PTHrP prevents chondrocyte premature hypertrophy by inducing cyclin-D1-dependent Runx2 and Runx3 phosphorylation, ubiquitylation and proteasomal degradation

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Summary

In chondrocytes, PTHrP maintains them in a proliferative state and prevents premature hypertrophy. The mechanism by which PTHrP does this is not fully understood. Both Runx2 and Runx3 are required for chondrocyte maturation. We recently demonstrated that cyclin D1 induces Runx2 protein phosphorylation and degradation. In the present studies, we tested the hypothesis that PTHrP regulates both Runx2 and Runx3 protein stability through cyclin D1. We analyzed the effects of cyclin D1 on Runx3 protein stability and function using COS cells, osteoprogenitor C3H10T1/2 cells and chondrogenic RCJ3.1C5.18 cells. We found that cyclin D1 induced Runx3 degradation in a dose-dependent manner and that both Myc-tagged Runx3 and endogenous Runx3 interact directly with CDK4 in COS and RCJ3.1C5.18 cells. A conserved CDK recognition site was identified in the C-terminal region of Runx3 by sequence analysis (residues 356-359). Pulse-chase experiments showed that the mutation of Runx3 at Ser356 to alanine (SA-Runx3) increased the half-life of Runx3. By contrast, the mutation at the same serine residue to glutamic acid (SE-Runx3) accelerated Runx3 degradation. In addition, SA-Runx3 was resistant to cyclin D1-induced degradation. GST-Runx3 was strongly phosphorylated by CDK4 in vitro. By contrast, CDK4 had no effect on the phosphorylation of SA-Runx3. Although both wild-type and SE-Runx3 were ubiquitylated, this was not the case for SA-Runx3. Runx3 degradation by cyclin D1 was completely blocked by the proteasome inhibitor PS1. In C3H10T1/2 cells, SA-Runx3 had a greater effect on reporter activity than SE-Runx3. The same was true for ALP activity in these cells. To investigate the role of cyclin D1 in chondrocyte proliferation and hypertrophy, we analyzed the growth plate morphology and expression of chondrocyte differentiation marker genes in *Ccnd1*-knockout mice. The proliferating and hypertrophic zones were significantly reduced and expression of chondrocyte differentiation marker genes and ALP activity were enhanced in 2-week-old *Ccnd1*-knockout mice. PTHrP significantly suppressed protein levels of both Runx2 and Runx3 in primary chondrocytes derived from wild-type mice. By contrast, the suppressive effect of PTHrP on Runx2 and Runx3 protein levels was completely abolished in primary chondrocytes derived from *Ccnd1*-knockout mice. Our findings demonstrate that the cell cycle proteins cyclin D1 and CDK4 induce Runx2 and Runx3 phosphorylation, ubiquitylation and proteasomal degradation. PTHrP suppresses Runx2 and Runx3 protein levels in chondrocytes through cyclin D1. These results suggest that PTHrP might prevent premature hypertrophy in chondrocytes, at least in part by inducing degradation of Runx2 and Runx3 in a cyclin-D1-dependent manner.

Key words: PTHrP, Cyclin D1, Runx2, Runx3, Chondrocyte

Introduction

Endochondral bone formation, including chondrocyte proliferation, maturation, terminal differentiation and apoptosis, is one major type of bone formation in the vertebrate skeleton. Numerous signaling molecules, cell cycle regulatory proteins, and transcription factors precisely control the balance between chondrocyte proliferation and differentiation.

Runx2 is a critical transcription factor that promotes chondrocyte maturation. In *Runx2*-knockout (*Runx2*$^{-/-}$) mice, the formation of hypertrophic chondrocytes is severely impaired in some skeletal elements including the femur and the humerus (Inada et al., 1999). Targeted expression of *Runx2* in non-hypertrophic Col2a1-expressing chondrocytes accelerates chondrocyte differentiation and rescues the chondrocyte phenotype in *Runx2*$^{-/-}$ mice (Takeda et al., 2001; Ueta et al., 2001). By contrast, over expression of a dominant-negative *Runx2* in Col2a1-expressing chondrocytes inhibits chondrocyte maturation (Takeda et al., 2001; Ueta et al., 2001). These results indicate that Runx2 plays an important role in chondrocyte maturation and also suggests that Runx2 acts not only in hypertrophic chondrocytes but also in Col2a1-expressing proliferating chondrocytes.

Runx3, which also belongs to the Runt-domain family of transcription factors, is crucial for gastric epithelial cell growth, neurogenesis of the dorsal root ganglia and CD8-lineage T cell differentiation (Li et al., 2002; Levanon et al., 2002; Taniuchi et al., 2002; Woolf et al., 2003). In addition, Runx3 also plays an

important role in the process of endochondral bone development. In *Runx2*$^{+/-}$/*Runx3*$^{-/-}$ mice, chondrocyte maturation and vascular invasion into the cartilage were delayed compared with that of *Runx2*$^{+/-}$ mice during embryogenesis, indicating that, in addition to Runx2, Runx3 is required for endochondral ossification (Yoshida et al., 2004). *Runx3* expression was first detected at embryonic day (E)12.5 during mouse embryonic skeletal development, mainly in proliferating and prehypertrophic chondrocytes. This expression pattern partially overlaps that of *Runx2* (Stricker et al., 2002; Yoshida et al., 2004).

Although chondrocyte maturation is delayed in *Runx2*-deficient mice, terminal differentiation of chondrocytes does occur in these mice (Inada et al., 1999; Kim et al., 1999). Further evidence demonstrates that chondrocyte hypertrophy is completely absent in *Runx2/3* double-knockout (dKO) mice. At E18.5, chondrocytes of *Runx2/3* dKO mice express *Col2a1*, but not *Col10a1*, a marker of hypertrophy. Mineralization is observed in restricted regions of the limbs, vertebrae and ribs of *Runx2*$^{-/-}$ newborns, but is completely absent throughout entire skeletons of *Runx2/3* dKO newborns (Yoshida et al., 2004). These findings indicate that both Runx2 and Runx3 are required for chondrocyte hypertrophy and maturation.

During the G1-S cell cycle transition, it is 'decided' whether cells will proceed with proliferation or withdraw from the cell cycle for differentiation (Bartek et al., 1996; Galindo et al., 2005). Cyclin D1 (CCND1) is the regulatory subunit of the cyclin-D1–CDK4 holoenzyme that interacts with and phosphorylates the retinoblastoma (Rb) protein to promote the transition of cells from G1 into S phase. In cartilage, cyclin D1 expression is limited specifically to proliferating chondrocytes (Long et al., 2001). *Ccdn1*-deficient mice display a reduced proliferating zone in the growth plate (Beier et al., 2001), indicating that cyclin D1 is required for chondrocyte proliferation.

Parathyroid hormone-related protein (PTHrP) is a critical growth factor in regulation of chondrocyte function in the growth plate. It maintains chondrocytes in a proliferative state and prevents premature chondrocyte hypertrophy. Targeted disruption of the *Pthrp* (also known as *Pth1h*) or *Pthrp receptor* (*Pthr*; also known as *Pth1r*) genes leads to early cessation of chondrocyte proliferation and subsequent acceleration of chondrocyte differentiation (Karaplis et al., 1994; Kobayashi et al., 2002; Lanske et al., 1996). Conversely, mice overexpressing *Pthrp* in chondrocytes exhibit almost exclusive proliferation of chondrocytes in bones and a profound delay in endochondral ossification (Weir et al., 1996). The mechanism of PTHrP action in chondrocytes is still poorly understood. In vitro studies using chondrocyte cell lines suggest that PTHrP activates cyclin D1 gene expression and promotes chondrocyte proliferation through transcription factors ATF2 and CREB (Beier et al., 2001). Recent studies demonstrate that PTHrP inhibits Runx2 mRNA expression in chondrocytes (Guo et al., 2006). Considering the fact that Runx2 can be phosphorylated and degraded by cyclin-D1–CDK4 in a ubiquitin-proteasome-dependent manner (Shen et al., 2006a), we hypothesize that PTHrP may regulate the functions of Runx2 and Runx3 through cyclin D1 during the process of chondrocyte development.

In the present studies, we found that cyclin-D1–CDK4 induced phosphorylation and degradation of Runx3. In postnatal *Ccdn1*-knockout mice, chondrocyte proliferation was reduced and accompanied by accelerated chondrocyte maturation. PTHrP downregulated protein levels of both Runx2 and Runx3 and these effects were completely abolished in *Ccdn1*-deficient chondrocytes. Our data suggests that one important mechanism by which PTHrP prevents chondrocyte maturation is through the targeting of Runx2 and Runx3 proteins for degradation by cyclin D1.

Results

Cyclin D1 interacts directly with Runx3

To determine whether cell cycle proteins regulate the expression of Runx3, a Myc-Runx3 expression plasmid was cotransfected with either a cyclin D1 or a cyclin A expression plasmid into COS cells. Runx3 protein expression was detected by western blotting. As shown in Fig. 1A, overexpression of cyclin A had no significant effect on Runx3 protein levels. On the contrary, Runx3 protein levels decreased in a dose-dependent manner upon overexpression of cyclin D1, suggesting that cyclin D1 induces Runx3 degradation. Cyclin D1 interacts with CDK4 in early G1 phase of the cell cycle to phosphorylate various target proteins. To determine whether CDK4 interacts with Runx3, an immunoprecipitation assay was performed. Myc-Runx3 and cyclin D1 expression plasmids were transfected into COS cells. The immunoprecipitation (IP) assay was performed using an anti-CDK4 antibody followed by western blotting with an anti-Myc antibody. An anti-cyclin-D1 antibody was used as a positive control. As shown in Fig. 1B, CDK4 interacts with Runx3, indicating that the cyclin-D1–CDK4 complex induces Runx3 degradation through direct interaction with Runx3. To further demonstrate the interaction of endogenous CDK4 and

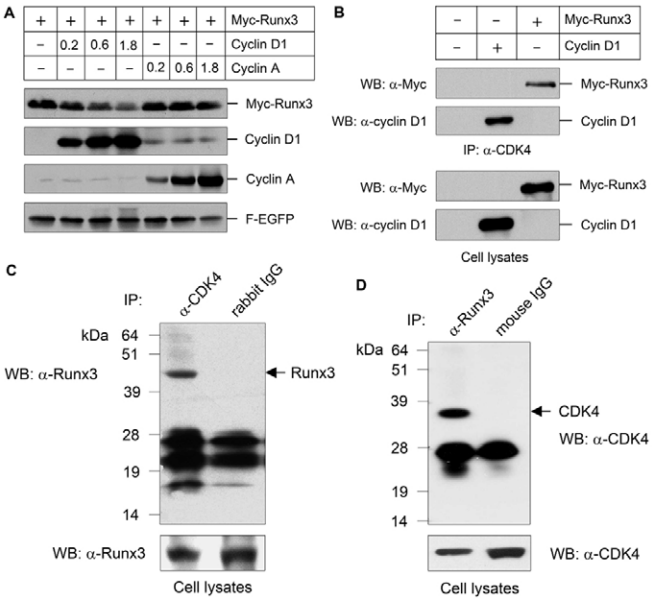

Fig. 1. Cyclin D1 induces Runx3 degradation. (A) A Runx3 expression plasmid was cotransfected with different amounts of cyclin A and cyclin D1 expression plasmids (0.2, 0.6 and 1.8 μg/6-cm culture dish) into COS cells. Western blotting was performed 48 hours after transfection. Cyclin D1 induced Runx3 degradation. By contrast, cyclin A had no effect on Runx3 degradation. (B) Myc-Runx3 and cyclin D1 expression plasmids were cotransfected into COS cells. Immunoprecipitation (IP) was performed using the anti-CDK4 antibody followed by western blotting (WB) with an anti-Myc or anti-cyclin D1 antibody. CDK4 interacts with Runx3 in COS cells. The interaction between cyclin D1 and CDK4 was also detected as a positive control. (C,D) Cell lysates were collected from chondrogenic RCJ3.1C5.18 cells and immunoprecipitation was performed using either an anti-CDK4 antibody or an anti-Runx3 antibody followed by western blotting using the anti-Runx3 or anti-CDK4 antibody. Interaction was detected between endogenous Runx3 and CDK4.

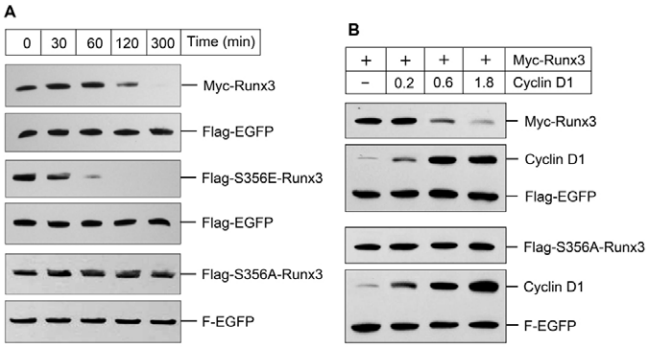

Fig. 2. Ser356 of Runx3 is critical for Runx3 stability. (A) Protein decay assay. Equal amounts of Myc-Runx3, FLAG-S356A-Runx3 or FLAG-S356E-Runx3 expression plasmid were transfected into COS cells. Cell lysates were extracted after treatment with 80 μg/ml cycloheximide for 0, 20, 60, 120 or 300 minutes and western blotting was performed. WT Runx3 protein levels were considerably reduced at 120 minutes after protein synthesis was inhibited by cycloheximide. FLAG-S356A-Runx3 remained stable during the entire 300 minute period after cycloheximide treatment. By contrast, FLAG-S356E-Runx3 expression began to be considerably reduced at 60 minutes after cells were treated with cycloheximide. (B) Myc-Runx3 and FLAG-S356A-Runx3 expression plasmids were cotransfected with different amounts of cyclin D1 plasmid (0.2, 0.6, and 1.8 μg/dish) into COS cells. Cell lysates were extracted 24 hours after transfection and western blotting was performed. WT Runx3 protein levels were considerably reduced by cyclin D1 in a dose-dependent manner. By contrast, S356A-Runx3 levels were not affected by the overexpression of cyclin D1.

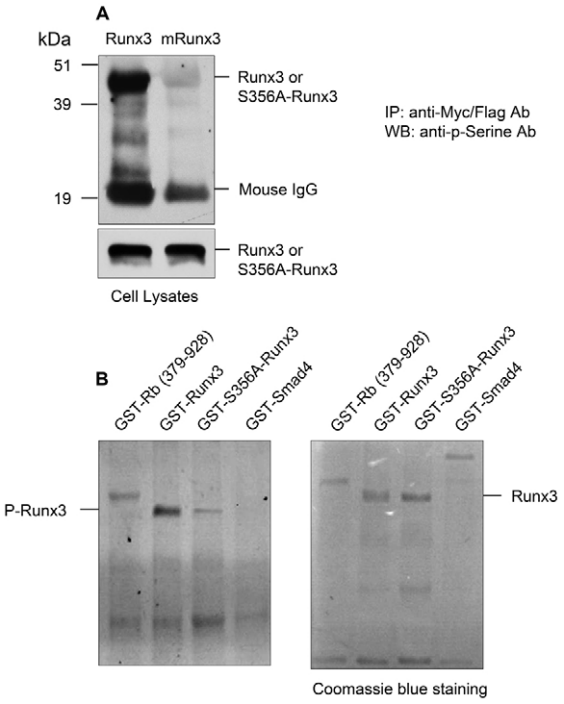

Fig. 3. Cyclin D1 induces Runx3 phosphorylation. (A) COS cells were transfected with equal amounts of Myc-Runx3 and FLAG-S356A-Runx3 plasmids and treated with PS1 (10 μM, 4 hours incubation). Cell lysates were extracted 24 hours after transfection. Phosphorylation of Runx3 was detected by western blotting (WB) using the anti-phosphoserine antibody after WT and mutant Runx3 protein was immunoprecipitated (IP) using either the anti-Myc or anti-FLAG antibody. Strong phosphorylation was detected in WT Runx3-transfected cells. However, only trace amount of phosphorylated mutant Runx3 (P-Runx3) was detected. (B) In vitro phosphorylation assay. CDK4 was immunoprecipitated from 300 μg C2C12 cell lysate using 1.5 μg anti-CDK4 antibody and was used in the Runx3 kinase assay (1 μM substrate). GST-Rb (379-928) was used as a positive control and GST-Smad4 was used as a negative control. GST-Runx3 exhibits a strong phosphorylation, whereas GST-S356A-Runx3 exhibits a weak phosphorylation. Left: Runx3 phosphorylation by CDK4. Right: protein amounts, detected by Coomassie blue staining.

Runx3 we performed coimmunoprecipitation assays using an anti-CDK4 antibody or an anti-Runx3 antibody, respectively, followed by immunoblotting with the anti-Runx3 or anti-CDK4 antibody. The cell lysates were collected from chondrogenic RCJ3.1C5.18 cells. The results demonstrated that endogenous CDK4 interacts with endogenous Runx3 in RCJ3.1C5.18 cells (Fig. 1C,D).

Cyclin D1 induces Runx3 phosphorylation

Our previous findings demonstrated that cyclin D1 phosphorylates Ser472 of mouse Runx2, inducing Runx2 degradation in a proteasome-dependent manner. A putative SP motif was also identified in the C-terminal region of human Runx3 (356-359) by sequence analysis. To determine the importance of Ser356 for Runx3 stability, mutant Runx3 (S356A and S356E) expression plasmids were constructed as previously described (Shen et al., 2006a) and protein decay assays were performed. Wild-type (WT) and mutant Runx3 expression plasmids were transfected into COS cells. Protein synthesis was stopped 36 hours after transfection, by the addition of the protein synthesis inhibitor cycloheximide. As shown in Fig. 2A, the expression levels of WT Runx3 began to decline after 120 minutes of cycloheximide treatment, while levels of S356A-Runx3 remained stable during the entire 300 minute period of cycloheximide treatment. By contrast, the protein levels of S356E-Runx3 began to decline significantly after only 60 minutes of cycloheximide treatment (Fig. 2A), indicating that Ser356 is crucial for Runx3 stability. To determine whether Ser356 mediates cyclin D1 degradation, WT or mutant Runx3 expression plasmids were cotransfected with different amounts of a cyclin D1 expression plasmid into COS cells. Western blotting was performed to detect changes in Runx3 protein levels. As shown in Fig. 2B, WT Runx3 protein levels were reduced by cyclin D1 in a dose-dependent manner. Expression levels of the S356A-Runx3, however, were not affected by cyclin D1, indicating that Ser356 of Runx3 is crucial for Runx3 degradation by cyclin D1.

To determine the importance of Ser356 in mediating Runx3 phosphorylation, WT and mutant Runx3 (S356A) expression plasmids were cotransfected with a cyclin D1 expression plasmid into COS cells. Immunoprecipitation was performed using anti-Myc or anti-FLAG antibodies followed by western blotting with an anti-phosphoserine antibody. As shown in Fig. 3A, WT Runx3 protein was phosphorylated by cyclin D1. By contrast, the phosphorylation of S356A-Runx3 was minimal compared with that of WT Runx3 (Fig. 3A). To confirm this finding, GST-Runx3 and GST-S356A-Runx3 fusion proteins were generated and used as substrates for in vitro phosphorylation assays with CDK4. GST-Rb (379-928) was used as a positive control and GST-Smad4 as a negative control (Matsuura et al., 2004). As shown in Fig. 3B, WT Runx3 was strongly phosphorylated by CDK4. Only very minor phosphorylation was detected when S356A-Runx3 was incubated with CDK4. These results demonstrate that Ser356 of Runx3 is crucial for phosphorylation induced by cyclin-D1–CDK4.

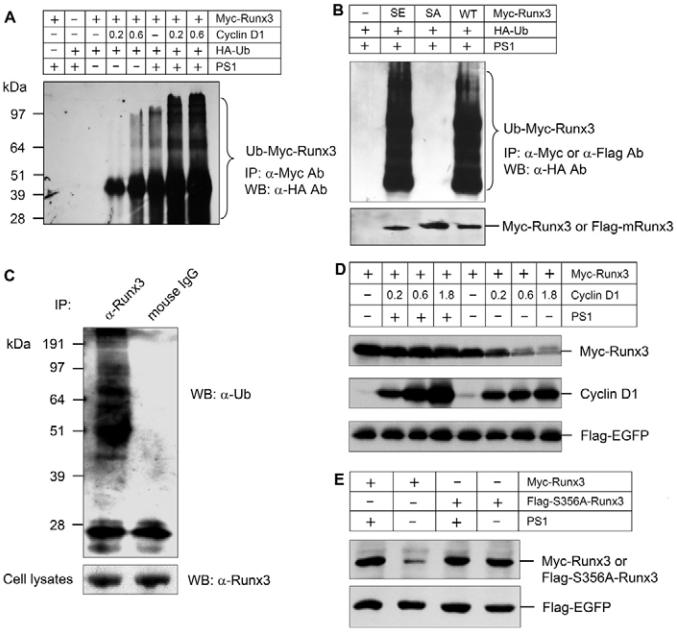

Fig. 4. Cyclin D1 induces ubiquitylation and proteasome degradation of Runx3. (A) Myc-Runx3 and HA-ubiquitin expression plasmids were cotransfected with different amounts of cyclin D1 expression plasmid (0.2 and 0.6 μg/6-cm culture dish) into COS cells in the presence or absence of PS1 (10 μM for 4 hour incubation). Cyclin D1 induced Runx3 ubiquitylation in a dose-dependent manner and this effect was further enhanced by the addition of PS1. (B) Myc-Runx3, FLAG-S356A-Runx3 or FLAG-S356E-Runx3 was cotransfected with HA-ubiquitin plasmid into COS cells in the presence of PS1 (10 μM, 4 hours incubation). Runx3 ubiquitylation was detected in the WT Runx3 and S356E-Runx3 groups but not in the S356A-Runx3 group. (C) To further determine the ubiquitylation of endogenous Runx3, chondrogenic RCJ3.1C5.18 cells were treated without or with MG132 (10 μM, 4 hour incubation) before cell lysates were collected. Immunoprecipitation was performed using the anti-Runx3 antibody followed by western blotting using the anti-ubiquitin antibody. Ubiquitylation of endogenous Runx3 was detected in the presence of MG132 in RCJ3.1C5.18 cells. (D) Myc-Runx3 expression plasmid was cotransfected with different amounts of cyclin D1 expression plasmid (0.2, 0.6 and 1.8 μg/dish) into COS cells. Cells were treated with PS1 (10 μM) for 4 hours after transfection. Cyclin D1 induced a dose-dependent degradation of Runx3 and treatment with PS1 completely reversed cyclin-D1-induced Runx3 degradation. (E) WT and mutant Runx3 (S356A) expression plasmids were transfected into COS cells. Cells were treated with PS1 (10 μM, 4 hour incubation) after transfection. The addition of PS1 increased the level of WT Runx3 protein but had not effect on mutant Runx3 (S356A) protein.

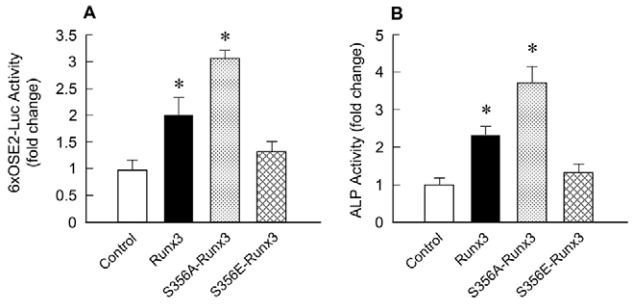

Fig. 5. Ser356 is critical for Runx3 function. (A) Wild-type (WT) and mutant Runx3 (S356E and S356A) expression plasmids were cotransfected with 6xOSE2-Luc reporter into C3H10T1/2 cells. Cell lysates were collected 24 hours after transfection and a luciferase assay was performed. The S356A-Runx3 increased activity and S356E-Runx3 decreased activity of the 6xOSE2-Luc reporter compared with the WT Runx3. **P*<0.05, compared with the WT Runx3; unpaired Student's *t*-test. (B) WT and mutant Runx3 (S356E and S356A) expression plasmids were transfected into C3H10T1/2 cells. ALP activity was measured using an ALP assay kit. S356A-Runx3 increased and S356E-Runx3 decreased ALP activity compared with the WT Runx3. **P*<0.05, compared with the WT Runx3; unpaired Student's *t*-test.

Cyclin D1 induces Runx3 ubiquitylation and proteasome degradation

To determine whether cyclin D1 mediates Runx3 ubiquitylation, Runx3 and cyclin D1 expression plasmids were cotransfected into COS cells in the presence or absence of proteasome inhibitor 1 (PS1). Immunoprecipitation was performed using an anti-Myc antibody and Runx3 ubiquitylation was detected by western blotting with an anti-HA antibody. As shown in Fig. 4A, ubiquitylation of Runx3 is increased in the presence of cyclin D1. Treatment of cells with PS1 further enhanced this effect. To determine the role of Ser356 during ubiquitylation, WT or mutant Runx3 expression plasmids were cotransfected with a cyclin D1 expression plasmid into COS cells followed by immunoprecipitation of the Runx3 proteins. As shown in Fig. 4B, ubiquitylation of both WT Runx3 and S356E-Runx3 was detected with enhanced ubiquitylation of S356E-Runx3. By contrast, no ubiquitylation of S356A-Runx3 was detected (Fig. 4B). To determine if endogenous Runx3 is ubiquitylated, chondrogenic RCJ3.1C5.18 cells were treated with or without proteasome inhibitor MG132. Runx3 protein was then immunoprecipitated using an anti-Runx3 antibody and the ubiquitylated Runx3 protein was detected by western blotting with an anti-ubiquitin antibody. Ubiquitylated Runx3 protein was detected in the presence of the proteasome inhibitor MG132 (Fig. 4C), suggesting that ubiquitylation of Runx3 could play a physiological role in chondrocytes.

To determine whether Runx3 degradation by cyclin D1 is proteasome dependent, Runx3 protein levels were assayed in COS cells treated with or without proteasome inhibitor PS1. The results demonstrated that cyclin D1 induced Runx3 degradation in a dose-dependent manner in the absence of PS1. However, Runx3 levels remained constant in the presence of PS1 (Fig. 4D), indicating that cyclin-D1-mediated Runx3 degradation is proteasome dependent. In addition to the increased levels of Runx3, cyclin D1 protein levels were also increased upon PS1 treatment. It is known that cyclin D1 protein stability is regulated by the ubiquitin-proteasome pathway mediated by a Skp1–Cul1–F-box (SCF) ubiquitin ligase (Lin et al., 2006; Feng et al., 2007). Treatment with PS1 may inhibit the degradation process of not only Runx3 but also cyclin D1, leading to the elevated levels of cyclin D1 protein. To further determine whether Ser356 is crucial for Runx3 degradation, WT and mutant Runx3 (S356A) expression plasmids were transfected into COS cells followed by treatment with or without PS1. As shown in Fig. 4E, PS1 completely rescued the expression of WT Runx3. Levels of S356A-Runx3 were unchanged in the two treatment groups, indicating that Ser356 is necessary for cyclin-D1-induced phosphorylation-dependent Runx3 degradation.

Mutations of *Runx3* alter its transcriptional activity

To determine whether Ser356 plays an important role in Runx3 transcriptional activity, WT and mutant Runx3 expression plasmids were cotransfected with the 6xOSE-Luc reporter into C3H10T1/2 cells. With respect to WT Runx3, S356A-Runx3 showed higher transactivation of the 6xOSE-Luc reporter, whereas S356E-Runx3 showed lower transactivation of the 6xOSE-Luc reporter (Fig. 5A).

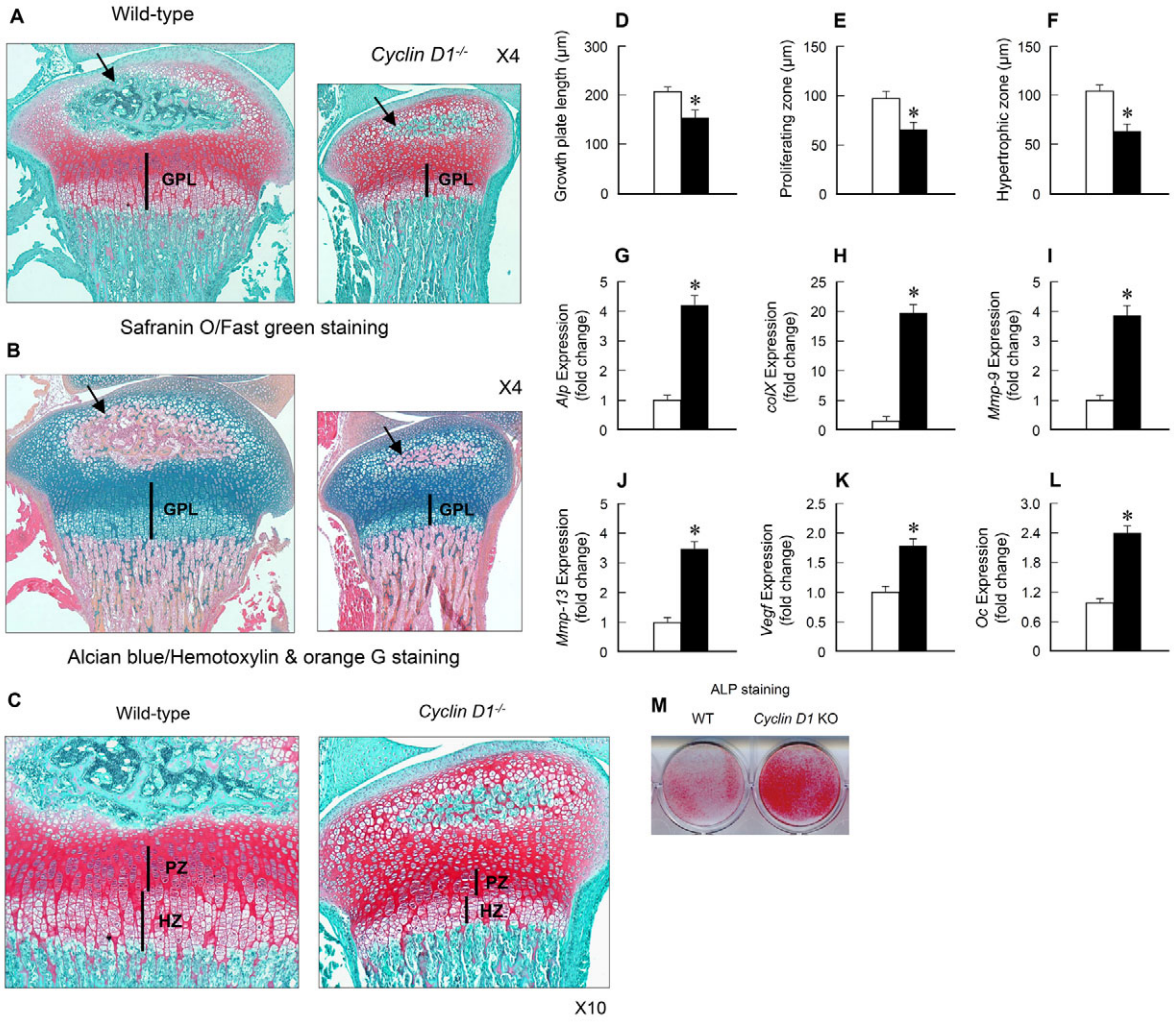

Fig. 6. Chondrocyte proliferation and differentiation are altered in *Ccdn1*-knockout mice. (A-F) Histological analyses, including Safranin O/Fast green (A,C) and Alcian blue/Hemotoxylin and Orange G (B) staining, showed that growth plate length (GPL; D) and the lengths of the proliferating zone (PZ; C,E) and the hypertrophic zone (HZ; C,F) were reduced (n=6) and the formation of the secondary ossification center was delayed (black arrows in A and B) in *Ccdn1*-knockout mice compared to their WT littermates. (G-M) Primary chondrocytes were isolated from 3-day-old *Ccdn1*$^{-/-}$ mice and WT littermates and expression of chondrocyte marker genes and alkaline phosphatase (ALP) staining were examined. The results demonstrated that the expression of chondrocyte marker genes, such as *Alp* (G), *collagen type X* (*ColX*; H), *Mmp9* (I), *Mmp13* (J), *Vegf* (K) and *osteocalcin* (*Oc*; L), and ALP activity (M) were significantly increased in *Ccdn1*-knockout chondrocytes (n=3). *P<0.05, compared with the WT littermate controls; unpaired Student's t-test.

Runx3 cooperates with Runx2 to induce chondrocyte differentiation and upregulation of the chondrocyte maturation marker gene, alkaline phosphatase (ALP) (Shen et al., 2006a). To further investigate the biological significance of Ser356 of Runx3, WT and mutant Runx3 expression plasmids were transfected into C3H10T1/2 cells followed by detection of ALP activity. As shown in Fig. 5B, overexpression of WT Runx3 significantly induced ALP activity (2.1-fold increase compared with the control). S356A-Runx3 further stimulated ALP activity (3.7-fold increase compared with the control). By contrast, S356E-Runx3 caused a reduction in ALP activity to 38% of that caused by WT Runx3. These results demonstrate that Ser356 is crucial for Runx3 function during chondrocyte maturation.

PTHrP downregulates Runx2 and Runx3 protein levels through cyclin D1

To better understand the role of cyclin D1 in cartilage development, 2-week-old homozygous *Ccdn1*-knockout mice and WT littermates were harvested for histological and histomorphometric analyses. Staining was performed using Safranin O and Fast green, Alcian blue and Hemotoxylin, and Orange G. Significant reduction in growth plate length (GPL), decreases in lengths of the proliferating and hypertrophic zones (PZ and HZ) and delay in the formation of the secondary ossification center were observed in the *Ccdn1*-knockout mice (Fig. 6A-F). The mRNA expression of *Alp* (4.2-fold), *ColX* (also known as *Col10a1*; 20-fold), *Mmp9* (3.8-fold), *Mmp13* (3.4-fold), *Vegf* (1.7-fold) and *osteocalcin* (*Oc*; also known as *Bglap1*; 2.4-fold) was significantly increased in *Ccdn1*-knockout mice (Fig. 6G-L). In addition, ALP staining showed that ALP activity was also increased (Fig. 6M). These results suggest that chondrocyte differentiation was accelerated when chondrocyte proliferation was inhibited. The reduction in the hypertrophic zone may be the result of a significant impairment in cell proliferation, resulting in fewer cells entering into the hypertrophic zone.

PTHrP maintains chondrocytes in a proliferative state. Accelerated chondrocyte hypertrophy and premature ossification

are observed in *Pthrp*-knockout mice (Lanske et al., 1996). The mechanism of PTHrP action in chondrocytes remains unclear. In the present studies, we examined whether PTHrP upregulates cyclin D1 and CDK4 expression in chondrocytes. Chondrogenic RCJ3.1C5.18 cells were cultured with 10^{-8} M PTHrP for different time periods (0-12 hours). Significant and time-dependent upregulations of the expression of cyclin D1 mRNA and CDK4 protein were observed by the treatment with PTHrP (Fig. 7A,B). Since PTHrP upregulates the expression of cyclin D1 and CDK4, which in turn induce phosphorylation and degradation of Runx2 and Runx3 proteins, we hypothesized that PTHrP downregulates Runx2 and Runx3 protein levels through cyclin D1. Primary chondrocytes were isolated from 3-day-old neonatal *Ccdn1*$^{-/-}$ mice and their WT littermates to assay the steady-state protein levels of Runx2 and Runx3. Much higher levels of Runx2 and Runx3 were detected in primary chondrocytes derived from *Ccdn1*$^{-/-}$-knockout mice than in those derived from WT mice (Fig. 7C,D). Treatment with PTHrP (48 hour incubation) significantly downregulated steady-state protein levels of Runx2 and Runx3 in WT chondrocytes. This downregulation was completely inhibited in primary chondrocytes derived from *Ccdn1*$^{-/-}$ mice (Fig. 7C,D). These findings strongly suggest that PTHrP regulates Runx2 and Runx3 protein levels in a cyclin-D1-dependent manner in chondrocytes.

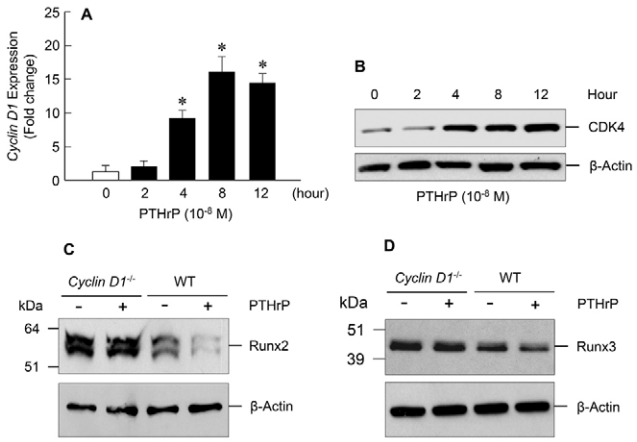

Fig. 7. PTHrP downregulates steady-state protein levels of Runx2 and Runx3 in a cyclin-D1-dependent manner. (A,B) The time-course effects of PTHrP on the expression of cyclin D1 mRNA and CDK protein were examined by real-time PCR and western blotting in chondrogenic RCJ3.1C5.18 cells. PTHrP (10^{-8} M) significantly increased the expression of cyclin D1 mRNA and CDK4 protein (4-12 hour treatment) in RCJ3.1C5.18 cells. (C,D) Effects of PTHrP on Runx2 and Runx3 protein levels were examined by western blotting in primary chondrocytes. PTHrP significantly downregulated Runx2 (C) and Runx3 (D) protein levels in WT chondrocytes. However, these effects were completely inhibited in *Ccdn1*-knockout chondrocytes. **P*<0.05, one-way analysis of variance followed by Dunnett's test, compared with the control group without PTHrP treatment.

Discussion

Phosphorylation-dependent and independent mechanisms of Runx2 ubiquitylation have previously been reported (Zhao et al., 2003; Shen et al., 2006a; Shen et al., 2006b). Several studies, each using different approaches, have indicated that the C-terminal Ser451 residue of human Runx2 is a critical site of phosphorylation that leads to a subsequent change in Runx2 activity (Wee et al., 2002; Qiao et al., 2006; Shen et al., 2006a; Rajgopal et al., 2007). Interestingly, the serine residue at this site is located within a cyclin-dependent kinase phosphorylation consensus motif [(S/T)PX(K/R)]. It is well established that the cell cycle is driven by the tightly regulated activity of the cyclin-dependent kinases (CDKs) and their cyclin partners. Levels of the cyclins oscillate throughout the cell cycle restricting activity of the CDKs to a defined period and, thereby, controlling the proliferative fate of the cell. For example, cyclin D1 expression is upregulated in response to a mitogenic signal received by a cell in quiescence or early G1 phase, resulting in the formation of active cyclin-D1–CDK4 and six complexes by mid-G1 phase (Sherr, 1996). We have recently demonstrated that cyclin-D1–CDK4 directly interacts with mouse Runx2 and phosphorylates Ser472 (equivalent to Ser451 of human Runx2). This phosphorylation induces Runx2 ubiquitylation and proteasome-dependent degradation leading to inhibition of Runx2 transcriptional activity (Shen et al., 2006a). In the present studies, we found that cyclin-D1–CDK4 is able to regulate Runx3 protein stability by a similar mechanism. Cyclin-D1–CDK4 phosphorylates Ser356 of human Runx3 leading to ubiquitylation and proteasome-dependent degradation of Runx3. Mutation of Ser356 to alanine inhibits Runx3 phosphorylation and degradation, thereby, enhancing Runx3 stability and transcriptional activity. By contrast, mutation of Ser356 to glutamic acid reduces the stability and transcriptional activity of Runx3 by mimicking constitutive phosphorylation of the protein. These data indicate that both the expression levels and functions of Runx2 and Runx3 are regulated by cyclin D1 and that the C-terminal SP motifs in Runx2 and Runx3 proteins are crucial for this regulation. Further investigation is needed to determine the ubiquitin E3 ligase that cooperates with cyclin D1 in regulation of ubiquitylation and proteasome-dependent degradation of Runx2 and Runx3.

Consistent with our findings, Galindo and colleagues demonstrated that in MC3T3 osteoblastic cells Runx2 levels are cell cycle-regulated with highest expression during G0-G1 phase and minimal expression during S and G2-M phases (Galindo et al., 2005). Furthermore, other cell cycle proteins, such as Cdc2 or cyclin B-CDK1, were also shown to phosphorylate Runx2 (Qiao et al., 2006; Rajgopal et al., 2007). Specifically, cyclin B-CDK1 was shown to regulate phosphorylation of Runx2 at Ser472 to promote DNA binding during mitosis. These findings suggest that Runx2 activity is regulated in multiple phases of the cell cycle by, presumably, different signaling pathways. For instance, when a cell receives mitogenic signals, cyclin D1 is upregulated, allowing phosphorylation and subsequent degradation of Runx2 and Runx3. When a cell receives differentiation cues, however, cyclin D1 is downregulated, allowing Runx2 and Runx3 stabilization and activation. In this manner, it may be possible for multiple Cdks to regulate the activity of Runx2 through the same phosphorylation site at different cell cycle phases or differentiation stages of the cell. Furthermore, the point at which cyclin D1 levels begin to drop and Runx2 and Runx3 protein levels begin to rise could be a key point in the developmental transition from chondrocyte proliferation to maturation.

Further supporting a role for cyclin D1 in regulation of Runx2 and Runx3 protein stability, we show here that the basal levels of both Runx2 and Runx3 are higher in primary chondrocytes from *Ccdn1*$^{-/-}$ mice than in those from WT mice. The cyclin-D1-deficient mice are smaller than their WT littermates and this can be attributed, in part, to a reduction in the size of the proliferating and hypertrophic zones of their growth plate (Beier et al., 2001;

Fantl et al., 1995; Sicinski et al., 1995). We also show an increase in expression of the chondrocyte differentiation marker genes *ColX*, *Mmp9*, *Mmp13*, *Vegf* and *Oc* as well as an increase in ALP activity in chondrocytes from *Ccdn1*$^{-/-}$ mice. This is probably a result of the increase in Runx2 and Runx3 protein levels in these cells.

It has been reported that cyclin D1 expression is specifically found in proliferating chondrocytes, as determined by in situ hybridization (Long et al., 2001), and that PTHrP stimulates cyclin D1 expression in proliferating chondrocytes (Beier et al., 2001). PTHrP is an auto/paracrine factor essential for endochondral bone development. In the growth plate, PTHrP mRNA is expressed in perichondrial cells and proliferating chondrocytes, whereas the protein is detected at the site of PTHrP receptor expression in a distinct region of non-proliferating prehypertrophic chondrocytes (Lee et al., 1995; Lee et al., 1996). Targeted disruption of the *PTHrP* gene in mice resulted in accelerated chondrocyte hypertrophy and ossification (Karaplis et al., 1994). Forced expression of PTHrP in chondrocytes, however, inhibited chondrocyte maturation and bone formation (Weir et al., 1996). This suggests that PTHrP maintains chondrocytes in a proliferative state and prevents premature chondrocyte hypertrophy (Karp et al., 2000). The exact mechanism by which PTHrP regulates chondrocyte proliferation and maturation is still unknown. One probable explanation is through regulation of the G1 to S phase cell cycle transition. For example, it was reported that crossing mice nullizygous for the cyclin-dependent kinase inhibitor *p57*^{Kip2} with *PTHrP*-knockout mice partially rescued the *PTHrP*-null phenotype. Specifically, the presence of proliferative chondrocytes was restored in most bones, as was the rate of chondrocyte hypertrophy (MacLean et al., 2004). These findings suggest that PTHrP may regulate chondrocyte proliferation through upregulation of cyclin-dependent kinase activity. Additionally, cyclin D1 mRNA was also shown to be upregulated in response to PTHrP signaling in chondrocytes (Beier et al., 2001). Furthermore, PTHrP was shown to negatively regulate Runx2 mRNA expression (Li et al., 2004). The effect of PTHrP on Runx2 protein stability, however, was not previously investigated. Here, we show that PTHrP reduces the steady-state protein levels of Runx2 and Runx3 in primary chondrocytes derived from wild-type mice and that this effect is completely abolished in *Ccdn1*-deficient chondrocytes. This suggests that PTHrP regulates the stability of Runx2 and Runx3 protein in a cyclin-D1-dependent manner. Taken together, it seems probable that PTHrP may regulate chondrocyte proliferation and hypertrophy through upregulation of the activity of cyclinD1-CDK4 and subsequent downregulation of Runx2 and Runx3 protein.

Interestingly, Guo and colleagues showed that although the femurs of *Runx2*$^{-/-}$ mice had no vascular invasion into the cartilage or mRNA expression of the hypertrophic markers *ColX* or *Oc*, femurs of *Runx2*$^{-/-}$ *Pthrp*$^{-/-}$ mice had some vascular invasion and some expression of both *ColX* and *Oc* mRNAs (Guo et al., 2006). This suggests that Runx2 is not the only target of PTHrP in regulation of chondrocyte maturation and that PTHrP must negatively regulate other factors that promote chondrocyte differentiation. One probable target is Runx3. The importance of Runx3 for completion of chondrocyte maturation is highlighted by the fact that, whereas chondrocytes from *Runx2*$^{-/-}$ mice will eventually undergo terminal differentiation, those from *Runx2*$^{-/-}$ *Runx3*$^{-/-}$ mice never differentiate (Yoshida et al., 2004). In osteoblasts, Runx2 is essential for differentiation but a role for Runx3 has not yet been defined. This difference could be due to the different expression patterns of Runx3 in chondrocytes and in osteoblasts. Nevertheless, it would be of interest to determine whether cyclin-D1–CDK4 also regulates Runx2 protein stability in osteoblasts. The data we present here support a model whereby, in chondrocytes, PTHrP is able to negatively regulate protein levels of both Runx2 and Runx3 in a cyclin-D1-dependent manner. Through direct interaction and phosphorylation, cyclin D1 promotes the ubiquitin-dependent proteasomal degradation of Runx2 and Runx3. Collectively, the data suggest a novel mechanism for regulation of chondrocyte maturation where PTHrP signaling, through cyclin D1, induces degradation of both Runx2 and Runx3. In this manner, PTHrP may prevent chondrocytes undergoing premature hypertrophy.

Materials and Methods

Cell culture and transfection

COS and C3H10T1/2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and RCJ3.1C5.18 cells were cultured in αMEM supplemented with 10% fetal calf serum at 37°C under 5% CO_2. DNA plasmids were transiently transfected into COS or C3H10T1/2 cells in 6-cm culture dishes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Empty vector was used to keep the total amount of transfected DNA constant in each group in all experiments. FLAG-EGFP plasmid was cotransfected as an internal control for transfection efficiency. Western blot and immunoprecipitation (IP) assays were performed 24 hours after transfection.

Western blotting and immunoprecipitation

Western blotting and immunoprecipitation (IP) were performed as described previously (Shen et al., 2006a; Shen et al., 2006b). The interaction between endogenous Runx3 and CDK4 and the ubiquitylation of endogenous Runx3 protein were determined in chondrogenic RCJ3.1C5.18 cells. For the Runx3 ubiquitylation assay, the proteasome inhibitor MG132 (10 μM) was added to the cell culture 4 hours before cells were harvested. The mouse anti-Runx3 monoclonal antibody (R3-6E9) was provided by Yoshiaki Ito (Oncology Research Institute, National University of Singapore, Singapore) (Ito et al., 2005). The mouse anti-cyclin D1 monoclonal antibody (DCS6) was purchased from Cell Signaling Technology (Danvers, MA). The rabbit anti-CDK4 (C-22) polyclonal antibody and the rabbit anti-ubiquitin (FL-76) polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

PCR-based site-directed mutagenesis

Myc-tagged human Runx3 cDNA (MRIP isoform, NCBI accession number: NM_004350) was amplified by PCR, sequenced, and cloned into pcDNA3 expression vectors (Stratagene, La Jolla, CA). Mutant Runx3 constructs (mRunx3-S356A and mRunx3-S356E) were generated using Stratagene QuikChange site-directed mutagenesis kit and cloned into pcDNA3 (Stratagene).

In vivo protein decay assay

Cells were seeded in 15-cm culture dishes, and equal amounts of Myc-Runx3, FLAG-SA-Runx3 and FLAG-SE-Runx3 were used for transfection. 24 hours after transfection, cells were trypsinized and split into five 10-cm dishes. 12 hours after recovery, cells were cultured in regular medium with 80 μg/ml cycloheximide (Calbiochem), for 0, 20, 60, 120 and 300 minutes before harvesting. Western blotting was performed to detect the decay of wild-type (WT) and mutant Runx3 proteins.

In vitro phosphorylation of Runx3 by CDK4

Glutathione *S*-transferase (GST)-Runx3 and GST-SA-Runx3 plasmids were generated by PCR-based cloning. GST-Rb-(379-928) and GST-Smad4 were used as controls. GST-tagged WT and mutant cDNAs were expressed in *Escherichia coli* and purified. The induction of protein expression was detected by Coomassie blue staining. To isolate soluble proteins, 100 ml of bacterial cultures were harvested, and pellets were resuspended in 10 ml of bacterial lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 15 μg/ml benzamidine, 2 mg/ml lysozyme) at 4°C for 20 minutes. Cell lysates were sonicated until no longer viscous and centrifuged at 18,000 ***g*** for 30 minutes at 4°C. The supernatant was incubated with 100 μl of GST beads. After columns were washed three times with sample buffer, the recombinant proteins were eluted with 5 mM reduced glutathione (Sigma) in 50 mM Tris-HCl, pH 8, 50 mM NaCl, and 1 mM dithiothreitol.

CDK4 was immunoprecipitated from 300 μg C2C12 cell lysate using 1.5 μg anti-CDK4 antibody. CDK4 was used to phosphorylate recombinant substrates for 40 minutes in 20-μl reactions containing 35 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 0.1% Tween 20, 1 mM dithiothreitol, 15 μM ATP at 30°C. After phosphorylation, the recombinant Runx3 or control proteins were directly denatured with sample buffer and subjected to SDS-PAGE followed by Pro-Q Diamond phosphoprotein staining (Invitrogen) according to the manufacturer's protocol.

Luciferase and ALP assays

The 6xOSE2-Luc reporter construct (Ducy et al., 1997) was cotransfected into C3H10T1/2 cells with Runx3 expression plasmids and different amounts of a cyclin D1 expression plasmid. Cell lysates were extracted 24 hours after transfection and luciferase activity was measured using a Promega Dual Luciferase reporter assay kit (Promega, Madison, WI). The ALP activity measurements were performed as described previously (Zhao et al., 2004; Shen et al., 2006a).

Ccdn1-knockout mice

Ccdn1-knockout mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Genotyping was performed following the protocols provided.

Histology

For histological analyses, mice were killed at day 14. Samples of long bones were fixed with 10% formalin. Fixed skeletal samples were subjected to decalcification for 2 weeks. Paraffin blocks were prepared by standard histological procedures. Paraffin sections were stained with Safranin O/Fast green and Alcian blue/Hemotoxylin and Orange G. Histomorphometry was performed using the OsteoMeasure system (OsteoMetrics, Atlanta, GA).

Primary chondrocyte isolation

Primary chondrocytes were isolated as described previously (Li et al., 2004; Chen et al., 2008). Briefly, sternae from 3-day-old neonatal *Ccdn1*$^{-/-}$ mice and their WT littermates were harvested, trimmed to remove any connective tissues, and subjected to digestion for 1 hour with pronase (2 mg/ml; Roche) in HBSS. Then sternae were washed with HBSS and subjected to digestion with collagenase D (Roche; 3 mg/ml) in DMEM. After washing the digested sternae twice in HBSS, they were digested with collagenase D (3 mg/ml) in DMEM to obtain purified primary chondrocytes.

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