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Elucidation of CPX-1 involvement in RANKL-induced osteoclastogenesis by a proteomics approach

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Abstract To identify proteins potentially involved in osteoclast differentiation, we conducted a proteomics-based analysis using the osteoclastogenesis model cell line RAW264.7. Total proteins from undifferentiated cells, committed pre-osteoclasts, and differentiated osteoclasts were resolved by two-dimensional gel electrophoresis. Protein spots showing differential expression levels were processed for peptide mass fingerprinting. Among them, we identified the metallocarboxypeptidase CPX-1, which was prominently increased in pre-osteoclasts and then decreased in mature osteoclasts. Results of reverse transcription polymerase chain reaction, Western blot, and confocal microscopy were in agreement with the proteomics data. Notably, the forced overexpression of CPX-1 led to the inhibition of osteoclast formation, but not pre-osteoclast generation. Therefore, the transient up-regulation pattern of CPX-1 expression may be important for the successful progression from pre-osteoclasts to mature osteoclasts.
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Key words: Osteoclast; RANKL; CPX-1; Proteomics

1. Introduction

Bone homeostasis is maintained through bone remodeling, which results from the coordinated action of bone resorption by osteoclasts and new bone formation by osteoblasts. Osteoclasts differentiate from hematopoietic myeloid precursors of the monocyte/macrophage lineage, and play pivotal roles in bone morphogenesis, remodeling, and resorption [1,2]. It is now widely accepted that the interaction between receptor activator of NF-κB ligand (RANKL), expressed by stromal cells/osteoblasts, and its receptor RANK, expressed on osteoclast precursors, is essential for osteoclastogenesis [3]. RANK, a member of tumor necrosis factor (TNF) family proteins, transduces biochemical signals by recruiting TNF receptor-associated factor proteins causing the activation of NF-κB and mitogen-activated protein kinases (MAPK) [4–8]. However, only a few target proteins of RANKL stimulation have been elucidated.

Members of the carboxypeptidase gene family have been implicated in diverse functions including neuroendocrine peptide processing as well as food digestion [9]. CPX-1 was identified as a novel member of a distinct subgroup of the metallocarboxypeptidase gene family [10]. Despite homology with carboxypeptidases, CPX-1 lacks several residues important for the catalytic activity and did not show enzyme activity when expressed in either the baculovirus system or a mouse cell line [10]. In addition, CPX-1 contains an N-terminal region of 160 amino acids with sequence similarity to the discoidin domain as do two other inactive metallocarboxypeptidases, CPX-2 and AEBP1/ACLP [10]. The discoidin domain has been found in mammalian milk proteins, blood coagulation factors, and receptor tyrosine kinases and has been suggested to function in cell aggregation, adhesion, and cell–cell recognition [11]. In this context, mice deficient in AEBP1/ACLP displayed defects in abdominal wall development and dermal wound healing [11]. In situ hybridization of CPX-1 mRNA revealed an abundant expression in developing skeletal structures and a role in development was proposed for CPX-1 [10]. However, studies on CPX-1 have been very limited and understanding of the exact function of CPX-1 needs further investigations.

In this study, we performed a proteomics-based analysis to find proteins potentially involved in RANKL-induced osteoclast differentiation and identified CPX-1 as a protein whose expression level is regulated during osteoclastogenesis.

2. Materials and methods

2.1. Materials

Recombinant soluble human RANKL was purchased from Peprotech EC (London, UK) and murine macrophage/colony-stimulating factor from R&D Systems (Minneapolis, MN, USA). Polyclonal antibodies against CPX-1 were generated by immunizing rabbits with a murine CPX-1 peptide (STKANETSERHVRLRC).

2.2. In vitro generation of osteoclasts and determination of osteoclast phenotype

In vitro osteoclast generation was conducted as previously described with a slight modification [12]. RAW264.7 murine monocyte/macrophage cells were incubated in α-MEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U of penicillin and 100 μg/ml streptomycin in the presence of 100 ng/ml RANKL. Cell phenotype was determined by staining for tartrate-resistant acid phosphatase (TRAP) activity (Acid-Phosphatase Kit; Sigma-Aldrich). After

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Abbreviations: RANKL, receptor activator of NF-κB ligand; TRAP, tartrate-resistant acid phosphatase; 2-DE, two-dimensional gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight

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2 days of culture in the presence of RANKL, more than 90% of cells were TRAP-positive mononuclear and cells at this stage were considered pre-osteoclasts. Mature TRAP-positive multinucleated osteoclasts were obtained after culturing for 3 days.

2.3. Two-dimensional gel electrophoresis (2-DE) and peptide mass fingerprinting

2-DE was carried out in a horizontal electrophoresis system, Multiphor II system (Amersham Pharmacia, Piscataway, NJ, USA). The first-dimensional isoelectric focusing was conducted for 72 kVh using Immobiline dry strips (24 cm long, non-linear gradient between pH 4 and 7, Amersham Pharmacia). After the first dimension separation, the IPG gel was equilibrated twice in equilibration buffer consisting of 50 mM Tris–HCl (pH 6.8), 6 M urea, 2% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.002% (w/v) bromophenol blue. 1% (w/v) dithiothreitol was included in the equilibration buffer for the first equilibration step and 2.5% (w/v) iodoacetamide for the second equilibration step. The equilibrated IPG gel was subjected to the second-dimensional SDS–polyacrylamide gel electrophoresis (PAGE). Proteins on the gel were visualized by silver staining. Protein spots were cut, subjected to reduced *S*-carboxymethylation, dehydrated in 100% acetonitrile, dried in a Speed-Vac, and soaked in digestion buffer containing trypsin. After overnight digestion, peptide fragments in the supernatant were desalted by Zip tips C18 (Millipore, Bedford, MA, USA). Peptide mass maps were obtained using a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (Voyager DE-STR, Applied Biosystems, Foster City, CA, USA) at an instrument resolution in excess of 6000 over the *m*/*z* range 700–3500 Da. Protein identification was carried out using the MS-fit search engine in ProteinProspector (University of California San Francisco) by sending a query of the subtracted peptide mass data.

2.4. Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA, USA), and then reverse-transcribed for cDNA synthesis according to the manufacturer's instructions (Invitrogen). PCR was carried out in a Perkin Elmer thermal cycler for 35 cycles consisting of 30 s denaturation at 94°C, 1 min annealing at 56°C, and 1 min extension at 72°C. The sequence of the sense primer was 5′-ATAGGGCCATGCAGGATAC-3′) and that of the antisense primer was 5′-TGGGAGTCTTGGTGAGTAG-3′. PCR products were visualized by subjecting to electrophoresis in a 2% agarose gel and staining with ethidium bromide.

2.5. Western blot analysis

Western blotting analyses were performed as previously described [13]. Cells were lysed in lysis buffer (10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 mM sodium orthovanadate, 200 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 5 μg/ml aprotinin, 1% NP-40). The protein concentrations of the cell lysates were determined by Bradford assay and then equal amounts of proteins were loaded into 8% SDS–PAGE. Gels were blotted onto nitrocellulose membrane and incubated with polyclonal anti-mCPX-1 antibody. The immunoreactive proteins were detected with ECL reagents after incubation with anti-rabbit IgG-horseradish peroxidase.

2.6. Confocal microscopy

Cells were fixed with 3.7% formaldehyde/phosphate-buffered saline (PBS) for 5 min and permeabilized in 0.5% saponin for 20 min. After blocking with 2% bovine serum albumin/PBS, cells were stained with polyclonal anti-mCPX-1 and Cy3-conjugated anti-rabbit IgG antibodies. Actin stress fibers were stained with fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma-Aldrich) to show cell entity in the photographed field. Fluorescent images were collected using a confocal microscope (Leica).

2.7. CPX-1 expression plasmid construction and RAW264.7 cell transfection

The pVL1393 plasmid harboring the full-length cDNA of mouse CPX-1 was generously provided by Dr. L.D. Fricker (Albert Einstein College of Medicine, Bronx, NY, USA). The CPX-1 cDNA was subcloned into pcDNA3.1(+) using *Bam*HI and *Eco*RI sites. RAW264.7 cells were transiently transfected with pcDNA3.1(+)-CPX-1 plasmid. Cells (1×10^5/well) were seeded onto six-well plates 24 h prior to transfection. The plasmid DNA was incubated for 20 min at room temperature in serum-free medium containing LipofectAMINE 2000 reagent (Invitrogen). The DNA–LipofectAMINE complexes were added dropwise to the cells. After 3–5 h incubation at 37°C, the cells were washed and incubated in fresh medium containing 10% FBS for 24 h. Cells were induced to differentiate to osteoclasts by treating with RANKL.

2.8. Statistical analysis

Statistical differences were analyzed by Student's *t*-test. All experiments were repeated three or four times, and results from a representative experiment are shown.

3. Results

To identify proteins differentially regulated during RANKL-induced osteoclastogenesis, we employed the model cell line RAW264.7. This type of cells has been well characterized to differentiate into functional osteoclasts when cultured in the presence of RANKL [14]. These cells became positive for TRAP, a frequently used marker of committed and differentiated osteoclasts, 2 days after incubation with RANKL (Fig. 1A, panel b). Most of the cells at this stage were mononuclear with a few cells in the process of fusion. TRAP-positive multinuclear cells were generated after culturing with RANKL for 3 days (Fig. 1A, panel c). For proteome analyses, cells at each stage shown in Fig. 1A were lysed and cellular proteins were subjected to 2-DE and MALDI-TOF analysis as described in Section 2. Several groups of protein spots were down- or up-regulated by RANKL treatment. Among the proteins identified by MALDI-TOF, we further analyzed a protein spot of which the best matched PMF was the metallocarboxypeptidase CPX-1 (GenBank accession number Q9Z100). On 2-DE gels this protein showed an approximate molecular mass of 80 kDa and p*I* of 6.5. The expression levels of this protein increased as cells changed to pre-osteoclasts and then decreased as cells further differentiated to multinuclear osteoclasts (Fig. 1B,C). Eleven peptides matched with less than 50 delta ppm covering 15% of the total sequence over eight regions (Fig. 1D). The theoretical molecular weight (80.9 kDa) and p*I* (6.41) values were closely matched with those estimated from 2-DE.

To evaluate the validity of the proteomics result, we first examined the expression levels of CPX-1 mRNA and protein during osteoclastogenesis. When determined by RT-PCR, CPX-1 mRNA expression was found to be elevated at the pre-osteoclast stage after culturing for 2 days with RANKL and then diminished in mature osteoclasts at day 3 of culture (Fig. 2A). This result indicates that changes in CPX-1 mRNA levels occur during osteoclast differentiation, which could be due to either transcriptional or posttranscriptional events affected by RANKL stimulation. To assess the protein levels, we raised rabbit antiserum toward mouse CPX-1 using a synthetic peptide. This antibody successfully recognized mouse CPX-1 expressed in human embryonic kidney cell line 293 by transient transfection (data not shown). Western blotting analyses with this antibody of cell lysates from the different stages of osteoclastogenesis showed that CPX-1 protein was also increased in pre-osteoclasts and declined in multinucleated osteoclasts (Fig. 2B). This pattern of CPX-1 protein expression was also observed during differentiation of osteoclasts from bone marrow cells (data not shown). Similarly, immunostaining of permeabilized cells with the anti-CPX-1

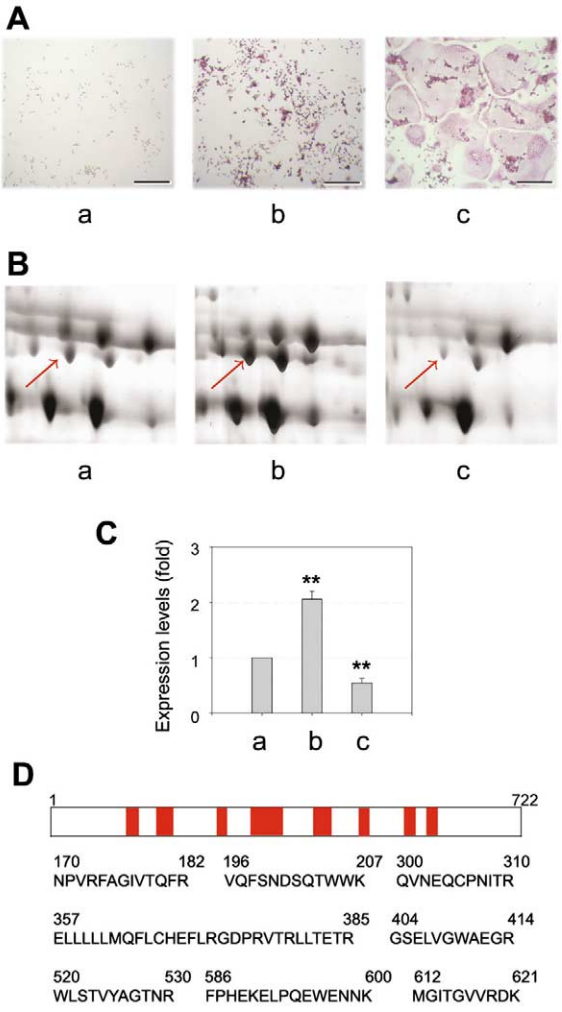

Fig. 1. Identification of CPX-1 by proteomics analysis. A: RAW264.7 cells were seeded at 1×10^5/well in six-well plates and incubated for 2 days (b) or 3 days (c) with 100 ng/ml RANKL. These cells and undifferentiated RAW264.7 cells (a) were stained for TRAP as described in Section 2. Bar, 200 μm. B: Cells at each stage were lysed and cellular proteins were resolved by 2-DE. The gels were silver-stained and scanned. Protein spots showing differential expression levels were subjected to trypsin digestion and mass spectrometry was performed using MALDI-TOF. The protein spots indicated by arrows were identified as CPX-1. C: The density of CPX-1 spots was measured from three gels. Data are expressed as means ± S.D. $^{**}P<0.02$ vs. undifferentiated cells. D: The amino acid sequences of peptides matching the database in the PMF analysis are shown.

antibody revealed a clear staining in pre-osteoclasts (Fig. 3, panel b) whereas differentiated osteoclasts had very low staining (Fig. 3, panel d). Overall these results support the proteomic identification of CPX-1 as a protein transiently up-regulated during osteoclastogenesis.

We next sought to examine the biological significance of CPX-1 in relation to osteoclast differentiation. The expression pattern of CPX-1 suggests that timely changes in CPX-1 levels may be important for osteoclastogenesis. With this assumption, we thought that a disturbance in the regulated expression of CPX-1 might interfere with the successful differentiation of osteoclasts. To test our hypothesis, we conducted a transient transfection of RAW264.7 cells with a CPX-1 expression plasmid and evaluated the formation of pre-osteoclasts as well as multinucleated osteoclasts in response to RANKL stimulation. The overexpression of CPX-1 was confirmed by Western blotting to be dependent on the amount of plasmid used (Fig. 4A). The CPX-1 transfection had little

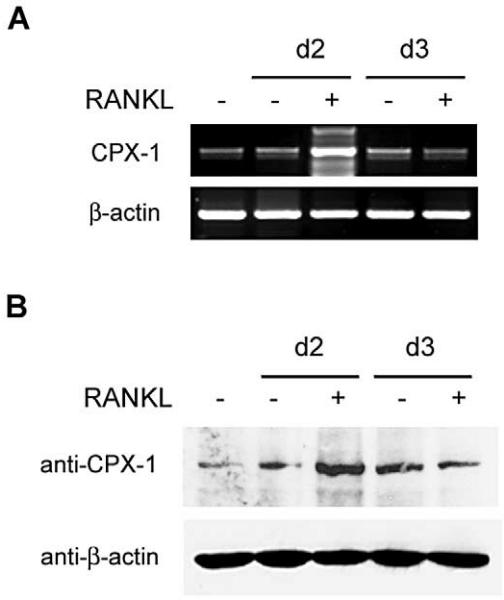

Fig. 2. The expression patterns of CPX-1 mRNA and protein during osteoclastogenesis. A: RAW264.7 cells were cultured in the presence or absence of RANKL (100 ng/ml) for 2 or 3 days. Total RNA was prepared and the CPX-1 mRNA levels were assessed by RT-PCR. B: Total lysates were obtained from cells cultured as in A. The lysate proteins were resolved by SDS–PAGE. Protein levels of CPX-1 were determined by Western blotting with a CPX-1 polyclonal antibody. The β-actin blot is shown as a loading control.

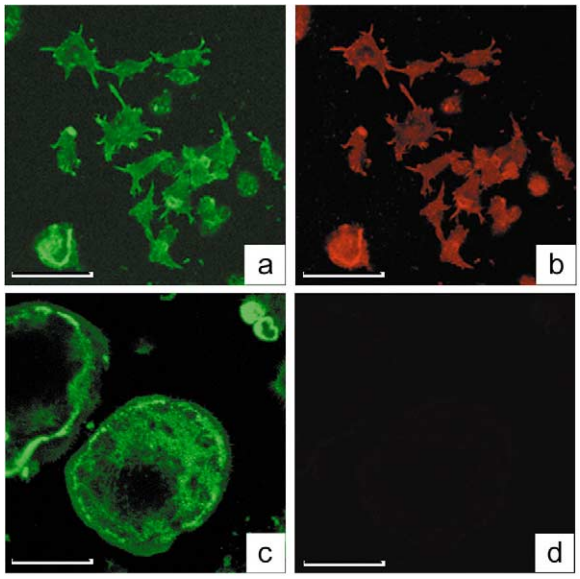

Fig. 3. Immunofluorescence microscopy for CPX-1 expression in osteoclasts. RAW264.7 cells were incubated for 2 (a and b) or 3 (c and d) days in the presence of 100 ng/ml RANKL. Cells were fixed and permeabilized prior to staining with rabbit antiserum to CPX-1. Subsequently cells were incubated with Cy3-conjugated anti-rabbit IgG and FITC-labeled phalloidin. Fluorescence images of cells were captured under a confocal microscope. CPX-1 staining patterns are shown in panels b and d. The actin cytoskeleton images of cells in the same fields are shown in panels a and c. Bar, 100 μm.

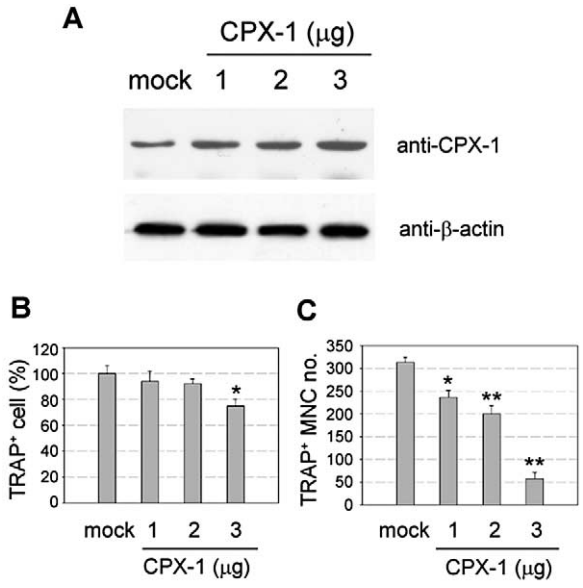

Fig. 4. Inhibition of osteoclast formation by CPX-1 overexpression. A: RAW264.7 cells were transfected with indicated amounts of murine CPX-1 plasmid or 3 μg of the vector DNA. Twenty-four hours after transfection, CPX-1 levels were examined by Western blotting. B: Transfected cells were incubated with 100 ng/ml of RANKL for 2 days and stained for TRAP. The percentage of TRAP-positive pre-osteoclasts was determined. $*P < 0.05$ vs. mock-transfected cells. C: After 3 days of RANKL treatment, osteoclast formation was analyzed by TRAP assay. The numbers of TRAP-positive multinuclear cells are presented. Data are expressed as means ± S.D. $*P < 0.05$; $**P < 0.02$ vs. mock-transfected cells.

effect on the generation of pre-osteoclasts except the highest amount of plasmid, which showed a moderate inhibition (Fig. 4B). However, the generation of multinucleated osteoclasts was significantly affected at all doses of transfected DNA. The number of differentiated osteoclasts decreased by 22.6%, 35.5%, and 82.3% at 1, 2, and 3 μg DNA, respectively (Fig. 4C). These findings imply that the controlled expression of CPX-1 is important for osteoclast differentiation and the down-regulation of CPX-1 after pre-osteoclast stage is necessary for successful progression of the multinucleation step during osteoclastogenesis.

4. Discussion

Using a proteomic analysis, we found that the expression of CPX-1 is regulated during osteoclastogenesis. The protein level of CPX-1 is very low in undifferentiated progenitor cells, while it increases as cells change to committed pre-osteoclasts. The CPX-1 level then decreases as cells further differentiate to multinuclear mature osteoclasts. This expression timing implies that the CPX-1 gene may have a role specifically at the pre-osteoclast stage.

It was shown that CPX-1 mRNA was abundantly expressed in primordial cartilage and skeletal structures of embryonic and fetal mouse tissues [10]. In thorax, CPX-1 mRNA was detected in multiple developing skeletal structures including chondrocytes and perichondrial cells of the ribs, vertebrae, and long-bone primordia [10]. This expression pattern led to the suggestion that CPX-1 may have a function in skeletal development. CPX-1 may regulate osteoclastogenesis during skeletal development. However, studies on CPX-1 have been scarce and more investigations are needed to obtain supporting evidence.

The structural characteristics of CPX-1 include a carboxypeptidase homology domain and a discoidin-like domain [10]. Despite the presence of homology to other carboxypeptidases, CPX-1 failed to show the catalytic activity [10]. These features are also found in two other metallocarboxypeptidase family proteins, CPX-2 and AEBP1/ACLP [15]. The lack of enzymatic function has drawn attention to the discoidin-like domain in exploring roles of this subgroup of the carboxypeptidase family.

The slime mold protein discoidin I is secreted and functions in cell aggregation in *Dictyostelium*. AEBP1/ACLP, an inactive carboxypeptidase group protein with discoidin-like domain, is secreted into the extracellular matrix (ECM) of collagen-rich tissues including the vasculature, dermis, and the developing skeleton [11]. In adipogenesis of 3T3-L1 cells, a concurrent decrease in AEBP1/ACLP expression and ECM stability was observed during the early phase of differentiation [15]. The discoidin domain of discoidin domain receptor proteins binds various types of collagen [16]. These findings have led to the postulation that extracellular discoidin domain-containing proteins bind collagen via the discoidin domain and facilitate ECM stabilization. One possibility for the role of CPX-1 in osteoclastogenesis is the regulation of ECM stability through its discoidin domain. It is likely that ECM components are destabilized in order for cells to undergo morphological changes for fusion. CPX-1, which may facilitate ECM stabilization, then needs to be down-regulated for the progression of multinucleation during osteoclastogenesis. Interference with this timely down-regulation of CPX-1 by ectopic overexpression might cause a disturbance in the formation of multinuclear osteoclasts (Fig. 4). In line with this notion, we observed an augmenting effect of the CPX-1 antibody on multinucleation, which might have been exerted through neutralizing effects of the antibody (data not shown).

Intracellular functions of the discoidin domain have also been reported. AEBP1/ACLP, whose expression decreases during adipocyte differentiation, has a transcription repressor activity [17]. The transcription repression by AEBP1/ACLP is greatly attenuated by deletion of its discoidin domain [18]. However, whether the deletion caused conformational changes and/or loss of recruitment of an interacting protein that is directly involved in repression or whether the discoidin domain itself has a repressor activity needs to be clarified. In either case, the possibility can be considered that CPX-1 has a transcription repressor activity and its discoidin domain functions in an analogous way to that of AEBP1/ACLP. Independent of the transcription repressor function, AEBP1/ACLP can regulate MAPK activation during adipogenesis [18]. The MAPK modulation appears to be mediated by the discoidin domain through binding to MAPK and protection from a MAPK phosphatase [18]. Whether the discoidin domain of CPX-1 has a similar function during osteoclastogenesis is an intriguing question to be addressed.

In summary, we report a regulated expression of the metallocarboxypeptidase family member CPX-1 during osteoclast differentiation driven by RANKL. Furthermore, our results suggest that CPX-1 expression needs to be controlled for successful progression of multinucleation during osteoclastogenesis. Our findings raise a potential importance of CPX-1 for the differentiation of osteoclasts and warrant more studies to

understand the molecular mechanism by which CPX-1 functions in osteoclasts.

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