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CHIP mediates down-regulation of nucleobindin-1 in preosteoblast cell line models

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ABSTRACT

Nucleobindin-1 (NUCB1), also known as Calnuc, is a highly conserved, multifunctional protein widely expressed in tissues and cells. It contains two EF-hand motifs which have been shown to play a crucial role in binding Ca^{2+} ions. In this study, we applied comparative two-dimensional gel electrophoresis to characterize differentially expressed proteins in HA-CHIP over-expressed and endogenous CHIP depleted MC3T3-E1 stable cell lines, identifying NUCB1 as a novel CHIP/Stub1 targeted protein. NUCB1 interacts with and is down-regulated by CHIP by both proteasomal dependent and independent pathways, suggesting that CHIP-mediated down-regulation of nucleobindin-1 might play a role in osteoblast differentiation. The chaperone protein Hsp70 was found to be important for CHIP and NUCB1 interaction as well as CHIP-mediated NUCB1 down-regulation. Our findings provide new insights into understanding the stability regulation of NUCB1.

1. Introduction

CHIP (carboxyl terminus of Hsc70-interacting protein), also as known as Stub1 (STIP1 homology and U-box containing protein 1), was first characterized as a tetratricopeptide (TPR)-containing and Hsc/Hsp70-interacting protein in the human heart [1]. CHIP possesses intrinsic E3 ubiquitin ligase activity which is dependent on the highly conserved *C*-terminal U-box motif [2]. To date, multiple proteins involved in various biological processes have been identified as substrates of CHIP, including p53, Smad proteins, ER-α, eIF5A, Src-3, p65 and TLR4 [3–8]. CHIP plays important roles in bone development. It was reported to regulate TGF-β/BMP signaling by enhancing ubiquitination and degradation of Smad proteins [4,9–11]. CHIP was also shown to promote Runx2 ubiquitination and degradation, thereby negatively regulating osteoblast differentiation [12]. These findings were confirmed by Guo et al., in a study using miR-764-5p to repress the translation of CHIP, resulting in promotion of osteoblast differentiation in progenitor cells [13]. Under inflammatory conditions, CHIP was found to be up-regulated by TNF-α, and then the osteogenic activity of osteoblasts were suppressed via CHIP mediated enhanced ubiquitination and degradation of osterix [14]. Since bone formation is a complicated process involving multiple molecules, we hypothesize that more targets of CHIP remain to be identified.

Similar to other E3 ubiquitin ligases, CHIP binds to its substrate, and mediates its ubiquitination and degradation. E3 ligase substrate screening strategies are, therefore, based on these particular steps and characteristics. Yeast two hybrid screens, co-immunoprecipitation followed by mass spectrometry, and bioinformatic approaches are utilized to identify new proteins interacting with E3 ligases of interest [2,15], the majority of which are found to be substrates of E3. In vitro ubiquitination screens using a protein microarray platform can be applied to identify proteins ubiquitinated by E3 ubiquitin ligases [16,17]. A combination of stable isotope labeling by amino acids in cell culture, affinity purification and mass spectrometry was used by several groups for enrichment and identification of ubiquitinated proteins [18–20]. Comparative two-dimensional [21–24] or high-throughput quantitative fluorescence microscopy [25] were applied to identify differentially expressed putative targets of E3.

In our study, we applied comparative two-dimensional gel electrophoresis to characterize differentially expressed proteins in HA-CHIP over-expressed and endogenous CHIP depleted MC3T3-E1 stable cell lines. Three candidate proteins were identified by mass spectrometry.

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One of the proteins, nucleobindin-1 (NUCB1), interacted with CHIP. Protein levels of NUCB1 and CHIP were negatively correlated during osteoblast differentiation.

Our results confirm that this approach can be utilized to discover new candidate substrates. Furthermore, our findings suggest that CHIP-mediated down-regulation of NUCB1 might play a role in osteoblast differentiation.

2. Materials and methods

2.1. Cell lines and culture conditions

The preosteoblast cell line MC3T3-E1 and 293T cells were cultured in minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS). Stable cell lines were maintained in MEM containing 400 μg/mL G418. All cells were grown in a humidified incubator at 37 °C and 5% CO_2.

2.2. Constructs and transfections

Human influenza hemaglutinin (HA) tagged wild type and mutated CHIP constructs, HA-CHIP, HA-CHIP (K30A), HA-CHIP (H260Q), and Hsp70 constructs used for over-expression in 293T cells, and vector-based siRNA targeting Hsp70 or EGFP were kindly supplied by Dr. Zhijie Chang. The HA-CHIP (K30R) expression construct was generated by introducing a GCG (A) to AGG (R) substitution into the CHIP (K30A) gene sequence using an overlap extension PCR strategy. The NUCB1-FLAG expression construct was generated by inserting the ORF from a plasmid kindly supplied by Dr. Marc Pouliot with an added C-terminal FLAG tag into pEF/Bos/Neo at *Eco*RI/*Not*I sites.

293T cells were grown to 50% confluence in 6-well plates and transfected with 2.5 μg of plasmid DNA using jetPRIME DNA&RNA transfection reagent (Polyplus) according to the manufacturer's instructions. Cells were harvested 48 h post-transfection unless otherwise noted.

2.3. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was carried out as previously described [26]. Briefly, 10 million cells were resuspended in 500 μL lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 65 mM DTT) containing protease inhibitors (Roche) and Nuclease Mix (GE), and incubated on ice for 30 min, after which the lysates were centrifuged for 30 min at 12,000 rpm at 4 °C to remove debris. The protein concentration of the supernatant was determined using a 2-D Quant kit (GE), and 1 mg protein for each sample was used to rehydrate a 24-cm immobilized pH gradient strip (pH 4–7) and then focused for separation in the first dimension. Separation in the second dimension was performed by 12.5% SDS-PAGE. For each independent experiment, two-dimensional gel electrophoresis for protein extracts from the mock MC3T3-E1 cell lines, HA-CHIP over-expressing MC3T3-E1 (OE) cell lines and MC3T3E1 stable cell lines with endogenous CHIP knock-down (KD) were processed in parallel for direct comparison. Each two-dimensional gel electrophoresis run was repeated in triplicate. Gels were stained with Coomassie Brilliant Blue G-250 (Amresco) and scanned using ImageScanner (Amersham Biosciences). Image analysis was carried out using ImageMaster 2D Platinum software (GE). Protein spots were manually excised from the gel, digested and subjected to Q-TOF analysis.

2.4. Western blotting assay

Cells were lysed in 2× SDS-PAGE loading buffer, resolved by SDS-PAGE and electrotransferred onto PVDF membranes (Millipore). The following primary antibodies were used in this study: anti-NUCB1 (Sigma, SAB4200040), anti-cytochrome *b*5 (Santa Cruz, sc-33,174), anti-Filamin A (Bethyl, A301-135A), anti-HA tag (Santa Cruz, sc-7392), anti-β-Actin (Santa Cruz, sc-1616R), anti-FLAG tag (Sigma, F1804, F7425), anti-Hsp70 (Santa Cruz, sc-24), anti-COX1 (Santa Cruz, sc-17540), anti-caspase 3 (Santa Cruz, sc-7272), anti-caspase 8 (Cell Signaling, #9746), and anti-endogenous CHIP (gift from Dr. Zhijie Chang). Horseradish peroxidase-conjugated antibodies to rabbit (GE, NA934), mouse (GE, NA931) or goat (Santa Cruz, sc-2020) were used as the secondary antibodies and SuperSignal West Dura Chemiluminescent Substrate (Pierce) was used for ECL detection. Signal acquisition was accomplished with a digital imaging system (CLiNX Science Instruments). All band densities were analyzed using ImageJ software.

2.5. Quantitative PCR

Total RNA was extracted, and reverse transcribed as previously described [27]. SYBR Premix DimerEraser (Takara, DRR091A) was used with a Lightcycler 2.0 Real-time PCR System (Roche) with the following parameters: 95 °C (10 min), followed by 40 cycles of 95 °C (15 s), 55 °C (15 s) and 72 °C (15 s). Primer sequences were as follows: *Gapdh*, forward 5′-tcgtcccgtagacaaaatgg-3′, reverse 5′- ttgaggtcaatgaaggggtc-3′; *Nucb1*, forward 5′-actgatgctgtctgctgtgc-3′, reverse 5′-cacgttgatgacctcctgg-3′; *cytochrome b*5, forward 5′-ccgacgtcctcaaaattctc-3′, reverse 5′- ctgggtga tcctgcatcata-3′; *filamin alpha*, forward 5′-aggtcacaggtcctcgtctt-3′, reverse 5′-gcctttggctactaccttgc-3′.

2.6. Co-immunoprecipitation

Two million cells were harvested and lysed on ice for 30 min in 600 μL whole cell lysis buffer (20 mM Tris/HCl [pH 8.0], 120 mM NaCl, 0.5% NP-40) supplemented with protease inhibitor cocktail (Roche) and PMSF (Sigma). Following centrifugation at 13,000 × *g* for 10 min at 4 °C, the supernatant was collected as whole cell lysate. Subsequently, 20 μL of anti-FLAG M2 agarose beads (Sigma) was added and incubated for 2 h at 4 °C. Precipitated proteins were eluted with 30 μL of 2× SDS-PAGE loading buffer and analyzed by Western blot.

2.7. In vivo ubiquitination assay

The in vivo ubiquitination assay was performed as described [27].

2.8. Induction of MC3T3-E1 preosteoblast differentiation

Forty thousand MC3T3-E1 preosteoblast cells were seeded into 100 mm dishes and cultured with alpha-MEM (Minimum Essential Media) supplemented with 10% FBS (fetal bovine serum), 50 μg/mL L-ascorbic acid (Sigma), and 10 mM β-glycerophosphate (Sigma) for up to 21 days. Media were changed every three days.

2.9. Statistical analysis

One-way ANOVA and post hoc multiple comparisons were performed using SPSS10. Statistical significance was assessed at $P < 0.05$.

3. Results

3.1. Comparative proteomics analysis reveals news targets for CHIP

To identify novel substrates for CHIP, the wild type MC3T3-E1 cell line, MC3T3-E1 cell lines stably over-expressing HA-CHIP by transfection of a HA-CHIP expression plasmid and selected by G418 (MC3T3-E1 OE, clone 2# and clone 8#), and MC3T3-E1 cell lines with depletion of endogenous CHIP by stable transfection of a short hairpin RNA (shRNA) targeting endogenous CHIP mRNA (MC3T3-E1 KD, clone 9# and clone 20#) were subjected to comparative proteomics analysis. For each independent experiment, two-dimensional gel electrophoresis of protein extracts from the wild type MC3T3-E1 cell line, one MC3T3-E1