The PcG protein HPC2 inhibits RBP-J-mediated transcription by interacting with LIM protein KyoT2

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Received 15 October 2004; revised 27 December 2004; accepted 13 January 2005

Available online 26 January 2005

Edited by Gianni Cesareni

Abstract The DNA-binding protein recombination signal-binding protein-Jk (RBP-J) plays a key role in transcriptional regulation by targeting the intracellular domain of Notch (NIC) and the Epstein–Barr virus nuclear antigen 2 (EBNA2) to specific promoters. In the absence of the Notch signaling, RBP-J acts as a transcriptional suppressor through recruiting co-suppressors such as histone deacetylase (HDAC). KyoT2 is a LIM domain protein that suppresses the RBP-J-mediated transcriptional activation. In the current study, we show that the polycomb group (PcG) protein HPC2, which functions as a transcriptional suppressor, is a candidate of KyoT2-binding proteins. To confirm the physical and functional interaction between KyoT2 and HPC2, we carried out yeast two-hybrid, GST-pull down, co-immunoprecipitation, as well as mammalian two-hybrid assays. Our results showed HPC2 and KyoT2 interacted both in vitro and in vivo, probably through the C-terminal fragment of HPC2 and LIM domains of KyoT2. In addition, we also found that overexpression of HPC2, not only inhibited transactivation of a RBP-J-dependent promoter by NIC, but also transactivation by RBP-J–VP16, a constitutively active form of RBP-J. Taken together, our results suggested that KyoT2 might inhibit the RBP-J-mediated transactivation through NIC by recruiting co-suppressors such as HPC2.

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Keywords: Notch; RBP-J; KyoT2; HPC2; Transcription

1. Introduction

The Notch signaling pathway is an evolutionarily conserved pathway participating in cell fate determination steps in development (reviewed in [1]). Notch was identified in \textit{Drosophila} decades ago and encodes a type I transmembrane protein receptor that contains multiple structural motifs. The extracellular domain of Notch contains 36 EGF-like repeats and 3 LIN-12/Notch repeats. The intracellular domain consists of a RAM (recombination signal-binding protein-Jk (RBP-J)-associating molecule) domain, 6 ankyrin/CDC10 repeats, nuclear localization sequences, a transactivation domain, and a C-terminal PEST domain. Genetic and biochemical analyses have demonstrated that when Notch is triggered by association with its ligands, a proteolysis reaction occurs within the transmembrane domain of the receptor, and the intracellular domain of Notch (NIC) is released. The released NIC translocates into the nuclear and serves as a transcriptional activator of downstream genes. However, because NIC does not possess a DNA-binding activity, it requires a DNA-binding protein, Su (H) (Suppressor of Hairless) in \textit{Drosophila} and RBP-J in mammals, to mediate its transcriptional activation activity [1,2]. In mammals, although multiple members of Notch family (Notch 1–4 in human and mouse) and their ligands (Delta1 and Jagged1 and 2 in mouse) have been identified, evidence has shown that RBP-J is the intranuclear target of all four types of the Notch receptors [3,4]. RBP-J recognizes a consensus sequence C/TGTGGGAA that exists in multiple differentiation-regulating genes such as members of the mouse Hes (Hairy and enhancer of split) family [5]. NIC activates promoters recognized by RBP-J through replacement of transcriptional suppressors by the CDC10/ankyrin repeats, and through recruitment of two conserved histone acetyltransferases, PCAF (p300/CREB-associated factor) and GCN5, by the internal transactivation domain located downstream to the CDC10/ankyrin repeats [6,7]. In addition to NIC, RBP-J also mediates transactivation of the Epstein–Barr (EB) virus nuclear antigen 2 (EBNA2), a crucial molecule involved in cell immortalization and transformation by the EB virus [8,9]. On the other hand, in the absence of transactivators like NIC or EBNA2, RBP-J functions as a transcriptional suppressor [10]. Multiple molecules have been proposed to participate in the transcriptional suppression by RBP-J, such as histone deacetylase (HDAC), SMRT/N-CoR (silencing mediator for retinoid and thyroid receptor/nuclear receptor co-repressor), CIR (CBF1 interacting co-repressor), SAP50, and MINT (MSX2-interacting nuclear target protein) [11–16]. Although, these molecules have been identified to negatively regulate Notch signaling, however, the molecular mechanism of the RBP-J-mediated transcriptional suppression is still elusive. KyoT2 is a LIM domain protein and interacts with RBP-J through a binding motif on its C-terminus generated by alternative mRNA splicing [17]. Previous studies had shown that

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Abbreviations: RBP-J, recombination signal-binding protein-Jk; NIC, intracellular domain of Notch; EBNA2, Epstein–Barr virus nuclear antigen 2; PcG, polycomb group; HDAC, histone deacetylase; cDNA, complementary DNA; RT-PCR, reverse transcription polymerase chain reaction

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doi:10.1016/j.febslet.2005.01.022
KyoT2 inhibits transactivation of promoters containing the RBP-J recognition sites by blocking interaction between RBP-J and transactivators such as NIC and EBNA2. However, our recent results have suggested that in addition to act as a competitor for binding sites, KyoT2 may regulate RBP-J through its LIM domains, which have been shown to function as a protein–protein interaction interface [18–20]. Thus, through interaction with LIM domains of KyoT2, RING1, a member of the polycomb group (PcG) proteins, might suppress transactivation of RBP-J and regulate Notch signaling during mammalian development [21].

HPC2, another member of the PcG proteins, was also identified as a candidate of KyoT-interacting molecule in the yeast two-hybrid screening [21]. In this study, we investigated the physical and functional interaction of KyoT2 with HPC2. We showed that HPC2 physically interacts with KyoT2 both in vitro and in vivo. Overexpression of HPC2 suppressed transactivation of an RBP-J-dependent promoter by NIC, as well as transcription activity of a constitutively active RBP-J, the RBP-J–VP16 fusion protein. Our data further suggested that KyoT2 might inhibit the RBP-J-mediated transactivation by NIC through recruiting HPC2, in addition to RING1.

2. Materials and methods

2.1. RT-PCR

The coding sequence of human HPC2 complementary DNA (cDNA) was amplified by reverse transcription polymerase chain reaction (RT-PCR) from total RNA of Hela cells using the Trizol reagent (Invitrogen). The primers used for PCR were 5′-CCATGGAGCTGCCAGCTGTTGGCGAG-3′ and 5′-CTTCCGGCTACACCGTCACGTACTCC-3′. The amplified fragment was cloned into a T-vector (Promega, Germany) and confirmed by DNA sequencing.

2.2. Yeast two-hybrids assay

All bait plasmids, including pGBK-T7–KyoT1, pGBK-T7–KyoT2, pGBK-T7–KyoT2–LIM1, and pGBK-T7–KyoT2–LIM2, for the yeast two-hybrid assay were described previously [21]. The full-length HPC2 cDNA was inserted in frame into pGADT7 to construct a prey plasmid (pGADT7–HPC2). Prey plasmids with truncated HPC2 (pGADT7–HPC2–N with amino acids 1–374, and pGADT7–HPC2–C with amino acids 375–588) were generated by restriction digestion and ligation, and confirmed by sequencing. Plasmids were used to transform the yeast strain AH109 in combinations as described in the results by the LiAc method, and cloned grown were tested for nutritional phenotypes. Single clone was tested by liquid β-galactosidase assay for β-galactosidase activity.

2.3. Cell culture and transfection

HEK293 and 293T cells were maintained in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine (Gibco BRL). Cells were plated onto 6 well plates at a density of 5 × 10^5 cells per well and cultured at 37 °C with 5% CO₂ overnight for transfection. The transfection was performed at 90% cell confluence with a total amount of 2 μg DNA (for details, see Section 3) per well using Lipofectamine™ 2000 (Invitrogen), following the manufacturer’s protocol.

2.4. GST-pull down assay

The GST–KyoT2 fusion protein was produced as described previously [21]. The coding region of full-length HPC2 cDNA was inserted into pCMV2-Flag, and the generated plasmid (pCMV2-Flag-HPC2) was used to transfect 293T cells. Harvested cell extracts were incubated with the purified GST–KyoT2 or GST (used as a negative control) protein, respectively, and the protein–protein interaction was assayed by pulling down with the glutathione–Sepharose beads (Sigma), followed by immunoblotting with an anti-Flag antibody (M2, Sigma).

2.5. Co-immunoprecipitation

Plasmids pCMV-KyoT2-Myc, pCMV2-Flag-RING1, and pCMV2-Flag-RBP-J were described previously [21]. Plasmids were transfected into 293T cells using Lipofectamine™ 2000 as shown in Section 3. Sixty hours after transfection, cells were collected and lysed using the phospho-lysis buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 0.5% NP40, 1 mg/ml BSA, and 0.1 mM PMSE). Immunoprecipitation was carried out with an anti-Myc (9E10, Santa Cruz) antibody. After washing extensively with the phospho-lysis buffer, co-precipitated proteins were analyzed by SDS–PAGE followed by Western blotting using the anti-Flag antibody, or antibodies against NIC (K0403) [17, provided by T. Honjo), HPC2 (a gift from BQ Jin, unpublished), or RING1 (C-20) (Santa Cruz). Expression of the Myc-tagged proteins was detected by Western blotting of the cell lysates using the anti-Myc antibody.

2.6. Reporter assays

For mammalian two-hybrid experiments, the full-length KyoT2 cDNA and HPC2 cDNA were inserted in frame into multiple cloning sites of pCMX–GAL4–DBD and pCMX–VP16 (NLS) (generously provided by T. Honjo), to generate plasmids pCMX–GAL4–DBD–KyoT2 and pCMX–VP16–HPC2, respectively. The plasmids were co-transfected with a reporter construct (TK MH100 × 4 luc), in which the luciferase gene was under control of a promoter containing multiple recognition sites of the GAL4 DNA-binding domain. Cells were lysed 48 h after transfection with a hypotonic buffer (91 mM K₂HPO₄, 9 mM KH₂PO₄, 10% glycerol, 1 mM DTT, 10% Triton X-100), and the level of the luciferase in cell lysates was examined as described. Transfection efficiency was calibrated by co-transfection with pSVβ-gal, followed by examining the β-galactosidase activity in cell lysates. Each experiment was repeated at least three times and data were analyzed with the Student’s t test.

Transactivation of the RBP-J-responsive promoter was detected using reporter assay with the reporter construct pGa981-6, which contains a hexamerized 50 bp EBNA2 responsive element of the TP-1 promoter and is strictly dependent on RBP-J [3]. Expression vectors for KyoT2 (pEFBOS-KyoT2), RING1 (pEFBOS–RING1), HPC2 (pEFBOS–HPC2) and NIC (pEFBOS–NIC) were constructed by insertion of the full-length cDNA of KyoT2, RING1, HPC2, as well as NIC into pEFBOS-neo vector, respectively. Cells were collected 48 h after transfection with different plasmids and the luciferase activity was examined as above. pSVβ-gal was included in each transfection as an internal control of the transfection efficiency.

2.7. CHIP assays

Chromatin immunoprecipitation (CHIP) assay was carried out using a kit from Upstate (Milton Keynes, UK) according to the manual provided by the supplier. Briefly, NIH3T3 cells were transfected with expression vectors for Myc-NIC or Myc-KyoT2 plus Flag-HPC2, together with pGa981-6. Forty-eight hours after transfection, cells were crosslinked with 1% formaldehyde, disrupted and ultra-sonicated. The cell lysates were immunoprecipitated with anti-Myc, anti-Flag, or anti-RBP-J, with a premunine serum as a control. The crosslinking of the immunoprecipitates were reversed by heating up, and bound DNA was amplified using primers to the promoter of pGa981-6 containing RBP-J-recognition sequence. The sequence of the primers was 5′-gtagacgactgtgg-3′ and 5′-ttccaggtgcctc-3′. The amplified fragments were analyzed using 3% agarose gel electrophoresis.

3. Results

3.1. KyoT2 interacts with HPC2 through LIM domains in yeast

In a screening of KyoT2-interacting proteins using the yeast two-hybrid system with KyoT2 as bait [21], we identified HPC2 as another candidate of KyoT2-binding proteins. To confirm the interaction between KyoT2 and HPC2, and to identify potential domains of KyoT2 and HPC2 responsible...
for their interaction, we performed yeast two-hybrid assays with full-length KyoT1, KyoT2, and HPC2, as well as their truncated derivatives (Fig. 1). When full-length KyoT1 or KyoT2 and different truncated HPC2 fragments were used to transform yeast AH109, positive interactions were detected between KyoT2 and HPC2 as well as KyoT1 and HPC2, suggesting that KyoT2 interacted with HPC2 through its LIM domain(s), because KyoT1 contains four LIM domains without an RBP-J-binding motif [17]. This was further confirmed by yeast two-hybrid assays using single LIM domains (LIM1 or LIM2 from the N-terminal) of KyoT2 as baits. Both of the LIM domains of KyoT2 showed positive interaction with HPC2. On the other hand, while the C-terminal fragment of HPC2 showed strong positive interaction with KyoT2, its N-terminal fragment showed much weaker interaction with KyoT2 in yeast. These results indicated that the LIM domains of KyoT2 and the C-terminal fragment of HPC2 might be mainly responsible domains for the interaction between KyoT2 and HPC2 in yeast.

3.2. Physical interaction between KyoT2 and HPC2

Direct in vitro interaction of KyoT2 with HPC2 was verified by a GST-pull down assay. The GST and GST–KyoT2 fusion protein were generated in Escherichia coli and purified, and were used to interact with the HPC2 protein expressed by transfection of cultured 293T cells. As shown in Fig. 2A, a clear interaction of HPC2 with GST–KyoT2 was observed. No interaction could be detected between GST and HPC2. This result suggested that HPC2 directly interacted with KyoT2.

In vivo interaction of KyoT2 with HPC2 was tested by co-immunoprecipitation assays. Cultured 293T cells were transfected with expression vectors of the Myc-tagged KyoT2 and the Flag-tagged HPC2, or the Flag-tagged RBP-J as a positive control. The KyoT2 protein was immunoprecipitated using an antibody against the Myc tag. Co-immunoprecipitated proteins were detected by Western blotting using the anti-Flag antibody. As shown in Fig. 2B, both Flag-HPC2 and Flag-RBP-J were co-precipitated with Myc-KyoT2. Moreover, we examined interactions between Myc-tagged KyoT2 and endogenous HPC2, as well as endogenous RBP-J and RING1. Cell lysates from Myc-KyoT2-transfected cells were examined by Western blotting using anti-RING1, anti-RBP-J, or anti-HPC2 antibodies. Endogenous RING1, RBP-J, and HPC2 were all detected in the precipitated proteins (Fig. 2C). These results indicated that HPC2 was able to interact with KyoT2 in vivo.

To further examine whether KyoT2 and HPC2 interact in mammalian cells, we employed the mammalian two-hybrid assay. KyoT2 was fused to the GAL4 DNA-binding domain and HPC2 was fused to the VP16 transactivation domain, to generate pCMX–GAL4DBD–KyoT2 and pCMX–VP16–HPC2, respectively. Then pGAL4DBD–KyoT2 was co-transfected into HEK-293 cells together with increasing amounts of pCMX–VP16–HPC2, as well as the reporter plasmid TK MH100 × 4 luc. Luciferase activity in cell lysates was examined 48 h after transfection. The results showed that, while transfection only with the GAL4DBD–KyoT2 or VP16–HPC2 expression vector did not activate expression of the luciferase in cells, co-transfection of the two vectors stimulated increasingly higher luciferase activity in cell lysates (Fig. 2D). This result suggested that KyoT2 and HPC2 interacted in mammalian cells.

3.3. HPC2 formed a complex with RBP-J through KyoT2 in cells

Since KyoT2 binds RBP-J through its RBP-J-binding motif on the C-terminal [17], and binds HPC2 through its LIM domains on the N-terminal as shown above, we questioned whether HPC2 might form a complex with RBP-J through KyoT2. To access this question, we tested the formation of a three-molecule complex of KyoT2, HPC2 and RBP-J in cells using the co-immunoprecipitation assay. We co-expressed the Flag-tagged HPC2 and the Flag-tagged RBP-J in 293T cell with the Myc-tagged KyoT2. Immunoprecipitation was carried out using the anti-Myc antibody 60 h after transfection, and co-precipitated proteins were detected with the anti-Flag antibody after Western blotting. As shown in Fig. 3A, Flag-RBP-J and Flag-HPC2 could be simultaneously co-precipitated with KyoT2, suggesting that KyoT2, HPC2, and RBP-J might form a three-molecule complex in cells. In addition, as we have shown that RING1 also associates with KyoT2, we looked at if HPC2 and RING1 could bind to KyoT2 simultaneously by the co-immunoprecipitation experi-
Fig. 2. Interaction between KyoT2 and HPC2 in vitro and in vivo. (A) GST-pull down assay. GST and GST–KyoT2 were generated in *E. coli* and purified, and were incubated with Flag-HPC2 expressed in transfected cells. Protein interaction was examined by Western blotting after pulling down with the glutathione–Sepharose 4B beads. (B) Immunoprecipitation assay. Vectors for expression of the Myc-tagged KyoT2 and the Flag-tagged HPC2 or the Flag-tagged RBP-J were used to transfect 293T cells. Cell lysates were prepared 60 h after transfection and were immunoprecipitated with the anti-Myc antibody, followed by Western blotting with the anti-Flag antibody. Lower panel, Western blotting of cell lysates with the anti-Myc antibody. One-tenth of the cell extract used for immunoprecipitation was run as the input. (C) Immunoprecipitation assay. Cells were transfected with pCMV-KyoT2-Myc and cell lysates were immunoprecipitated with the anti-myc antibody, followed by Western blotting with anti-RING1 (left), anti-RBP-J (middle), or anti-HPC2 (right). Myc-tagged KyoT2 in cell lysates was detected using anti-Myc. One-twentieth of the cell extract used for immunoprecipitation was run as the input. (D) Mammalian two-hybrid assay. HEK-293 cells were co-transfected with expression vectors of GAL4–DBD–KyoT2 (0.2 μg) and increasing amounts (0.2, 0.4, and 0.8 μg) of expressing vectors of VP16–HPC2 and the luciferase reporter plasmid (0.4 μg). Luciferase activity was detected in cell lysates 48 h after transfection.

Fig. 3. (A) Co-immunoprecipitation of HPC2, RBP-J, and KyoT2. Expression vectors of the Myc-tagged KyoT2 and the Flag-tagged RBP-J and HPC2 were used to transfect 293T cells. Cell lysates were prepared 60 h after transfection and were immunoprecipitated with the anti-Myc antibody. Co-precipitated proteins were detected with the anti-Flag antibody after Western blotting. (B) Co-immunoprecipitation of HPC2, RING1, and KyoT2. Cells were transfected with expression vectors of the Myc-tagged KyoT2 and the Flag-tagged RING1 and HPC2, and immunoprecipitation was performed as above. One-tenth of the cell extract used for immunoprecipitation was run as the input.
The result showed that Flag-tagged HPC2 and RING1 could be simultaneously immunoprecipitated with Myc-tagged KyoT2 (Fig. 3B), suggesting that a ternary complex containing HPC2, RING1, and KyoT2 be formed in cells.

3.4. HPC2 suppressed transactivation of RBP-J by NIC

Our earlier works have shown that RING1 inhibited the RBP-J-mediated transactivation by NIC through association with LIM protein KyoT2 [21]. In the current study, our data showed that KyoT2 might also recruit HPC2 to RBP-J through its LIM domains. Because both HPC2 and RING1 are members of the PcG complex and have transcriptional suppression activity, we assumed that HPC2 might also suppress the RBP-J-mediated transactivation through association with KyoT2. In order to answer this question, we transfected HEK293 cells using vectors expressing KyoT2 and/or HPC2, together with the luciferase reporter plasmid pGa981-6 [3]. The results showed that expression of NIC with pGa981-6 led to strong transcriptional activation. Transactivation of the RBP-J-dependent promoter was slightly inhibited by low amount of KyoT2 alone (Fig. 4A) [17,21]. Low amount of HPC2 also slightly inhibited RBP-J-mediated transactivation by NIC, suggesting that there is endogenous KyoT2 protein in transfected cells, as confirmed by RT-PCR (data not shown). This transactivation was strongly suppressed by co-expression of HPC2 in a dose-dependent manner in the presence of KyoT2 (Fig. 4A). Because HPC2 (and RING1) does not associate directly with RBP-J and suppresses RBP-J (our unpublished results) [21], these results suggested that KyoT2 might suppress the RBP-J-mediated transcription by recruiting the PcG protein HPC2.

3.5. KyoT2 suppressed transactivation of the RBP-J-dependent promoter by RBP-J–VP16

KyoT2 has been shown to suppress transactivation of NIC by competitively binding to a site on RBP-J. However, our previous and current work suggested that KyoT2 might also suppress transcription by recruiting co-suppressors such as RING1 [21] and HPC2 through its LIM domains. To further demonstrate this assumption, we tested the effect of HPC2 on transactivation of the RBP-J-dependent promoter by the constitutively active RBP-J, RBP-J–VP16. As shown in Fig. 4B, in the presence of KyoT2, HPC2 suppressed transactivation activity of RBP-J–VP16 in a dose-dependent manner.

3.6. KyoT2 and HPC2 bind to the promoter harboring RBP-J recognition sites

The PcG proteins including HPC2 suppress transcription through forming large PcG complexes on promoters [22–28]. We employed the CHIP assay to test whether HPC2 could be recruited to RBP-J-binding promoters through KyoT2. Cells were transfected with plasmids as well as pGa981-6, as indicated in Fig. 5, and a CHIP assay was performed using specific antibodies. The precipitated DNA was amplified using primers targeting the TP-1 promoter sequences recognized by RBP-J. The results showed that while no positive band was amplified when precipitated with a preimmune serum, the TP-1 promoter sequence was amplified in precipitates with anti-Myc-NIC, anti-Myc-KyoT2, anti-Flag-HPC2, as well as anti-RBP-J (Fig. 5), suggesting that these molecules could form complexes on the promoter sequence of pGa981-6, the RBP-J responsible reporter construct.

4. Discussion

As the key transcription factor in the Notch signaling pathway, RBP-J mediates transactivation of all the four Notch receptors. In the absence of transactivators, RBP-J suppresses transcription. So far, sophisticated molecular mechanisms involving multiple co-activators and co-suppressors have been suggested to explain the transcriptional regulation of RBP-J. In the current study, we suggested that in addition to other molecules, PcG protein HPC2 might be another player in the...
regulation of the Notch-RBP-J signaling pathway. We showed that HPC2 interacted with the LIM protein KyoT2, and might in turn form a molecular complex with RBP-J. We also provided evidence that HPC2 suppressed transactivation of the RBP-J-dependent promoter by NIC when overexpressed in cells with KyoT2. Given that KyoT2 binds to RBP-J at the same site as NIC [17], we suggested that KyoT2 suppressed the RBP-J-mediated transcription by two ways, namely, competition with transactivators for binding sites and recruitment of co-suppressors such as RING1 [21] and HPC2 (this study).

RING1 and HPC2 are members of the PcG family proteins that are parts of a cellular memory system responsible for the stable inheritance of gene activity [22–24]. The PcG genes have been first identified in Drosophila as suppressors of the homeotic gene activity [25], and then it has been proposed that PcG proteins suppress gene activity via the formation of multimeric protein complexes (reviewed in [26]). In vertebrate, human HPC2 can interact with a RING finger protein RING1 and other PcG proteins to form a Polycomb repressive complex [27]. Moreover, it has been reported recently that members of PcG proteins are also recruited to promoters and suppresses transcription [28]. In this study, we provided evidence that HPC2 might be recruited to RBP-J-dependent promoters by KyoT2, and suppressed their transactivation. However, more detailed studies are necessary to elucidate whether HPC2 regulates the Notch pathway upon transactivation, or acts in a transcriptional memory machinery to maintain the transcription status established during Notch signaling.

Although both RING1 and HPC2 possess transcriptional suppression domains [22,23], the molecular mechanism by which they suppress RBP-J is unclear. Two mechanisms might be proposed. One is that HPC2 and/or RING1 are recruited by KyoT2 to RBP-J associating with RBP-J-dependent promoters, and suppress transcription mediated by RBP-J. Both HPC2 and RING1 are nuclear-localized proteins, and they suppress transcription when recruited promoters [23,28]. The result of the CHIP assay (Fig. 5) favors that at least HPC2 might be recruited to RBP-J-recognizing promoter and suppress transcription. However, recent studies have shown that the PcG protein HPC2 also functions as a SUMO E3 ligase [29], which catalyzes sumoylation of target proteins, and modify their specific subcellular locations as well as their functions [30]. HPC2 may thus also modulate KyoT2 and/or RBP-J through sumoylation of these proteins, and regulate their functions. Further studies are underway to test this possibility.

Acknowledgments: We specially thank Dr. T. Honjo and Dr. B.Q. Jin for plasmids and antibodies. We thank Yan-Xia Zhang and Ling Zhang for secretary assistance and technical support. This work was supported by grants from National Natural Science Foundation of China (30200148, 30300550, 30300110, 30425015) and from the Ministry of Science and Technology of China (001CB509906).

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