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CHIP functions an E3 ubiquitin ligase of Runx1

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ABSTRACT

Runx1 is a key factor in the generation and maintenance of hematopoietic stem cells. Improper expression and mutations in Runx1 are frequently implicated in human leukemia. Here, we report that CHIP, the carboxyl terminus of Hsc70-interacting protein, also named Stub1, physically interacts with Runx1 through the TPR and Charged domains in the nucleus. Over-expression of CHIP directly induced Runx1 ubiquitination and degradation through the ubiquitin–proteasome pathway. Interestingly, we found that CHIP-mediated degradation of Runx1 is independent of the molecular chaperone Hsp70/90. Taken together, we propose that CHIP serves as an E3 ubiquitin ligase that regulates Runx1 protein stability via an ubiquitination and degradation mechanism that is independent of Hsp70/90.

Introduction

Runx1 (also known as AML1, CBFα2, or PEBP2αB) is a member of the Runt-related transcription factors [1], which encodes the DNA-binding α-chain partner of the heterodimeric CBF (Core binding factor) complex. Runx1 plays a crucial role in hematopoietic development, angiogenesis, muscle sustainment, and neurogenesis [2–4]. Genetic ablation of Runx1 results in embryonic lethality and a complete lack of fetal liver hematopoiesis [5]. Runx1 is found to be mutated, by chromosomal translocations or point mutation, in human leukemia [6]. In addition, elevated expression of Runx1 has also been reported in several human leukemias [7].

The level of Runx1 is tightly regulated at both the transcriptional and post-translational level [8–12]. In particular, Runx1 activity is regulated through an ubiquitin–proteasome-mediated protein degradation mechanism [12,13]. To date, the Cdh1–APC complex is reported to mediate the degradation of phosphorylated or unphosphorylated Runx1, and the Cdc20–APC and Skp2–SCF complexes are found to promote the degradation of only phosphorylated Runx1 [12], however, the E3 ligase for Runx1 remains to be identified.

CHIP/Stub1 was originally identified as a co-chaperone protein and a U-box containing E3 ligase [14]. CHIP has been reported to play a critical role in quality control for misfolded or unfolded proteins and also in the regulation of protein stability by promoting their ubiquitination and degradation (see review [15]). Depletion of CHIP in the mouse demonstrated shortened lifespan and a higher sensitivity to stress [16]. We previously reported that CHIP regulates BMP and TGF-β signals by enhancing Smad protein ubiquitination and degradation [17,18]. Interestingly we recently found that CHIP promotes Runx2 ubiquitination and degradation, thereby negatively regulating osteoblast differentiation [19]. Here, we demonstrate that CHIP also interacts with Runx1 and regulates its degradation and ubiquitination by its E3 ligase activity. Our data provide new insights into the regulation of Runx1 stability.

Materials and methods

Antibodies and reagents. Anti-HA(A-7), anti-Myc(9E10), and anti-GFP(FL) antibodies were purchased from Santa Cruz. Anti-β-Actin(AC-15) antibody and recombinant Heat Shock Protein Hsp90 from bovine brain (H6774) were purchased from Sigma. MG132 was purchased from Calbiochem.

Plasmids. Plasmids of pEF-HA-Runx1 was kindly supplied by Drs. Yoshiaki Ito and Huang Gang. His-tagged E1 and UbcH5a were kindly provided by Drs. Dieter A Wolf and Kazuhiro Iwai, respectively. Myc-CHIP, Myc-CHIP(K30A), Myc-CHIP(H260Q), Myc-His-Ub, GST-CHIP, His-CHIP, His-Smurf1, His-Ubi, and His-Hsp70 were constructed in our lab [18,19]. The CHIP deletions were constructed by PCR amplification and subcloned into pGEX-5x-3 and pACT2, respectively.

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Cell culture and transfection. HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% FBS (GIBCO), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C in the presence of 5% CO_2. Cells were transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

Co-immunoprecipitation, protein degradation and Western blot experiments. HEK293T cells were transfected with the indicated expression plasmids and were harvested in lysis buffer (50 mM Tris–Cl, pH7.6, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 10% glycerol, 1 mM DTT, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) on ice for 30 min. Whole-cell lysates were incubated with 2 μg of the indicated antibodies and 30 μl of protein G-Sepharose beads (Santa Cruz) for 8 h at 4 °C. Precipitated proteins were eluted with 2× SDS–PAGE loading buffer and analyzed by Western blot. For the degradation assay, cells were lysed in 2× SDS–PAGE loading buffer. Separation of nuclear and cytoplasmic proteins was as previously described [19].

Pull-down assay. HEK293T cells transfected with the indicated expression plasmids were lysed in cell lysis buffer (described above). Lysates were then incubated with GST-CHIP or CHIP-deletions bound to GST-beads (about 10 μg of protein) for 8 h at 4 °C. The bound proteins were analyzed by Western blot.

In vivo ubiquitination assay. HEK293T cells were transfected with the indicated expression plasmids and harvested into buffer A (6 M guanidine–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole, pH8.0) and then sonicated. Whole-cell lysates were incubated with 50 μl of equilibrated (50%) Ni–NTA–agarose for 3 h at room temperature. Beads were washed with buffer A 2 times, followed by another two washes with buffer A/buffer T1 (1:3) and then one wash with buffer T1 (25 mM Tris–Cl, 20 mM imidazole, pH6.8). Precipitated proteins were eluted with 2× SDS–PAGE loading buffer (containing 250 mM imidazole) and subjected to SDS–PAGE followed by Western blot.

In vitro ubiquitination assay. The ubiquitination assay was performed as described [18]. In brief, the reaction mixture (20 μl) containing 5 mM HA-Runx1, 0.1 μM E1, 2.5 μM UbcH5a, 5 μM CHIP or 1 μM Smurf1, 2 μg/μl of His-Ubiquitin, 1 μg Hsp70 protein and 1 μg Hsp90 protein and 2 μl of 10 × ATP regenerating system (10 mM ATP, 100 mM creatine phosphate (Fluka), 40 mM magnesium acetate, 100 unit/ml creatine kinase (Sigma)) in 50 mM Tris–HCl (pH7.3), 100 mM NaCl, 2 mM dithiothreitol was incubated for 2 h at 30 °C.

Results

CHIP associates with Runx1

In a previous study, we observed that CHIP regulates Runx2 stability through the ubiquitin–proteasome pathway [19]. As Runx2 shares a highly conserved amino acid sequence with Runx1 [20], we investigated whether CHIP was also able to interact with Runx1. The physical interaction between CHIP and Runx1 was examined by co-immunoprecipitation experiments in HEK293T cells cotransfected with Myc-tagged CHIP, and HA-tagged Runx1 expression vectors. The results show that CHIP was detected in a complex immunoprecipitated with anti-HA antibody, and that Runx1 was detected with an anti-Myc antibody precipitated complexes when both HA-Runx1 and Myc-CHIP were co-expressed (Fig. 1A). This result indicates that CHIP associates with Runx1 in mammalian cells.

Runx1 is located in the nucleus [21] and CHIP is reported to be distributed in both the cytoplasm [14] and nucleus [22]. To determine whether the interaction of Runx1 and CHIP occurs in the nucleus, we separated nuclear and cytoplasmic proteins to perform immunoprecipitation experiments. The majority of HA-Runx1 is present in the nucleus and Myc-CHIP is present in both the cytoplasm and the nucleus (data not shown), which is consistent with our previous report [19]. HA-Runx1 was precipitated in Myc-CHIP protein complexes only in nuclear extracts (Fig. 1B) and we did not observe any association of CHIP with

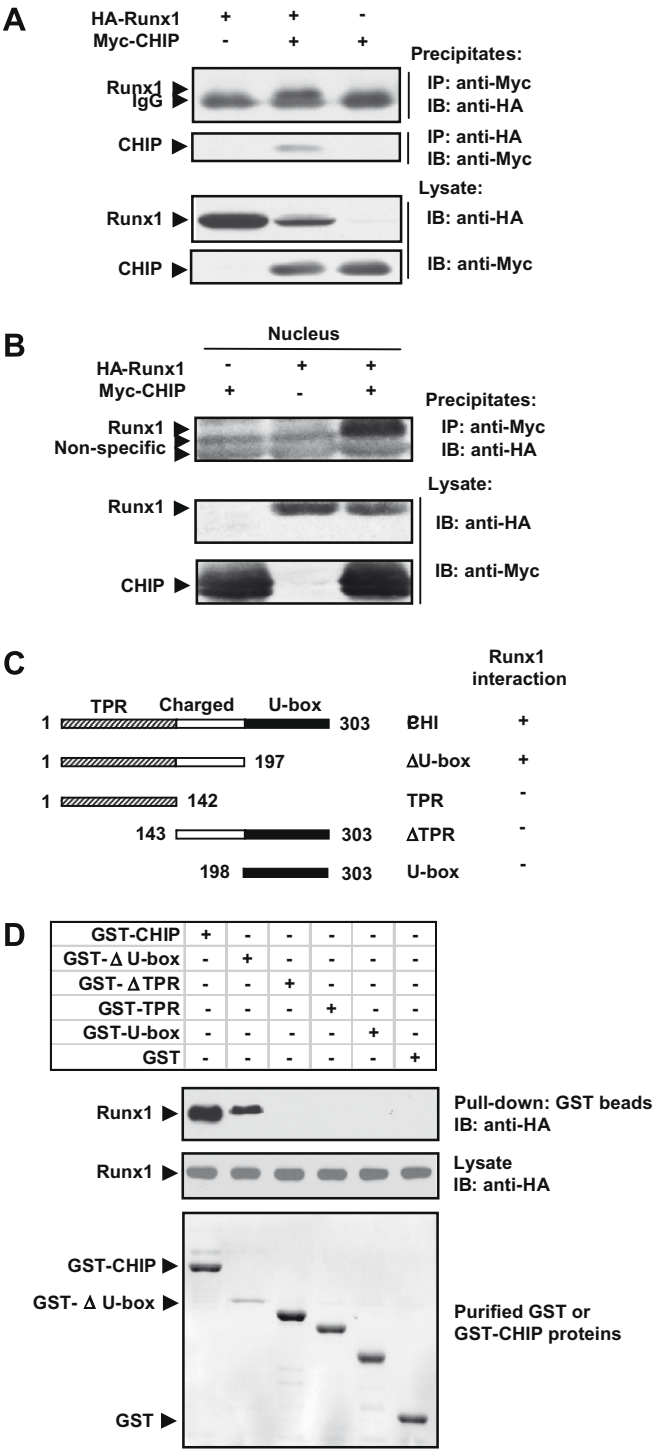

Fig. 1. CHIP associates with Runx1. (A) CHIP interacts with Runx1 *in vivo*. (B) Interaction of CHIP with Runx1 occurs in the nucleus of mammalian cells. Nuclear extracts were used in co-immunoprecipitation assays. (C) A schematic diagram shows the protein structures of CHIP and its truncated mutants. (D) TPR and Charge domains are both required for the interaction of CHIP and Runx1.

Runx1 in cytoplasmic fractions (data not shown). These results suggest that CHIP is capable of interacting with Runx1 in the nucleus of mammalian cells.

The TPR and Charged domains are required for the CHIP-Runx1 interaction

CHIP contains a U-box domain for the E3 ubiquitin ligase activity, a TPR domain responsible for chaperone binding and a Charged domain rich in charged residues [14]. To elucidate which domain of CHIP binds to Runx1, a series of GST- and Myc- tagged CHIP deletion mutants were generated (Fig. 1C). An *in vitro* binding assay (Fig. 1D) and a co-immunoprecipitation assay in transfected HEK293T cells (data not shown) revealed that the TPR domain, U-box domain and Charged domain plus U-box domain failed to interact with Runx1 but that the TPR domain plus Charged domain retained the ability to associate with Runx1. These data imply that the TPR together with the Charged domain of CHIP are indispensable in mediating the interaction of CHIP with Runx1.

CHIP directly induces Runx1 ubiquitination for degradation

The interaction of CHIP with Runx1 suggests that CHIP may mediate Runx1 degradation as CHIP has been reported to be an E3 ubiquitin ligase mediating the degradation of several substrates (see review [15]) including Runx2 [19]. To examine this hypothesis, HA-Runx1 was co-expressed with increasing amounts of HA-CHIP protein in 293T cells. Immunoblotting results demonstrate that the levels of HA-Runx1decreased with the increasing Myc-CHIP protein levels (Fig. 2A, lanes 1–4). The level of HA-Runx1 was restored by the addition of MG132, a proteasome inhibitor, when Myc-CHIP was expressed at the highest dosage (Fig. 2A, lanes 5–6). In these experiments, we observed that the protein levels of co-expressed GFP remained unchanged with increasing levels of CHIP (Fig. 2A). These results suggest that CHIP specifically mediates Runx1 degradation though the ubiquitin–proteasome pathway.

We further examined the turn-over rate of Runx1 in the presence of over-expressed CHIP protein with the addition of cycloheximide. The data showed that Runx1 is destabilized by coexpression of CHIP (Fig. 2B, upper panel). A quantitative analysis demonstrated that the protein turn-over rates of Runx1 is dramatically increased when CHIP is over-expressed (Fig. 2B, bottom panel). These data suggest that CHIP negatively regulates the stability of Runx1.

To obtain direct evidence that CHIP has an E3 ubiquitin ligase activity toward Runx1, we performed an *in vitro* ubiquitination assay with the purified recombinant proteins. The presence of ubiquitinated Runx1 was detected as high molecular mass smear by Western blotting with an anti-HA antibody. The data showed that when E1, E2 (UbcH5a) and CHIP were added to the reaction system containing purified HA-Runx1, a strong smear was observed (Fig. 2C, lane 2), while no smear could be seen when CHIP was removed from the E1 and E2 reaction mixture (Fig. 2C, lane 1), indicating that CHIP functioned as an E3 to mediate the ubiquitination of Runx1 in conjunction with the E1 and E2 enzymes.

Since Smurf1 has been reported to function similarly to CHIP to enhance Runx2 degradation [19,23], we addressed whether CHIP and Smurf1 have a similar effect on the ubiquitination of Runx1. The result showed that Smurf1 has little ability to induce Runx1 ubiquitination, compared to CHIP (Fig. 2C, compare lane 4 with lane 2). These results suggest that CHIP plays a role as a specific E3 ligase in Runx1 ubiquitination.

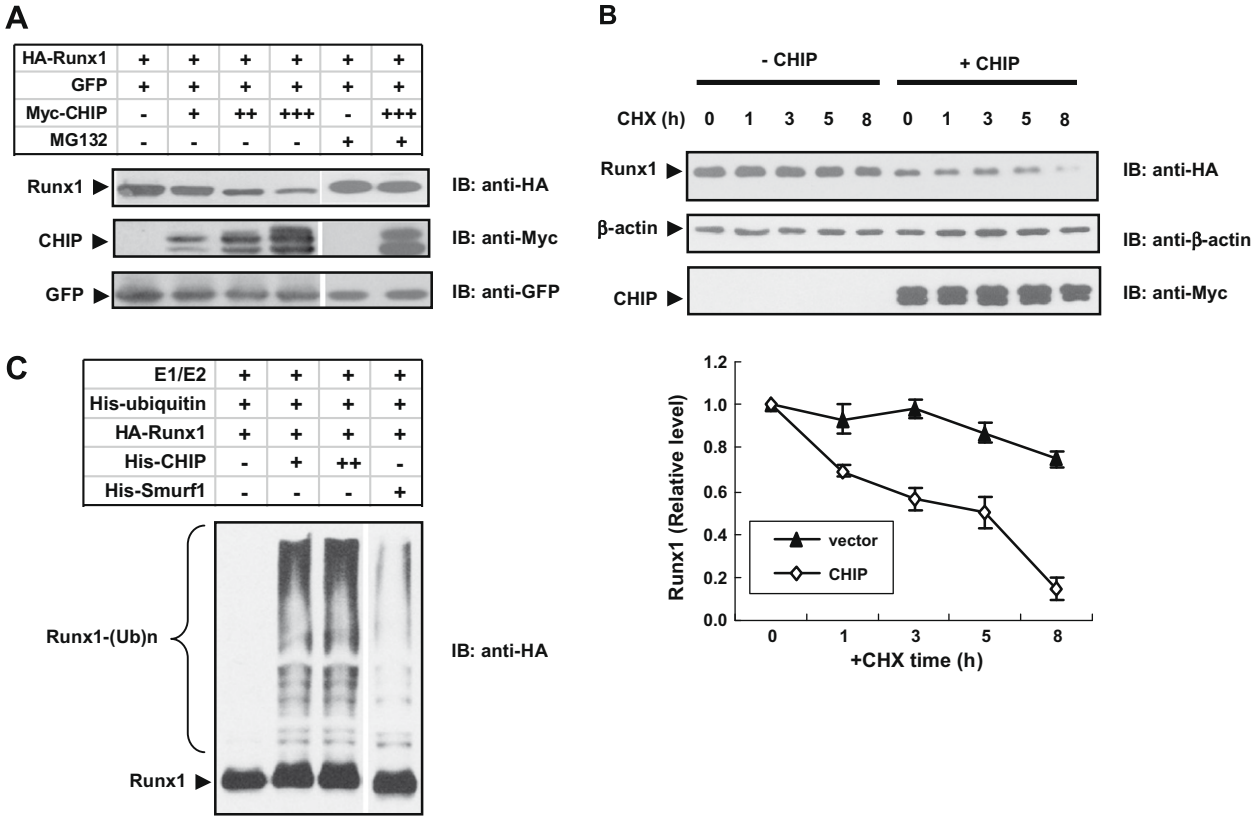

Fig. 2. CHIP directly induces Runx1 degradation and ubiquitination. (A) CHIP mediates the degradation of Runx1 in a dose dependent manner. (B) CHIP accelerates the turnover of Runx1 protein. The Runx1 protein levels at different time points after treatment with cycloheximide (CHX) (5 μM) were measured. Quantitative presentation of the results with standard errors (three repetitions) is shown in the bottom panel. (C) CHIP directly induces Runx1 ubiquitination *in vitro*.

In addition, if CHIP directly mediates Runx1 ubiquitination, then disruption of its U-box domain should abolish its activity toward Runx1. To test this hypothesis, we expressed CHIP(H260Q), a point mutant of CHIP in the U-box domain which fails to bind its cognate E2. The results showed that CHIP(H260Q) caused a marked decrease in Runx1 ubiquitination compared to wild-type CHIP in an *in vivo* ubiquitination assay (Fig. 3C, compare lane 3 with lane 1), suggesting that the E3 ligase activity of CHIP is necessary for regulating Runx1 ubiquitination. These results confirmed that CHIP functions as a direct E3 ubiquitin ligase on Runx1.

CHIP mediates ubiquitination and degradation independent of molecular chaperons Hsp70/90

Many studies have revealed that CHIP ubiquitinates substrates and promotes their degradation in a chaperone-dependent manner [24]. Previously we demonstrated that CHIP-mediated Runx2 ubiquitination and degradation is also dependent of chaperon activity as a mutation of the TPR domain (K30A), which causes a loss of the ability of CHIP to interact with Hsp70/90, impaired the ability of CHIP to mediate the ubiquitination of Runx2 [19]. Accordingly, we examined whether CHIP also teams with molecular chaperones to regulate Runx1 ubiquitination and degradation. We co-expressed CHIP(K30A) with HA-Runx1 to examine the interaction of CHIP(K30A) with Runx1. To our surprise, CHIP(K30A) was found to interact with Runx1 as strongly as wild-type or CHIP(H260Q) did. Interestingly, we observed that CHIP(K30A) has an effect similar to wild-type CHIP on the degradation of Runx1 (Fig. 3B, lane 3), while, as expected, CHIP(H260Q) has less of an effect on Runx1 degradation (Fig. 3B, lane 4). In an *in vivo* ubiquitination experiment, we observed that CHIP(K30A) retains the ability (slightly enhances) to ubiquitinate Runx1 while CHIP(H260Q) activity is almost abolished (Fig. 3C). To determine whether chaperones play a role in Runx1 ubiquitination *in vitro*, we examined Runx1 ubiquitination with the addition of Hsp70/90 protein in an *in vitro* ubiquitination experiment. The results showed that there was no difference in the levels of ubiquitinated Runx1 with or without the addition of Hsp70/90 (Fig. 3D). These results indicate that the down-regulation of Runx1 protein stability by CHIP does not require the Hsp70/90 chaperones.

Discussion

Runx1 has been reported to be associated with various forms of human leukemia [6]. Runx1 activity is regulated at various levels, including transcription [8], phosphorylation [9], acetylation [10], and ubiquitin-mediated degradation [11,12]. Despite the importance of degradation, however to date, no single E3 ligase has been reported to mediate the ubiquitination and degradation of Runx1. In this study, we show that CHIP interacts with Runx1 and mediates the degradation of Runx1 independent of chaperons Hsp70/90; therefore, we identify CHIP as a potential E3 ligase for Runx1.

Runx1 is subject to a rapid degradation process through the ubiquitin–proteasome system so it is often difficult to detect it as an in-

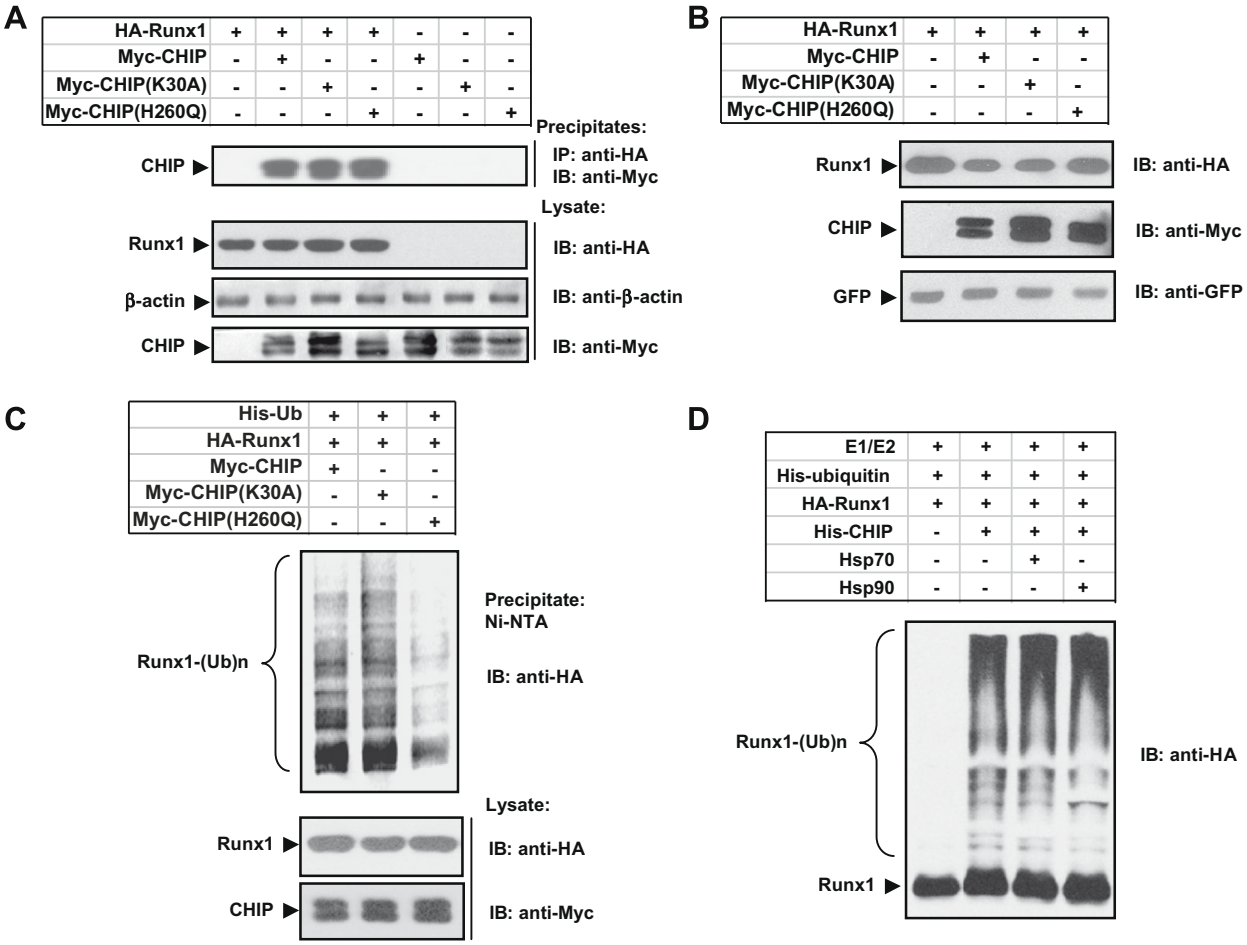

Fig. 3. CHIP mediates Runx1 degradation and ubiquitination independent of chaperons Hsp70/90. (A) CHIP(K30A), a mutant of CHIP at TPR domain, interacts with Runx1. (B) CHIP(K30A) mediates degradation of Runx1. (C) CHIP(K30A) retains the ability to mediate Runx1 ubiquitination. (D) Hsp70/90 protein has no effect on the level of ubiquitinated Runx1 induced by CHIP.

tact protein within cells [1]. Imai et al. reported that the release of mSin3A, which functions as an inhibitor, promotes the proteasome-mediated degradation of Runx1 in an ERK-dependent phosphorylation manner [11]. Cdh1–APC, Cdc20–APC and Skp2–SCF complexes have been reported to degrade Runx1 [12], however, since both Cdc20 and Cdh1 proteins serve as substrate-targeting subunits for APC, the interaction of Cdc20 and Cdh1 with Runx1 appears to mediate the formation of the APC complex to recruit Runx1. Therefore, Cdc20 or Cdh1-mediated Runx1 ubiquitination is most likely an indirect event. In our study, we found that CHIP, an E3 ligase, directly interacts with Runx1 to mediate its ubiquitination and subsequently degradation (Figs. 1 and 2). Our study provides a new mechanism by which Runx1 protein is degraded directly through CHIP via the ubiquitin–proteasome pathway.

CHIP has been reported to function as an E3 ligase that is dependent on Hsp70/90. Many reports have demonstrated that CHIP recognizes substrates with the assistance of chaperones [24]. If CHIP fails to interact with a chaperone, it loses its ability to mediate the degradation of substrates (e.g., its function on GR and ErbB2). To our surprise, we observed that the CHIP(K30A) mutant, which has lost the ability to interact with Hsp70/90, retains an ability to mediate Runx1 ubiquitination and degradation (Fig. 3). These results are in consistent with that fact that the presence of Hsp70/90 did not affect CHIP-mediated Runx1 ubiquitination (Fig. 3D). These results suggest that the role of CHIP in Runx1 degradation is quite different from that of its other substrates including the highly conserved Runx2 [19]. Recently, Jason et al. reported that CHIP-mediated degradation of BER, an enzyme in the base excision repair proteins involved in the regulation of DNA damage-dependent stabilization [25], occurs in a manner similar to that described in this report. Our findings expand the role of CHIP in the chaperone-independent mechanism of protein degradation.

Runx1 mutation and abnormal expression is related to the human leukemia diseases [6]. Recently, CHIP has been reported to play a critical role in the metastasis of breast cancer [26]. Our unpublished data also indicate that the overexpression of CHIP can inhibit tumor growth in a nude mice model (data not shown), therefore, it would be of particular interest to investigate whether CHIP plays a role in the human leukemia.

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