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SIAH-1 interacts with mammalian polyhomeotic homologues HPH2 and affects its stability via the ubiquitin–proteasome pathway

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ABSTRACT

Polycomb Group (PcG) genes encode proteins that form large multimeric and chromatin-associated complexes implicated in the stable repression of developmentally essential genes. HPH2, the *Homo sapiens* polyhomeotic homologue 2, functions as one of the subunits of PcG complex 1. In our study, SIAH-1, an E3 ligase, could directly associate with HPH2 both *in vitro* and *in vivo*. Both the cysteine-rich region of SIAH-1 and the PxVxAxP motif of HPH2 were essential for the interaction. HPH2 was co-localized with SIAH-1 in nuclei. Furthermore, SIAH-1 was able to facilitate the ubiquitination and degradation of HPH2 via ubiquitin–proteasome pathway *in vivo*. The ubiquitination activity was severely impaired in the SIAH-1 mutant that either lost E3 ligase activity or had weakened binding ability with HPH2, strongly suggesting that SIAH-1 was the direct E3 ligase of HPH2. Thus, our results propose a novel role of SIAH-1 in regulating the expression level of HPH2 through the ubiquitin–proteasome pathway.

1. Introduction

PcG genes encode proteins that form large multimeric complexes implicated in the maintenance of transcriptional repression of target genes such as *Hox* cluster genes during the development [1,2]. These complexes are deeply involved in epigenetic phenomena, especially the acquisition of specific histone marks on their target genes. In *Drosophila*, PcG products form two distinct complexes, PcG complex 1 which contains Pc, Psc, Ph and Scm, and PcG complex 2 which consists of Esc, E(z) and Pho. PcG complex 2 is responsible for recruiting DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) [3,4] during methylation of lysine 27 of histone H3 (H3K27) [5]. Methylated H3K27 is bound specifically and decoded by PcG complex 1 for transcriptional repression [6–8]. PcG complex 1 has E3 ubiquitin ligase activity. Mono-ubiquitination of lysine 119 of histone H2A by PcG complex 1 correlates with transcriptional silencing [9–11]. More recently, PcG has been linked to cancer progression and poor prognosis, as well as activity of cancer stem cells [12].

Drosophila polyhomeotic (*ph*) encoding one subunit of PcG complex 1, has highly conserved homologues in zebrafish (*ph2α* and *ph2β*), mouse (*Mph1, Mph2* and *Mph3*) and human (*HPH1* and *HPH2*). *In situ* hybridizations revealed that zebrafish *Ph2α* and *Ph2β* are involved in spatio-temporal generation of somites and anterior–posterior (A–P) specification of individual somites. Abnormal embryo epibolic movements as well as a thick tailbud or incomplete covering of the yolk plug would happen when the *ph2α* expression was inhibited [13,14]. During the development of nervous system, *Mph2*, the mouse orthologue of human *HPH2*, appears to repress expression of *Cdkn2a* genes by directly associating with chromatin. *Mph2*-deficient mice showed posterior transformations of the axial skeleton and premature senescence of embryonic fibroblasts, which was associated with repression of *Hox* gene cluster and *Cdkn2a* genes [15]. Human HPH2 associates with Bmi1, Ring1 and Ring2 to form the complex that is involved in the ubiquitination of histone H2A and silencing *HoxC5* genes [16]. However, comparing to the progress in understanding the role of HPH2 in development regulation, the mechanisms underlying the regulation of HPH2 are still not clear.

Polyubiquitination of proteins and subsequent proteasomal degradation are essential for controlling protein levels in cells. Ubiquitin, an evolutionarily conserved 76-residue polypeptide, attaches to target proteins by a three-step enzymatic cascade which requires ubiquitin-activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). E3 ligases, serving as the specific recognition factors to both E2 and target proteins, play a key role in the ubiquitin-mediated proteolytic cascade. SIAH, a human homologue of *Drosophila* Sina (seven-in-absentia), functions as an E3 ubiquitin ligase and is evolutionally conserved from fly to mammals. Humans have two highly conserved SIAH proteins, SIAH-1 and SIAH-2, mainly differing in their N-terminus [17]. Both of them contain an N-terminal RING finger domain responsible for E3 activity to associate with E2 [18,19], and their C-terminal

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regions are involved in the interaction with substrate proteins. SIAH-1 is responsible for degradation of several target proteins with diverse functions, such as membrane receptor DCC [20], presynaptic protein synphilin-1 [21], developmental regulator Numb [22], microtubule-associated motor protein Kid [23], transcriptional regulators OBF-1 [24], etc.

In this study, we report that SIAH-1 was able to facilitate the ubiquitination and degradation of HPH2 via the ubiquitin–proteasome pathway. Our results suggest a novel mechanism of SIAH-1 in regulating the expression level of HPH2 via the ubiquitin–proteasome pathway.

2. Materials and methods

2.1. Plasmid construction

ORF of human SIAH-1 (NM_001006610) was amplified by PCR from human liver cDNA library, further cloned in-frame into the pDBLeu vector (Invitrogen) for yeast two-hybrid screen, into bacterial pGEX-5x-1 vector (New England Biolabs) to generate GST-fusion protein, into pCMV-Myc vector (Clontech) for expressing in mammalian cells, or into pEGFP-C1 vector (Clontech) for localization assay. All SIAH-1 deletion mutants SIAH-1Δ1 and SIAH-1Δ2 were amplified from full-length SIAH-1 and introduced into the pGEX-5x-1 vector to generate GST-fusion proteins. Two SIAH-1 point mutants, SIAH-1m and SIAH-1R, were generated by site-directed mutagenesis PCR and inserted into the pCMV-Myc vector.

ORF of full length human HPH2 (NM_004427) was amplified from pPC86-HPH2 plasmid and cloned into the pEF-FLAG or pDs-Red-C1 (Clontech) vector, respectively. HPH2 deletion mutants (HPH2Δ1 and HPH2Δ2) were amplified from full-length HPH2 and cloned in-frame into the pEF-FLAG vector. The PxVxAxP motif (aa 25–30) was deleted to generate HPH2Δ, which was further cloned into pEF-FLAG vector.

2.2. Yeast two-hybrid screen

Two-hybrid screen was performed in the ProQuestTM two-hybrid system (Invitrogen). pDBLeu-SIAH-1 was used as bait to screen the human fetal brain library pPC86.

2.3. Cell culture, transfection and immunoprecipitation

HEK293T and HeLa cells were obtained from American Type Culture Collection (Rockville, MD). Plasmids were introduced into cells using Lipofectamine reagent (HEK293T) or Lipofectin 2000 (HeLa) (Invitrogen). Cells were treated with or without 50 μM MG132 (Sigma) as indicated, and were harvested at 24 h posttransfection.

For immunoprecipitation, harvested cells were washed with ice-cold PBS and lysed in cell lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 1% Nonidet P-40, 1 mM DTT, 5 mM EDTA pH 8.0, 2 μg/ml Antipain–HCl, 10 μg/ml Leupeptin, 1 μg/ml Pepstain A,10 μg/ml Aprotinin, 1 mM PMSF). For detection of ubiquitinated HPH2, cell lysis buffer was supplemented with 0.1% (w/v) deoxycholate and 100 μM MG132. Cell lysates were pre-cleared with protein A/G-agarose beads (Santa Cruz) at 4 °C for 45 min and were immunoprecipitated with indicated antibodies at 4 °C for 6 h. Immunoprecipitation time was reduced to 1 h for detection of ubiquitination. The bead-conjugated complexes were washed with cell lysis buffer, boiled in SDS sample buffer and subjected to Western blot analysis.

2.4. GST pull down assay

Both GST and GST-SIAH-1 fusion proteins were expressed in *Escherichia coli* strain BL21 and purified according to the manufacture's protocol (Amersham Pharmacia Biotech). Briefly, whole cell lysates from transfected HEK293T cells were incubated with GST-fusion proteins immobilized on the beads for 6 h at 4 °C. The bead-conjugated complexes were washed with cell lysis buffer, boiled in SDS sample buffer and subjected to Western blot analysis.

2.5. Western blot

Protein samples were separated by SDS–PAGE and then transferred to PVDF membranes (Millipore). After blocked with 5% non-fat milk, the membranes were incubated with primary antibodies at 4 °C overnight, followed by incubation of horseradish peroxidase-conjugated secondary antibody. Immunoreactivity was visualized by enhanced chemiluminescence (Millipore). Specific antibodies we used included: anti-Myc antibody (Santa Cruz); anti-FLAG and anti-HA antibodies (Sigma); anti-GAPDH antibody (Kangcheng).

2.6. Subcellular localization assay

Transfected HeLa cells were washed with PBS and fixed with 4% paraformaldehyde (pH 7.4) in PBS for 20 min. After permeabilization with 0.5% Tween 20 in PBS for 10 min, cells were stained with 1 μg/ml Hoechst 33342 (Beyotime) for 10 min at 37 °C. Fluorescent images were acquired using an Olympus IX71 microscope.

3. Results

3.1. HPH2 bound to SIAH-1 in vitro and in vivo

Yeast-two-hybrid screen was performed using SIAH-1 as the bait to screen the human fetal brain library. Among 42 positive preys, 10 encode HPH2, human homologue of *Drosophila* polyhomeotic (data not shown). Interaction between SIAH-1 and HPH2 was further verified by *in vitro* and *in vivo* binding assays. In the GST pull down assay, HPH2 protein expressed in HEK293T could efficiently bind to GST-SIAH-1, but not to GST tag (Fig. 1A). In HEK293T cells, exogenous FLAG-HPH2 could be detected in the immunoprecipitates from the cells co-transfected with Myc-SIAH-1, but not from the cells co-transfected with empty vector (Fig. 1B). Taken together, these data strongly suggest that HPH2 could bind to SIAH-1 *in vitro* and *in vivo*.

3.2. Identification of regions involved in the HPH2–SIAH-1 interaction

It has been reported that the N-terminal RING domain of SIAH-1 was required for its E3 ligase activity, while the C-terminal sequences may regulate its substrate binding ability [19]. Therefore, a series of SIAH-1 deletion mutants were generated and applied to GST pull down assay to identify the binding regions between HPH2 and SIAH-1 (Fig. 2A, left panel). SIAH-1Δ1 (129–313 aa) exhibited similar HPH2-binding to the full length protein, suggesting that the region spanning amino acid 1–128, including RING domain, is dispensable for the interaction. However, SIAH-1Δ2 (167–313 aa), which suffers extra deletion of cysteine-rich region comparing to SIAH-1Δ1, showed severely impaired interaction with HPH2 (Fig. 2A, right panel), indicating that the cysteine-rich region is essential for the interaction.

Most substrates of SIAH-1, like DCC, OBF-1 and Kid, contain a consensus PxAxVxP motif that contributes to their interaction with SIAH-1 [25,26]. HPH2 harbors a PxAxVxP motif from amino acid 25–31 (PQAIVKP). As shown in Fig 2B, deletion of PxAxVxP motif (HPH2Δ) completely abolished the interaction between HPH2 and SIAH-1, further suggesting that PxAxVxP motif of HPH2 is also indispensable for the interaction. In contrast, HPH2Δ1, in which N-

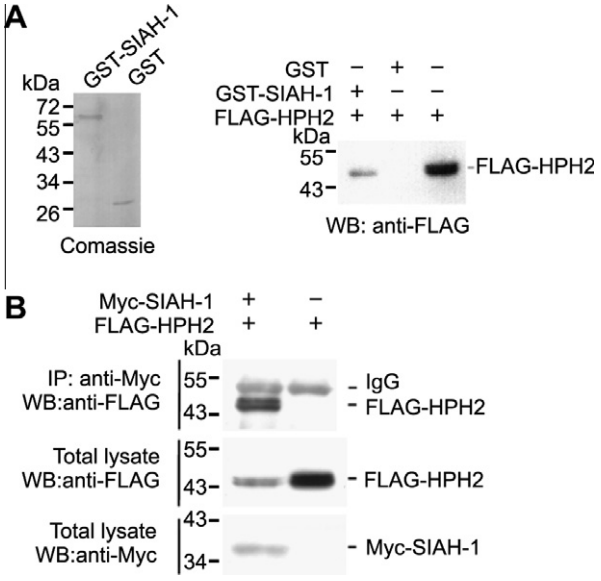

Fig. 1. HPH2 interacted with SIAH-1 *in vitro* and *in vivo*. (A) GST-SIAH-1 interacted with FLAG-HPH2 in GST pull down assay. Left panel: Recombinant GST-SIAH-1 and GST proteins were purified from bacterial. Right panel: GST-SIAH-1, but not GST, could bind to FLAG-HPH2 expressed in HEK293T cells. (B) Myc-SIAH-1 interacted with FLAG-HPH2 in HEK293T cells. FLAG-HPH2 was detected in the anti-Myc immunoprecipitates from cells co-transfected with Myc-SIAH-1 and FLAG-HPH2.

terminus (amino acid 1–24) was deleted, or HPH2Δ2, in which C-terminus (amino acid 218–324) was deleted, bound to SIAH-1 as well as wild type HPH2, implying that neither the N-terminus nor the C-terminus of HPH2 is crucial for the interaction.

3.3. SIAH-1 regulated HPH2 degradation via the ubiquitin–proteasome pathway

Since HPH2 harbors the consensus PxAxVxP motif, HPH2 is probably another substrate of SIAH-1. To demonstrate this hypothesis, HPH2 was co-transfected into HEK293T cells with gradually increasing amount of SIAH-1. Immunoblot analysis of cell lysates revealed that the protein levels of HPH2 reduced gradually when the expression of SIAH-1 increased (Fig. 3A). Importantly, the reduction of HPH2 could be blocked by the proteasome inhibitor MG132 (Fig. 3A, lane 5). These data suggests that SIAH-1 can effectively down-regulate the protein level of HPH2 in the proteasome-dependent pathway.

SIAH-1 promotes proteasome-dependent proteolysis by ubiquitinating its substrates. To address the question whether SIAH-1 can facilitate the ubiquitination of HPH2, FLAG-HPH2 and Myc-SIAH-1 were co-transfected into HEK293T cells. Cells were pre-treated with or without MG132 before being subjected to immunoblot analysis. In the cell lysate pre-treated with MG132, a ladder of bands recognized by anti-FLAG antibody probably represented the poly-ubiquitinated forms of FLAG-HPH2, while no ladder was shown in the cell lysate without MG132 treatment (Fig. 3B). To provide more evidence that FLAG-HPH2 might be ubiquitinated by SIAH-1, HA-tagged ubiquitin (HA-Ub) was co-transfected with FLAG-HPH2 into HEK293T cells, followed by the treatment with MG132. FLAG-HPH2 was immunoprecipitated and HPH2-conjugated ubiquitins were detected by anti-HA blot (Fig. 3C, middle lane). Moreover, in the cells co-transfected with Myc-SIAH-1, FLAG-HPH2 and HA-Ub, the amount of HA-Ubs conjugated with HPH2 remarkably increased (Fig. 3C, right lane), demonstrating that SIAH-1 could facilitate the ubiquitination of HPH2.

The interactive relationship between SIAH-1 and HPH2 was also demonstrated by fluorescent localization assay in HeLa cells expressing GFP-SIAH-1 and RFP-HPH2. As shown in Fig. 3D, RFP-HPH2 alone was specifically localized in nucleus (Fig. 3D, a–c). Since SIAH-1 tends to be self-ubiquitinated and degraded, GFP-SIAH-1 alone could hardly be observed (Fig. 3D, d–f). However, after treatment with MG132, GFP-SIAH-1 could be detected both in cytoplasm and nucleus, especially around the nuclear envelope (Fig. 3D, g–i). RFP-HPH2 was invisible when co-transfected with GFP-SIAH-1 into cells, probably due to SIAH-1 mediated degradation (Fig. 3D, j–m). However, both proteins could be detected and co-localized in nucleus when cells were pre-treated with MG132 (Fig. 3D, n–q). These results suggest that SIAH-1 interacts with HPH2 in nucleus and thereby facilitates the degradation of HPH2.

3.4. E3 ligase activity and HPH2-binding capacity of SIAH-1 are required for the ubiquitination and degradation of HPH2

Since SIAH-1 is able to facilitate the ubiquitination and degradation of HPH2 *in vivo*, we wondered if HPH2 is the direct substrate of

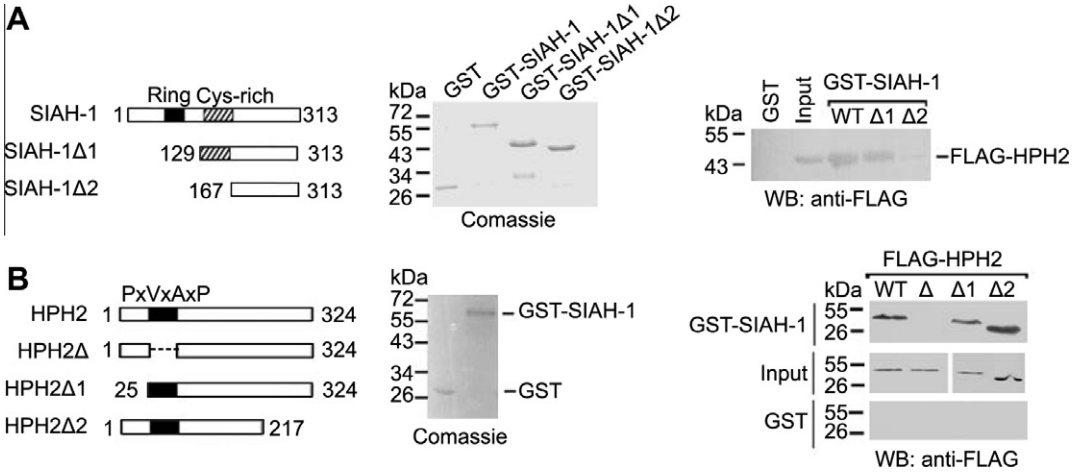

Fig. 2. Identification of regions involved in SIAH-1–HPH2 interaction. (A) Mapping of the HPH2-binding region inside SIAH-1. Left panel: Schematic representation of different SIAH-1 truncated mutants. Among different GST-fused SIAH-1 mutant proteins purified from bacterial (middle panel), SIAH-1Δ2 lost the binding ability with FLAG-HPH2, while that of SIAH-1Δ1 was not affected (right panel). (B) Mapping of the SIAH-1-binding region inside HPH2. Left panel: Schematic representation of different HPH2 mutants. Wild type and mutant HPH2 were transfected into HEK293T cells and cell lysates were incubated with GST or GST-SIAH-1 (middle panel) for pull down assay. Only HPH2Δ mutant without the PxVxAxP motif exhibited impaired binding ability with SIAH-1 (right panel).

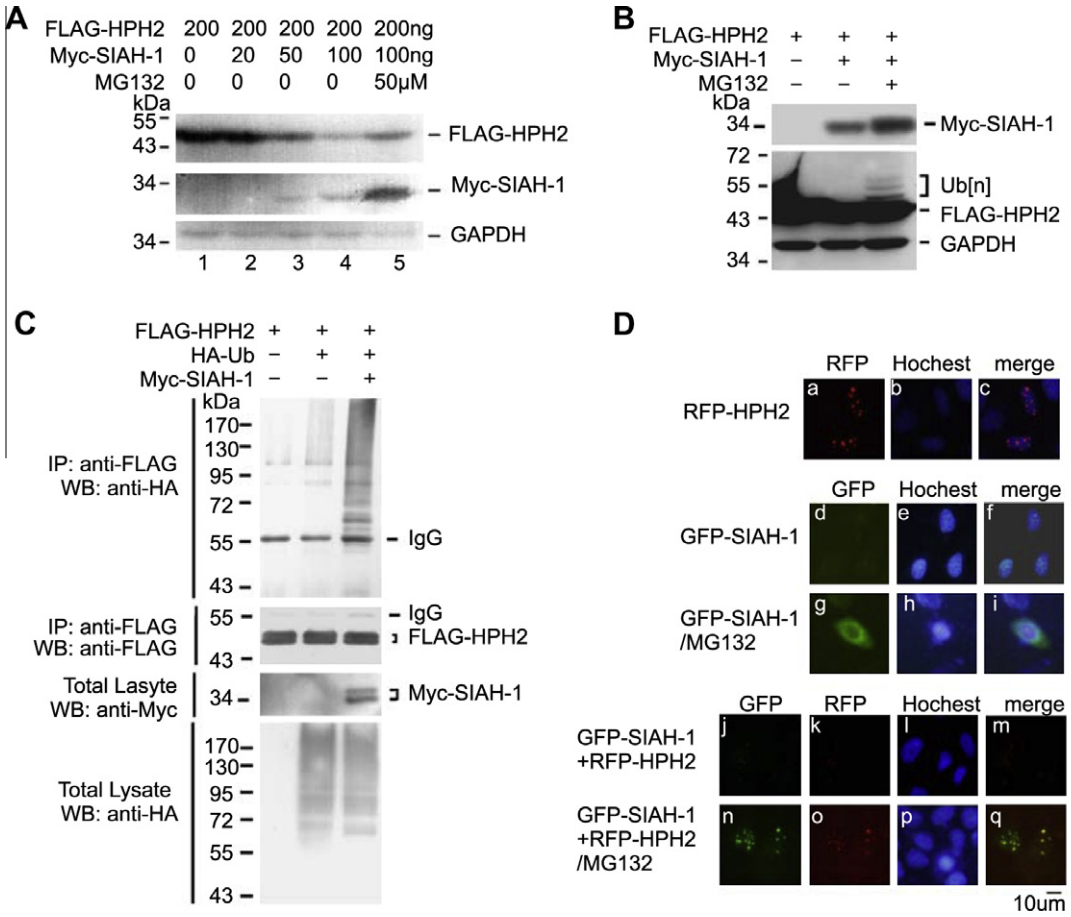

Fig. 3. SIAH-1 regulated HPH2 degradation via the ubiquitin–proteasome pathway. (A) Western blot analysis showed that SIAH-1 down-regulated the protein level of HPH2 and MG132 treatment could inhibit this regulatory function. GAPDH was used as the loading control. (B) Cells were co-transfected with FLAG-HPH2 and Myc-SIAH-1. With the treatment of MG132, anti-FLAG immunoblot recognized a ladder of bands probably presenting the poly-ubiquitinated HPH2 mediated by SIAH-1. (C) HPH2 could be poly-ubiquitinated by SIAH-1 with HA-tagged ubiquitin. HA-ubiquitin was detected in the anti-FLAG immunoprecipitates from cell lysates expressing both FLAG-HPH2 and SIAH-1 in the presence of MG132. (D) Co-localization of HPH2 and SIAH-1 in HeLa cells. Nuclei were stained with Hochest33342 (blue). (a–c) Cells expressing RFP-HPH2 (Red) alone. (d–i) Cells expressing GFP-SIAH-1 (Green) alone and pre-treated with or without MG132 as indicated. (j–q) Cells were co-transfected with pEGFP-SIAH-1 and pDsRed-HPH2 and pre-treated with or without MG132 as indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

SIAH-1. One way to address this question is to investigate whether the E3 ligase activity and HPH2-binding capacity of SIAH-1 are required for the ubiquitination and degradation of HPH2.

To this end, two SIAH-1 mutants were constructed. SIAH-1m contains three Cys to Ser substitutions at amino acid positions 159, 161 and 166 in the cysteine-rich region, leading to a weakened binding ability with HPH2. SIAH-1R carries two Cys to Ser substitutions at amino acid positions 72 and 75 in the RING finger domain. This mutation interrupts the cross-brace structure inside the RING finger domain and leads to reduced E3 ligase activity [19].

As expected, SIAH-1m exhibited weakened interaction with HPH2 in the immunoprecipitation assay while the binding ability of the SIAH-1R mutant was not affected (Fig. 4A). Interestingly, SIAH-1R mutant seemed to bind to even more HPH2 than wild type SIAH-1, but it could be partially explained by the different expression levels of HPH2.

E3 ligase activities of SIAH-1 mutants were further assessed by SIAH-1 self-ubiquitination assay (Fig. 4B, lane 2). As predicted, the ubiquitination level of SIAH-1R was almost undetectable (Fig. 4B, lane 6), which means SIAH-1R lost the E3 ligase activity. Surprisingly, there were more ubiquitins conjugated to SIAH-1m (Fig. 4B, lane 4). Previous work reported that Cys-rich region was involved in the oligomerization of SIAH proteins, which promoted the degradation of oligomer itself [22,27]. In the case of SIAH-1m, although the mutations inside the Cys-rich region did not affect the E3 activity, it might delay the degradation process mediated by oligomerization, leading to the accumulation of the poly-ubiquitinated SIAH-1m.

When cells were co-transfected with HPH2 and SIAH-1m, the reduction of HPH2 level achieved by wild type SIAH-1, was abolished (Fig. 4C). Also, SIAH-1R failed to down-regulate the protein level of HPH2. The data showed that the function to facilitate the degradation of HPH2 was impaired in both SIAH-1m and SIAH-1R mutants.

In cells co-transfected with either SIAH-1m or SIAH-1R, HPH2-conjugated HA-Ubs were reduced significantly in comparison with that in cells transfected with wild type SIAH-1 (Fig. 4D). It means the SIAH-1-dependent ubiquitination of HPH2 was abolished in both mutants.

Since both the HPH2-binding capacity and the E3 ligase activity of SIAH-1 were necessary for SIAH-1 mediated ubiquitination and degradation of HPH2, SIAH-1 is most likely the direct E3 ligase of HPH2.

4. Discussion

As one of the basal subunits of PcG complex 1, HPH2 was shown to be indispensable for the early developmental events. Knockout

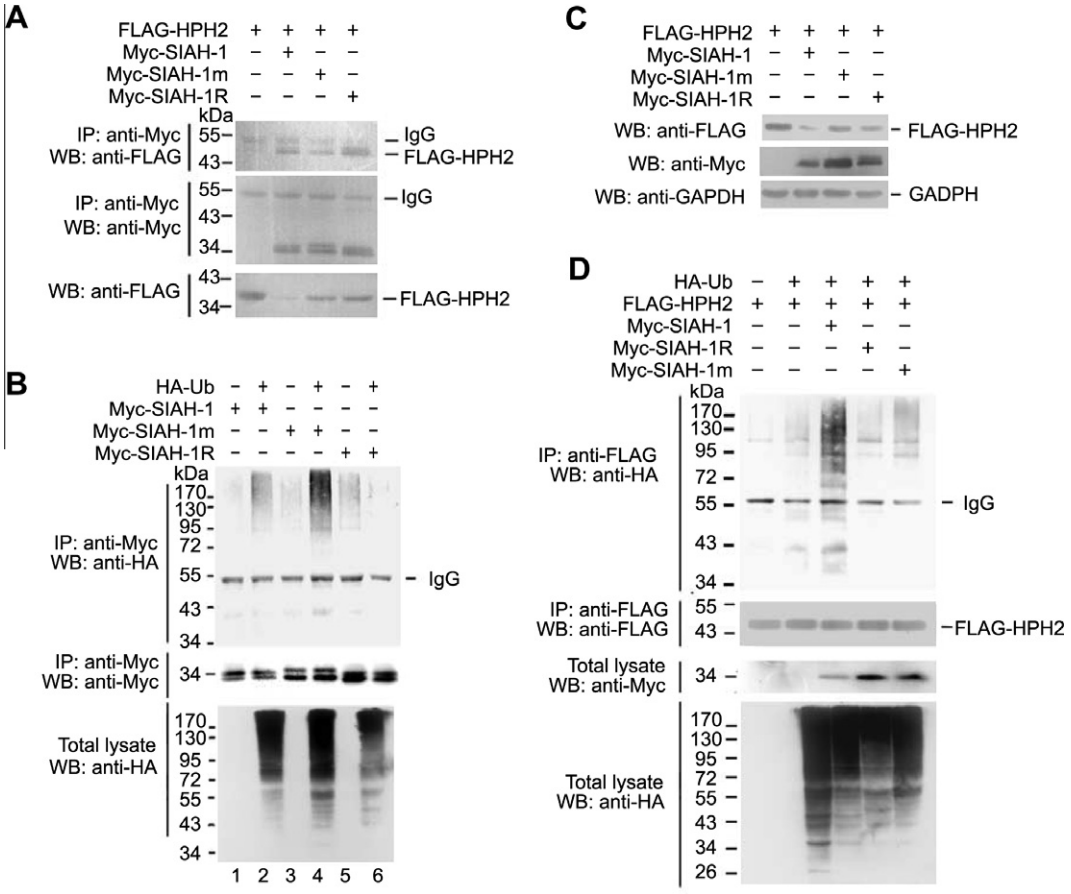

Fig. 4. SIAH-1m and SIAH-1R failed to regulate the ubiquitination and degradation of HPH2. (A) Co-immunoprecipitation assay between SIAH-1 (wild type and mutants) and HPH2. SIAH-1m, but not SIAH-1R showed weakened interaction with HPH2. (B) Anti-HA immunoblot revealed that auto-polyubiquitination was abolished in SIAH-1R. (C) Western blot analysis showed that SIAH-1m and SIAH-1R failed to down-regulated the protein levels of HPH2. GAPDH was used as the loading control. (D) Ubiquitination assay was performed by co-transfection of HA-Ubiquitin into cells. Anti-HA immunoblot showed that SIAH-1m and SIAH-1R could not promote the ubiquitination of HPH2 as wild type SIAH-1 did.

of HPH2 homologue in either zebrafish or mice caused anterior–posterior development abnormality [14,15]. On the other hand, Sina/SIAH family targets multiple proteins involved in the developmental processes for degradation. For example, Sina regulates R7 photoreceptor development by ubiqutinating and degrading proteins that inhibit R7 differentiation [28]. Among the substrates of human SIAH-1, Numb is associated with cell differentiation and Notch signaling pathway. Two other substrates, synaptophysin and group 1 mGluR, are linked to the nerve system development [29,30]. In this report, we found that HPH2 was a new target of SIAH-1, bringing insights into the novel role of SIAH-1 during early development. Clarification of the potential relationship between HPH2 and other substrates of SIAH-1 would be an interesting subject in future.

HPH1 is the other human homologue of *Drosophila* Polyhomeotic (Ph). Like HPH2, HPH1 is the subunit of PcG complex 1. Synergistic effect was observed in mice with both HPH1 and HPH2 homologues knocked-out, which implied the functional overlapping of two proteins [15]. In human cells, HPH1 and HPH2 interacted with each other through their C-terminal regions and were co-localized with Bmi1 in nucleus [31]. HPH1 shares low sequence similarity with HPH2 except for a short stretch of sequence at both N and C terminus. Noticeably, the PxAxVxP motif is well conserved at the N-terminus of HPH1. As we showed above, HPH2 binds to SIAH-1 through this motif. It is likely that HPH1 could interact with SIAH-1 by using the same region. Considering the functional similarity between HPH2 and HPH1, it is reasonable to speculate that SIAH-1 is also the E3 ligase for HPH1. In the future, it will be interesting to test the possibility that SIAH-1 affects the stability and activity of the PcG complex 1 by regulating both HPH1 and HPH2.

5. Conclusion

Here, we showed that SIAH-1, an E3 ligase, directly associates with HPH2 *in vitro* and *in vivo*. Both the cysteine-rich region of SIAH-1 and the PxVxAxP motif of HPH2 were required for the interaction. SIAH-1 facilitates the ubiquitination and degradation of HPH2 *in vivo*, which proposes a novel role of SIAH-1 in regulating the expression level of HPH2 via the ubiquitin–proteasome pathway.

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