed by white arrowheads). Yet, we still found a dramatic decrease of Ci when En and Pc were knocked down simultaneously (Figure 4H–I”), which is similar with that caused by Pc RNAi alone. Thus, we conclude that the downregulation of Ci transcription in Pc RNAi region was not due to derepression of En.

Fsh–Pc–Sce complex is required for Ci transcription

Since Fsh, Pc, and Sce can form a complex and all of them can positively regulate Ci transcription, we sought to answer whether the intact Fsh–Pc–Sce complex is essential for Ci transcription. To evaluate this possibility, we carried out ChIP assays with anti-Pc and anti-Sce antibodies to examine their binding to Ci locus. As shown in Figure 5A–C, just like Fsh (Figure 1F), both Pc and Sce bind to Ci locus with a peak around the TSS, suggesting that Fsh, Pc, and Sce may function together as a complex to regulate Ci transcription. Since we demonstrated that the E domain of Fsh is essential for interaction between Fsh and Pc (Figure 2C and D), truncated Fsh protein with the E domain deletion (Fsh-ΔE) should be unable to regulate Ci transcription if the intact Fsh–Pc–Sce complex is required for Ci transcription. For this purpose, we then generated Fsh-ΔE transgenic fly and tested its effect on Ci expression. Fsh-ΔE failed to activate Ci-lacZ expression as what WT Fsh did (Figure 5D–F’), suggesting the indispensable role of Fsh–Pc–Sce complex in regulating Ci transcription. To further confirm this point, we tested whether Fsh could still induce Ci transcription in the circumstance of either Pc or Sce knockdown. Fsh-mediated Ci induction was markedly impaired due to loss of the Pc or Sce as indicated by reduced Ci-lacZ levels (compare Figure 5D–D’ to F’–F” and G–G’), further suggesting the important role of the Fsh–Pc–Sce complex in Ci transcriptional regulation. Mechanistically, through ChIP assays, we found that knocking down Pc or Sce could both decrease the binding of Fsh at Ci locus (Figure 5H), suggesting that Pc and Sce may interact with Fsh to facilitate the binding of Fsh to Ci locus, resulting in Ci transcription. On the contrary, fs(1)h RNAi does not change the binding capacity of Pc or Sce to the promoter regions of Ci (Supplementary Figure S3), suggesting that Fsh is not required for Pc and Sce binding to Ci locus. Collectively, we conclude that the Fsh–Pc–Sce complex is required for Ci expression (Supplementary Figure S4).

Discussion

Through an RNAi-based genetic screen, we identified a direct and positive role of Fsh in regulating Ci transcription (Figure 1). Following biochemistry assays identified a physical interaction between Fsh and Pc–Sce complex (Figure 2). We then showed that Pc and Sce are required for Ci transcription, and such regulation is unlikely mediated by depression of En in Pc mutants (Figures 3 and 4). Importantly, combining the genetic interaction and biochemistry data, we conclude that the Fsh–Pc–Sce complex is required for transcription of Ci (Figure 5). Meanwhile, we also demonstrate that Pc, Sce, or fs(1)h knockdown could produce Hh signaling-related wing phenotypes (Supplementary Figure S5).

The evolutionarily conserved Hh pathway plays a vital role in embryonic tissue-patterning (Ingham et al., 2011; Briscoe and Therond, 2013; Yao and Chuang, 2015) and adult tissue homeostasis maintenance (Petrova and Joyner, 2014). Aberrant Hh signaling is responsible for several congenital syndromes and human cancers (di Magliano and Hebrok, 2003; Kim et al., 2015). As the transcription factor of Hh signaling pathway, Ci directly controls the expression of many downstream target genes. Therefore, it must be precisely regulated to ensure the proper Hh signaling activity. During the past decades, great progress has been achieved in investigations focusing on Ci protein, such as post-translational modifications regulating its stability and/or activity (Jiang and Struhl, 1998; Aikin et al., 2008; Li et al., 2014; Hsia et al., 2015; Zhou et al., 2015; Lv et al., 2016b), and partners regulating its target gene expression (Akimaru et al., 1997; Zhang et al., 2013a). However, the transcriptional regulation of Ci itself is poorly investigated. Here, we show that loss of Fsh leads to the reduction of Ci during normal developmental process. This finding is consistent with a recent finding showing that Brd4, the mammalian homolog of Fsh, positively regulates Gli transcription (Long et al., 2014; Tang et al., 2014), suggesting that both homologs may regulate Ci/Gli transcription in a very similar way. Yet the mechanism by which Fsh controls Ci expression is largely unknown. In present study, we further demonstrate that Fsh, Pc, and Sce form a complex to function together in regulating Ci transcription.
Figure 4 The reduced Ci level caused by loss-of-Pc in the wing pouch seems not to be mediated by En. (A) Immunofluorescence analysis of Ci (red) and En (blue) in a representative wing disc carrying PcXT109 clones (GFP-negative regions). (B–B”) Ci level was dramatically reduced whereas no obvious staining of En was observed in clones located in A compartment of the wing pouch. (C–C”) A derepression of En was also observed in the clone located in the wing hinge. (D–I”) Immunofluorescence analysis of Ci (red) and En (blue) in representative wing discs with indicated genotypes. (D–G”) Compared with control, knockdown of En did decrease the level of En and increase the level of Ci, especially in the region pointed by white arrowheads. (H–I”) Yet, a dramatic decrease of Ci was found even En and Pc were simultaneously knocked down (both yellow and white arrowheads), which is similar with that caused by Pc RNAi alone.
Figure 5 Fsh–Pc–Sce complex is required for Ci transcription. (A–C) ChIP analyses on Ci locus. (A) Diagram shows the PCR amplicons at Ci locus used for following ChIP–qPCR assay. (B and C) ChIP assays were performed with IgG, anti-Sce, or anti-Pc antibodies in WT wing discs. Relative enrichment to IgG control is shown. (D–G') Immunofluorescence analysis of Ci-lacZ (blue) in representative wing discs carrying indicated clones (Flag-positive regions). Ci-lacZ level was upregulated in Fsh overexpression clones (arrowheads, D–D'), but not FshΔE overexpression clones (arrowheads, E–E'). The increase of Ci-lacZ level in Fsh overexpression clones (arrowheads, D–D') was blocked by further knocking down Pc or Sce (arrowheads, F–G'). (H) ChIP assays were performed with anti-Fsh antibody in wing discs of indicated genotypes. Relative enrichment to input is shown. Data are represented as mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, unpaired, two-tailed Student's t-test.
Fsh is a general co-activator whereas PcG proteins are defined as classical repressors. Yet the co-localization of Fsh and PcG proteins has been detected in cultured cells (Kockmann et al., 2013). It is very interesting to figure out the biological function of such unexpected co-localization. In the present study, we demonstrate that such unexpected co-localization is probably due to the physical interaction between Fsh and Pc–Sce complex which results in activated Ci expression in vivo. It will be interesting to test whether this is a general mechanism regulating other critical genes for animal development in the future.

We also noticed that although both Fsh-L and Fsh-S contain the ET domain which is essential for their interacting with Pc, we found that Fsh-S but not Fsh-L showed regulation capacity to Ci transcription (Figure 1G–H). To answer whether this phenotype discrepancy between Fsh-L and Fsh-S is due to their binding affinity difference with Pc and Sce, we performed co-IP assays to examine whether Fsh-L has impaired interaction. As shown in Supplementary Figure S6, Fsh-L and Fsh-S showed comparable binding capacity to Pc and Sce. Therefore, we speculated that there may be an inhibitory domain in Fsh-L which prevents its activation function.

Taken together, we identify an Fsh–Pc–Sce complex as a positive regulator for Ci transcription and propose a molecular model of how the transcriptional factor Fsh collaborates with epigenetic modulators to regulate Ci transcription. These findings will shed new light on the crosstalk between genetic and epigenetic regulation on gene expression. Although PRC1 and PRC2 are classical complexes/units of PcG proteins in transcriptional regulation, subcomplex may also be formed to modulate different target gene expressions (Gutierrez et al., 2012). It is thus very important and interesting to systematically investigate the functions of different PcG proteins in transcriptional regulation, positive or negative.

**Materials and methods**

**Drosophila strains**

Fly stocks were cultured under standard conditions. AP4, AP4GFP, Ci-lacZ, and AG4 have been described (flybase) (Zhang et al., 2013b). Following mutants were kind gifts from Dr Jürg Müller, FRT82 SceΔ/TM6B, FRT82 Sce3SM/TM6B (Fritsch et al., 2003; Scheuermann et al., 2010; Gutierrez et al., 2012; Franke et al., 1995). PcAT109 was a kind gift from Dr Rongwen Xi (Li et al., 2010). FRT82 Sce1/TM6B, Gal80Δ0, and Act5c-Gal4 were obtained from Bloomington; uas-dsRNA Sce and uas-dsRNA Pc were obtained from NIG. Since the progeny of Act5c-Gal4; uas-dsRNA Fsh is lethal, Gal80Δ was introduced. Flies were cultured at 18°C for 5 days, and then transferred to 29°C for 48 h before dissected for RT-qPCR assay. Mutant clones were generated with Flp-mediated mitotic recombination (Theodosiou and Xu, 1998).

**Immunostaining of wing discs**

Wing discs from third instar larvae were dissected for immunostaining with the standard protocol as described previously (Lv et al., 2016a). Wing discs with indicated genotypes were fixed in 4% formaldehyde/PBS for 30 min at room temperature, washed and incubated with a specific combination of primary and secondary antibodies in 0.1% Triton X-100/PBS, and mounted in 40% glycerol. Pictures were taken with the confocal microscope (LAS SP8; Leica) using a 40×/1.25 NA oil objective (Leica). Antibodies used in this study were anti-Ci antibody (DSHB), anti-lacZ antibody (Cappel), anti-Ubx antibody (DSHB), and anti-CD2 antibody (AbD Serotec). The secondary antibodies were bought from Millipore.

**RT-qPCR**

Total RNA was isolated from wing discs following standard protocol. Approximately 500 ng of total RNA was used for reverse transcription with ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, FSQ-301). Real-time PCR was performed on CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD) with SYBR® Green Realtime PCR Master Mix (TOYOBO, QPK-201). The 2−ΔΔCT method was used for relative quantification. The primer pairs used are listed below. Rpl32-F, CTAAGCTGCTCAAAATGGG, Rpl32-R, AGGAACCTCTGAATCCGG TG; Sce-F, GTTATCCTGAATGGCACC, Sce-R, AAGCCTGGTACA GCTTAGG; Pc-F, GGGAAATTTACAACTCCTGCT, Pc-R, ATGTACAA TCATTCCGCA; Ubx-F, CGAGGAAAATCGTCGACAGAC, Ubx-R, CA GATGAAACATTGTTTTCCAC, fsh(1)F, CCGCAGAAGAGATGGACT CC, fsh(1)R, TAATCGTCTCCACTGTGCGC; CI-F, CTTTGTCTCAGGCC TTATTC, CI-R, AGCAGAAATATGCCGTAAGACC.

**ChIP–qPCR**

ChIP assays were performed as previously described (Orlando et al., 1997; Papp and Muller, 2006; Lv et al., 2016a) but with some modifications. Third instar larvae with certain genotypes were dissected and cross-linked in 1% formaldehyde/PBS for 20 min at room temperature. Wing discs were dissected and sonicated in 250 μl sonication buffer (50 mM Hapes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and proteinase inhibitor cocktail). After centrifugation, supernatant was incubated with 2 μg of the indicated antibodies for overnight. 20 μl protein A/G PLUS agarose (Santa Cruz) was then added and incubated for another 2–4 h on a rotator at 4°C. Beads were washed for three times with ChIP wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 150 mM NaCl, and 20 mM Tris-CI, pH 8.0), and once with ChIP final wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 500 mM NaCl, and 20 mM Tris-CI, pH 8.0). Genomic DNA was eluted with elution buffer (1% SDS and 100 mM NaHCO3) at 65°C for 30 min. NaCl was added to a final concentration of 200 mM for reversing cross-link at 65°C for 4 h or overnight. Then, EDTA and protease K were added to final concentrations of 5 mM and 0.25 mg/ml, respectively. The resulting mixture was incubated at 55°C for 2 h. Genomic DNA was purified with a DNA purification kit (QIAGEN) and sent for real-time PCR. Antibodies used were rabbit anti-Pc antibody (Santa Cruz), rabbit anti-H3K27me3 antibody (Millipore), rabbit anti-Sce antibody (generated in this study with full-length Sce protein as antigen, Abclonal technology), rabbit anti-Fsh antibody (kind gifts from Drs Igor B Dawid and Der-Hwa Huang).
(Chang et al., 2007), rabbit anti-Pol II antibody (Abcam), and control rabbit IgG (Santa Cruz). Primer pairs used in this study are listed below. Ci-a-F, CCCGTGAAAATCGTAGTACGAG, Ci-a-R, CATATGCTTCCGAGCT; Ci-b-F, CAAAGCTACCTGCTAGCACC, Ci-b-R, CTCAAAGGAGGAAACCGGCTC; Ci-c-F, CCCCAATATACCCATAAGTACGG, Ci-c-R, TGGCAATAGCATTCTTGTACG; Ci-d-F, ATAAATATTGTATCGCCGAGG, Ci-d-R, CAGTAAACAGTCATGGTGC; Ci-e-F, CACCAATGACCTGTCTACTG, Ci-e-R, GTGATTGTTAGATGGACGGTT; Ci-f-F, AAGTGGAAATTATATGCGTGC, Ci-f-R, CTTTAATGATGGAAACGCGTC; Ci-g-F, AAGGACTGCTATTCTGTGGTGC, Ci-g-R, AAGGCCTTCTCGAATATGC; Ci-h-F, TATTGCTATATTGCGAAGTAAAGCC, Ci-h-R, TATTTAATCCATACATTTCTAACATTCA.

DNA constructs

Full-length Sce cDNA was obtained from Drosophila melanogaster embryonic cDNA, and then inserted into a pUAST-attb vector. pUAST-Fsh-Flag was provided by Dr Der-Hwa Huang (Haynes et al., 1989; Chang et al., 2007). pUAST-VE-Ptc has been reported previously (Lv et al., 2016a).

Cell culture, transfection, immunoprecipitation, GST pull-down, and western blot analysis

Embryo-derived S2 cells were cultured as standard protocols. Transfection, immunoprecipitation, and western blot analysis were carried out using standard protocols. In brief, cells were lysed in standard RIPA buffer, and lysates were first incubated with 20 μl anti-Flag M2 beads (Sigma) for 2 h. Beads were washed three times and half of the beads were eluted with Flag peptide (Sigma) solved in PBS for 1 h. The eluted samples were then subjected to 2nd-round IP with anti-V5 antibody for 2 h, then incubated with Protein A/G PLUS beads (Santa Cruz) for 1 h and washed for three times, and boiled in SDS loading buffer. GST pull-down was performed as described previously (Lv et al., 2016b). GST-Sce was affinity purified from Escherichia coli, and incubated with S2 cell lysates for 1 h. After three times washing, the products were analyzed by WB. Anti-Flag and anti-V5 antibodies used for co-IP–WB were purchased from Sigma. Anti-Fsh antibody was provided by Drs Igor B Dawid and Der-Hwa Huang (Haynes et al., 1989; Chang et al., 2007).

Supplementary material

Supplementary material is available at Journal of Molecular Cell Biology online.

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