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Calcium/calmodulin-dependent protein kinase IV (CaMKIV) enhances osteoclast differentiation via the up-regulation of Notch1 protein stability

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ABSTRACT

The Notch signaling pathway plays a crucial role in the regulation of cell fate decision, and is also a key regulator of cell differentiation, including bone homeostasis, in a variety of contexts. However, the role of Notch1 signaling in osteoclast differentiation is still controversial. In this study, we show that Receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclast differentiation is promoted by the Notch1 intracellular domain (Notch1-IC) and Ca^{2+}/Calmodulin dependent protein kinase IV (CaMKIV) signaling. Notch1-IC protein level was augmented by CaMKIV through escape from ubiquitin dependent protein degradation. In addition, CaMKIV remarkably increased Notch1-IC stability, and the kinase activity of CaMKIV was essential for facilitating Notch1 signaling. CaMKIV directly interacted with Notch1-IC and phosphorylates Notch1-IC, thereby decreasing proteasomal protein degradation through F-box and WD repeat domain-containing 7 (Fbw7). We also found that Notch1-IC prevented inhibition of osteoclast differentiation by KN-93 but not the phosphorylation deficient form of Notch1-IC. These results suggest that phosphorylated Notch1-IC by CaMKIV increases Notch1-IC stability, which enhances osteoclast differentiation.

1. Introduction

Receptor activator of NF-κB ligand (RANKL) stimulates osteoclast differentiation in uncommitted cells of the monocyte-macrophage lineage by interacting with its cell-surface receptor, Receptor activator of NF-κB (RANK) [1,2]. RANKL-RANK signaling is essential for osteoclast differentiation because both *RANKL*$^{-/-}$ and *RANK*$^{-/-}$ mice do not develop osteoclasts [3,4]. The RANKL-RANK signaling pathway activates transcription factors NF-κB, Fos, AP-1, and Nuclear factor of activated T cells (NFATc1) [5–11]. The activity of these transcription factors leads to the induction of osteoclast-specific genes, such as tartrate-resistant acid phosphatase (TRAP), cathepsin-K, MMP9 and calcitonin receptor [12–14]. RANKL induced CaMKIV activation is required for the induction of NF-kB, c-Fos and NFATc1, which leads to induction of osteoclast differentiation [15–17]. Intracellular Ca^{2+} signaling plays a critical role in a broad array of cell functions, including transcription, cell cycle, apoptosis, exocytosis, and motility, and often has multiple roles in cells [18]. Calcium regulates many functions by forming a complex with calmodulin (CaM), upon the binding of Ca^{2+}/CaM increases its affinity for its targets, which include the Ca^{2+}/CaM dependent Ser-Thr kinase (CaMK) family [19,20]. These kinases have the general consensus phosphorylation sequence R-X-X-S/T, Phosphorylation of CaMKIV by CaMKK increases CaMKIV kinase activity, and activated CaMKIV translocates to the nucleus [18]. Nuclear CaMKIV triggers the disruption of subnuclear domains containing class II histone deacetylases (HDACs) and acts as a silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), a broad-specificity co-repressor which represses nuclear hormone receptors and CBF1 [21]. These data suggest the possibility that CaMKIV enhances Notch signaling [21]. Some studies have recently demonstrated that after RANKL stimulation, mRNA and protein expression of Hes1 were up-regulated in Raw264.7 cells [22–24]. Notch signaling in bone metabolism has been shown to play a role in somitogenesis during skeletal development, because several Notch family deficient mice die during embryogenesis before bone formation [25–28]. Notch1 deficiency promotes osteoclastogenesis indirectly by enhancing the ability of osteoblast lineage cells to stimulate osteoclastogenesis. This is achieved by decreasing the osteoprotegerin/RANKL expression ratio [29].

Notch, a highly conserved transmembrane protein, plays an essential role in the regulation of cell fate determination including bone homeostasis [30–32]. Several downstream targets of Notch signaling also have been identified, including the Enhancer of split [E(spl)] complex genes and the mammalian homologues of the Hairy and E(spl) genes, Hes1, Hes5, Hes7, Hey1, Hey2, and HeyL [33–42]. Asagiri group demonstrated the requirement for NFATc1 in the generation of osteoclasts in vivo by transferring NFATc1-deficient hematopoietic stem cells into c-Fos-deficient mice [43]. Induction of Notch signaling by Jagged1 or by ectopic expression of intracellular domain enhances NFATc1 promoter activity and expression and promotes osteoclastogenesis [23]. Multiple lines of evidence have indicated that Notch signaling dysfunction

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results in bone disease. For example, presenilin 1-deficient mice and Delta3 mutant mice (Pudgy) exhibit axial skeletal defects [44–46]. Notch activation by Delta1 reduces the surface levels of the Macrophage colony-stimulating factor (M-CSF) receptor, c-Fms, in osteoclast precursor cells and enhances the expression of osteoprotegerin (OPG) in stromal cells, resulting in the downregulation of osteoclast differentiation [47]. Despite these observations, the precise mechanisms underlying the connection between Notch signaling and the RANKL-CaMKIV mediated osteoclastogenesis pathways are still largely unknown.

In this study, we demonstrate that Notch1-IC is regulated by CaMKIV during osteoclast differentiation. The transcriptional activity and the protein level of Notch1-IC were increased by CaMKIV via inhibition of Notch1-IC proteasomal degradation through Fbw7. We also determined that the up-regulated Notch1-IC by CaMKIV accelerates osteoclast differentiation. Collectively, our finding suggests that CaMKIV is crucial for the regulation of Notch1-IC in osteoclast differentiation.

2. Materials and methods

2.1. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells (ATCC No. CRL-1573) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 7% fetal bovine serum (FBS, Gibco-BRL) and 1% penicillin/streptomycin (Gibco-BRL), Presenilin 1/2 double-positive (PS1/2$^{+/+}$), and Presenilin 1/2 double-negative (PS1/2$^{-/-}$) cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator with an atmosphere containing 5% CO_2. The mouse macrophage-like cell line Raw264.7 cells (ATCC No. TIB-71) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator with an atmosphere containing 5% CO_2. For plasmid DNA transfection, cells were plated at a density of 2×10^6 cells/100-mm-diameter dish (SPL), grown overnight, and transfected with the appropriate expression vectors in the presence of the indicated combinations of plasmid DNAs by using the calcium phosphate method or Lipofectamine (Invitrogen) [17,48].

2.2. Osteoclast differentiation and TRAP staining

RAW264.7 cells were plated at 1×10^5 cells/well in 96-well plates in DMEM with 10% FBS and were incubated overnight in a humidified incubator. The medium was then replaced with α-minimal essential medium (α -MEM, Gibco-RBL) containing 10% FBS and 20 ng/ml RANKL (R&D systems) or RANKL plus either 5 μM KN93 (Sigma), or 5 μM DAPT (Sigma). Culture media were replaced at least every second day with fresh media containing the RANKL and appropriate concentrations of CaMK inhibitor. After 4–5 days, cells were fixed with fixative solution (Sigma) for 5 min, and then washed three times with PBS, and incubated with mixtures containing naphthol AS-BI phosphate (Sigma), Fast Garnet GBC Base Solution (Sigma), and Sodium Nitrite Solution (Sigma) in presence of Tartrate (Sigma) (Leukocyte Acid Phosphatase Assay Kit, Sigma). TRAP-positive multinucleated cells containing three or more nuclei were recorded by light microscopy [16,49].

2.3. Site-directed mutagenesis

Site-directed mutagenesis of Notch1-IC cDNA was performed using a QuikChange kit (Stratagene), and the mutagenic primers were S2063A (5′ CGCCCGTGAGGGCgcCTATGAGACTGCC-3′, S2173A (5′-CACGGAGG AAGAAGgCC CAGGATGGCAAG-3′) (mismatches with the Notch1-IC cDNA template are indicated by lowercase letters). The mutations were verified by automatic DNA sequencing [48].

2.4. Luciferase Reporter assay

The HEK293 cells, PS1/2$^{+/+}$, and PS2$^{-/-}$ cells were cotransfected with 4XCSL, Hes1, and Hes5-Luc and β-galactosidase together with the indicated vector constructs. The cells were lysed in chemiluminescent lysis buffer (18.3% of 1 M K_2HPO_4, 1.7% 1 M KH_2PO_4, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM dithiothreitol [DTT]) and assayed for luciferase activity using a luciferase assay kit (Promega). The activity of the luciferase reporter protein in the transfected cells was normalized in reference to the β-galactosidase activity in the same cells [50]. The data are expressed as the means ± S.D. of triplicates from one of three independent experiments. All of these results are representative of at least three independent experiments.

2.5. GST-pull down assay

The recombinant GST-Notch1-IC protein was expressed in the *E. coli* BL21 strain using the pGEX system, as indicated. The GST fusion protein was then purified with GSH–agarose beads (Sigma) in accordance with the manufacturer's instructions. An equal amount of GST or GST–Notch1-IC fusion protein was incubated with the lysates of the HEK293 cells, which had been transfected for 4 h with combinations of expression vectors at 4 °C, with rotation. After incubation, the beads were washed three times in ice-cold phosphate-buffered saline (PBS, pH 7.4) and boiled with 20 μl of Laemmli sample buffer. The precipitates were then separated via SDS-PAGE, and the pull-down proteins were detected via immunoblotting with specific antibodies [48].

2.6. Immunoblot analysis

After 48 h of transfection, the cultured HEK293 cells were harvested and lysed in RIPA buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 2 g/ml each of leupeptin and aprotinin] for 30 minutes at 4 °C. The cell lysates were subjected to 20 minutes of centrifugation at 12,000 *g* and 4 °C. The resultant soluble fraction was boiled in Laemmli buffer and subjected to SDS-PAGE. After gel electrophoresis, the separated proteins were transferred via electroblotting onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with Tris-buffered saline solution (pH 7.4) containing 0.1% Tween 20 and 5% nonfat milk. The blotted proteins were then probed with anti-Myc antibody (9E10), anti-HA (12CA5) antibody, or anti-FLAG M2 antibody (Sigma), and β-actin (Santa Cruz) followed by incubation with anti-mouse horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). Membranes were also probed with anti-Notch1-IC (Santa Cruz), anti-CaMKIV (Abcam) followed by incubation with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Santa Cruz). The blots were developed using enhanced chemiluminescence (Anigene).

2.7. Protein stability assay

In the protein stability assay, cells were seeded at a density of 50–60% confluence and incubated overnight. The cells were treated with 0.1 mM cycloheximide for 0, 1, 2, 4 or 8 h to block the synthesis of new proteins. Cells were collected at each time point and the total lysates were then lysed in RIPA or Laemmli buffer. Protein levels of Notch1 were determined by immunoblotting with anti-Myc at dilutions of 1:3000 [51].

2.8. Coimmunoprecipitation assays

The cells were lysed in 1 ml of radioimmunoprecipitation Assay (RIPA) buffer for 30 min at 4 °C. After centrifugation at 12,000 ×*g* for 20 min, supernatants were subjected to immunoprecipitation with the

appropriate antibodies coupled to protein A-agarose beads. The resulting immunoprecipitates were washed three times with phosphate-buffered saline (PBS; pH 7.4). Laemmli sample buffer was then added to the immunoprecipitated pellets. The pellets were heated at 95 °C for 5 min and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed with the indicated antibodies [52].

2.9. Immunocomplex kinase assay

To analyze the kinase activity, confluent cells were harvested and lysed in lysis buffer. Cell lysates were then subjected to 10 min of centrifugation at 12,000 ×*g* and 4 °C. The soluble fraction was incubated for 1 h with appropriate antibodies against the indicated protein kinases at 4 °C. The immunocomplexes were then coupled to protein G-agarose during an additional 1 h of incubation at 4 °C, after which they were pelleted by centrifugation. The immunopellets were rinsed three times with lysis buffer and then twice with 20 mM HEPES, at a pH of 7.4. Immunocomplex kinase assays were conducted by incubation of the immunopellets for 30 min at 30 °C with 2 μg of substrate proteins in 20 μl reaction buffer containing 0.2 mM sodium orthovanadate, 10 mM $MgCl_2$, 2 μCi [γ^{32}P] ATP, 20 mM HEPES (pH 7.4). Phosphorylated substrates were then visualized by SDS-PAGE and quantified using a Fuji FLA 7000 phosphorimager [50].

2.10. Immunofluorescence staining

Assays were conducted as previously described with HEK293 cells plated at a density of 1×10^5 cells per well onto coverslips (Fisher) [48]. The cultured cells were fixed with 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS), and then permeabilized with 0.1% Triton X-100 in PBS. Cells were blocked in 1% BSA in PBS. anti-Myc antibody and anti-FlagM2 antibody (Sigma) were used as the primary antibodies at a dilution of 1:100 and the cells were washed three times in PBS. Mouse secondary antibodies conjugated to Alexa Fluor 488, and Alexa Fluor 532 (Invitrogen) was added and the DNA dye ToPro3 was used for nuclear localization. The stained cells were evaluated for localization using confocal microscopy (Leica TCS SPE). For each experiment, at least 300 cells were examined, and the images shown here represent the typical staining pattern observed for a majority of cells.

2.11. Cloning and preparation of recombinant proteins

A full-length mouse CaMKIV gene, a mouse Notch1 gene, and deletion mutants were constructed via PCR and inserted into either the mammalian expression vector p3XFlag-CMV (Sigma) or the bacterial expression vector pGEX4T-3 (Amersham Pharmacia). The Notch1-IC deletion mutants constructed for the present study were Notch1-IC-RAM-ANK (amino acid residues 1744 to 2110), Notch1-IC-RAM (amino acid residues 1744 to 1870), Notch1-IC-OPA (amino acid residues 2076 to 2369), and Notch1-IC-PEST (amino acid residues 2369 to 2531). The Myc-tagged Notch1-IC (pCS2-Myc-Notch1-IC), 4xCSL-Luc, Hes1-Luc and Hes5-Luc were a kind gifts from Raphael Kopan (Washington University, St. Louis, MO). Expression of the recombinant glutathione S-transferase (GST) fusion proteins within the transformed bacteria was induced using 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma). GST fusion proteins were purified with glutathione (GSH)-agarose beads (Sigma), in accordance with the manufacturer's instructions.

3. Results

3.1. CaMKIV and Notch1 are required for RANKL-induced osteoclast differentiation

RANKL is a ligand for osteoprotegerin and functions as a key factor for osteoblast differentiation and activation. To confirm the effect of RANKL on osteoclast differentiation, Raw264.7 cells were stimulated with recombinant RANKL. The number of large multinucleated tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts increased after RANKL treatment, suggesting that RANKL was sufficient to promote osteoclast differentiation (Fig. 1A). The CaMKIV protein is expressed in osteoclast precursor cells and activated after RANKL stimulation and thereby crucial for osteoblast differentiation and function [15]. To clarify the role of endogenous CaMKIV in the regulation of RANKL induced osteoclastogenesis, we assessed the effects of the CaMKIV inhibitor on the RANKL induced osteoclast differentiation. Coincident with previous report, inhibition of CaMKIV by the general CaMKs inhibitor KN-93 suppressed the formation of TRAP-positive multinucleated osteoclast (Fig. 1B). The role of endogenous Notch1 in the RANKL-induced osteoclast differentiation was further evaluated by the knockdown of Notch1 using N-[N-(3,5-difluorophenylacetyl-l-alanyl)]-S-phenylglycine t-butylester (DAPT), which is a gamma-secretase inhibitor. DAPT was shown to efficiently block the presenilin/gamma-secretase complex [53] and, consequently, it efficiently prevents activation of the Notch response [54,55]. We then assessed whether DAPT regulates RANKL-induced osteoclast differentiation. Inhibition of Notch1-IC generation using DAPT suppressed the formation of TRAP-positive multinucleated osteoclast (Fig. 1B). The role of endogenous Notch1 in RANKL-induced osteoclast differentiation was further evaluated by the knockdown of Notch1 using short interference Notch1. Unlike what was observed in the control short interference plasmid-transfected cells, the cells transfected with short interference Notch1 showed low expression levels of Notch1 (data not shown). TRAP-positive multinucleated cells, which were stimulated by RANKL, were down regulated through the knock-down of Notch1 expression (Fig. 1C). These results indicate that the level of CaMKIV and Notch1-IC might affect RANKL-induced osteoclast differentiation.

3.2. CaMKIV increases the transcriptional activity of Notch1 target genes

To determine whether CaMKIV is involved in regulating the transcriptional activation of Notch1 target genes, a reporter assay was performed with HEK293 cells using luciferase reporter genes. In this experiment, two types of luciferase reporter genes were evaluated under the control of the Hes1 promoter (Hes1-Luc) and the artificial four-time repeat of the CSL (4XCSL-Luc) binding sequence. We investigated the effect of CaMKIV on Notch1-IC transcriptional activity. The HEK293 cells were transfected with 4XCSL-Luc and either Notch1-IC or an empty vector. As expected, Notch1-IC-mediated transcriptional activity was increased in these samples. The Notch1-IC induced 4XCSL luciferase reporter activity was increased by cotransfection with CaMKIV in a dose-dependent manner (Fig. 2A). We also observed similar results using the Hes1-Luc reporter systems (Fig. 2B). To determine the effect of CaMKIV in the absence and presence of endogenous Notch1-IC, a transcription reporter assay was performed using PS1/2$^{+/+}$ and PS1/2$^{-/-}$ MEF cells. The transcriptional activity of Notch1-IC after CaMKIV treatment gradually increased in both PS1/2$^{+/+}$ and PS1/2$^{-/-}$ MEF cells. (Fig. 2C, D). These data demonstrate that CaMKIV could activate Notch1 reporter activity despite the absence of Notch1-IC generation, suggesting that CaMKIV, at least in part, could regulate Notch1 target gene expression in a Notch1-IC independent manner. However, the absolute value of three reporter systems was higher in PS1/2$^{+/+}$ MEF cells than PS1/2$^{-/-}$ MEF cells, suggesting that Notch1-IC and CaMKIV has a synergic effect on Notch1 reporter activity. To investigate whether the transcriptional activation of endogenous Notch1 target genes by CaMKIV occurs through a RBP-Jk dependent manner, a transcription reporter assay was performed using the dominant negative form of RBP-Jk (RBP-Jk-DN). In the luciferase reporter gene assay with PS1/2$^{-/-}$ cells, RBP-Jk DN was co-transfected with CaMKIV, and the effect of this transfection on the transcriptional activation of Notch1 target genes was then assessed using 4XCSL-Luc, Hes1-Luc. The transcriptional activity of Notch1-IC was increased by CaMKIV, and down-regulated by cotransfection with

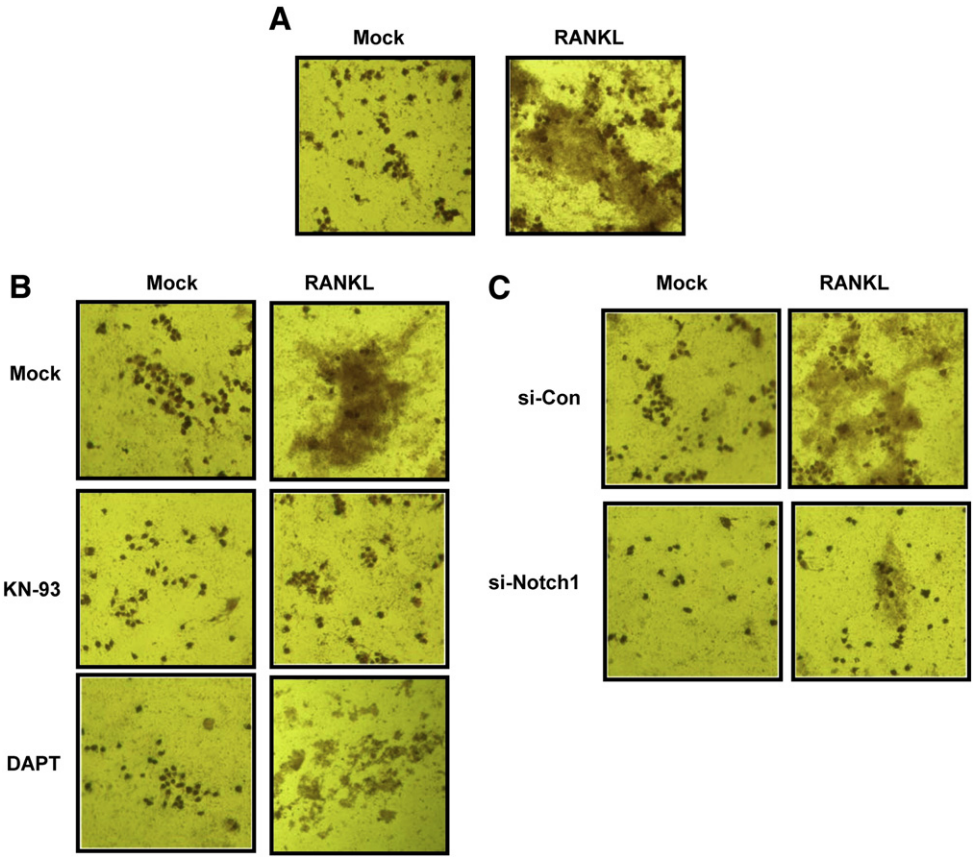

Fig. 1. CaMKIV and Notch1 are required for RANKL-induced osteoclast differentiation. (A) Raw264.7 cells were plated in 96-well plates and cultured in complete medium in the presence of 20 ng/ml of RANKL. (B) Raw264.7 cells were plated in 96-well plates and cultured in complete medium in the presence of 20 ng/ml of RANKL, 5 μM of KN93, and 5 μM of DAPT as indicated. (C) Raw264.7 cells were transfected with expression vectors for si-Control or si-Notch1 for 48 h and plated in 96-well plates and cultured in complete medium in the presence of 20 ng/ml of RANKL. (A-C) After 4–6 days, the cells were fixed with 3.8% paraformaldehyde and osteoclasts were detected by staining for Tartrate-resistant acid phosphatase (TRAP). All results were representative of at least three independent experiments.

CaMKIV and RBP-Jk DN (Fig. 2E). Similar results were also observed using the Hes5-Luc reporter systems (data not shown). These results indicated that the increase in the transcriptional activity of Notch1-IC by CaMKIV was independent of Notch1-IC and dependent on RBP-Jk.

3.3. Notch1-IC interacts directly with CaMKIV in intact cells

Given that our results suggest that Notch1-IC is a target of CaMKIV, we next investigated whether these two proteins interact physically in intact cells. In the in vitro binding studies, purified GST and GST-Notch1-IC proteins were immobilized on GSH-agarose. Cell lysates expressing Flag-CaMKIV were incubated either with immobilized GST or with GST-Notch1-IC on GSH-agarose. The interaction between GST-Notch1-IC and CaMKIV was detected on bead complexes (Fig. 3A). HEK293 cells were cotransfected with vectors encoding Flag-tagged CaMKIV and Myc-tagged Notch1-IC and were then subjected to coimmunoprecipitation analysis. Immunoblot analysis of Flag immunoprecipitates from the transfected cells with an anti-Myc antibody revealed that Flag-CaMKIV physically associated with Myc-Notch1-IC in the cells (Fig. 3B). We examined whether endogenous CaMKIV and Notch1-IC could interact in intact cells. Immunoblot analysis of the CaMKIV immunoprecipitates using an anti-Notch1-IC antibody indicated that endogenous CaMKIV and Notch1-IC were physically associated in intact cells (Fig. 3C). We also examined whether endogenous CaMKIV and Notch1-IC could interact during RANKL-induced osteoclast differentiation. Immunoblot analysis of the CaMKIV immunoprecipitates using an anti-Notch1-IC antibody indicated that endogenous CaMKIV and Notch1-IC were physically associated during RANKL-induced osteoclast differentiation in Raw264.7 cells (Fig. 3D). Immunofluorescence staining was performed to verify the subcellular localization of CaMKIV and Notch1-IC. HEK293 cells were transfected with vector encoding Myc-tagged Notch1-IC, Flag-tagged CaMKIV. Notch1-IC is localized mainly in the nucleus, and CaMKIV is distributed in both the nucleus and cytoplasm. We then attempted to assess the subcellular localization of CaMKIV within HEK293 cells in the presence of Notch1-IC. When the cells were coexpressed with Notch1-IC, CaMKIV had slightly accumulated in the nucleus (Fig. 3E). These data indicate that Notch1-IC and CaMKIV may exist within the same compartment. Notch1-IC has a CDC 10/Ankyrin domain that includes a RAM domain and seven Ankyrin repeats, an OPA domain and a PEST domain within its structure. We investigated which of these domains might be involved in the interaction between Notch1-IC and CaMKIV. We used a variety of Flag-tagged Notch1 deletion mutant: Notch1-IC-N (CDC 10/ANK domain), Notch1-IC-ΔNΔC (OPA domain), or Notch1-IC-C (PEST domain). We performed coimmunoprecipitation using three Notch1-IC deletion mutants and endogenous CaMKIV. Our results show that CaMKIV bound to the Notch1-IC-N mutant that contained RAM-ANK but not to the mutants that contained either the OPA or PEST domains (Fig. 3F). In these experiments, we demonstrated that Notch1-IC physically interacts with CaMKII via the N-terminal CDC 10/Ankyrin domain.

3.4. CaMKIV up-regulates the Notch1-IC protein stability

To observe the effects of CaMKIV on the molecular interactions between Notch1-IC and RBP-Jk, coimmunoprecipitation was performed by cotransfection of Myc-Notch1-IC, HA-RBP-Jk, and Flag-CaMKIV.

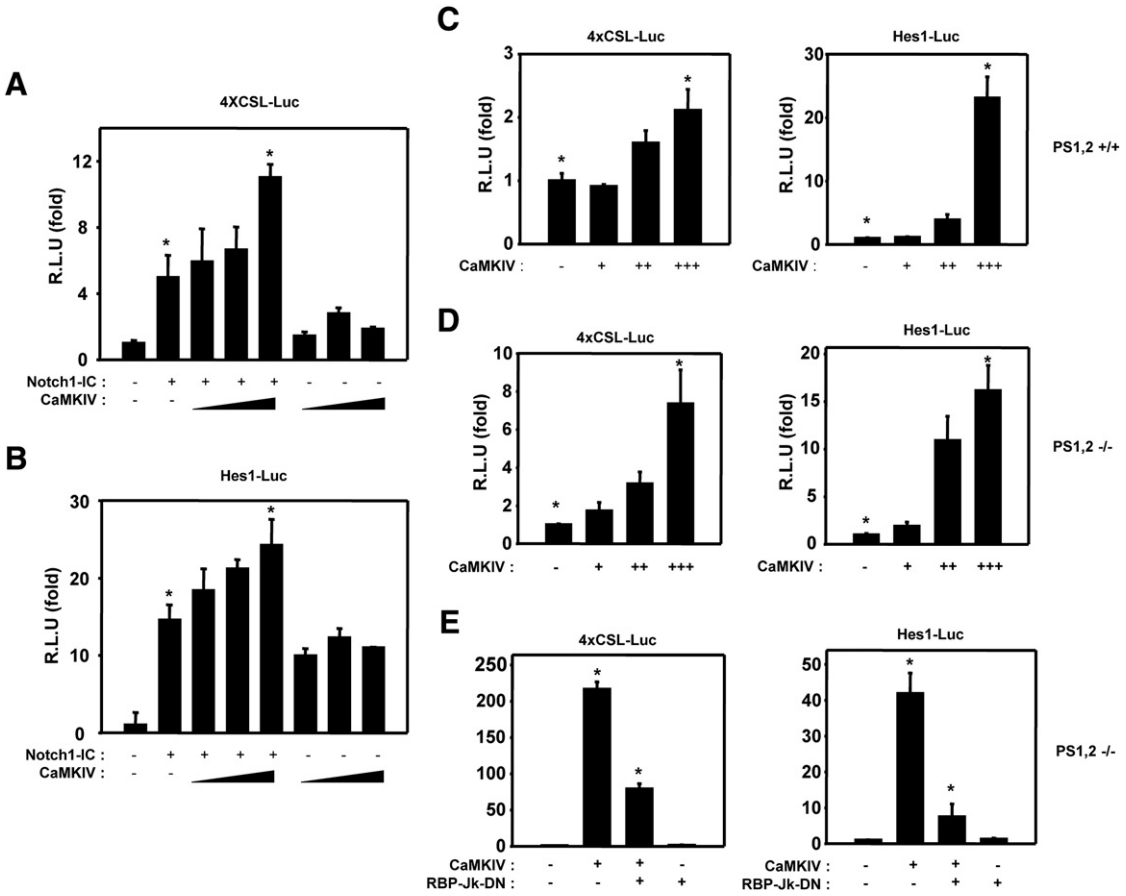

Fig. 2. CaMKIV increases the transcriptional activity of Notch1 target genes. (A) HEK293 cells were transfected with expression vectors for 4xCSL-Luc and β-galactosidase, along with Notch1-IC and CaMKIV, as indicated. (B) HEK293 cells were transfected with expression vectors for Hes1-Luc and β-galactosidase, along with Notch1-IC and CaMKIV, as indicated. (C, D) PS1/2$^{+/+}$ and PS1/2$^{-/-}$ MEF cells were transfected with expression vectors for 4xCSL-Luc and Hes1-Luc, and β-galactosidase, along with CaMKIV as indicated. (E) PS1/2$^{-/-}$ cells were transfected with expression vectors for 4xCSL-Luc and Hes1-Luc, and β-galactosidase, along with CaMKIV and the dominant negative form of RBP-Jk (RBP-Jk-DN). (A–E) After 48 h of transfection, the cells were lysed and the luciferase activity determined. ANOVA, $^{*}P<0.001$.

Notch1-IC and RBP-Jk were coimmunoprecipitated, but when they were cotransfected with CaMKIV, the band intensity corresponding to the interaction between Notch1-IC and RBP-Jk was enhanced (Fig. 4A). Immunoprecipitation was performed on cell lysates using an anti-HA antibody and immunoblotting was performed with the anti-Myc antibody. Surprisingly, in the cell lysate immunoblot, the level of Notch1-IC protein was up-regulated upon cotransfection with CaMKIV (Fig. 4A), which shows that CaMKIV may regulate the steady state level of Notch1-IC protein.

Next, HEK293 cells were subjected to western blot analysis to determine whether CaMKIV plays a role in the regulation of the Notch1-IC protein level. When HEK293 cells were cotransfected with Notch1-IC and CaMKIV, the Notch1-IC protein level was found to be increased by CaMKIV treatment in a dose-dependent manner (Fig. 4B). To determine whether the kinase activity of CaMKIV plays a role in the regulation of the Notch1-IC protein level, cells were cotransfected with Myc-tagged Notch1-IC, Flag-tagged CaMKIV and Flag-tagged CaMKIV K75E. The Notch1-IC protein level was shown to increase upon cotransfection with CaMKIV but was decreased upon cotransfection with the inactive form of CaMKIV (CaMKIV K75E) (Fig. 4C). This result showed that the kinase activity of CaMKIV is essential for the regulation of the Notch1-IC protein level. We determined the protein stability of Notch1-IC using cycloheximide, an inhibitor of protein translation, in the presence and absence of CaMKIV. After cycloheximide treatment, the amount of Notch1-IC was analyzed by immunoblotting. The level of Notch1-IC protein gradually decreased, where approximately half of the protein was degraded after 1 h in the absence of CaMKIV (Fig. 4D). Upon cycloheximide treatment, the level of Notch1-IC protein slowly decreased, where approximately half of the protein was degraded after more than 6 h in the presence of CaMKIV (Fig. 4D). This result demonstrated that Notch1-IC was slowly turned over in the presence of CaMKIV.

3.5. CaMKIV positively regulates Notch1 signaling through an E3 ligase, Fbw7

Given that our data suggest that CaMKIV regulates Notch1-IC protein stability. We next investigated whether CaMKIV regulates Notch1 protein stability through Fbw7. HEK293 cells were cotransfected with vectors coding for Myc-tagged Notch1-IC, Flag-tagged CaMKIV, and His-tagged Ubiquitin, and were then subjected to ubiquitylation analysis. Immunoblot analysis of the His immunoprecipitates from the transfected cells using an anti-Myc antibody showed that CaMKIV reduces the ubiquitylation of Notch1-IC in a dose-dependent manner (Fig. 5A). Generally, the ubiquitylation of proteins leads to their rapid degradation, and Notch1-IC is degraded by the ubiquitin-proteasome system in the nucleus. Notch1-IC is ubiquitinated by the F-box protein Fbw7/Sel-10/hCdc4/Ago, which was first isolated during the genetic screening of negative regulators of Notch in *C. elegans* [56]. We expected that CaMKIV might act as a mediator for the positive regulation of Notch1-IC by Fbw7. When Fbw7 was cotransfected with Notch1-IC and CaMKIV, the transcriptional activation of Notch1 was increased

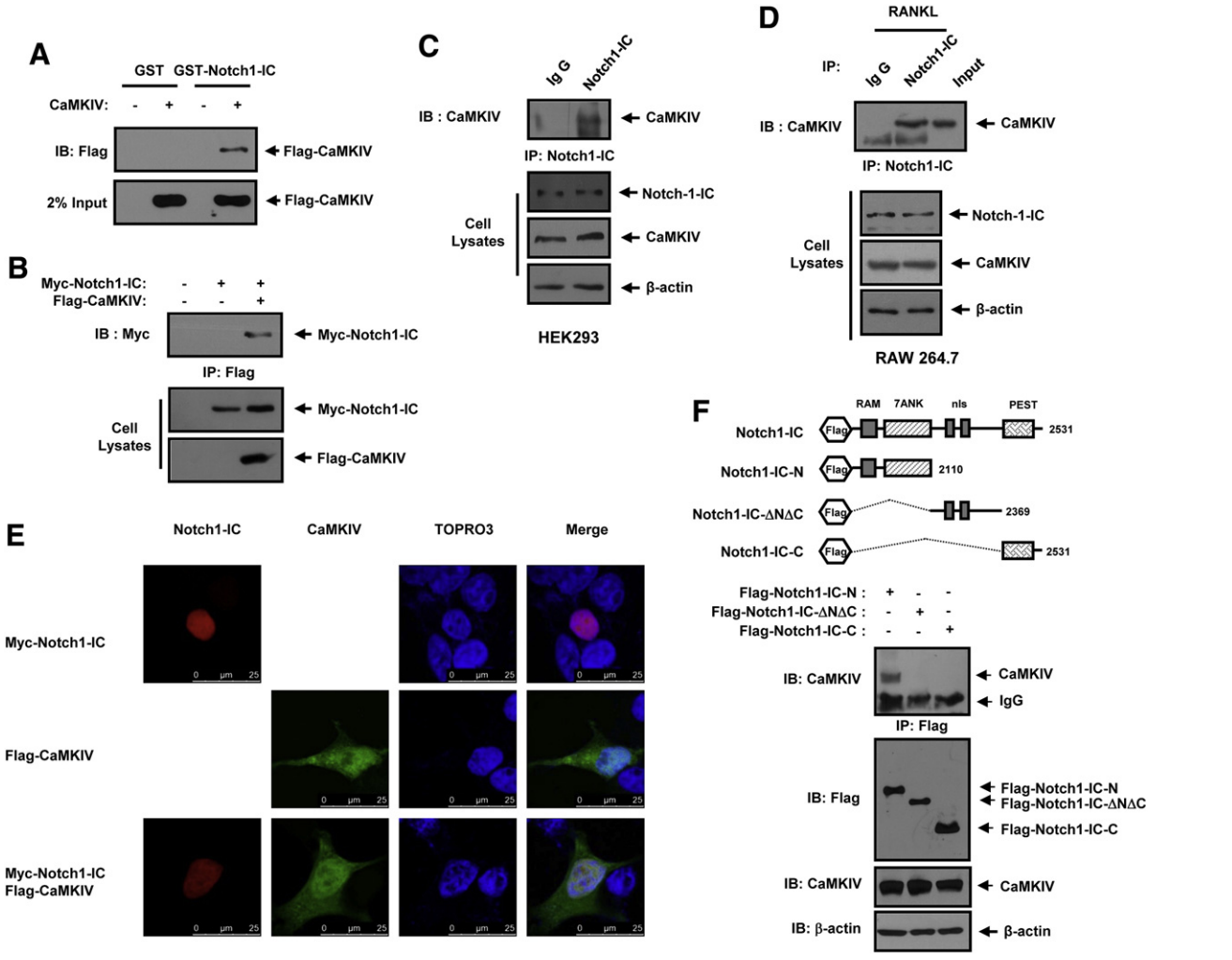

Fig. 3. Notch1-IC interacts directly with CaMKIV in intact cells. (A) The Flag-CaMKIV expressing cell lysates were subjected to GST pull-down experiments with immobilized GST, GST-Notch1-IC. Proteins bound to GST, GST-Notch1-IC were analyzed via immunoblotting with anti-Flag antibody. (B) HEK293 cells were transfected for 48 h with expression vectors encoding Myc-Notch1-IC and Flag-CaMKIV, as indicated. After transfection, the cell lysates were subjected to immunoprecipitation with anti-Flag antibody. The immunoprecipitates were then immunoblotted with anti-Myc antibody, respectively. (C) HEK293 cells were lysed and subjected to immunoprecipitation with IgG and anti-Notch1-IC antibodies, as indicated. The immunoprecipitates were immunoblotted with anti-CaMKIV antibody. (D) Raw264.7 cells were cultured in complete medium in the presence of 20 ng/ml of RANKL. After 2 days, the cells were lysed and subjected to immunoprecipitation with IgG and anti-Notch1-IC antibodies, as indicated. The immunoprecipitates were immunoblotted with anti-CaMKIV antibody. (E) The cells were fixed and CaMKIV and Notch1-IC were stained with Alexa Fluor 488 (green) and Alexa Fluor 555 (red) and examined by confocal microscopy. White Scale bar, 25 μm. (F) HEK293 cells were transfected for 48 h with the indicated combinations of expression vectors for Flag-Notch1-IC, Flag-Notch1-IC-N, Flag-Notch1-IC-ΔNΔC, Flag-Notch1-IC-C. The cell lysates then were subjected to immunoprecipitation with anti-Flag antibody, and the resulting precipitates were subjected to immunoblotting analysis with anti-CaMKIV antibody.

(Fig. 5B). These results revealed that CaMKIV contributed to Fbw7-independent Notch1-IC degradation in Notch1-IC reporter activity (Fig. 5B). To determine the effects of CaMKIV on the molecular interactions between the constitutively active form of GFP-tagged Notch1 (ΔEN1-GFP) and Fbw7, coimmunoprecipitation was performed by cotransfection of ΔEN1-GFP, Myc-Fbw7, and Flag-CaMKIV. Notch1-IC and Fbw7 coimmunoprecipitated but when they were cotransfected with CaMKIV, a band of Notch1-IC that interacted with Fbw7 decreased (Fig. 5C). HEK293 cells were cotransfected with vectors coding for Myc-tagged Notch1-IC, HA-tagged ubiquitin, GFP-tagged Fbw7, and Flag-tagged CaMKIV, and were then subjected to ubiquitylation analysis. Immunoblot analysis of the Myc immunoprecipitates from the transfected cells with an anti-HA antibody showed that Fbw7 facilitated the ubiquitylation of Notch1-IC, and CaMKIV decreases Fbw7-dependent Notch1-IC ubiquitylation (Fig. 5D). These results suggest that CaMKIV improves Notch1-IC protein stability by inhibiting the interaction between Fbw7 and Notch1-IC.

3.6. CaMKIV-mediated phosphorylation of Notch1-IC inhibits the degradation of Notch1-IC

Given that our results suggest that Notch1 is a target of CaMKIV, we next investigated whether CaMKIV phosphorylates Notch1-IC. CaMKIV preferentially phosphorylates substrate protein serine and threonine residues that lie in an R-X-X-S/T. We conducted a kinase assay with Flag-CaMKIV and purified GST-Notch1-IC. The Flag-CaMKIV immunocomplexes prepared from cells catalyzed the phosphorylation of purified recombinant GST-Notch1-IC, but not GST-Fbw7 (Fig. 6A). In silico studies have shown that the Notch1-IC contains possible conserved serine residues in vertebrates; these serine residues are accessible and located in the Ankyrin domain, and in front of the C-terminus of Notch1-IC. This motif is present at amino acids 2060–2063, and 2170–2173 of Notch1-IC. Furthermore, via site-directed mutagenesis, we determined that the replacement of serine 2063, serine 2173, and serine 2063/2173 of Notch1-IC with alanine, caused a reduction in the in

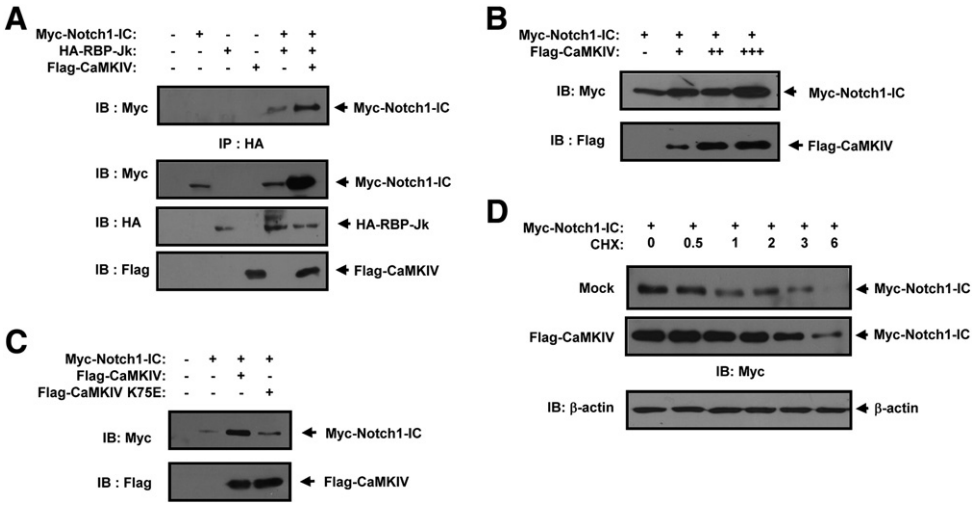

Fig. 4. CaMKIV up-regulates the Notch1-IC protein stability. (A) HEK293 cells were transfected for 48 h with expression vectors encoding the indicated combinations of Myc-Notch1-IC, HA-RBP-Jk, and Flag-CaMKIV. Cell lysates were subjected to immunoprecipitation with anti-HA antibody and the immunoprecipitates were immunoblotted with anti-Myc antibody. (B) HEK293 cells were transfected for 48 h with expression vectors encoding the indicated combinations of Myc-Notch1-IC and Flag-CaMKIV. (C) HEK293 cells were transfected for 48 h with expression vectors encoding the indicated combinations of Myc-Notch1-IC , Flag-CaMKIV, and Flag-CaMKIV K75E (catalytic inactive form). (D) HEK293 cells were transfected with expression vectors encoding Myc-Notch1-IC and Flag-CaMKIV in the indicated combinations. HEK293 cells were treated with 100 μM cycloheximide (CHX) for the indicated periods of time, and the cell lysates were immunoblotted with anti-Myc antibodies.

vitro phosphorylation of the recombinant protein by the CaMKIV immunoprecipitates (Fig. 6B). Moreover, we demonstrated that the Notch1-IC mutant (S2173A) is resistant to CaMKIV-induced inhibition of Notch1-IC degradation, which implies that the CaMKIV-induced phosphorylation of Notch1-IC is crucial for the inhibition of the Notch1 protein degradation (Fig. 6C). We then attempted to characterize the involvement of phosphorylation in the poly-ubiquitylation of Notch1-IC by Fbw7. HEK293 cells were co-transfected with vectors coding for Myc-tagged Notch1-IC,

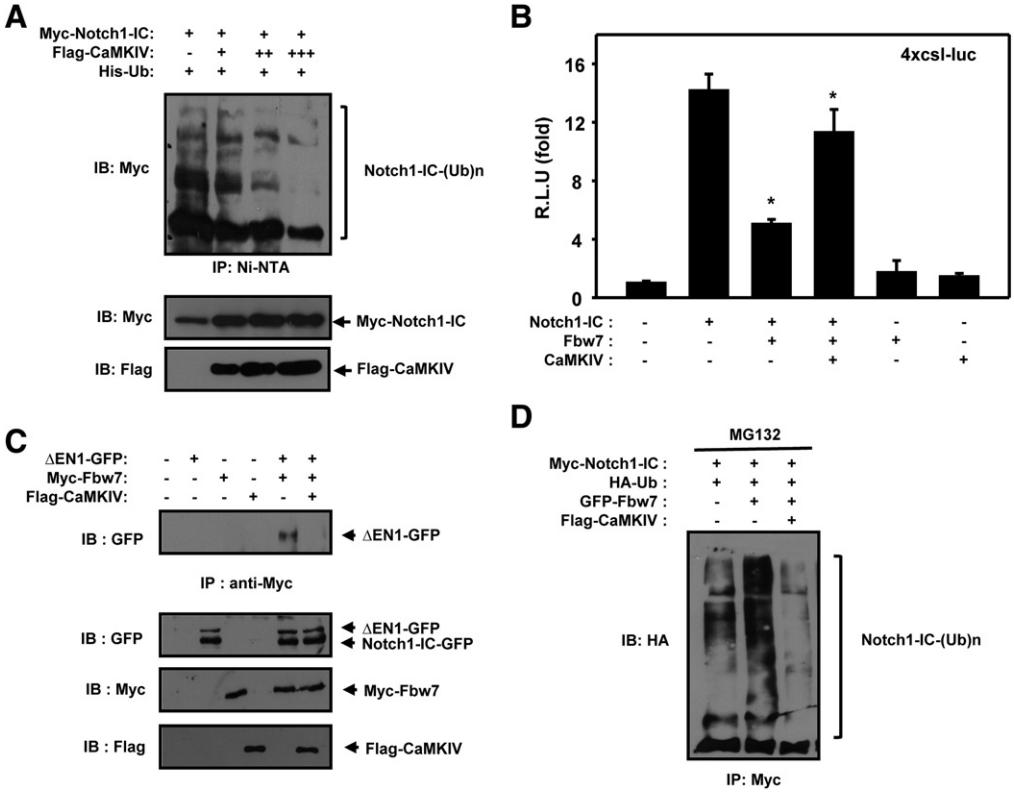

Fig. 5. CaMKIV positively regulates Notch1 signaling via an E3 ligase, Fbw7. (A) HEK293 cells were transfected with expression vectors encoding Myc-Notch1-IC, Flag-CaMKIV, and His-Ub as indicated. After 48 h of transfection, the cell lysates were incubated with Ni^{2+}−NTA agarose beads to precipitate Histidinated (hence ubiquitinated) proteins. The immunoprecipitates were then immunoblotted with an anti-Myc antibody. (B) HEK293 cells were transfected with expression vectors encoding Notch1-IC, CaMKIV, and Fbw7 with the 4×CSL-Luc reporter in the indicated combinations. After 48 h of transfection, the cells were lysed and the luciferase activity determined. ANOVA, $^{*}P<0.001$. (C) HEK293 cells were transfected for 48 h with expression vectors encoding the indicated combinations of ΔEN1-GFP and Myc-RBP-Jk, and Flag-CaMKIV. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody, and the immunoprecipitates were immunoblotted with anti-GFP antibody. (D) HEK293 cells were transfected with expression vectors encoding Myc-Notch1-IC, HA-Ub, GFP-Fbw7, and Flag-CaMKIV as indicated. After 42 h of transfection, the cells were treated with MG132 (5 μM) for 6 h as indicated, and the cell lysates were subjected to immunoprecipitation with anti-Myc antibodies. The immunoprecipitates were then immunoblotted with an anti-HA antibody.

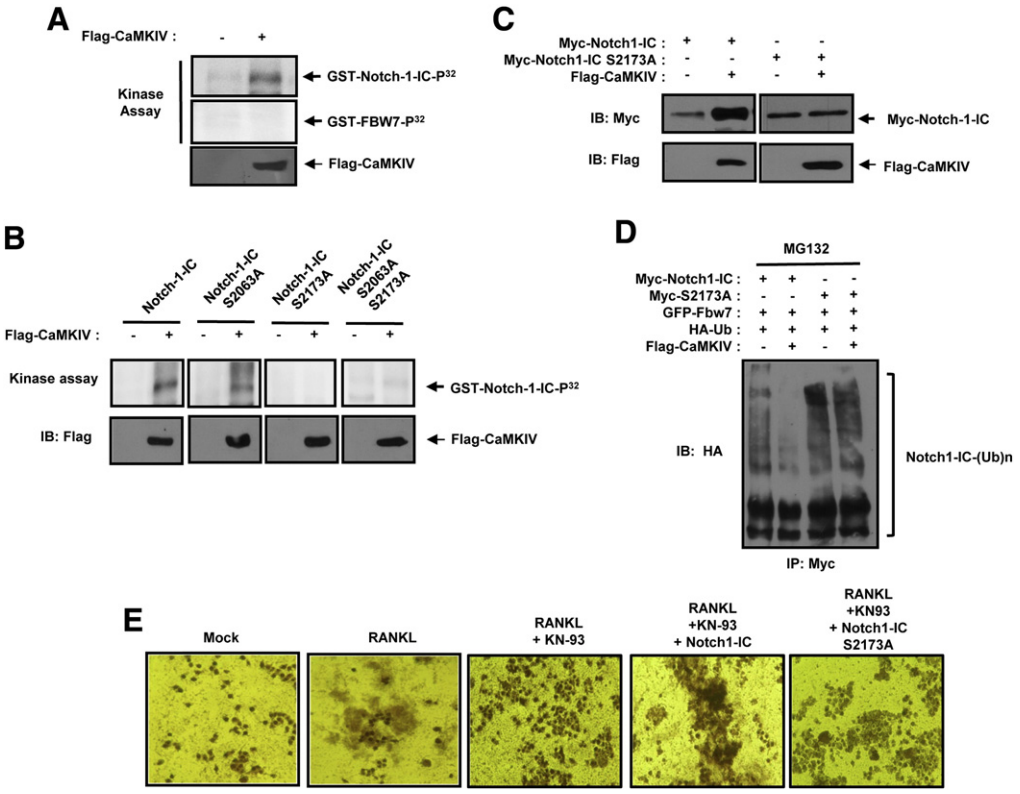

Fig. 6. CaMKIV-mediated phosphorylation of Notch1-IC inhibits the degradation of Notch1-IC. (A) HEK293 cells were transfected with expression vectors encoding Flag-CaMKIV as indicated. After 48 h of transfection, the cell lysates were subjected to immunoprecipitation with an anti-Flag antibody, and the resulting precipitates were examined for CaMKIV kinase activity by an immune complex kinase assay using GST-Notch1-IC. (B) HEK293 cells were transfected with expression vectors encoding Flag-CaMKIV as indicated. After 48 h of transfection, the cell lysates were subjected to immunoprecipitation with an anti-Flag antibody, and the resulting precipitates were examined for CaMKIV kinase activity by an immune complex kinase assay using GST-Notch1-IC, GST-Notch1-IC (S2063A), GST-Notch1-IC (S2173A), and GST-Notch1-IC (S2063A/S2173A). (C) HEK293 cells were transfected with expression vectors encoding Myc-Notch1-IC, Myc-Notch1-IC (S2173A), and Flag-CaMKIV as indicated. After 48 h of transfection, the cell lysates were subjected to immunoblotting with an anti-Myc and anti-Flag antibody. (D) HEK293 cells were transfected with expression vectors encoding Myc-Notch1-IC, Myc-Notch1-IC (S2173A), HA-Ub, GFP-Fbw7, and Flag-CaMKIV as indicated. After 42 h of transfection, the cells were treated with MG132 (5 μM) for 6 h as indicated, and the cell lysates were subjected to immunoprecipitation with anti-Myc antibodies. The immunoprecipitates were then immunoblotted with an anti-HA antibody. (E) Raw264.7 cells were transfected with expression vectors for Notch1-IC, Notch1-IC (S2173A) and treated CaMKIV inhibitor KN-93 as indicated. After 24 h, cells were cultured in complete medium in the presence of 20 ng/ml of RANKL. After 4–5 days, the cells were fixed with 3.8% paraformaldehyde and osteoclasts were detected by staining for Tartrate-resistant acid phosphatase (TRAP).

Myc-tagged Notch1-IC (S2173A), HA-tagged Ubiquitin, GFP-tagged Fbw7, and FLAG-tagged CaMKIV, and were then subjected to ubiquitylation analysis. Immunoblot analysis of the anti-Myc antibody immunoprecipitates from the transfected cells with an anti-HA antibody showed that CaMKIV and the wild type Notch1-IC reduced the ubiquitylation of Notch1-IC and that mutant Notch1-IC increased the ubiquitylation of Notch1-IC (Fig. 6D). These results showed that the phosphorylation of Notch1-IC by CaMKIV is crucial to its augmentation of Notch1-IC stability. To determine the effect of phosphorylation of Notch1-IC by CaMKIV on osteoclast differentiation, we performed TRAP staining. RANKL-induced osteoclast differentiation was reduced by KN93, and recovered by over-expression of Notch1-IC, but not recovered by over-expression of Notch1-IC (S2173A) (Fig. 6E). These results showed that phosphorylation of Notch1-IC by CaMKIV plays critical role in the RANKL-induced osteoclast differentiation.

4. Discussion

RANKL stimulates osteoclast differentiation in uncommitted cells of the monocyte-macrophage lineage by interacting with its cell-surface receptor, receptor activator of NF-κB (RANK) [1,2]. Recent reports have shown that the CaMKIV protein was expressed in osteoclast precursor cells and activated after RANKL stimulation In addition CaMKIV, has been shown to be crucial in regulating osteoclast differentiation and acquisition of bone-resorbing activity [15–17]. Recent reports have shown that RANKL induces mRNA and protein expression of Hes1, a downstream target gene of the Notch1 signaling [22,24]. However, the mechanism behind this regulation has not been clearly established. In this study, we demonstrated that RANKL-induced CaMKIV enhanced the transcriptional activity of Notch1-IC, and the kinase activity of CaMKIV was essential for this function. CaMKIV augmented Notch1-IC protein stability by suppressing the proteasomal degradation of Notch1-IC proteins, which induced osteoclast differentiation.

Although multiple lines of evidence indicate that Notch signaling dysfunction results in bone disease, the specific role for Notch in regulating osteoclastogenesis is still controversial. The possible involvement of Notch signaling in osteoclastogenesis was examined through in vitro and in vivo studies. Immobilized Delta-1 and constitutive active form of Notch1 caused increased expression of RANKL and OPG genes and strongly inhibited M-CSF gene expression, which negatively regulates osteoclastogenesis [47]. Osteoblast specific deletion of both Notch1 and Notch2, and specific deletion of presenilin 1 and presenilin 2, results in the induction of RANKL expression and suppression of OPG expression [57,58]. On the other hand, Notch 2, Jagged 1 and Hes 1 are up-regulated during RANKL-induced osteoclastogenesis [22–24]. Gamma-secretase inhibitor or Notch 2 short hairpin RNA suppresses RANKL-induced osteoclastogenesis [23]. These results imply that Notch signaling positively regulates osteoclastogenesis. Our results demonstrated that a gamma-secretase inhibitor or Notch1 short hairpin RNA suppressed RANKL-induced osteoclastogenesis, suggesting that

Notch1 signaling may also be involved in the regulation of osteoclast differentiation. We also found that the CaMKs inhibitor inhibits RANKL-induced osteoclast differentiation. These results suggest that CaMKIV and Notch1 signaling play a critical role in the regulation of osteoclastogenesis.

Several recent studies have shown that ubiquitination mediated protein degradation plays a role in the regulation of Notch1 signaling through degradation of Notch1 ligands, Notch1 itself, and RBP-Jk [48,51,59,60]. This study provides the first evidence that CAMKIV is an endogenous positive regulator of the Notch signaling pathway by preventing the degradation of the Notch1-IC proteins. When CAMKIV activity was enhanced by RANKL, the Notch1 transcriptional activity was significantly increased in HEK293 cells. On the other hand, when CAMKIV was overexpressed, the Notch1 transcriptional activity was markedly increased in a dose-dependent manner. Our findings provide unequivocal evidence that CAMKIV is an endogenous signaling agent that up-regulates Notch1-IC transcriptional activity. Our result showed that *in vivo* and *in vitro* binding between CaMKIV and Notch1-IC, and the N-terminal domain of Notch1-IC, was critical to the binding of CaMKIV. When Notch1-IC interacts with RBP-Jk, co-repressors dissociated, and formed a transcriptional active complex that activated RBP-Jk-dependent transcription [30]. Our result showed that interaction between Notch1-IC and RBP-Jk was enhanced by CaMKIV due to improved Notch1-IC protein stability.

Our findings reveal that CaMKIV kinase activity plays a crucial role in the proteasomal degradation of Notch1-IC. Notch1-IC and Fbw7 contain a conserved consensus site that can bind to CaMKIV, and Notch1-IC is phosphorylated by CaMKIV. Furthermore, we demonstrated that CaMKIV mediated Notch1-IC phosphorylation of serine 2173 resulted in an increase of the Notch1-IC protein level and decrease in the ubiquitylation of Notch1-IC. Therefore, phosphorylation of Notch1-IC by CaMKIV is a possible mechanism that regulates Notch1-IC signaling. However, in our earlier report, we showed that phosphorylation on S2173 of Notch1-IC by ILK decreased the stability of Notch1-IC through Fbw7 [48]. This raised the question of how does the same phosphorylation site have two different functions? We assumed that each signaling event occurred through different co-factors. These different binding partners might have a different effect on the same protein. This is still an unknown mechanism, and must be intensively investigated. This study suggests a novel role of CaMKIV in increasing Notch signaling. Because CaMKIV activation increases Notch1-IC phosphorylation, it is expected to reduce Notch1-IC ubiquitylation and degradation.

A recent report has shown that the CaMKIV protein was expressed in osteoclast precursor cells and activated after RANKL stimulation. In addition, CaMKIV was shown to be crucial in regulating osteoclast differentiation, and acquisition of bone-resorbing activity [15,16]. We have shown that RANKL induced osteoclast differentiation was reduced by KN93, DAPT. Notch signaling negatively or positively regulates osteoclast differentiation [22,23,29,44]. Especially, the Notch-dependent Hes1 mRNA and protein expression level was increased in RANKL stimulated osteoclast cells [22,23]. Our data demonstrated that RANKL induced osteoclast differentiation was suppressed by KN93, and recovered by Notch1-IC. However, the phosphorylation deficient form of Notch1-IC could not rescue osteoclast differentiation, suggesting that RANKL-induced CaMKIV mediated phosphorylation of Notch1-IC is critical for osteoclast differentiation. Previous reports have shown that activated Notch signaling enhance the expression of NFATc1

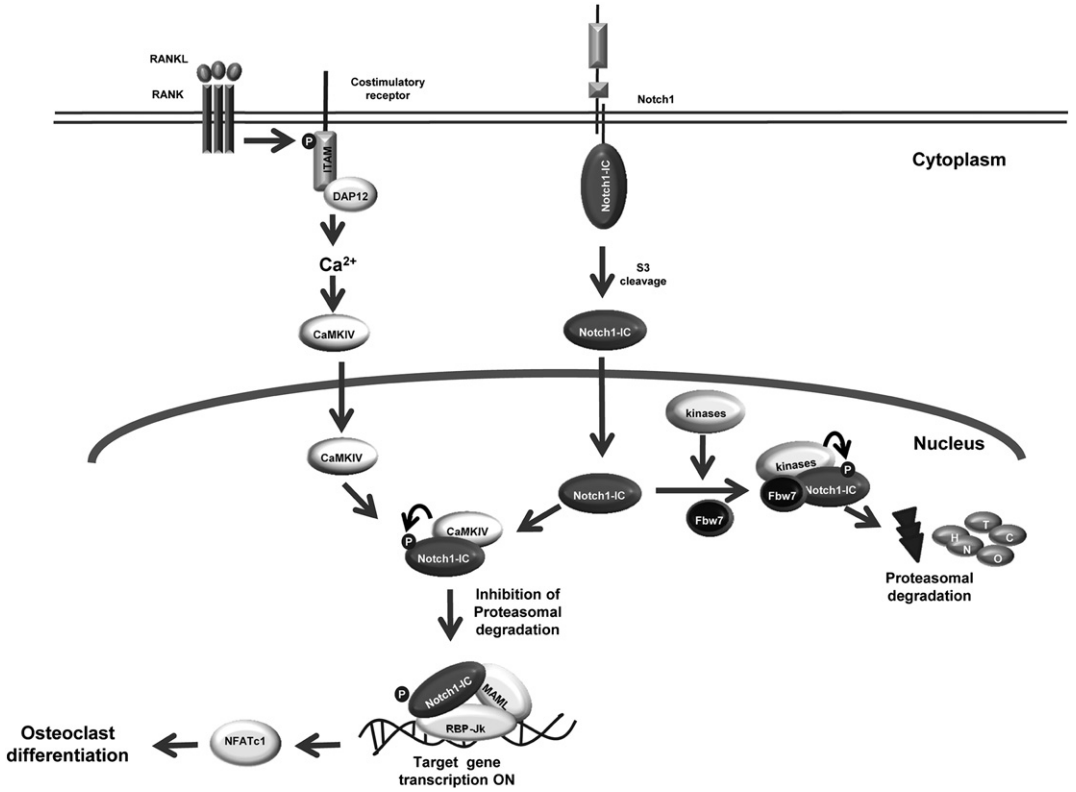

Fig. 7. Schematic diagram for regulation of osteoclast differentiation by CaMKIV and Notch1-IC. Notch1 proteins are activated by a series of cleavages that release its intracellular domain (Notch1-IC), followed by its nuclear translocation. The nuclear translocation of Notch1-IC recruits the transcription activator complex and drives transcription of target genes. The stability of Notch1-IC is regulated through its PEST (polypeptide enriched in proline, glutamate, serine and threonine) domain. Binding of cyclin-dependent kinase 8 (CDK8) and other kinases phosphorylate the Notch1-IC and facilitate Fbw7 dependent ubiquitylation and proteasomal degradation. After stimulation of RANKL, RANK cooperates with costimulatory receptors to trigger an increase in Ca^{2+} concentration. An increased intracellular Ca^{2+} concentration stimulates Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV). CaMKIV interacts with Notch1-IC and phosphorylates Notch1-IC. Notch signaling enhances NFATc1 promoter activity and expression and promotes osteoclastogenesis [23]. Surprisingly, phosphorylation of Notch1-IC by CaMKIV protects against the degradation of Notch1-IC via Fbw7 and thereby increases osteoclast differentiation.

through RBP-Jk dependent mechanism [23]. These results suggest that Notch1-IC acts as a positive regulator of osteoclast differentiation. Therefore, our results demonstrated that CaMKIV increase RANKL-induced osteoclast differentiation by increasing Notch1-IC stability.

In summary, we have found that phosphorylation of Notch1-IC by CaMKIV inhibits the proteasomal degradation of Notch1-IC through Fbw7 (Fig. 7). The findings of this study indicate that up-regulated Notch1-IC by CaMKIV accelerates osteoclast differentiation, and inhibitors of CaMKIV and Notch1-IC could be therapeutic targets of bone diseases, such as osteoporosis.

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