Acta Biochim Biophys Sin 2014, 46: 136–140 | © The Author 2013. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmt136.
Advance Access Publication 26 December 2013

Original Article

RNF20 promotes the polyubiquitination and proteasome-dependent degradation of AP-2α protein

Peng Ren[1†], Zhifeng Sheng[2†], Yijun Wang[1,3], Xin Yi[1], Qiuzhi Zhou[1], Jianlin Zhou[1], Shuanglin Xiang[1], Xiang Hu[1*], and Jian Zhang[1*]

[1]Key Laboratory of Protein Chemistry and Developmental Biology of Ministry of Education, College of Life Science, Hunan Normal University, Changsha 410081, China
[2]Institute of Metabolism and Endocrinology, The Second Xiang-Ya Hospital, Central South University, Changsha 410011, China
[3]Department of Biochemistry and Molecular Biology, Changsha Medical University, Changsha 410219, China
[†]These authors contributed equally to this work.
*Correspondence address. Tel/Fax: +86-731-88872792; E-mail: huxiang@hunnu.edu.cn (X.H.)/Tel/Fax: +86-731-88872792; zhangjian@hunnu.edu.cn (J.Z.)

Transcription factor activator protein 2α (AP-2α) is a negative regulator of adipogenesis by repressing the transcription of CCAAT/enhancer binding protein (*C/EBPα*) gene. During adipogenesis, AP-2α is degraded, leading to transcriptional up-regulation of *C/EBPα*. However, the mechanism for AP-2α degradation is not clear. Here, using immunoprecipitation assay and mass spectrometry, we identified ring finger protein 20 (RNF20) as an AP-2α-interacting protein in 3T3-L1 preadipocytes. RNF20 has been proved to be an E3 ubiquitin ligase for both histone H2B and tumor suppressor ErbB3-binding protein 1 (Ebp1). In this study, we demonstrated that RNF20 co-localized and interacted with AP-2α, and promoted its polyubiquitination and proteasome-dependent degradation. Over-expression of RNF20 inhibited the activity of AP-2α and rescued the *C/EBPα* expression which was inhibited by AP-2α. These results suggested that RNF20 may play roles in adipocyte differentiation by stimulating ubiquitin–proteasome-dependent degradation of AP-2α.

Keywords AP-2α; RNF20; E3 ubiquitin ligase; ubiquitin–proteasome-dependent degradation; C/EBPα

Received: August 12, 2013 Accepted: October 29, 2013

Introduction

Transcription factor activator protein 2α (AP-2α) belongs to the AP-2 family of transcription factors that consists of five different proteins in humans and mice: AP-2α, AP-2β, AP-2γ, AP-2δ, and AP-2ε (reviewed in [1,2]). Among these, AP-2α is the first discovered and best characterized member. AP-2α is involved in various physiological and pathological processes, including the regulation of cell proliferation, differentiation, apoptosis, and carcinogenesis [1,2]. Recently, AP-2α has been found to play roles in adipocyte differentiation. In 3T3-L1 preadipocytes, AP-2α represses the transcription of CCAAT/enhancer binding protein (*C/EBPα*) gene [3,4], which is an important regulator of adipogenesis [5]. Upon induction of differentiation, both protein level and DNA binding activity of AP-2α decline, allowing the expression of *C/EBPα* gene [3,4]. However, it is unknown how the stability and activity of AP-2α protein are regulated during adipocyte differentiation.

Many studies have demonstrated that the stability and activity of AP-2α protein can be regulated by its interacting partners through physical interaction or chemical modification [1,6]. For example, potassium channel tetramerization domain containing 1 (KCTD1) interacts with AP-2α and inhibits its transactivity; the protein kinase A-mediated phosphorylation of AP-2α stimulates the effect of AP-2 on the apolipoprotein E (*ApoE*) promoter [7]; our previous study has demonstrated that casein kinase 2 phosphorylates AP-2α and increases its stability [8]. Here, to understand the regulatory mechanism of AP-2α in adipogenesis, we screened its interacting partner in 3T3-L1 preadipocytes using immunoprecipitation (IP) assay followed by mass spectrometry, and identified ring finger protein 20 (RNF20) as an AP-2α-interacting partner. Furthermore, we demonstrated that RNF20 promoted the polyubiquitination and degradation of AP-2α.

Materials and Methods

Plasmids and antibodies

The mouse RNF20 expression plasmid pHA-RNF20 was purchased from GeneCopoeia (Rockville, USA). The AP-2α expression plasmid pMyc-AP-2α, pEGFP-Myc-AP-2α, and the

reporter plasmid pA2-Luc which contains three copies of AP-2 binding sequence from human metallothionein IIa (hMT-IIa) promoter region, were described previously [9]. The His-tagged ubiquitin mammalian expression plasmid pHis-UB was a gift from Prof. Marsh JL (University of California, Los Angeles, USA) [10]. The β-galactosidase expression plasmid pCMV-β was purchased from Clontech (Palo Alto, USA). All of the antibodies used in this study were purchased from Santa Cruz (Santa Cruz, USA).

Cell culture, transfection, and luciferase assay

All cell lines used in this study were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). 3T3-L1 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Carlsbad, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL), 2 mM L-glutamine, and 100 U/ml penicillin–streptomycin at 37°C in a 5% CO_2 incubator. The 3T3-L1 cells were grown in a 24-well plate and transfected with 0.1 μg of pA2-luc, 0.1 μg of pCMV-β, and increasing amount of pHA-RNF20 (0, 0.1, 0.2, 0.4 μg) using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) at 80% confluence. At 36 h post-transfection, the cells were lysed to measure the luciferase activity using the luciferase assay system (Promega, Madison, USA) as previously described [9]. The luciferase activities were normalized according to β-galactosidase activities.

Co-immunoprecipitation and mass spectrometry

The AP-2α-interacting partners were screened by IP assay followed by mass spectrometry as described previously [11]. Cell extracts from 3T3-L1 preadipocytes were immunoprecipitated using anti-AP-2α antibody or IgG. The immunoprecipitated proteins of anti-AP-2α antibody or IgG were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by silver staining. The protein bands which only existed in immunoprecipitated proteins of anti-AP-2α antibody, but not in those of IgG, were digested with trypsin and analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) mass spectrometry. The mass spectrometry data were used to search against the protein sequence database using the MASCOT database search engine.

Co-immunoprecipitation and western blot analysis

Co-immunoprecipitation (co-IP) and western blot analysis were performed as previously described [9]. Briefly, HEK293 cells were transfected with pMyc-AP-2α and pHA-RNF20 plasmids. At 24 h post-transfection, cells were lysed in RIPA buffer [50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS] with protease inhibitors. Cell lysates (800 μg) were immunoprecipitated with rabbit anti-HA polyclonal antibody or control pre-immune rabbit IgG. Immunoprecipitated Myc-AP-2α was electrophoresed on 10% SDS–polyacrylamide gel, transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, USA), and detected with the anti-Myc monoclonal antibody and horseradish peroxidase-conjugated goat anti-mouse secondary antibody. A total of 80 μg of cell extract was used as positive control (Input). The signal was visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, USA).

Subcellular localization analysis by fluorescence microscopy

The 3T3-L1 cells were seeded on glass coverslips and treated as described previously [9]. The pEGFP-Myc-AP-2α and pHA-RNF20 plasmids were co-transfected into 3T3-L1 cells. At 24 h post-transfection, the cells were fixed. The mouse anti-HA monoclonal antibody and Texas Red-conjugated anti-mouse IgG (red) were used to detect HA-RNF20 fusion protein, whereas enhanced green fluorescent protein (EGFP) fluorescence was examined directly by fluorescence microscopy, nuclei were stained with Hoechst 33258 (Sigma, St Louis, USA).

Detection of ubiquitinated proteins

The ubiquitinated AP-2α proteins in 3T3-L1 were detected according to Tansey's method [12]. The pMyc-AP-2α and pHis-Ub were co-transfected with pHA-RNF20 or empty vector into 3T3-L1 cells. At 24 h post-transfection, cells were harvested. The total ubiquitinated proteins were isolated from cell extract using Ni-nitrilotriacetate (NTA) agarose (Qiagen, Hilden, Germany). The isolated proteins and input samples (whole-cell extracts) were analyzed by western blot analysis using anti-Myc monoclonal antibody.

Statistical analysis

Data were presented as mean ± standard deviation from three independent experiments. Microsoft Excel was used for statistical analysis. Student's *t*-test was performed to evaluate the significance of difference between samples. $P < 0.05$ was considered significant difference.

Results

RNF20 interacts with AP-2α

To search for the partners of AP-2α, we conducted IP with cell extracts from 3T3-L1 preadipocytes using anti-AP-2α antibody or IgG. The immunoprecipitated proteins were resolved on SDS–PAGE, followed by silver staining. The protein bands (**Supplementary Fig. S1**) which only existed in immunoprecipitated proteins of anti-AP-2α antibody, but not in those of IgG, were excised, digested with trypsin, and analyzed by MALDI-TOF mass spectrometry. After searching against the protein sequence database using the MASCOT database search engine, we identified RNF20 as one of the potential partners of

AP-2α (**Supplementary Table S1**). Then, we used anti-tag co-IP assay to further determine the interaction between RNF20 and AP-2α. We co-transfected HA-tagged RNF20 expression plasmid pHA-RNF20 and Myc-tagged AP-2α expression plasmid pMyc-AP-2α into HEK293 cells. At 24 h post-transfection, cells were harvested, and cell lysates were precipitated with either anti-HA polyclonal antibody or control IgG, and the precipitated complex was detected for the presence of Myc-AP-2α by western blot analysis using anti-Myc monoclonal antibody. As shown in **Fig. 1A**, Myc-AP-2α could be detected in immune complexes precipitated by anti-HA monoclonal antibody, but not by IgG, indicating that RNF20 can interact with AP-2α.

To further confirm the interaction between RNF20 protein and AP-2α protein, we examined whether these two proteins share the same subcellular location in the cell by immunofluorescence staining. The HA-tagged RNF20 expression plasmid pHA-RNF20 and EGFP-tagged AP-2α expression plasmid pEGFP-AP-2α were transfected into 3T3-L1 cells. As shown in **Fig. 1B**, both EGFP-AP-2α and HA-RNF20 fusion proteins were localized in the nucleus of 3T3-L1 cells, and the merged image showed the co-localization of EGFP-AP-2α and HA-RNF20 proteins.

RNF20 promotes AP-2α polyubiquitination

RNF20 has been proved to be an E3 ubiquitin ligase, which mediates H2B monoubiquitination [13] and ErbB3-binding protein 1 (Ebp1) polyubiquitination [14]. To investigate whether RNF20 also mediated AP-2α ubiquitination, we co-transfected pHis-Ub and pMyc-AP-2α into 3T3-L1 cells with or without pHA-RNF20. The His-ubiquitinated proteins were isolated from cell extracts using NTA agarose. Isolated proteins and input samples (whole-cell extracts) were analyzed by western blot analysis using anti-Myc monoclonal antibody. The ubiquitin conjugation was detected in the RNF20-containing samples, but few in the samples without RNF20 (**Fig. 1C**), which indicated that RNF20 promoted AP-2α polyubiquitination.

RNF20 induces the proteasome-dependent degradation of AP-2α

Ubiquitinated proteins are usually targeted for degradation in the proteasome. Therefore, we assessed whether E3 ubiquitin ligase RNF20 negatively regulated the level of AP-2α protein. The identified amount of pMyc-AP-2α plasmid was co-transfected with the increasing amounts of pHA-RNF20 plasmid, and the expression of Myc-AP-2α was detected by

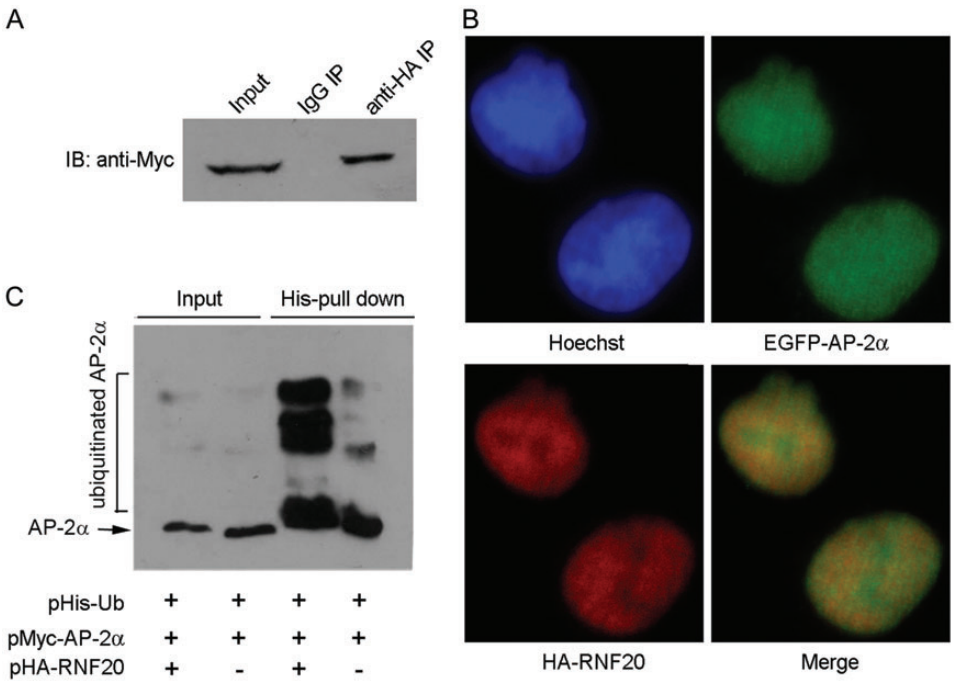

Figure 1. RNF20 interacts and co-localizes with AP-2α, and promotes its polyubiquitination (A) HEK293 cells were transfected with pMyc-AP-2α and pHA-RNF20 plasmids. At 24 h after transfection, cell extracts (800 μg) were prepared and IP with rabbit anti-HA polyclonal antibody or control pre-immune rabbit IgG. Immunoprecipitated Myc-AP-2α was detected by immunoblotting analysis with the anti-Myc monoclonal antibody. A total of 80 μg of cell extract was used as positive control (Input). (B) The pEGFP-Myc-AP-2α and pHA-RNF20 plasmids were co-transfected into 3T3-L1 cells. At 24 h post-transfection, the mouse anti-HA monoclonal antibody and Texas Red-conjugated anti-mouse IgG (red) were used to detect HA-RNF20 fusion protein, whereas EGFP fluorescence was examined directly by fluorescence microscopy, nuclei were stained by Hoechst 33258. The merged image showed the co-localization of HA-RNF20 and EGFP-AP-2α. (C) The pMyc-AP-2α and pHis-Ub were co-transfected with pHA-RNF20 or empty vector into 3T3-L1 cells. The His-ubiquitinated proteins were isolated from cell extracts using NTA agarose. Isolated proteins and input samples (whole-cell extracts) were analyzed by western blot analysis using anti-Myc monoclonal antibody.

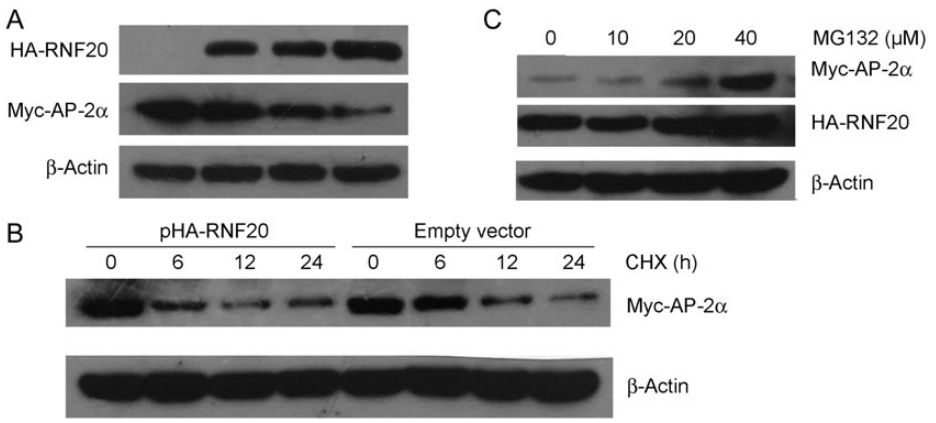

Figure 2. RNF20 promotes the proteasome-dependent degradation of AP-2α protein (A) HEK293 cells were cultured on 6 cm dishes and transfected with 1 μg of pMyc-AP-2α and increasing amount of pHA-RNF20 (0, 1, 2, 4 μg) and harvested at 24 h post-transfection. (B,C) HEK293 cells were cultured on 6 cm dishes and transfected with 2 μg of pMyc-AP-2α and 4 μg of pHA-RNF20. At 8 h post-transfection, transfected cells were incubated with either CHX (50 μg/ml) for the indicated time (B), or CHX plus different concentration of MG132 for 6 h (C). The amounts of Myc-AP-2α and HA-RNF20 fusion proteins were detected by western blot analysis using anti-Myc and anti-HA antibody, respectively.

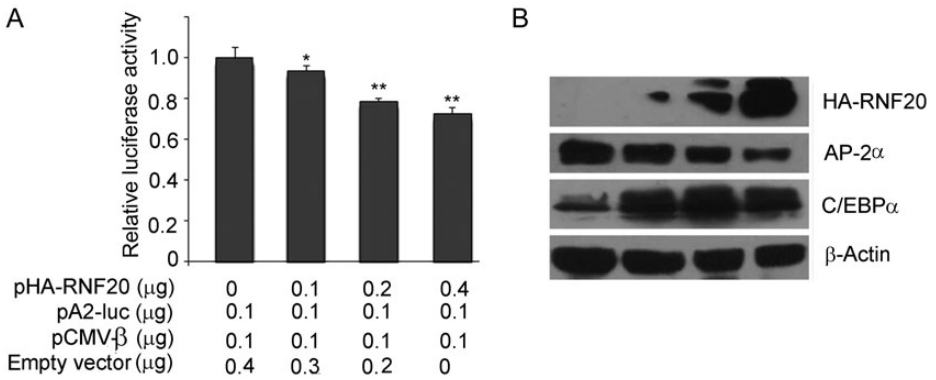

Figure 3. RNF20 inhibits the activity of AP-2α and rescues the expression of C/EBPα inhibited by AP-2α (A) 3T3-L1 cells were cultured on 24-well plates and transfected with pA2-luc and increased amount of pHA-RNF20 as indicated. Luciferase activities were measured at 36 h post-transfection. Data were represented as the percent activity relative to that observed in cells without pHA-RNF20. $*P < 0.05$; $**P < 0.01$. (B) 3T3-L1 cells were cultured on 6 cm dishes and transfected with increased amount of pHA-RNF20 (0, 1, 2, 4 μg). HA-RNF20 fusion protein and endogenous AP-2α, C/EBPα, and β-Actin proteins were detected by western blot analysis.

western blot analysis at 24 h post-transfection. As expected, with an increase of HA-RNF20 protein, the level of Myc-AP-2α protein decreased dramatically (**Fig. 2A**). To determine whether the reduced level of the AP-2α protein was resulted from protein degradation, we co-transfected pMyc-AP-2α into HEK293 cells with pHA-RNF20 or empty vector, and then treated cells with protein translation inhibitor cycloheximide (CHX) for the indicated time (0, 6, 12, 24 h). Myc-AP-2α fusion protein in cells transfected with pHA-RNF20 was much more rapidly degraded than that in cells with empty vector (**Fig. 2B**). However, treatment with the proteasomal inhibitor MG132 efficiently rescued the protein level of AP-2α (**Fig. 2C**). These results indicated that RNF20 could induce the proteasome-dependent degradation of AP-2α.

RNF20 inhibits the transactivity of AP-2α and rescues the C/EBPα expression inhibited by AP-2α

AP-2α functions as either transcription activators or transcription repressors, depending on the promoter and cellular context of their target genes. For example, AP-2α activates the transcription of *hMT-IIa* [15], but represses that of *C/EBPα* gene [3,4]. To assess the effect of RNF20 on the transactivity of AP-2α, we transfected pHA-RNF20 and the reporter plasmid pA2-Luc, which contained three copies of AP-2 binding sequence from hMT-IIa and pHA-RNF20 into 3T3-L1 cells. Luciferase assay demonstrated that transfection of RNF20 significantly decreased the luciferase activity of pAP2-luc in a dose-dependent manner (**Fig. 3A**), suggesting that RNF20 inhibits the transactivity of AP-2α.

The transcription of *C/EBPα* gene has been shown to be repressed by AP-2α in preadipocyte 3T3-L1 cells [3,4]. We transfected RNF20 into 3T3-L1 cells and found that, with the increase of HA-RNF20 expression, the expression of endogenous AP-2α was decreased and the expression of C/EBPα protein was increased (**Fig. 3B**), suggesting that RNF20 increased the expression of C/EBPα by stimulating the degradation of AP-2α.

Discussion

The RING domain is a type of zinc-finger domain that comprises 40–60 residues and is identified by the presence of a Cys-X2-Cys-X9-39-Cys-X1-3-His-X2-3-Cys-X2-Cys-X4-48-Cys-X2-Cys motif (where X can be any amino acid; histidines and cysteines are sometimes exchanged) [16]. A number of RING-containing proteins function as E3 ubiquitin ligases (E3). The RNF20, also known as Bre1, was first discovered as the major histone H2B-specific ubiquitin ligase that targets lysine 120 for monoubiquitination [17]. RNF20 selectively enhances or suppresses the expression of distinct subsets of genes via H2B ubiquitination [18–21]. A recent study has proved that RNF20 also mediates the polyubiquitination of the tumor suppressor Ebp1 as an E3 ligase [14]. In this study, we identified RNF20 as an AP-2α-interacting partner in preadipocyte 3T3-L1 cells, and the over-expression of RNF20 promoted the polyubiquitination and proteasome-dependent degradation of AP-2α, suggesting that RNF20 could also act as an E3 ligase for AP-2α. Moreover, we showed that, through stimulating the polyubiquitination and proteasome-dependent degradation of AP-2α, RNF20 increased the expression of C/EBPα, which is a key regulator of adipocyte differentiation. Our results indicated that RNF20 may also play roles in adipocyte differentiation.

Supplementary Data

Supplementary data are available at *ABBS* online.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (31071150), China Postdoctoral Science Foundation (2012M511380), Hunan Postdoctoral Science Foundation (2012RS4016), and E-Institutes of Shanghai Municipal Education Commission (E03003).

References

1. Eckert D, Buhl S, Weber S, Jager R and Schorle H. The AP-2 family of transcription factors. Genome Biol 2005, 6: 246.
2. Pellikainen JM and Kosma VM. Activator protein-2 in carcinogenesis with a special reference to breast cancer—a mini review. Inter J Cancer 2007, 120: 2061–2067.
3. Jiang MS, Tang QQ, McLenithan J, Geiman D, Shillinglaw W, Henzel WJ and Lane MD. Derepression of the C/EBPalpha gene during adipogenesis: identification of AP-2alpha as a repressor. Proc Natl Acad Sci USA 1998, 95: 3467–3471.
4. Holt EH and Lane MD. Downregulation of repressive CUP/AP-2 isoforms during adipocyte differentiation. Biochem Biophys Res Commun 2001, 288: 752–756.
5. Wu Z, Rosen ED, Brun R, Hauser S, Adelmant G, Troy AE and McKeon C, *et al*. Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. Mol Cell 1999, 3: 151–158.
6. Kerschgens J, Renaud S, Schutz F, Grasso L, Egener-Kuhn T, Delaloye JF and Lehr HA, *et al*. Protein-binding microarray analysis of tumor suppressor AP2alpha target gene specificity. PLoS ONE 2011, 6: e22895.
7. Garcia MA, Campillos M, Marina A, Valdivieso F and Vazquez J. Transcription factor AP-2 activity is modulated by protein kinase A-mediated phosphorylation. FEBS Lett 1999, 444: 27–31.
8. Ren K, Xian S, He F, Zhang W, Ding X, Wu Y and Yang L, *et al*. CK2 phosphorylates AP-2alpha and increases its transcriptional activity. BMB Rep 2011, 44: 490–495.
9. Ding X, Luo C, Zhou J, Zhong Y, Hu X, Zhou F and Ren K, *et al*. The interaction of KCTD1 with transcription factor AP-2alpha inhibits its transactivation. J Cell Biochem 2009, 106: 285–295.
10. Steffan JS, Agrawal N, Pallos J, Rockabrand E, Trotman LC, Slepko N and Illes K, *et al*. SUMO modification of Huntingtin and Huntington's disease pathology. Science 2004, 304: 100–104.
11. Zhou Q, Wang Y, Liu Q, Li L, Zhang Hg, Zhou J and Ding X, *et al*. Separation and identification of AP-2α interacting proteins from mice pre-adipocyte cells. Acta Laser Biol Sin 2011, 20: 199–202.
12. Tansey WP. Detection of ubiquitylated proteins in mammalian cells. Cold Spring Harb Protoc 2006, http://cshprotocols.cshlp.org/content/2006/6/pdb.prot4616.abstract.
13. Zhu B, Zheng Y, Pham AD, Mandal SS, Erdjument-Bromage H, Tempst P and Reinberg D. Monoubiquitination of human histone H2B: the factors involved and their roles in HOX gene regulation. Mol Cell 2005, 20: 601–611.
14. Liu Z, Oh SM, Okada M, Liu X, Cheng D, Peng J and Brat DJ, *et al*. Human BRE1 is an E3 ubiquitin ligase for Ebp1 tumor suppressor. Mol Biol Cell 2009, 20: 757–768.
15. Imagawa M, Chiu R and Karin M. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell 1987, 51: 251–260.
16. Budhidarmo R, Nakatani Y and Day CL. RINGs hold the key to ubiquitin transfer. Trends Biochem Sci 2012, 37: 58–65.
17. Hwang WW, Venkatasubrahmanyam S, Ianculescu AG, Tong A, Boone C and Madhani HD. A conserved RING finger protein required for histone H2B monoubiquitination and cell size control. Mol Cell 2003, 11: 261–266.
18. Shema E, Tirosh I, Aylon Y, Huang J, Ye C, Moskovits N and Raver-Shapira N, *et al*. The histone H2B-specific ubiquitin ligase RNF20/hBRE1 acts as a putative tumor suppressor through selective regulation of gene expression. Genes Dev 2008, 22: 2664–2676.
19. Arora M, Zhang J, Heine GF, Ozer G, Liu HW, Huang K and Parvin JD. Promoters active in interphase are bookmarked during mitosis by ubiquitination. Nucleic Acids Res 2012, 40: 10187–10202.
20. Shema E, Kim J, Roeder RG and Oren M. RNF20 inhibits TFIIS-facilitated transcriptional elongation to suppress pro-oncogenic gene expression. Mol Cell 2011, 42: 477–488.
21. Wang E, Kawaoka S, Yu M, Shi J, Ni T, Yang W and Zhu J, *et al*. Histone H2B ubiquitin ligase RNF20 is required for MLL-rearranged leukemia. Proc Natl Acad Sci USA 2013, 110: 3901–3906.