RANKL Up-regulates Brain-type Creatine Kinase via Poly(ADP-ribose) Polymerase-1 during Osteoclastogenesis*

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Receptor activator of nuclear factor κB ligand (RANKL) is the key regulator for osteoclast formation and function. During osteoclastogenesis, RANKL-stimulated signals differentially modulate expression of a large number of proteins. Using proteomics approaches, we identified that brain-type cytoplasmic creatine kinase (Ckb) was greatly induced in mature osteoclasts. Ckb has been shown to contribute to osteoclast function. However, the mechanisms of Ckb regulation and the contribution of other isoforms of creatine kinase during RANKL-induced osteoclastogenesis are unknown. We found that Ckb was the predominant isoform of creatine kinase during osteoclastogenesis. Real-time PCR confirmed that RANKL induced ckb mRNA expression by over 40-fold in primary mouse bone marrow macrophages and Raw 264.7 cells. The RANKL-responsive region was identified within the −0.4- to −0.2-kb 5′-flanking region of the ckb gene. Affinity binding purification followed by mass spectrometry analysis revealed that poly(ADP-ribose) polymerase-1 (PARP-1) bound to the −0.4/−0.2-kb fragment that negatively regulated expression of ckb in response to RANKL stimulation. Electrophoretic mobility shift assays with PARP-1-specific antibody located the binding site of PARP-1 to the TTCCCA consensus sequence. The expression of PARP-1 was reduced during RANKL-induced osteoclastogenesis, concurrently with increased expression of Ckb. Consistently, knockdown of PARP-1 by lentivirus-delivered shRNA enhanced ckb mRNA expression. The activity of PARP-1 was determined to be required for its inhibitory effect on the ckb expression. In summary, we have demonstrated that PARP-1 is a negative regulator of the ckb expression. Down-regulation of PARP-1 is responsible for the up-regulation of ckb during RANKL-induced osteoclastogenesis.

Osteoclasts are multinucleated bone-resorbing cells that play a crucial role in physiological bone remodeling, as well as in pathological bone resorption such as osteoporosis and periodontal disease (1, 2). To decalcify bone and degrade the organic extracellular matrix, osteoclasts are required to maintain an overall intracellular and extracellular ionic milieu with a large number of plasma membrane and intracellular transport systems, including the vacuolar-type H+ -ATPase, a highly conductive chloride channel, chloride bicarbonate exchangers, and accessory pumps (3). Mature osteoclasts exhibit high citric acid cycle activity and active mitochondrial respiration to generate high levels of ATP, which are used to maintain ATPase activities and ultimately lead to bone resorption (3−5). Recent studies have demonstrated that a large amount of ATP is used by vacuolar-type H+-ATPases in mature osteoclasts to pump protons into an extracellular resorption area (3), which is critical for the bone resorption function (6, 7).

Creatine kinase isoenzymes regulate ATP homeostasis in subcellular compartments by the transfer of phosphates between creatine and adenine nucleotides. The mitochondrial isoform generates creatine phosphate, which is shuttled to cytosolic isoforms localized to specific subcellular regions to provide ATP at sites where high and fluctuating energies are required (8). There are two mammalian creatine kinase cytosolic isoforms, the muscle (Ckm) and brain (Ckb) forms, as well as two mitochondrial isoforms, the ubiquitous (Ckmtu) and the sarcomeric (Ckmts) forms. Ckb may coordinate with Ckmtu to function as a subcellular, site-specific regulator of ATP regeneration rather than a pan-cellular generator of ATP (9). Ckb is present in a range of tissues, including brain, retina, uterus, kidney, and testes, to execute the function of energy maintenance and regulation (10, 11). Studies using Ckb null mice have demonstrated that Ckb deficiency does not affect basal bone turnover; however, it protects mice from bone loss stimulated by ovariectomy or systemic lipopolysaccharide challenge (12). Furthermore, Ckb deficiency does not affect osteoclast differentiation but reduces osteoclast activity in vitro (12). The mechanism may be related to decrease in regeneration of ATP, which is needed for cytoskeleton reorganization and bone resorption (12, 13). Therefore, understanding the modulation of the Ckb expression in osteoclasts may provide new insights into therapeutic strategy for osteolytic bone disorders.

Transcriptional regulation of ckb has been reported in rat and human cells (14−20). Multiple binding sites for diverse nuclear transcription factors have been identified within the ckb promoter. For instance, expression of ckb was found to be

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3 The abbreviations used are: Ckm, cytoplasmic creatine kinase muscular isoform; Ckb, cytoplasmic creatine kinase brain isoform; Ckmtu, creatine kinase mitochondrial isoform; RANKL, receptor activator of NF-κB ligand; PARP, poly(ADP-ribose) polymerase; NFAT, nuclear factor of activated T-cells; TRAP, tartrate-resistant acid phosphatase; Cath K, cathepsin K; CTR, calcitonin receptor; BMM, bone marrow macrophage.
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regulated by transcriptional factors activator protein-2 and nuclear factor Y in U87-MG glioblastoma cells (16, 17). The sequences between −75/−45 and −568/−523 bp at human ckb promoter have been characterized as estrogen-responsive regions, albeit the binding site for the estrogen receptor is only present in the −568/−523-bp region (18–20). In addition, other transcriptional factors, such as myocyte enhancer factor 2 and Sp1, have also been shown to initiate or sustain the transcription of ckb (18, 21). Nevertheless, the regulation of ckb in osteoclasts has yet to be determined.

The receptor activator for nuclear factor κB ligand (RANKL), a member of the tumor necrosis factor (TNF) superfamily, is a potent inducer of osteoclast differentiation, function, and survival. RANKL exerts its effects by binding to its receptor RANK, which interacts with various TNF receptor-associated factors to initiate intracellular signaling pathways, including nuclear factor κB (NF-κB), activator protein 1 (AP-1), and nuclear factor of activated T-cells (NFAT) c1 during RANKL-mediated osteoclastogenesis (22–25). NFATc1 plays an integral role in the RANKL-induced transcriptional program during the late stage of differentiation. AP-1 transcription factors are important transcriptional partners of NFATc1 and are critical for the induction of NFATc1 (26). RANKL-induced NF-κB, NFATc1, and AP-1 signaling pathways play essential roles in activating the expression of osteoclast-specific genes, such as tartrate-resistant acidic phosphatase (TRAP), cathepsin K (Cath K), and calcitonin receptor (CTR), thus promoting osteoclast differentiation and function.

In the present studies, we investigated the molecular mechanisms of Ckb regulation during RANKL-induced osteoclastogenesis. We found that the poly(ADP-ribose) polymerase-1 (PARP-1) was a negative regulator of ckb at the basal level and during RANKL-induced osteoclastogenesis. The effect of PARP-1 on the regulation of ckb was characterized with a PARP-1-specific inhibitor and by PARP-1 knockdown. Furthermore, with the use of a luciferase reporter system and electrophoretic mobility shift assays, the PARP-1 binding domain was identified at 5′ of the ckb gene. These studies provide important insights into molecular regulation of Ckb and its function during RANKL-induced osteoclastogenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Primary mouse bone marrow macrophages (BMMs) were isolated from long bones of 6–8-week-old C57BL/6 mice, and osteoclast differentiation was induced by GST-RANKL (100 ng/ml) in the presence of macrophage colony-stimulating factor (M-CSF) as we described previously (27). The Raw 264.7 cells obtained from ATCC (Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (Mediatech) containing 10% fetal bovine serum. Osteoclastogenesis was induced by 50 ng/ml RANKL and determined by TRAP staining according to the manufacturer’s instructions (Sigma).

**Knockdown of PARP-1 in BMMs with Lentivirus-delivered shRNA**—Four lentiviral constructs expressing a 21-nucleotide PARP-1 short hairpin RNA (shRNA) targeting murine PARP-1 gene (GenBank accession number NM_007415) were purchased from Open Biosystems (Huntsville, AL). Each construct was packed into lentivirus-like particles pseudotyped with the vesicular stomatitis virus glycoprotein as we previously described (28). Transduction was performed by incubating BMMs with recombinant lentivirus, and stably transduced cells were selected with puromycin (2 μg/ml).

**Western Blot Analysis**—Western blot analysis was performed as we previously described (27). Goat polyclonal anti-Ckb, mouse monoclonal anti-NFATc1, and rabbit polyclonal anti-PARP-1 antibodies were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (RDI-TRK5G4-6C5) was obtained from Research Diagnostics Inc.

**Assessments of Gene Expression by RT-PCR and Quantitative Real-time PCR**—Total RNA was isolated from indicated cells with TRIzol reagents (Invitrogen), reverse-transcribed, and amplified with primers for mouse ckb, TRAP, CTR, and Cath K as we previously described (27). Expression of GAPDH was used as a loading control. Quantitative real-time PCR was performed and analyzed using SYBR Green I supermix (Bio-Rad) in a Bio-Rad i-Cycler. Primers for PCR reactions were as follows: ckb, 5′-ACGCGCTCAAGAGCATGACTGA-3′ and 5′-TGCGATGAGTATGCAGGT-3′; TRAP, 5′-GACGATGGGCGCTGACTTCA-3′ and 5′-GCGCTTGGAGATTCTTAGGT-3′; Cath K, 5′-ACAGGGCCTAGCTTGAAGA-3′ and 5′-GATGCCAAGCTTGCGTCAT-3′; CTR, 5′-GCAAACGTCTGGCTGATG-3′ and 5′-GAAGCAGTGAATAGTCCCA-3′. Standard curves were generated for all PCR assays.

**Dual-Luciferase Reporter Assays**—A series of deletion constructs from the 5′-flanking region of the ckb gene (−3.5 to −0.2 kb) were cloned into pGL3-Basic (Promega) at the XhoI/HindIII sites. Genomic DNA from C57BL/6 mice was amplified using the following primers located at indicated positions according to the ATG translation start site. All inserts were verified by DNA sequencing. Forward primers are as follows: −3.5 kb, 5′-TACTCGAGAAGACCCCTCATTGGCTTCC-3′; −1.4 kb, 5′-TACTCGAGAAGATCCTGCTAGGAGATCC-3′; −0.9 kb, 5′-TACTCGAGAATGGAAGCTCGCTGCA-3′; −0.4 kb, 5′-TACTCGAGTCTCTGCTCCACATTGCC-3′; −0.2 kb, 5′-TACTCGAGGATCGAGCTGGAAGCTGCTGGCA-3′; 0 kb, 5′-TACTCGAGTGCAAGCGATC-3′. Reverse primer is as follows: +21 bp, 5′-TAAAGCTTATGGAATCCCTGCTTGGCAG-3′.

Plasmid transfection and luciferase reporter assays were performed as described previously (29). Briefly, Raw 264.7 cells were seeded in 48-well plates and transiently transfected with 0.2 μg of ckb-Luc reporter plasmid using FuGENE HD (Roche Applied Science). A plasmid encoding Renilla luciferase gene driven by the cytomegalovirus (CMV) promoter was used to control for transfection efficiency. Five hours after transfection, cells were treated with 50 ng/ml RANKL for the indicated time. Luciferase activities were determined with the Dual-Luciferase assay kit (Promega).

**Nuclear Extract Preparation and Biotin-Streptavidin Pull-down Assays**—Raw 264.7 cells were treated with or without 50 ng/ml RANKL for 72 h, and nuclear extracts were prepared as described (30). After lysis, the nuclear pellet was resuspended in nuclear extract buffer containing 0.4 M NaCl, 20 mM HEPES pH 7.9, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 10% glycerol, 5 mM...
NaF, 1 mM Na2VO4, and Complete protease inhibitor cocktail. The insoluble debris was removed by centrifugation, and the supernatant was used in the following experiments.

A 0.2-kb probe spanning from −0.4 to −0.2 kb of the ckb promoter was amplified with biotinylated primers: forward, 5′-biotin-CTCTCACATGACCTCCCTCCTACAAC-3′, and reverse, 5′-TAGCGGTCCCAGACTAGTT-3′. Biotin-labeled probe was incubated with nuclear extracts and captured by streptavidin-conjugated agarose. The pulled down proteins were separated by electrophoresis. Coomassie Blue staining followed by mass spectrometry analysis (DE-Pro MALDI-TOF mass spectrometer, Applied Biosystems) was performed to identify the DNA-bound proteins, which were further confirmed by Western blot analysis.

Electrophoretic Mobility Shift Assays (EMSAs)—EMSAs to analyze protein-DNA interactions were performed as described previously (27) using oligonucleotides containing PARP-1 binding elements, wild type (WT): 5′-GCCATCTGGCTTCCCAAGCTCAGTT-3′, or mutant (lowercase), 5′-GCCATCTGGCTaatattGACTCAGTT-3′. For supershift assays, nuclear extracts were preincubated with anti-PARP-1 serum (ALX-210-302-R100; Enzo Life Sciences) or control rabbit IgG for 20 min before the addition of the labeled probe. For competition experiments, an excessive amount of unlabeled probe (100-fold) was added to the binding reactions.

Statistical Analysis—Each experiment was repeated at least three times; all results are expressed as means ± S.D. Statistical analysis was performed as described previously (27). Differences between two groups were identified by Student’s t tests. For multiple groups, one-way analysis of variance and Student Newman-Keuls tests were used to identify differences. Significance was defined as p < 0.05.

RESULTS

RANKL Induces Expression of Ckb during Osteoclastogenesis—To determine RANKL-regulated proteins during osteoclastogenesis, whole cell lysates from BMMs or mature osteoclasts (Fig. 1A) were subjected to SDS-PAGE analysis. A drastic up-regulation of a protein with a molecular mass at 46 kDa was identified in mature osteoclasts (Fig. 1B). Mass spectrometry analysis identified this 46-kDa protein as brain-type creatine kinase (Ckb), which was confirmed by Western blot analysis using anti-Ckb specific antibody (Fig. 1C). The increased Ckb expression was concurrent with the up-regulation of osteoclast marker genes, including TRAP, Cath K, and CTR (Fig. 2B, upper panel). The increased ckb expression was concurrent with the up-regulation of osteoclast marker genes, including TRAP, Cath K, and CTR (Fig. 2D).

To precisely map the RANKL-responsive elements, luciferase reporters containing different lengths of the 5′-flanking region (−3.5, −1.4, −0.9, −0.4, −0.2, and 0 kb) were generated, and RANKL-induced luciferase activity was determined (Fig. 2C).

FIGURE 1. Expression of Ckb during RANKL-induced osteoclastogenesis. BMMs were treated with (RANKL) or without (Control) RANKL for 72 h. A, osteoclastogenesis was depicted by TRAP staining. B, protein extracts were subjected to SDS-PAGE analysis followed by Coomassie Blue staining. The arrow indicates the RANKL-up-regulated protein, which was identified as Ckb by mass spectrometry analysis. C, Western blotting analyses of Ckb in BMMs (upper) or Raw 264.7 cells (lower) with or without exposure to RANKL for 72 h. GAPDH was used as a loading control. D, quantitative analysis of Ckb production determined by Western blotting analysis in C. E and F, the mRNA expression of creatine kinase isoforms was determined by RT-PCR (E) and quantitative real-time PCR (F). Representative pictures of three independent experiments are shown. *, p < 0.01.
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PARP-1 Negatively Regulates ckb by Binding to Its Promoter Region—To identify the transcription factors that regulate the ckb expression in response to RANKL, we isolated nuclear proteins bound to a biotinylated 0.2-kb fragment (−0.4 to −0.2 kb). A protein at a molecular mass of ~120 kDa was pulled down both at the basal level and upon RANKL treatment (Fig. 3A). Decreased binding of the protein to the probe was observed when cells were exposed to RANKL (Fig. 3A), indicating that this protein may have a negative role in mediating RANKL-induced ckb expression. Mass spectrometry analysis identified the protein as PARP-1, the founding member of the PARP family (31). Western blotting analysis further confirmed the specific binding of PARP-1 to the 0.2-kb fragment at the basal level, which was dramatically inhibited in RANKL-induced mature osteoclasts (Fig. 3B, upper panel). NFATc1, the key transcription factor for osteoclastogenesis, did not bind to the 0.2-kb fragment (Fig. 3B, bottom panel), albeit it was induced by the RANKL treatment, confirming the binding specificity of PARP-1.

Bioinformatic analysis of the −0.4/−0.2-kb fragment revealed a putative PARP-1 binding sequence located between −265 and −261 bp. This region contains TTCCC A, the known PARP-1 consensus sequence within the TRAP and Tcirg1 promoter regions (32–34). An EMSA assay using a probe (−271/−246 bp) containing the putative PARP-1 binding element demonstrated a specific binding (Fig. 3C, lanes 2 and 3), which was abolished with the addition of a 100-fold molar excess of the unlabeled probe (lane 4). Mutation of the consensus sequence abolished the binding (Fig. 3C, lanes 6 and 7). This binding was dramatically decreased in nuclear extracts from RANKL-treated cells (Fig. 3C, lane 3), further supporting the notion that this region is involved in negative regulation of the ckb promoter activity. The binding specificity of PARP-1 was also characterized with the use of a PARP-1 antibody. A supershifted complex was identified in the presence of PARP-1 antibody (Fig. 3D, lane 4), which was not observed when the control IgG was used (Fig. 3D, lane 5), confirming the specificity and functional importance of PARP-1 binding in the ckb transcription.

Functional Engagement of PARP-1 in RANKL-induced Ckb Expression—To further characterize the role of PARP-1 in RANKL-induced Ckb expression, we determined the produc-
tion level of PARP-1 during RANKL-induced osteoclastogenesis. Consistent with increased expression of ckb observed in Fig. 2, production of the Ckb protein increased continuously during RANKL-induced osteoclastogenesis. The expression of PARP-1 protein (120 kDa) decreased gradually in response to RANKL (Fig. 4). Two proteins at lower molecular mass, one at 70 kDa (arrow) and the other at 89 kDa (asterisk), were found to react with PARP-1 antibody as well (Fig. 4). The expression of these two proteins increased during RANKL-induced osteoclastogenesis concomitantly with a decrease in the intact PARP-1 protein, implying the cleavage of PARP-1 during RANKL-induced osteoclastogenesis.

To determine whether poly(ADP-ribosyl)ation activity of PARP-1 was responsible for its effect on the ckb expression, we examined the effect of PJ34, a known inhibitor for PARP-1 poly(ADP-ribosyl)ation activity. We found that PJ34 dose-dependently induced the expression of ckb in Raw 264.7 cells as well as BMMs (Fig. 5A). The level of the induced ckb in BMMs was less than that in Raw 264.7 cells, which may reflect different sensitivities of these two cell types in response to treatment with PARP inhibitors.

Furthermore, we generated BMMs with PARP-1 knockdown using lentivirus-delivered shRNAs specific for PARP-1. As shown in Fig. 5B, four shRNAs targeting different regions of PARP-1 mRNA exhibited differential inhibition on the expression of PARP-1. Inhibition of PARP-1 by shRNA was found to increase the expression of ckb as demonstrated by real-time PCR (Fig. 5C). Importantly, the expression of PARP-1 (Fig. 5B) was inversely related to the expression of ckb (Fig. 5C), indicating that PARP-1 negatively regulated ckb mRNA expression.

**DISCUSSION**

The function of Ckb in regulating osteoclast bone resorption activity in mice has been reported, which may be related to decrease in regeneration of ATP that is required for cytoskeleton reorganization and bone resorption (12, 13). In humans, elevation of serum levels of Ckb has been found in patients with type 2 autosomal dominant osteopetrosis. The patients have increased osteoclasts without any bone resorption activity (35, 36). The present studies determined the molecular mechanisms underlying the regulation of Ckb in osteoclasts, which may shed light on a molecular mechanism of the elevation in serum levels of Ckb in these osteopetrosis patients with increased osteoclasts.

We examined different isoforms of creatine kinase in osteoclasts and their response to RANKL. We demonstrated that Ckb was the predominant isoform in osteoclasts and that RANKL induced the Ckb expression during osteoclastogenesis. The −0.4/−0.2-kb region of the ckb gene was responsible for RANKL-induced ckb transcription. Although there was no report on gene regulation of murine ckb, previous studies have identified several transcription factors that bind to the 5′-flank-
ing region of \textit{ckb} and promote \textit{ckb} transcription in rat and human cells (16, 18, 21, 37, 38). However, no binding sites of these previously reported transcription factors were found within this RANKL-responsive \(-0.4/-0.2\)-kb region.

PARP-1 was identified to bind to the RANKL-responsive region. PARP-1 is an abundant and ubiquitous nuclear enzyme (31). PARP-1 was originally characterized as a key factor in DNA repair pathways (31, 39, 40). PARP-1 has a DNA binding domain in the amino terminus containing two zinc finger motifs and a third zinc binding domain, as well as a central auto-modification domain (31, 41). These domains allow PARP-1 to bind directly to specific DNA sequences or structures in the regulatory regions of genes (42–44). PARP-1 has also been shown to modulate chromatin structure and composition by competing with histone for binding to nucleosomes or by PARYlation (45, 46). In addition, PARP-1 acts as a promoter-specific co-regulator (a co-activator or a co-repressor) for many different transcriptional regulators (39, 47–49). In HeLa cells, PARP-1 has been shown to inhibit estrogen receptor \(\alpha\)-activated gene transcription (39, 40). In BMMs, PARP-1 was found to inhibit the expression of TRACP and Tcirg1 genes (32–34). Consistent with these observations, we demonstrated that PARP-1 bound directly to the \textit{ckb} promoter region. The binding was further determined to be responsible for a negative regulation of the \textit{ckb} transcription by PARP-1.

The PARP-1 binding site was localized to the \(-265/-261\) bp of the \textit{ckb} promoter. The binding sequence TTCCCA, which was responsible for the negative regulation of \textit{ckb} by PARP-1, is similar to the PARP-1 consensus element identified by Beranger \textit{et al.} (32–34) on TRACP and Tcirg1 genes. PARP-1 binding sequences have been reported to regulate other genes, such as CATTCCCT in the cardiac troponin T gene promoter (42) and TTGAXXACAA in the human T cell leukemia virus type I gene promoter (50). These different PARP-1 binding sequences have only a few nucleotides in common, supporting the idea that PARP-1 may have fewer conserved binding sites to execute its diverse functions.

We found that the expression of intact PARP-1 was decreased during RANKL-induced osteoclastogenesis, which was inversely associated with RANKL-induced \textit{ckb} expression. Increased cleaved forms of PARP-1 were observed concomitantly with decreased intact PARP-1 in response to RANKL, indicating that RANKL induced cleavage of PARP-1 during osteoclastogenesis. In response to RANKL, increased cleaved PARP-1 fragment was predominantly present at a molecular mass of \(\sim 70\) kDa (Fig. 4, \textit{arrow}). A fragment at \(\sim 89\) kDa was detected with PARP-1 antibody as well but at a much lower level, which might be the PARP-1 fragment cleaved by caspase-3 or -7 during apoptotic cell death (51, 52) (Fig. 4, asterisk). Further investigations are warranted to characterize the mechanisms of RANKL-induced cleavage of PARP-1 and the function of the cleaved PARP-1 in regulating gene expression.

Inhibition of PARP-1 poly(ADP-ribosyl)ation activity with a specific inhibitor for PARP-1 cleavage was found to increase the \textit{ckb} mRNA expression, indicating that the poly(ADP-ribosyl)ation activity of PARP-1 is required for its inhibitory effect. These results are consistent with previous findings demonstrating that inhibition of PARP-1 poly(ADP-ribosyl)ation activity increases NFAT-dependent transactivation in T cells (53). With the use of lentivirus-delivered shRNA, we further confirmed that specific knockdown of PARP-1 increased expression of \textit{ckb}.

In summary, we identified that \textit{ckb} was the predominant isoform of creatine kinase and up-regulated by RANKL during osteoclastogenesis. RANKL-induced expression of \textit{ckb} is mediated by PARP-1, which directly bound to \(-265/-261\) bp 5′ of the \textit{ckb} gene and negatively regulated RANKL-induced \textit{ckb} transcription. RANKL induced cleavage of PARP-1 during osteoclastogenesis. Inhibition of cleavage of PARP-1 or knockdown of PARP-1 was shown to increase the expression of \textit{ckb} transcription at the basal level and in response to RANKL. Taken together, these results suggest that PARP-1 negatively regulates the \textit{ckb} expression at the basal level and that RANKL treatment induces degradation of PARP-1 and thus increases the \textit{ckb} expression.

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