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Calpain modulates cyclin-dependent kinase inhibitor 1B (p27^{Kip1}) in cells of the osteoblast lineage

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Abstract

The ubiquitously expressed calpains-1 and -2 belong to a family of calcium-dependent intracellular cysteine proteases. Both calpains are heterodimers consisting of a large catalytic subunit and a small regulatory subunit encoded by the gene *Capn4*. Ablation of the calpain small subunit eliminates calpain activity and leads to embryonic lethality. We previously created osteoblast-specific *Capn4* knockout mice to investigate a physiological role for the calpain small subunit in cells of the osteoblast lineage. Deletion of *Capn4* reduced trabecular and cortical bone mainly due to impaired proliferation and differentiation of cells of the osteoblast lineage. To further investigate an underlying mechanism by which osteoblast-specific *Capn4* knockout mice develop an osteoporotic bone phenotype, we established osteoblastic cell lines stably expressing either *control* or *Capn4* RNA interference (RNAi) for this study. *Capn4*-knockdown cells showed reduced cell proliferation, accumulation of total and phosphorylated cyclin-dependent kinase inhibitor 1B (p27^{Kip1}) on Serine (S) 10, reduced phosphorylation of retinoblastoma protein (Rb) on Threonine (T) 821. Moreover, ablation of *Capn4* increased 27^{Kip1} mRNA levels likely due to stabilized binding of Akt to protein phosphatase 2A (PP2A), which presumably results in reduced phosphorylation of Akt on S473 and forkhead Box O (FoxO) 3A on T32. Collectively, calpain regulates cell proliferative function by modulating both transcription and degradation of p27^{Kip1} in osteoblasts. In conclusion, calpain is a critical modulator for regulation of p27^{Kip1} in cells of the osteoblast lineage.

Keywords

calpain; calcium; osteoblast; osteoporosis

Introduction

Osteoporosis is a bone disease that leads to an increased risk of bone fracture, thus may significantly affect life expectancy and quality of life. Osteoporotic bone is characterized by low bone mineral density, disrupted bone microarchitecture, and abnormal amount and composition of bone matrix proteins [1].

Calpains are calcium-dependent intracellular cysteine proteases. Both calpains-1 and -2 consist of an 80-kDa large catalytic subunit encoded by the genes *Capn1* and *Capn2*, respectively, and a 28-kDa common small regulatory subunit encoded by the gene *Capn4*

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Disclosures

None

[2]. Deletion of the calpain small subunit eliminates calpain activity and leads to embryonic lethality, suggesting an essential role of *Capn4* during embryonic development [3].

Several lines of evidence have suggested that calpain plays a crucial role in parathyroid hormone (PTH)-mediated cellular functions in osteoblasts; PTH induces osteoblastic retraction likely caused by a calpain-dependent proteolytic modification of cytoskeletal organization [4–7]. PTH also stimulates activities of calpains-1 and -2 [5, 6] and pretreatment of MC3T3-E1 osteoblastic cells with calpain inhibitors blocks PTH-stimulated cell proliferation and differentiation [7, 8].

We previously showed that the calpain small subunit binds to the intracellular C-terminal tail of the receptor for PTH and PTH-related peptide (PTHrP) (PTH1R), and critically modulates ligand-mediated PTH1R signaling [9]. To investigate a role of the calpain small subunit in cells of the osteoblast lineage *in vivo*, we then generated osteoblast-specific *Capn4* knockout mice. Deletion of *Capn4* exhibited reduced trabecular and cortical bone mainly due to reduced proliferation and differentiation of cells of the osteoblast lineage [10]. However, we failed to provide the underlying molecular mechanism by which deletion of the calpain small subunit modulates osteoblast function. In our more recent study using chondrocyte-specific *Capn4* knockout mice, we found that deletion of *Capn4* reduces cell proliferation, at least in part, through accumulation of p27^{Kip1} protein in cells of the chondrocyte lineage [11]. Therefore, to further test our hypothesis that calpain also critically modulates p27^{Kip1} in cells of the osteoblast lineage, we established osteoblast cell lines stably expressing either *control* or *Capn4* RNAi and examined whether and how knockdown of *Capn4* modulates p27^{Kip1} protein levels in cells of the osteoblast lineage *in vitro* in this study.

Materials and Methods

Cell lines and antibodies

Mouse osteoblastic cells, MC3T3 Subclone4 (MC4) (ATCC, Manassas, VA), stably expressing either *control* or *Capn4* microRNAs are established as we described previously [11]. *Control* and *Capn4* microRNAs were commercially available from Invitrogen Corp. (Carlsbad, CA). Four monoclonal cell lines each were established, and knockdown of the calpain small subunit was assessed by calpain activity assay as we described previously [10]. MC4 stable cell lines were cultured in α minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin-streptomycin (Invitrogen). Mouse p27^{Kip1} siRNAs (Invitrogen) were also used to knockdown p27^{Kip1} protein as we described previously [11]. Antibodies against PP2A, total (t)-Akt, t-FoxO3A, phosphorylated (p)-Akt (S473), p-FoxO3A (T32) (Cell Signaling Technology Inc., Danvers, MA), cyclin D, cyclin E, p27^{Kip1}, cyclin-dependent kinase 2 (cdk2) and 4 (cdk4) (Santa Cruz Biotechnology, Santa Cruz, CA), p-retinoblastoma protein (Rb) (T821) (Invitrogen), and p-p27^{Kip1} (S10) (Abcam Inc., Cambridge, MA) were purchased.

Osteoblast apoptosis assay *in vitro*

To assess apoptosis of established cell lines, cells were stained with Annexin V-phycoerythrin and 7-amino actinomycin D using Guava PCA Nexin kit and analyzed by Guava Personal Cytometer (Guava Technology Inc., Hayward, CA) as described previously [10, 11].

Flow cytometry

Cell cycle analysis was performed using flow cytometric analysis as we described previously [10]. MC4 cells stably expressing control or *Capn4* microRNA were serum starved (1% FBS) for 2 days and then stimulated by serum replacement (10% FBS) for 10 h. Cells were labeled with 10 μ M bromodeoxyuridine (BrdU) for the last 1 h, harvested, and stained with anti-BrdU fluorescein isothiocyanate antibody for BrdU and propidium iodine for DNA as recommended by the manufacturer (Becton Dickinson and Company [BD], San Jose, CA). A total of 10,000 events were analyzed for each sample. The percentage of cells in G1, S, and G2/M phases of the cell cycle was determined by use of a BD LSRII analyzer, followed by analysis using FlowJo (Tree Star Inc., Ashland, OR) software.

Calpain activity assay

Calpain activity assay was performed as we described previously [10]. In brief, cell lysates (10 μ g/sample) were incubated with 200 μ M calpain substrate, *N*-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Bachem, Torrance, CA) in a buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 3mM Ca^{2+} at 37°C for 20 min. Fluorescence was measured at 460 nm with excitation at 355 nm by using a Vector3 plate reader, and data were analyzed using Wallac software (PerkinElmer Life and Analytical Science Inc., Waltham, MA). 7-Amino-4-methylcoumarine (Bachem) was used as a standard.

Real-time quantitative (q) RT-PCR

Real-time qRT-PCR was performed as described previously [10]. Samples were run in duplicate and the results were normalized to *GAPDH* expression. Primer sequences for *GAPDH*, *Cyclin D1*, *Cyclin E1*, and *p27^{Kip1}* were as follows: *GAPDH*, (forward, 5'-AACTACATGGTC TACATGTTCCA-3' and reverse 5'-CCATTCTCGGCCTTGACTGT-3'); *Cyclin D1*, (forward, 5'-GGGGACAACCTCTTAAGTCTCAC-3' and reverse, 5'-CCCAATAAAAGACCAATCTCTC-3'); *Cyclin E1* (forward, 5'-CACAACATCCAGACCCACAC-3' and reverse, 5'-ATGGCAGGTTTGGTCATTCT-3'); and *p27^{Kip1}* (forward, 5'-TAGAGATGGCACAGGGTTCC-3' and reverse, 5'-CCCATCCAATTTCGACAACTT-3').

Western blot and immunoprecipitation analyses

Western blot analysis was performed as described previously [10] with antibodies against the following proteins: cyclin D, cyclin E, cdk2, cdk4, p27^{Kip1}, p-retinoblastoma protein (T821), p-p27^{Kip1} (S10), t-Akt, p-Akt (S473), t-FoxO3A, and p-FoxO3A (T32). In some experiments, blots were incubated in Restore Western blot stripping buffer (Thermo Scientific, Rockford, IL) and reprobed with other primary antibodies. The intensity of the selected protein bands was semi-quantified using FluorChem SP (Alpha Innotech Corp., San Leandro, CA). The relative density of p-Akt (S473)/t-Akt and p-FoxO3A(T32)/t-FoxO3A at time 0 was set as 1 in control and *Capn4*-kdn cells, respectively. A rate constant for dephosphorylation of p-Akt (S473) and p-FoxO3A (T32) was calculated using one phase exponential decay curve fit program of Prism 4 Software (GraphPad Software Inc., La Jolla, CA).

For immunoprecipitation analysis, MC4 cells stably expressing either *control* or *Capn4* RNAi were harvested, and cell lysates were precleared with 0.5 μ g/ml normal mouse IgG agarose (Santa Cruz) for 2 h at 4°C with agitation. One milligram of protein of the supernatant was incubated with 2 μ g of primary antibodies to Akt for 1 h at 4°C and then with 40 μ l of protein A/G agarose (Santa Cruz) overnight at 4°C. Samples were washed,

dissolved in sample buffer, and resolved on a 10% SDS gel followed by Western blot analysis using anti-PP2A antibody. Some samples were also used for phosphatase activity assay.

PP2A phosphatase activity

PP2A phosphatase activity was measured using Immunoprecipitation Phosphatase Assay Kit (Millipore) with modification using 2 nM okadaic acid, a PP2A inhibitor [12].

Statistics

Data were calculated from three and more independent experiments and expressed as the mean $\pm$ SE. Statistical analysis was performed using the unpaired Student's T-test or analysis of variance. *p* values less than 0.05 were accepted as significant.

Results

Osteoblast cell lines stably expressing *Capn4* were established

To test a role of *Capn4* in cell proliferation of the osteoblast lineage, we created MC3T3 Subclone4 (MC4) cell lines stably expressing either *control* or *Capn4* microRNAs. The average calpain activities of established cells lines, MC4-*Capn4* microRNA 1 and 2, were 50% and 35% vs. MC4-*Control* microRNA (control) lines 107 $\pm$ 2% (*n*=4), respectively. We used MC4-*Capn4* microRNA 2 (*Capn4*-knockdown [kdn]) cell line, whose calpain activity was reduced to approximately 35% of that of control cells, in most of our experiments.

Deletion of *Capn4* in cells of the osteoblast lineage increases p27^{Kip1} protein levels by reducing calpain-mediated degradation of p27^{Kip1}

Stable deletion of *Capn4* in osteoblasts showed impaired cell growth assessed by cell number (Fig. 1a). Proliferation of control and *Capn4*-kdn cells was assessed by BrdU-incorporation followed by flow cytometry; the number of BrdU-positive cells was reduced by approximately 30% in *Capn4*-kdn cells compared with control cells (% cells in S phase; control 24 $\pm$ 3 % vs. *Capn4*-kdn 16 $\pm$ 2 %). No difference was observed in apoptosis between control and *Capn4*-kdn osteoblastic cells (% of apoptotic cells; control, 8.8 $\pm$ 0.3% vs. *Capn4*-kdn, 7.1 $\pm$ 1.1%). Collectively, knockdown of *Capn4* impairs cell proliferation of the osteoblast lineage as we reported previously [11].

To identify a mechanism by which knockdown of *Capn4* reduces cell proliferation of the osteoblast lineage, we next examined mRNA expression of cell cycle proteins, *cyclin D1* and *cyclin E1*. Expression of *cyclinD1* mRNA was significantly reduced in *Capn4*-kdn MC4 osteoblastic cells (Fig. 1b); this result was consistent with our reports that mRNA expression of *c-fos*, a downstream target of PTH and a positive regulator of cyclin D transcription, is impaired by ablation of *Capn4* in cells of the osteoblast [10] and chondrocyte lineages [11]. Levels of various cell cycle proteins were next examined. Despite reduced *cyclin D1* mRNA levels (Fig. 1b), increased protein levels of p27^{Kip1} (Fig. 1c, 1d, and 1e) and cyclin D (Fig. 1d and 1e) were detected by Western blot analysis. Notably, p27^{Kip1} and cyclin D are calpain substrates [13, 14]. We also observed reduced phosphorylation of Rb on T821 in *Capn4*-kdn cells compared with control cells (Fig. 1d and 1e). There were no significant differences in protein levels of cdk4 (Fig. 1d and 1e), cyclin E, cdk 2, and p21^{Cip1} (data not shown) between control and *Capn4*-kdn osteoblasts. Thus, these results suggest that, despite increased cyclin D protein levels, increased p27^{Kip1} effectively blocks a cyclin D-cdk4 complex and reduces phosphorylation of Rb, resulting in impaired cell cycle transition at G1/S as we described previously in cells of the chondrocyte lineage [11].

Moreover, phosphorylation of p27^{Kip1} on S10 was previously shown by others and us to be associated with calpain-mediated degradation of p27^{Kip1}; ablation of *Capn4* impairs phosphorylation of p27^{Kip1} on S10 [11] and removal of p-S10 p27^{Kip1} through a calpain-mediated degradation pathway [15]. Deletion of *Capn4* in osteoblastic cells also showed approximately twofold increased levels of p-S10 p27^{Kip1} protein (Fig. 1d and 1e), suggesting impaired calpain-mediated degradation of p-S10 p27^{Kip1} protein in osteoblasts. Next, we tested whether additional knockdown of p27^{Kip1} rescues an impaired cell growth phenotype of cells of the osteoblast lineage. We observed significantly normalized cell number in *Capn4* and p27^{Kip1}-double knockdown cells compared with in *Capn4*-knockdown cells (Fig. 1f). These results were consistent with our previous report in cells of the chondrocyte lineage [11]. Collectively, knockdown of *Capn4* in cells of the osteoblast lineage increases p27^{Kip1} protein levels, at least partially, by reducing calpain-mediated removal of p27^{Kip1}. Moreover, reduction of p27^{Kip1} protein levels can, at least partially, rescue an impaired cell proliferative function of *Capn4*-kdn osteoblasts.

Deletion of *Capn4* in cells of the osteoblast lineage increases p27^{Kip1} protein levels by increasing transcription of p27^{Kip1}

Bertoli et al showed that p27^{Kip1} protein levels are regulated by calpain-mediated modification of Akt/ FoxO3A phosphorylation. Ablation of *Capn4* reduces phosphorylation of Akt and FoxO3A, and hypophosphorylated FoxO3A translocates from cytoplasm to nucleus, where it presumably increases p27^{Kip1} transcription through the direct binding of FoxO3A to the promoter of p27^{Kip1} and, consequently, p27^{Kip1} protein levels in embryonic fibroblasts [16]. Therefore, we next examined whether knockdown of *Capn4* alters p27^{Kip1} mRNA levels and modifies phosphorylation of Akt and FoxO3A in cells of the osteoblast lineage. Our data showed that knockdown of *Capn4* increases p27^{Kip1} mRNA expression (Fig. 2a) and more rapidly reduces phosphorylation of Akt and FoxO3A than controls upon serum starvation (Fig. 2b) in proliferating osteoblasts prior to reaching confluence. We also semi-quantified the intensity of the protein bands corresponding to p-Akt (S473) and p-FoxO3A (T32) that was then normalized by t-Akt and t-FoxO3A protein levels, respectively, at various time points (Fig. 2c). The average rate constant for the dephosphorylation of p-Akt (S473) was 0.092±0.018 in control cells vs. 0.171±0.007 in *Capn4*-kdn cells; that for p-FoxO3A (T32) was 0.044±0.009 in control cells vs. 0.121±0.008 in *Capn4*-kdn cells. Thus, these results suggest that p-Akt and p-FoxO3A were likely dephosphorylated upon serum starvation approximately 2- and 3-times faster, respectively, in *Capn4*-kdn osteoblasts than controls. Collectively, p27^{Kip1} protein levels are also regulated by calpain-mediated modification of Akt/ FoxO3A signaling and a consequent increase of p27^{Kip1} mRNA levels in cells of the osteoblast lineage.

We next examined how deletion of *Capn4* modifies phosphorylation of Akt. PP2A is a heterotrimer consisting of a scaffolding subunit, a variable regulatory subunit, and a catalytic subunit [17]. PP2A regulatory subunits B56 α and γ are calpain substrates, and ablation of *Capn4* increases the stability of PP2A, which thereby increases dephosphorylation of Akt in embryonic fibroblasts [16]. Our co-immune precipitation assay showed an increased intensity of PP2A protein band (Fig. 3a) and significantly increased PP2A activity (Fig. 3b) of the protein complex precipitated with anti-Akt antibody. Collectively, these results suggest that calpain modifies p27^{Kip1} mRNA expression, at least in part, by modifying phosphorylation of Akt/FoxO3A in cells of the osteoblast lineage.

Discussion

Calpains are cysteine proteases, which have numerous diverse substrates and regulate various cellular functions [2]. We previously showed that osteoblast-specific *Capn4* knockout mice exhibited significantly reduced trabecular and cortical bone at least partially

due to reduced cell proliferation of the osteoblast lineage *in vivo* [10]. However, the limitation of our report was that we failed to identify a specific molecular target of calpain responsible for their bone phenotype.

p27^{Kip1} has been shown to be one of those critical target molecules of calpain and that calpain activity uniquely modulates levels of p27^{Kip1} *in vitro* [11, 13, 15, 16, 18]. Consistent with our previous report in cells of the chondrocyte lineage [11], we here showed that ablation of *Capn4* reduces cell growth by decreased cell proliferation of the osteoblast lineage. Knockdown of *Capn4* increases p27^{Kip1} protein levels by reducing removal of p-S10-p27^{Kip1}, a target of calpain-mediated degradation of p27^{Kip1}, resulting in reduced phosphorylation of Rb. Additional ablation of p27^{Kip1} protein, at least partially, rescues the impaired cell growth phenotype of the *Capn4*-kdn osteoblastic cells. It was also shown previously that regulatory subunits of PP2A are calpain substrates; therefore, knockdown of calpain stabilizes PP2A protein in a protein complex assembled with Akt, which thereby promotes dephosphorylation of Akt and FoxO3A in embryonic fibroblasts [16]. The promoter region of p27^{Kip1} contains binding sequences for FoxO3A [16]. Therefore, we next examined whether calpain regulates p27^{Kip1} protein levels by increasing p27^{Kip1} mRNA levels in cells of the osteoblast lineage. Knockdown of calpain increases the intensity and activity of PP2A in a PP2A-Akt protein complex, resulting in more rapid dephosphorylation of Akt and then FoxO3A in osteoblasts upon serum-starvation. Collectively, calpain is also a critical modulator for p27^{Kip1} in cells of the osteoblast lineage. Figure 4 shows a hypothetical scheme of calpain-mediated regulation of p27^{Kip1} in cells of the osteoblast lineage. Knockdown of calpain increases PP2A protein stability and activity assembled with Akt, resulting in reducing phosphorylation of Akt and then FoxO3A. Underphosphorylated FoxO3A translocates to nucleus and increases p27^{Kip1} mRNA levels through direct binding to the p27^{Kip1} promoter and increases p27^{Kip1} protein levels (left). On the other hand, knockdown of calpain decreases calpain-mediated degradation of p27^{Kip1} (right). Increased p27^{Kip1} inactivates mainly cyclin D-cdk4, resulting in reduced phosphorylation of Rb and impaired cell cycle transition at G1/S.

The mechanisms by which intermittent administration of PTH shows an anabolic effect on bone have been intensively studied. Investigators reached various conclusions depending on cell lines, dose and frequency of PTH administration, cell density, and culture condition *in vitro* studies [19–25]. However, a line of evidence has suggested that upregulation of p27^{Kip1} and cyclin-dependent kinase inhibitor 1A (p21^{Cip1}) proteins [26, 27] through PTH-mediated increase of protein kinase A (PKA) signaling is positively associated with an anabolic action of PTH in bone [26]. Because we have showed previously that ablation of calpain significantly decreases PTH-mediated PKA signaling [9, 10], calpain may uniquely increase p27^{Kip1} protein levels, at least partially, through a mechanism independent of a PTH-PKA signaling pathway. Further studies will be necessary to demonstrate how calpain-mediated modulation of p27^{Kip1} in bone affects anabolic action of PTH *in vivo*; however, our results at least suggest that calpain inhibition can be a critical therapeutic target for osteoporosis because it could increase an efficiency of anabolic action of PTH by uniquely increasing p27^{Kip1} protein levels in cells of the osteoblast lineage.

In conclusion, our current report suggests that calpain regulates p27^{Kip1} protein levels by modifying p27^{Kip1} protein degradation and p27^{Kip1} transcription via phosphorylation of Akt/FoxO3A, in cells of the osteoblast lineage.

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References

1. WHO ; Report of a WHO Study Group. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. World Health Organ Tech Rep Ser. 1994; 843:1–129. [PubMed: 7941614]
2. Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. *Physiol Rev.* 2003; 83:731–801. [PubMed: 12843408]
3. Arthur JS, Elce JS, Hegadorn C, Williams K, Greer PA. Disruption of the murine calpain small subunit gene, *Capn4*: calpain is essential for embryonic development but not for cell growth and division. *Mol Cell Biol.* 2000; 20:4474–4481. [PubMed: 10825211]
4. Tram KK, Murray SS, Lee DB, Murray EJ. PTH-induced osteoblast contraction is mediated by cysteine proteases. *Kidney Int.* 1993; 43:693–699. [PubMed: 8384280]
5. Tram KK, Spencer MJ, Murray SS, Lee DB, Tidball JG, Murray EJ. Identification of calcium-activated neutral protease activity and regulation by parathyroid hormone in mouse osteoblastic cells. *Biochem Mol Biol Int.* 1993; 29:981–987. [PubMed: 8508148]
6. Murray EJ, Tram KK, Murray SS, Lee DB. Parathyroid hormone-induced retraction of MC3T3-E1 osteoblastic cells is attenuated by the calpain inhibitor N-Ac-Leu-Leu-norleucinal. *Metabolism.* 1995; 44:141–144. [PubMed: 7869906]
7. Murray EJ, Grisanti MS, Bentley GV, Murray SS. E64d, a membrane-permeable cysteine protease inhibitor, attenuates the effects of parathyroid hormone on osteoblasts in vitro. *Metabolism.* 1997; 46:1090–1094. [PubMed: 9284902]
8. Murray SS, Grisanti MS, Bentley GV, Kahn AJ, Urist MR, Murray EJ. The calpain-calpastatin system and cellular proliferation and differentiation in rodent osteoblastic cells. *Exp Cell Res.* 1997; 233:297–309. [PubMed: 9194492]
9. Shimada M, Mahon MJ, Greer PA, Segre GV. The receptor for parathyroid hormone and parathyroid hormone-related peptide is hydrolyzed and its signaling properties are altered by directly binding the calpain small subunit. *Endocrinology.* 2005; 146:2336–2344. [PubMed: 15691895]
10. Shimada M, Greer PA, McMahon AP, Bouxsein ML, Schipani E. In vivo targeted deletion of calpain small subunit, *capn4*, in cells of the osteoblast lineage impairs cell proliferation, differentiation, and bone formation. *J Biol Chem.* 2008; 283:21002–21010. [PubMed: 18515801]
11. Kashiwagi A, Schipani E, Fein MJ, Greer PA, Shimada M. Targeted Deletion of *Capn4* in Cells of the Chondrocyte Lineage Impairs Chondrocyte Proliferation and Differentiation. *Mol Cell Biol.* 2010; 30:2799–2810. [PubMed: 20368361]
12. Begum N, Ragolia L. cAMP counter-regulates insulin-mediated protein phosphatase-2A inactivation in rat skeletal muscle cells. *J Biol Chem.* 1996; 271:31166–31171. [PubMed: 8940115]
13. Patel YM, Lane MD. Mitotic clonal expansion during preadipocyte differentiation: calpain-mediated turnover of p27. *J Biol Chem.* 2000; 275:17653–17660. [PubMed: 10749891]
14. Choi YH, Lee SJ, Nguyen P, Jang JS, Lee J, Wu ML, Takano E, Maki M, Henkart PA, Trepel JB. Regulation of cyclin D1 by calpain protease. *J Biol Chem.* 1997; 272:28479–28484. [PubMed: 9353308]
15. Delmas C, Aragou N, Poussard S, Cottin P, Darbon JM, Manenti S. MAP kinase-dependent degradation of p27Kip1 by calpains in choroidal melanoma cells. Requirement of p27Kip1 nuclear export. *J Biol Chem.* 2003; 278:12443–12451. [PubMed: 12529328]
16. Bertoli C, Copetti T, Lam EW, Demarchi F, Schneider C. Calpain small-1 modulates Akt/FoxO3A signaling and apoptosis through PP2A. *Oncogene.* 2009; 28:721–733. [PubMed: 19029949]
17. Xu Y, Xing Y, Chen Y, Chao Y, Lin Z, Fan E, Yu JW, Strack S, Jeffrey PD, Shi Y. Structure of the protein phosphatase 2A holoenzyme. *Cell.* 2006; 127:1239–1251. [PubMed: 17174897]
18. Yajima Y, Kawashima S. Calpain function in the differentiation of mesenchymal stem cells. *Biol Chem.* 2002; 383:757–764. [PubMed: 12108540]
19. Partridge NC, Opie AL, Opie RT, Martin TJ. Inhibitory effects of parathyroid hormone on growth of osteogenic sarcoma cells. *Calcif Tissue Int.* 1985; 37:519–525. [PubMed: 3933791]

20. MacDonald BR, Gallagher JA, Russell RG. Parathyroid hormone stimulates the proliferation of cells derived from human bone. *Endocrinology*. 1986; 118:2445–2449. [PubMed: 3009159]
21. Finkelman RD, Mohan S, Linkhart TA, Abraham SM, Boussy JP, Baylink DJ. PTH stimulates the proliferation of TE-85 human osteosarcoma cells by a mechanism not involving either increased cAMP or increased secretion of IGF-I, IGF-II or TGF beta. *Bone Miner*. 1992; 16:89–100. [PubMed: 1315602]
22. Scutt A, Duvos C, Lauber J, Mayer H. Time-dependent effects of parathyroid hormone and prostaglandin E2 on DNA synthesis by periosteal cells from embryonic chick calvaria. *Calcif Tissue Int*. 1994; 55:208–215. [PubMed: 7987735]
23. Tam VK, Clemens TL, Green J. The effect of cell-matrix interaction on parathyroid hormone (PTH) receptor binding and PTH responsiveness in proximal renal tubular cells and osteoblast-like cells. *Endocrinology*. 1998; 139:3072–3080. [PubMed: 9645678]
24. Nasu M, Sugimoto T, Kaji H, Chihara K. Estrogen modulates osteoblast proliferation and function regulated by parathyroid hormone in osteoblastic SaOS-2 cells: role of insulin-like growth factor (IGF)-I and IGF-binding protein-5. *J Endocrinol*. 2000; 167:305–313. [PubMed: 11054645]
25. Swarthout JT, Doggett TA, Lemker JL, Partridge NC. Stimulation of extracellular signal-regulated kinases and proliferation in rat osteoblastic cells by parathyroid hormone is protein kinase C-dependent. *J Biol Chem*. 2001; 276:7586–7592. [PubMed: 11108712]
26. Onishi T, Hruska K. Expression of p27Kip1 in osteoblast-like cells during differentiation with parathyroid hormone. *Endocrinology*. 1997; 138:1995–2004. [PubMed: 9112398]
27. Qin L, Li X, Ko JK, Partridge NC. Parathyroid hormone uses multiple mechanisms to arrest the cell cycle progression of osteoblastic cells from G1 to S phase. *J Biol Chem*. 2005; 280:3104–3111. [PubMed: 15513917]

Fig. 1.

a Reduced calpain activity decreases cell growth of the osteoblast lineage. MC3T3 Subclone 4 (MC4) cells stably expressing either *control* or *Capn4* microRNA were established. Efficiency of RNAi was assessed by calpain activity. Calpain activities of *Capn4-1* and *Capn4-2* knockdown (kdn) cells were 50 and 35% of that of control cells ($107 \pm 2\%$), respectively. **b** Knockdown of *Capn4* significantly reduces *cyclin D1* but not *cyclin E* mRNA levels in proliferating cells of the osteoblast lineage. **c** Knockdown of *Capn4* increases levels of p27^{Kip1} protein in osteoblasts. **d** Knockdown of *Capn4* increases cyclin D, total (t)- and phosphorylated (p)-Serine (S)10-p27^{Kip1} protein levels, and decreases phosphorylation of retinoblastoma protein (Rb) on T821. Cells were serum-starved for 3 d before isolating cell lysates. # and ## indicate that the intensity of protein bands of *Capn4-kdn* cells were higher and lower than that of control cells, respectively. **e** Semi-quantitative representation of protein expression of various cell cycle proteins. The density of the protein bands shown in Fig. 1d was next analyzed using FluorChem SP. The density of the protein bands corresponding to each cell cycle protein isolated from control cells was set as 1. **f** Transient knockdown of p27^{Kip1}, at least partially, rescues a reduced cell growth phenotype of *Capn4-kdn* osteoblasts. At some time points, error bars were within symbols. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

Fig. 2.

a Knockdown of *Capn4* increases *p27^{Kip1}* mRNA expression in proliferating osteoblasts. **b** Knockdown of *Capn4* more rapidly reduces phosphorylation of Akt on S473 and FoxO3A on T32 than controls upon serum starvation in osteoblasts. Two independent representative experiments were shown as experiments 1 (exp.1) and 2 (exp.2). **c** Knockdown of *Capn4* promotes dephosphorylation of p-Akt and p-FoxO3A in osteoblasts. The density of the p-Akt, t-Akt, p-FoxO3A, and t-FoxO3A protein bands (data from b) of control and *Capn4*-kdn cells, respectively, were analyzed at 0, 5, 15, and 30 min after serum depletion. The relative density of p-Akt (S473)/t-Akt and p-FoxO3A (T32)/t-FoxO3A at time 0 was set as 1 in control and *Capn4*-kdn cells, respectively. In some time points, error bars were within symbols.

Fig. 3.

a Deletion of *Capn4* increases the intensity of the PP2A protein assembled with Akt in osteoblasts. # indicates that the intensity of protein bands of *Capn4*-kdn cells (C4-kdn) was higher than that of control cells (Cont). **b** Deletion of *Capn4* increases the activity of PP2A assembled with Akt in osteoblasts. One mg of whole cell lysates was immuno-precipitated with anti-Akt-antibody. The intensity of protein band assembled with Akt was assessed by Western blot analysis (a) and its activity of PP2A was determined using Immunoprecipitation Phosphatase Assay (b). *, $p < 0.05$.

Fig. 4.

A hypothetical scheme of calpain-mediated regulation of p27^{Kip1} protein in cells of the osteoblast lineage. Calpain modulates p27^{Kip1} protein levels by increasing its transcription and reducing degradation in cells of the osteoblast lineage. Knockdown of calpain increases PP2A protein stability and activity assembled with Akt, resulting in reducing phosphorylation of Akt and then FoxO3A. Underphosphorylated FoxO3A translocates to nucleus and increases *p27^{Kip1}* mRNA levels through direct binding to the *p27^{Kip1}* promoter and increases p27^{Kip1} protein levels (left). On the other hand, knockdown of calpain also decreases calpain-mediated degradation of p27^{Kip1} (right). Increased p27^{Kip1} inactivates mainly cyclin D-cdk4, resulting in reduced phosphorylation of Rb and impaired cell cycle transition at G1/S in osteoblasts.